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ADVANCES IN THE APPLICATION OF GM PHAGES IN RESEARCH, DEVELOPMENT AND INDUSTRY

INVENTORY OF THE CURRENT DEVELOPMENTS IN GENETICALLY MODIFIED BACTERIOPHAGES AND OUTLOOK ON CHARACTERISTICS RELEVANT FOR RISK ASSESSMENT

ONAFHANKELIJKE WETENSCHAPPELIJKE ADVISERING EN SIGNALERING VAN DE RIJKSOVERHEID

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Advances in the application of GM phages in research, development and industry

Inventory of the current developments in genetically modified bacteriophages and outlook on characteristics relevant for risk assessment

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Foreword

Bacteriophages or phages are viruses that infect and multiply in bacterial hosts. Since the discovery of these bacteria-eaters by the French-Canadian microbiologist Félix d'Hérelle in 1917, bacteriophages have been studied in depth. The field of phage biology has expanded largely and over recent decades many new discoveries were made. Genetic modification of phages is increasingly used in order to adapt phages for applications in medical, biotechnological and agricultural fields. For example, adapting phages to fight infections with multidrug resistant bacterial pathogens is receiving increased attention. However, risk assessment of wild type and genetically modified phages has not been thoroughly developed.

The COGEM has therefor commissioned a research project to broaden its knowledge on phage biology, applications of phages as well developments on genetic modification of phages. In addition, the status of risk assessment of phages was addressed. The project was carried out by Perseus who thoroughly evaluated the literature on bacteriophage research. Meetings with Perseus were insightful and pleasant. The advisory committee provided regular feedback in meetings with the researchers of Perseus and suggestions were incorporated after evaluation by Perseus. The advisory committee is pleased with the resulting report which provides an in-depth overview of the developments in bacteriophage biology, applications and genetic modifications as well as the current status on risk assessment.

Dr. de Cock, J.J.P.A. (Hans)

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Summary

Bacteriophages have emerged as highly promising and versatile tools with applications spanning various sectors, including human and veterinary medicine, research and development, diagnostics, food safety, environmental protection, and agriculture. As natural predators of bacteria, bacteriophages offer a targeted approach to combating bacterial infections and addressing the challenges of antibiotic resistance. Genetic modification presents opportunities to customize bacteriophages for specific applications, enhancing their efficacy and safety. Moreover, their utility can be significantly expanded by incorporating sequences of genes of interest, which can then be expressed for purposes such as vaccination, gene therapy, diagnostics, or material sciences.

This study aimed to provide the Commission on Genetic Modification (COGEM) with a comprehensive review of advancements in the genetic modification of bacteriophages, enabling them to anticipate requests for case-specific advice. It is based on a literature review, patent screening, analysis of selected published risk assessments, and consultation with experts.

The first part of the study reviewed **specific features of naturally occurring bacteriophages**, as these characteristics form the basis for their applications and provide the background for genetic modification.

In brief, bacteriophages infect bacteria and are abundant in the environment. They have a narrow bacterial host range, making them interesting candidates for specific antibacterial therapy. Different replication cycles have been described, including lytic, non-lytic, or lysogenic, each leading to the assembly of new phage particles. During the replication cycle, transfer of genetic information - also known as transduction - is an important feature. Generalized transduction involves random bacterial DNA being packaged inside phage capsids, which can potentially alter the genome of a new host cell upon injection. Specialized transduction, typically carried out by lysogenic phages, transfers specific bacterial genes, potentially increasing bacterial virulence by transferring antibiotic resistance or toxin genes. Lateral transduction occurs when a prophage replicates DNA before excision, leading to the transfer of large segments of bacterial DNA to other bacteria at higher frequencies than other forms of transduction.

Bacteria can resist phage infection by developing phage resistance. Such resistance strategies include altering receptors and limiting phage binding, preventing DNA injection, restriction-modification systems, the CRISPR-Cas immune system and bacterial suicide. In response to this, phages can modify their interactions with bacteria to break the resistance. It remains to be determined whether development of phage resistance will negatively impact potential phage applications by reducing the sensitivity of bacteria. Therefore, phage resistance is a factor to be taken into account when considering the risk of a phage. Other risk factors that should be considered when applying wildtype bacteriophages involve amongst others the transfer of undesirable genetic sequences between bacteria (e.g. toxin genes, virulence factors), interference with bacterial homeostasis, release and/or presence of endotoxins and other bacterial components, unwanted interactions at the level of the eukaryotic cell, and immunogenicity.

Currently, clearly defined criteria for risk classification are still rather limited. Therefore, risk assessment is mostly done case-by-case. Phages that have not been fully characterized are typically categorized as risk group 2 until their safety profile is thoroughly understood. Strictly lytic phages are preferred to mitigate risks such as creation of more virulent bacteria. Various laboratory assays and bioinformatic tools help determine phage characteristics (e.g. life cycle) and potential risks. Controversies exist regarding the transduction of antibiotic resistance genes by phages indicating a need to establish safe limits for transduction frequencies.

The study then reviewed **current applications of wild-type phages** in various fields, including human health, veterinary medicine, agriculture (such as food crop production, livestock, and aquaculture), food safety, wastewater treatment, environmental and equipment sanitation, and bacterial detection. Bacteriophages have regained interest due to a fast increase in antibiotic resistance among pathogenic bacteria leading to multidrug resistant (MDR) bacteria. While some applications are still in development, others are already commercially available. The availability of these applications varies significantly between regions, such as the US and Europe.

Different **challenges** were identified in view of future phage therapy. These include practical issues such as achieving sufficient concentrations of phages in targeted tissues, and the need to balance narrow host tropism with effective action, often requiring the use of phage cocktails tailored to specific bacterial strains. Additionally, societal aspects play an important role. Public opinion on bacteriophage therapy varies, with some people welcoming bacteriophages as a promising alternative to traditional antibiotics in the face of rising antibiotic resistance, while others express concerns about the safety, efficacy, and regulation of

bacteriophage therapy. In fact, a tailored legal framework for bacteriophages is lacking, and there is no consensus in which legal framework some bacteriophage applications should be handled.

The study then focused on **genetic modification of bacteriophages**. Three types of genetic modifications have been identified: (1) mitigation or control of risk factors (see above) associated with wildtype bacteriophages, (2) enhancing intrinsic characteristics such as antibacterial activity, or host tropism, and (3) introduction of entirely new functionalities such as specific cell targeting, markers or increasing stability of (bio)materials. Different techniques are available for efficient alteration of the genetic information of phages, for an efficient selection of the desired recombinant phages and to increase transformation efficiencies of the bacterial host. Cell-free transcription-translation systems allow creation of recombinant phage like particles from DNA *in vitro,* in a single test tube*,* overcoming the need for a highly competent host. Considering the rapid advancements in genetic modification techniques, it is anticipated that applications of genetically modified phages will eventually emerge.

The genetically modified phages identified in the current study are mostly in the development phase. No clinical or environmental application of genetically modified phages has reached the commercial phase yet. Only a few commercial applications were identified in R&D (e.g. vector system). Although this may be due to the fact that genetically modified phages are still much more recent than wildtype phages, a lack of thorough risk assessment criteria may also play a role. Also, there is a critical need for robust testing methodologies capable of identifying genetic and phenotypic alterations in phages, ensuring their stability and irreversibility and their effects on gene transfer. A repertoire of suitable tests may already be available, the parameters to be assessed, and the delineation of acceptance criteria remain limited, posing challenges to the comprehensive and objective assessment of the long-term risks associated with genetically modified phages. Addressing these limitations is essential to bolstering the efficacy and reliability of risk evaluation methodologies in the context of phage genetic modification. As for wildtype phages, also the lack of suitable regulatory frameworks for genetically modified phage products is a significant obstacle to their widespread adoption. There's limited guidance on how to conduct such assessments or whether it should differ from that of wildtype ones. There is thus an urgent need for tailored regulatory guidelines that thoroughly cover the risk assessment of modified phages. To establish robust evaluation frameworks for emerging technologies, collaboration is essential among regulatory bodies, industry stakeholders, and researchers. Additionally, it is crucial to consider public opinion into these discussions.

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Samenvatting

Bacteriofagen worden momenteel verkend als zeer veelbelovende en veelzijdige hulpmiddelen met mogelijke toepassingen in verschillende sectoren, waaronder de humane en veterinaire geneeskunde, onderzoek en ontwikkeling, diagnostiek, voedselveiligheid, milieubescherming en landbouw. Als natuurlijke vijanden van bacteriën bieden bacteriofagen een gerichte aanpak voor het bestrijden van bacteriële infecties en de uitdagingen van antibioticaresistentie aan te pakken. Daarenboven biedt genetische modificatie mogelijkheden om bacteriofagen aan te passen voor specifieke toepassingen, waardoor hun effectiviteit en veiligheid kunnen worden verbeterd. Bovendien kan hun bruikbaarheid aanzienlijk worden uitgebreid door sequenties van gewenste genen op te nemen, die vervolgens tot expressie kunnen worden gebracht voor onder andere vaccinatie, gentherapie, diagnostiek of materiaalwetenschappen.

Deze studie had als doel om de Commissie Genetische Modificatie (COGEM) een uitgebreid overzicht te bieden van ontwikkelingen op gebied van de genetische modificatie van bacteriofagen, zodat zij bij specifieke verzoeken voor advies omtrent het gebruik van genetisch gemodificeerde fagen zouden kunnen anticiperen. De studie is gebaseerd op een literatuurstudie, patentonderzoek, analyse van geselecteerde gepubliceerde risicobeoordelingen en overleg met experts.

Het eerste deel van de studie geeft een overzicht van **specifieke kenmerken van in de natuur voorkomende (wild type) bacteriofagen**, aangezien deze kenmerken de basis vormen voor hun toepassingen en de achtergrond bieden voor genetische modificatie.

Kort samengevat, infecteren bacteriofagen bacteriën, en zijn ze overvloedig aanwezig in het milieu. Ze hebben over het algemeen een beperkt gastheerbereik, wat hen interessante kandidaten maakt voor specifieke antibacteriële therapieën. Verschillende replicatiecycli zijn beschreven, waaronder lytisch, niet-lytisch of lysogeen, die elk leiden tot de assemblage van nieuwe fagenpartikels. Bij gegeneraliseerde transductie wordt willekeurig bacterieel DNA verpakt binnen de capsiden van de fagen. Dit bacterieel DNA kan mogelijk het genoom van een nieuwe gastheercel veranderen bij injectie. Bij gespecialiseerde transductie, meestal uitgevoerd door lysogene fagen, worden specifieke bacteriële genen overgedragen, wat mogelijk de bacteriële virulentie verhoogt indien antibioticaresistentie- of toxinegenen hierbij betrokken zijn. Laterale transductie treedt op wanneer een profaag DNA repliceert vóór excisie, wat leidt tot de overdracht van grote segmenten bacterieel DNA naar andere bacteriën met hogere frequenties dan waargenomen bij andere vormen van transductie.

Bacteriën kunnen resistentie tegen fagen ontwikkelen. Deze resistentiestrategieën omvatten het veranderen van receptoren en het beperken van faagbinding, het voorkomen van DNA-injectie, restrictie-modificatiesystemen, het CRISPR-Cas immuunsysteem en bacteriële zelfmoord. In reactie hierop kunnen fagen hun interacties met bacteriën aanpassen om de resistentie te doorbreken. Het moet nog worden vastgesteld of de ontwikkeling van fagendefensie de potentiële toepassingen van fagen negatief zal beïnvloeden door de gevoeligheid van bacteriën te verminderen. Daarom is fagendefensie een factor die in aanmerking moet worden genomen bij het overwegen van de risico's van een faag. Andere risicofactoren die in overweging moeten worden genomen bij het toepassen van wildtype bacteriofagen omvatten onder andere de overdracht van ongewenste genetische sequenties tussen bacteriën (bijvoorbeeld toxinegenen, virulentiefactoren), interferentie met bacteriële homeostase, vrijgave en/of aanwezigheid van endotoxinen en andere bacteriële componenten, ongewenste interacties op het niveau van de eukaryotische cel en immunogeniciteit.

Momenteel zijn duidelijk gedefinieerde criteria voor risicoclassificatie nog vrij beperkt. Daarom wordt risicobeoordeling meestal per geval gedaan. Fagen die niet volledig zijn gekarakteriseerd, worden doorgaans ingedeeld in risicogroep 2 totdat hun veiligheidsprofiel grondig is begrepen. Strikt lytische fagen worden geprefereerd om risico's zoals de creatie van virulentere bacteriën te verminderen. Verschillende laboratoriumtests en bio-informatica tools helpen bij het bepalen van fageneigenschappen (bijv. levenscyclus) en potentiële risico's. Er bestaan controverses over de transductie van antibioticumresistentiegenen door fagen, wat aangeeft dat er behoefte is aan het vaststellen van veilige limieten voor transductiefrequenties.

Deze studie onderzocht vervolgens de **huidige toepassingen van wildtype fagen** in verschillende gebieden, waaronder de menselijke gezondheid, diergeneeskunde, landbouw (zoals voedselproductie, veeteelt en aquacultuur), voedselveiligheid, afvalwaterbehandeling, sanering van omgeving en apparatuur, en bacteriële detectie. Bacteriofagen hebben opnieuw interesse gewekt vanwege een snelle toename van antibioticaresistentie bij pathogene bacteriën, wat leidt tot multiresistente (MDR) bacteriën. Hoewel sommige

toepassingen nog in ontwikkeling zijn, zijn andere al commercieel verkrijgbaar. De beschikbaarheid van deze toepassingen varieert aanzienlijk tussen regio's, zoals de VS en Europa.

Verschillende **uitdagingen** werden geïdentificeerd met het oog op toekomstige faagtherapie. Deze omvatten praktische kwesties zoals het bereiken van voldoende concentraties fagen in de doelweefsels en de noodzaak om een evenwicht te vinden tussen een nauw gastheerspectrum en effectieve werking, wat vaak vereist dat fagencocktails worden gebruikt die zijn afgestemd op specifieke bacteriestammen. Daarnaast spelen maatschappelijke aspecten een belangrijke rol. De publieke opinie over bacteriofaagtherapie varieert; sommige mensen verwelkomen bacteriofagen als een veelbelovend alternatief voor traditionele antibiotica in het licht van de toenemende antibioticaresistentie, terwijl anderen zich zorgen maken over de veiligheid, effectiviteit en regulering van bacteriofaagtherapie. In feite ontbreekt een op maat gemaakt juridisch kader voor bacteriofagen, en is er geen consensus over welk wettelijk kader voor sommige toepassingen van bacteriofagen moet worden gehanteerd.

In een laatste luik van de studie werd de **genetische modificatie van bacteriofagen** onderzocht. Drie typen genetische modificaties zijn geïdentificeerd: (1) mitigatie of beheersing van risicofactoren (zie hierboven) die gepaard gaan met wildtype bacteriofagen, (2) verbetering van intrinsieke eigenschappen zoals antibacteriële activiteit of gastheerspecificiteit, en (3) introductie van geheel nieuwe functionaliteiten zoals specifieke celdoelwitten, markers of het verhogen van de stabiliteit van (bio)materialen. Er zijn verschillende technieken beschikbaar voor de efficiënte aanpassing van de genetische informatie van fagen, voor een efficiënte selectie van de gewenste recombinante fagen en om de transformatie-efficiëntie van de bacteriële gastheer te verhogen. Celvrije transcriptie-translatie systemen maken het mogelijk om recombinante faagachtige deeltjes te creëren uit DNA *in vitro*, in een enkele reageerbuis, waardoor de noodzaak voor een zeer competente gastheer wordt omzeild. Gezien de snelle vooruitgang in genetische modificatietechnieken wordt verwacht dat toepassingen van genetisch gemodificeerde fagen uiteindelijk zullen opduiken.

De genetisch gemodificeerde fagen die in de huidige studie werden geïdentificeerd, bevinden zich in de voornamelijk in de ontwikkelingsfase. Er is nog geen klinische of milieutoepassing van genetisch gemodificeerde fagen die de commerciële fase heeft bereikt. Slechts enkele commerciële toepassingen werden geïdentificeerd in R&D (bv. vectorsystemen). Hoewel dit te wijten kan zijn aan het feit dat genetisch gemodificeerde fagen nog veel recenter zijn dan wildtype fagen, kan een gebrek aan grondige risicobeoordelingscriteria ook een rol spelen. Er is een kritieke behoefte aan robuuste testmethodologieën die genetische en fenotypische veranderingen in fagen kunnen identificeren, hun stabiliteit en onomkeerbaarheid kunnen waarborgen en hun effect op overdracht van genen. Hoewel er mogelijk al een repertoire van geschikte tests beschikbaar is, blijven de te beoordelen parameters en de afbakening van acceptatiecriteria beperkt, wat uitdagingen vormt voor de uitgebreide en objectieve beoordeling van de langetermijnrisico's die mogelijk gepaard gaan met genetisch gemodificeerde fagen. Het aanpakken van deze beperkingen is essentieel om de effectiviteit en betrouwbaarheid van risicobeoordelingsmethoden in de context van genetische modificatie van fagen te versterken.

Net als bij wildtype fagen vormt het ontbreken van geschikte regelgevende kaders voor genetisch gemodificeerde faagproducten een significant obstakel voor hun brede acceptatie. Er is beperkte duiding over hoe dergelijke beoordelingen moeten worden uitgevoerd of in hoeverre deze zouden moeten verschillen van die van wildtype fagen. Er is dus dringend behoefte aan op maat gemaakte regelgevende richtlijnen die de risicobeoordeling van gemodificeerde fagen grondig dekken. Om robuuste evaluatiekaders voor opkomende technologieën vast te stellen, is samenwerking tussen regelgevende instanties, industriële belanghebbenden en onderzoekers essentieel. Bovendien is het cruciaal om de publieke opinie in deze discussies te betrekken.

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Abbreviations

Abi system	Abortive-infection system
ABSA	American Biological Safety Association
acr	anti-CRISPR
AMG	Auxiliary metabolic gene
ATP	Adenosine triphosphate
BLA	Biologics license application (USA)
BRED	Bacteriophage recombineering of phage DNA
BRIP	Bacteriophage recombineering with infectious particles
COGEM	Commissie Genetische Modificatie (Netherlands Commission on Genetic Modification)
CFR	Code of Federal Regulations (USA)
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CsCl	Caesium chloride
DC	Dendritic cell
DGR	Diversity-generating retroelement
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EBSA	European Biosafety Association
EFSA	European Food Safety Association
EFSA BIOHAZ Panel	EFSA's Panel on Biological Hazards
EFSA FEEDAP Panel	EFSA's Panel on Additives and Products or Substances used in Animal Feed
ELISA	Enzyme Linked Immune Sorbent Assay
EMA	European Medicine Agency
EPA	Environmental Protection Agency (USA)
EPO	European Patent Office
EPS	Exopolysaccharide
EU	European Union
FDA	Food and Drug Administration
FSANZ	Food Standards Australia New Zealand
FSH	Follicle-stimulating hormone
GFP	Green Fluorescent Protein
GM	Genetically modified
GMM	Genetically modified microorganism
GMO	Genetically modified organism
GRAS	Generally Recognized as Safe
GTVV	Related recombinant viral or microbial product
HGMF	High-gradient magnetic fishing
HGT	Horizontal Gene Transfer
hsd	Host specificity determinant
IBDV	Infectious bursal disease virus
IND	Investigational new drug (USA)
kb	Kilobase
LH	Luteinizing hormone
LPS	Lipopolysaccharide
MDR	Multidrug-resistant

MHC	Major histocompatibility complex
MPS	Mononuclear phagocyte system
NAD+	Nicotinamide-adenine-dinucleotide
NHEJ	Non-homologous end joining
NIH	National Institutes of Health
Pac	Packaging signal
PAMP	Pathogen-associated molecular pattern
PEG	Polyethylene glycol
PCHS	Probiotic Cleaning Hygiene System
PCR	Polymerase chain reaction
PRR	Pattern-recognition receptor
qPCR	Quantitative polymerase chain reaction
QPS	Qualified presumption of safety
RBD	Receptor binding domain
RBP	Receptor binding protein
RES	Reticuloendothelial system
RNA	Ribonucleic acid
Sie	Superinfection exclusion
soc	Smaller outer capsid
tracrRNA	Trans-activating crRNA
tRNA	Transfer-RNA
TXTL system	Transcription-translation system
USDA	United States Department of Agriculture
UTI	Urinary tract infection
UV light	Ultraviolet light
VLP	Virus-like particle
VMP	Veterinary medicinal product
WHO	World Health Organisation
ZP	Zona pellucida

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Glossary

Adsorption	The specific binding of a virus to a cellular (host) receptor.
Bacterial resistance	Capacity of bacteria to withstand the effects of antibiotics or biocides that are intended to kill or control them
Bacteriophage	Or phages, are viruses that infect and replicate in bacterial cells, including cyanobacteria (cyanophages) and archaea with an extreme diversity in size, morphology, and genomic organisation
Cytokines	Small secreted proteins that mediate intercellular signalling
Dual use	Dual-use items are goods, software and technology that can be used for both civilian and military applications
Endocytosis	A cellular process whereby cells alter membrane shape or composition to internalize extracellular material, such as molecules, nanoparticles, or other agents
Filamentous phage	Named for their filamentous shape, a worm-like chain (long, thin and flexible, reminiscent of a length of cooked spaghetti), about 6 nm in diameter and about 1000-2000 nm long
Foodborne pathogens	Microorganisms including bacteria, viruses, or even parasites that are present in food and are the cause of major diseases such as food poisoning
Genetic adaptation	A biological characteristic with a heritable basis that improves reproduction and/or survival and results from evolution by natural selection
Genetic modification	The alteration of genetic material in a way that does not occur naturally by mating and/or natural recombination
Lipopolysaccharide (LPS)	A common microbe-associated molecular pattern found on the outer membranes of many Gram-negative bacteria
Lysogenic phage	After attachment and introduction of the phage's genome, the genome is integrated into the bacterial cell chromosome or maintained as an episomal element where, in both cases, it is replicated and passed on to daughter bacterial cells without killing them
Lysogenic conversion	The process of a bacterium acquiring a prophage. Often, the phage inserts specific characteristics that improve bacterial survival
Lytic phage	After attachment, introduction of the phage's genome and multiplication, the newly formed phage particles are released upon lysis of the host cell, thereby killing the host
Phage	*See* Bacteriophage
Phage display	Phage display is used to study protein-protein, protein-peptide, and protein-DNA interactions. It involves the use of filamentous bacteriophages such as M13 to display foreign peptides or proteins on their surface. For this, a gene encoding a protein of interest is inserted into a phage coat protein gene, causing the phage to display the protein on the outside. This technique allows screening of large libraries of proteins and amplification in the process called *in vitro* selection, which is analogous to natural selection. Phage display is also an effective way for producing large amounts of peptides, proteins and antibodies.
Phage resistance	Capacity of bacteria to withstand the antibacterial effects of bacteriophages
Phagocytosis	Cellular process whereby cells ingest larger particles (>0,5µm)
Pinocytosis	Uptake of extracellular fluids and smaller particles into a cell by the budding of small vesicles from the cell membrane
Prophage	A phage integrated in the bacterial genome
Prophylaxis	Administration of a medicinal product to an animal or group of animals before clinical signs of a disease, in order to prevent the occurrence of disease or infection

Tailed phage	Phage structured with a head, tail and adhesive device: the head (capsid) with the packed genome, the tail that allows the transfer of the genome during infection, and an absorption apparatus or adhesive device that fixes to the host cell and penetrates it to allow infection
Temperate phage	*See* Lysogenic phage
Transcytosis	The transport of macromolecules from one side of a polarized cell to the other
Tropism	In the context of infection, tropism determines which cells, tissues or hosts can become infected by a given (micro)organism. Such host tropism is determined by the biochemical receptor complexes on cell surfaces that are permissive or non-permissive to the docking or attachment of specific (micro)organisms (including bacteriophages)
Virome	Communities of viruses present in or on multicellular organisms.
Virulent phage	*See* Lytic phage
Wild type	Is the phenotype of the typical form of a species as it occurs in nature

1 Introduction

1.1 Background

The use of bacteriophages has been gaining attention for several years now. Bacteriophage therapy, especially as an alternative to antibiotics, is receiving significant interest, including in the media.

Until recently, bacteriophage therapy was more common in countries outside the EU, but in recent years, clinical studies on bacteriophage therapy have also been conducted within EU member states. Apart from clinical uses, bacteriophages have also been employed for years in combating bacteria in food.

While most experiments or applications involve the wild-type bacteriophages, there is a growing interest in utilising genetic modification in this field. Research is exploring whether genetic modification can enhance the effectiveness of phages in fighting bacterial infections and whether genetically modified (GM) phages can serve as vaccines.

The COGEM (Netherlands Commission on Genetic Modification) anticipates an increase in inquiries in the upcoming years regarding genetic modification and applications involving GM phages. To prepare for this, the COGEM wishes to conduct a survey on the advancements related to genetic modification of phages.

1.2 Purpose of this study

This study aims to assess the current advancements in genetic modification of phages to further support COGEM's advisory role. A literature review on genetic modification in phages was conducted to understand the possibilities and constraints of utilisation of phages as well as the potential risks introduced by genetic modification concerning e.g. a modified host range and virulence. Environmental risks related to phage use are outlined, along with suggested control measures.

This literature study covered various aspects, including the methods and techniques used in the genetic modification of phages, the specific properties or genes targeted in the research, and the potential future applications of GM phages. Furthermore, it explored phage mechanisms in transfer of genetic material.

2 Methods

2.1 Literature study

A literature review was undertaken compiling scientific information related to the development and use of genetically modified bacteriophages, as well as their potential risks.

In order to formulate **search strings**, the following keywords were selected:

- Bacteriophage
- Genetic* mod*
- Biosafe*
- Risk
- Adverse effect
- Safe*
- Environment*
- Biotech*
- Food*
- Medic*
- Therap*
- Agricult*
- Host tropism or range
- Detect, assay or test*
- Recombin*

After selecting keywords, search strings were composed using Boolean operators:

1) Bacteriophage AND (“Genetic* Mod*” OR “Genetic* Engine*”)
2) Bacteriophage AND (Biosafe* OR Environment* Risk OR “Adverse effect” OR Safe*)
3) Bacteriophage AND (“Genetic* Mod*” OR “Genetic* Engine*”) AND (Biosafe* OR Environment* Risk OR “Adverse effect” OR Safe*).
4) Bacteriophage AND (“Genetic* Mod*” OR “Genetic* Engine*”) AND (Biotech* OR Food OR Medic* OR Therap* OR Agricult*) AND Recombin*

Two electronic bibliographic multi-disciplinary databases were chosen to search for relevant publications: Web of Science™ core collection[1], and Scopus®[2]. Web of Science™ core collection consists of six online databases indexing scholarly books, peer reviewed journals, original research articles, reviews, editorials, chronologies, abstracts, as well as other items. Disciplines included in this index are agriculture, biological sciences, engineering, medical and life sciences, physical and chemical sciences, and many others. The database contains 1,4 billion cited references going back to 1900. Scopus® by Elsevier is an abstract and citation database of peer-reviewed literature, including scientific journals, books and conference proceedings, covering research topics across all scientific and technical disciplines, ranging from medicine and social sciences to arts and humanities. Scopus® is updated daily and includes over 71 million records and over 1,4 billion cited references after 1970.

The search was expected to result in the identification of publications in English or, if in another language, having a title, abstract or keywords in English. Initially, only original articles were searched for. In case the number of articles on a specific topic was large, relevant review articles were consulted.

The search was conducted on October 13, 2023. The search was restricted to the period 2013-2023 and resulted in the following numbers of publications:

[1] https://clarivate.com/products/web-of-science/databases/
[2] https://www.scopus.com

Search string	Web of Science	Scopus	Total after deduplication
Search string 1	343	1064	1129
Search string 2	1184	795	1683
Search string 3	42	26	62
Search string 4	31	120	132

In the first stage of **selection**, the title, keywords and abstract of the retrieved references were screened. This resulted in an important reduction of the number of potentially relevant publications. Of the selected references an attempt was made to retrieve a full text document, after which the full content was examined. The references of the included studies were manually screened to search for further papers. No language or publication restrictions were applied, and studies were not selected based on quality.

An **extraction** was performed of high-level data and the final set of publications was selected. The key findings of the selected, full text papers were then summarized including, but not limited to, information on the bacteriophages used for genetic modification, construction of de bacteriophages themselves (e.g. deletions, mutations, substitutions), application, and potential risks.

Additional publications were retrieved until March 2024, and were based on reference lists of publications identified in the primary literature study, on reference lists in COGEM and other advices, and on internet searches using terms relevant for the current study, often leading to publications dating back from 2013 or earlier.

2.2 Patent screening

In addition to literature searches and in order to have an idea of the current applications of bacteriophages that are in the pipeline, a patent search was conducted with “Espacenet[3]”, the largest single source of technical information in the world. The database offers free access to information about inventions and technical developments from 1782 to today, containing data on more than 140 million patent documents from around the world.

The search was conducted online on November 20, 2023. To get a broad coverage, the search string was set as “ntxt” all "bacteriopha*" AND (ntxt = "recomb*" OR ntxt = "modifi*").

Setting “ntxt” directs the search to take into consideration title, abstract and names. Using * as wild card, ensures that different endings of a word will be selected (e.g. recomb* will include recombinant, recombination, recombine, recombined…). The Boolean operators ensure that only specific sets are retrieved, even if they are not in a specific order.

The search provided several patent families in different systems. A patent family is a collection of patent applications covering the same or similar technical content.

In total 723 patent families were identified based on the search string. For further analysis, only those indicated at the European Patent Office were screened (see Supporting Information in Annex 1).

2.3 Precedents in risk assessment

Further insight in risk assessments underlying activities with genetically modified bacteriophages was obtained through an analysis of advices and recommendations related to (bio)safety issued by COGEM. Additionally, advices and recommendations were searched using the term ‘genetically modified (bacterio)phage’ on the websites of (inter)national organisations related to (bio)safety, including but not limited to the European Biosafety Association (EBSA), American Biological Safety Association (ABSA), National Institutes of Health (NIH), European Food Safety Authority (EFSA) and World Health Organisation (WHO).

[3] https://worldwide.espacenet.com/

2.4 Expert consultations

The study was further supported by scientific guidance and critical review by Prof. Dr. Isabelle Huys of the Clinical Pharmacology and Pharmacotherapy department of the Katholieke Universiteit (KU) Leuven, as well as Dr. Pieter-Jan Ceyssens, head of the unit Antibiotics and Resistance at Sciensano.

In addition, the Advisory Committee provided useful suggestions on studies and publications.

3 A general introduction to wild type bacteriophages

3.1 Introduction

Bacteriophages, also known as phages, are viruses that infect and replicate in bacterial cells, including cyanobacteria (cyanophages) (Martin and Kokjohn, 1999; Naureen *et al.*, 2020) and archaea (Clokie *et al.*, 2011; Naureen *et al.*, 2020). They are ubiquitous in the environment and are recognized as the most abundant biological agent on earth, approx. 10 times more abundant than bacteria, with a total amount of about 10^{30} - 10^{32} phage particles on earth (Comeau *et al.*, 2008; Moineau, 2013; Naureen *et al.*, 2020; Verheust *et al.*, 2010). They are extremely diverse in size, morphology, and genomic organisation. They are dynamic entities that interact with their bacterial hosts and their environment.

3.2 Structure of bacteriophages

Like other viruses, phages consist of a core of genetic material, surrounded by a protein capsid. Some phages also contain a lipid envelop. The genetic material may either be DNA or RNA and may be double-stranded or single-stranded. The shape of phages is closely related to their genome, i.e. a large genome indicates a large capsid and therefore a more complex organisation. There are three basic structural forms of phages: an icosahedral head with tail, an icosahedral head without a tail and a filamentous form. Furthermore, phages are classified according to their type of genome and host organism (Figure 1) (White and Orlova, 2019). Below, two typical phage structures are briefly discussed.

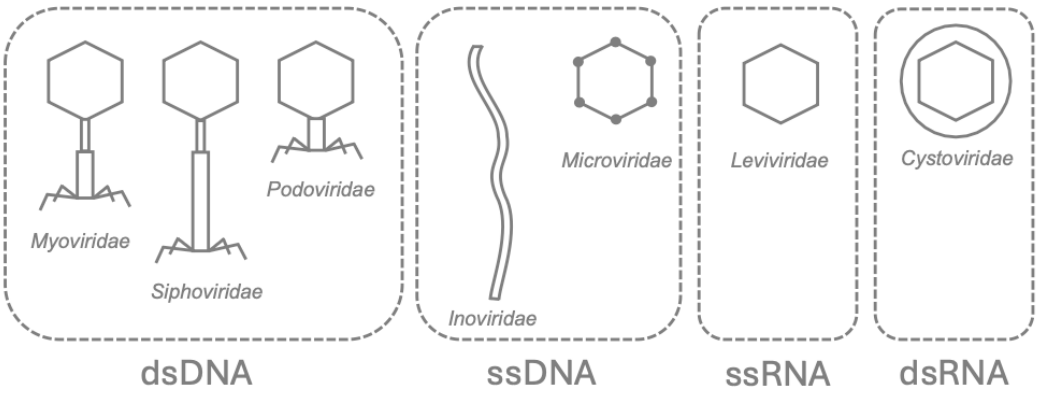

Figure 1. Families of bacteriophages according to their most common nucleic acid composition and shape (adapted from Dias *et al.*, 2013)

The most studied and largest group of phages are the **tailed phages** (White and Orlova, 2019). They consist of 3 parts: the head (capsid) with the packed genome, the tail that allows the transfer of the genome during infection, and an absorption apparatus or adhesive device that fixes to the host cell and penetrates it to allow infection. All contain double-stranded DNA (dsDNA) that ranges from 18 to 500 kb (Casjens, 2005). Phages with large genome sizes are referred to as giant phages. Examples are T4 phages or coliphages with a 169 kb genome, jumbo phages mostly belong to the tailed *Caudovirales* with genomes >200 kb (jumbo phage), and megaphages with genome sizes of about 500 kb (megaphage) (Iyer *et al.*, 2021). Their virion structure is more complex compared with smaller phages, including different virion sizes and specific substructures on their capsids, and tails. Due to their large genome size, they contain many genes (e.g. additional genes responsible for genome replication and nucleotide metabolism, paralogous genes for DNA polymerase and RNA

polymerase and even protein-modifying enzymes that might help hijacking the host-machinery) that do not exist in small genome phages (Iyer *et al.*, 2021; Yuan and Gao, 2017).

Tailed phages harbour numerous open reading frames in their genome. The genome includes information for the assembly of the capsid proteins, additional proteins that are responsible for switching cell molecular metabolism in favour of the phages, and information on the self-assembly process. For giant phages, the proteome size of up to 700 proteins and more is strongly positively and linearly correlated with genome size (Iyer *et al.*, 2021). Several genes allow these phages to be independent of host molecular machinery enabling them to have wide host options (Nazir *et al.*, 2021). Examples are multi-subunit DNA-dependent RNA polymerases, tubulin homologues, which helps form a nucleus-like compartment in the host that has been shown to protect the phage against host immune mechanisms, biosynthesis of NAD+, biosynthesis of capsular lipopolysaccharide, etc. (Iyer *et al.*, 2021).

During evolution extensive gene exchange has taken place among tailed phages leading to mosaicism of the genomes. However, not all genes in a given genome participate in mosaicism to the same degree (Hatfull and Hendrix, 2011). Groups of genes stay together like tail genes, head genes or lysis genes (core genes). A typical arrangement of genes in the genome may be groups of genes for the head, tail, integration, recombination, DNA replication and lysis. But the organisation, gene order and transcription pattern, of these groups may differ between phage types.

The capsids of the tailed phages often have fivefold or icosahedral symmetries. In many phages auxiliary or decoration proteins are attached to the capsid to enhance the capsid stability. The head-to-tail interface is a multi-protein complex, with dodecameric portal proteins within the capsid. The form of the tail may differ between families: *Siphoviridae* have long flexible tails, and *Podoviridae* have very short tails that mostly consist of the adhesive device. The *Myoviridae* have rigid long contractile tails. They are formed by several different proteins forming the inner rigid tube and the outer contractile sheath. Tail proteins form circular oligomeric rings with three- or sixfold rotational symmetry. Jumbo phages show a range of head morphologies which include regular icosahedra, icosahedra with elongated central triangles and icosadeltahedra. Both head and tail may have an array of morphological features, like whiskers and hair-like fibres.

Another typical example are the **filamentous phages**, which are part of the *Inoviridae* family, range in length from 800 nm to 4 µm (Hay and Lithgow, 2019). They are among the simplest viruses known. Examples are *E. coli* Ff and the *Pseudomonas* Pf1 phages. Both are ~6 nm in diameter, with the Ff phage virion approximately 1 µm long and the Pf1 virion approximately 2 µm long. The genome of a typical filamentous phage is a single-stranded, circular DNA. It consists of eleven genes. Genes VIII, III, VI, VII, and IX code for phage structural proteins. Genes II and V are needed for DNA synthesis and genes I and XI code for proteins that aid in phage assembly and extrusion (Straus and Bo, 2018). Protein pVIII forms the body of the phage, and its copy number is dependent on the length of the genome. Proteins pVII and pIX cap the leading (emergent) terminus of the nascent virion, while pIII and pVI cap the terminal end (Hay and Lithgow, 2019). The filament is comprised primarily of several thousand copies of identical major coat protein subunits (Straus and Bo, 2018). The ends of the virion are capped with minor coat proteins.

3.3 The interaction between phages and bacteria

3.3.1 Host tropism

Bacteriophages are bacterium-specific viruses that infect and destroy bacterial cells (Bordenstein and Bordenstein, 2016). Moreover, even within a bacterial species, bacteriophages may be highly specific and usually only infect a single strain within a species (Kowalska *et al.*, 2020a; Verheust *et al.*, 2010). The host range of a bacteriophage is the taxonomic diversity of hosts it can successfully infect (de Jonge *et al.*, 2019). Many model phages that have been studied in the past seem to exhibit a narrow host range (de Jonge *et al.*, 2019). However, some phages that are able to infect a large range of bacterial species have also been identified such as the well-known broad-host-range phages P1, PRD1, and Mu (Jensen *et al.*, 1998; Verheust *et al.*, 2010). Recent studies indicate that broad-host-range phages are potentially widely distributed in natural environments.

Host recognition specificity is conferred mainly by receptor binding proteins (RBPs) of the phage. RBPs are found in the phage's tail. RBPs recognize specific cell wall proteins or polysaccharides,

the host cell surface receptor. So-called monovalent phages bind to a single receptor (de Jonge *et al.*, 2019). They are more likely to have a narrow host range. Polyvalent phages can bind multiple different receptors and therefore may be able to infect a diversity of hosts. It is also possible that the phage species has a broad host range, but that individual particles have the capability to infect only a single host, referred to as host switching. The latter may be due to individual phage genotypes. Host specificity can be changed through mutations in the RBP gene. It is also possible that the genome in some phages codes for multiple RBPs, but only one RBP is expressed at any given time (monovalent phage). Polyvalent phages, on the contrary, may simultaneously express multiple RBPs. Schwarzer and colleagues (2012) studied Enterobacteria phage phi92 where five RBP genes for infection of several different *E. coli* and *Salmonella* strains are present. Phage Mu is one of the phages that has developed efficient strategies to broaden its host range by genetic adaptations. A recombinase that inverts the orientation of the receptor genes allows the synthesis of new receptors with new specificity (Kamp and Kahmann, 1981). *Bordetella* spp. bacteriophages have also adapted such a mechanism to broaden their host range (Doulatov *et al.*, 2004).

Apart from the RBPs, also other aspects may define the host range. For example, temperate phages (see further in section 3.3.2.3) need an integrase-mediated integration of their genome into that of their hosts (de Jonge *et al.*, 2019). Distinctive target sequences serve for the job and are therefore host specific. Targeting conserved integration sites in for example tRNA genes, may grant a broad host range. Instead of the phage genome integrating into the bacterial chromosome, it may form a plasmid and remain present as such.

Host specificity is also determined by the ability of the phages to leave their bacterial host. Indeed, for leaving the host after lytic replication, the endolysin which lyses the cells, and a holin which allows the endolysin access to the periplasm, are needed (de Jonge *et al.*, 2019). A compatible lysis system to efficiently exit is therefore needed. If such compatible system is not present in the host cell, the phage cannot leave the cell resulting in an inefficient replication cycle.

3.3.2 Life cycle of phages in bacteria

3.3.2.1 General description of the phage replication cycle

In general, the bacteriophage infection process starts with adsorption to the bacterial cell (Bertozzi Silva *et al.*, 2016). Phage adsorption to the bacterium is the interaction that dictates host range specificity (see also section 3.3.1). Adsorption usually consists of 3 steps: initial contact, reversible binding, and irreversible attachment. Phages, like other viruses, only passively find and contact their host by dispersion, diffusion, or flow. They lack the ability to actively locate host cells. Adsorption then involves either constituents of the bacterial cell wall (proteins, sugars) or protruding structures, like pili, flagella, and polysaccharide capsules. Sometimes tail fibers assist in the adhesion.

Binding occurs to receptors located in the walls of both Gram-positive and Gram-negative bacteria. Reversible binding allows the phage to search for the correct receptor. More than one receptor may be involved in the adsorption process (Bertozzi Silva *et al.*, 2016). Irreversible binding is sometimes mediated by an enzymatic cleavage that triggers conformational rearrangements in other phage molecules resulting in the transfer of the genetic material into to bacterium. In some cases, it may be that the phage proteins and host receptors involved in reversible adsorption are not the same as those involved in irreversible binding. Reversible binding may occur to moieties, which are more exposed and easier to access. It allows for more stability and increases the probability to find a cell receptor for irreversible binding.

The replication cycle will then follow different possible scenarios depending on the type of phage: the lytic phage infection, the non-lytic phage infection, and the lysogenic phage infection, as explained below. RNA phages (Leviviridae, Cystoviridae) have alternative, more complex, mechanisms to replicate their genomes but these will not be further discussed here and reference is made to Poranen *et al.* (2017) and Tars (2020).

As a final step in the replication cycle, there will be an assembly of the phage particle. The assembly of the bacteriophage starts with the coming together of the capsid proteins and the scaffold proteins (White and Orlova, 2019). The scaffold proteins assist in forming the shape of the capsid. The capsid/scaffold complex binds the portal protein to form the procapsid. Once formed, the scaffold proteins are ejected. The procapsid is then filled with bacteriophage DNA. The procapsid expands to its mature size. The stopper and the adaptor proteins complete the formation by binding to the

portal protein to prevent DNA leakage. The tail is preassembled separately and attached in *Myoviridae* and *Siphoviridae* or assembles at the stopper in the *Siphoviridae*.

3.3.2.2 *The life cycle of lytic phages*

During a lytic replication cycle (see Figure 2), a phage attaches to a susceptible host bacterium and introduces its genome into the bacterial cytoplasm. The host cell replication system may then be used by the bacteriophage to multiply. The viral DNA exists as a separate free-floating molecule within the bacterial cell and replicates separately from the host bacterial DNA. The ribosomes of the bacterial host are used to produce the phage's proteins. Replication will continue until phage-encoded proteins are activated. Newly generated viral genomes and proteins then assemble into multiple copies of the original phage. New bacteriophages are released upon lysis of the host cell due to activity of endolysin enzyme from the phages. They can then restart the cycle. A phage using the lytic replication cycle is referred to as a virulent phage.

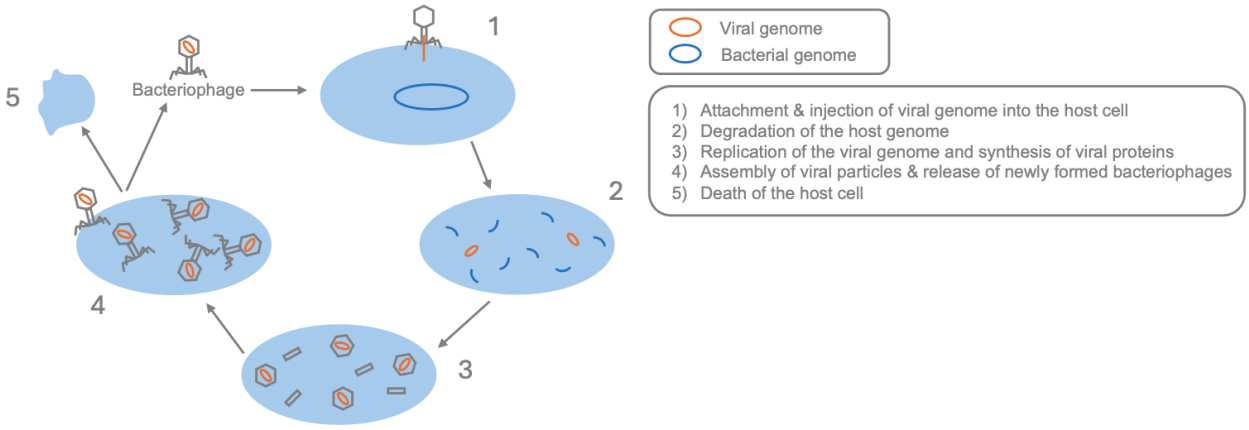

Figure 2. Stages of the bacteriophage lytic cycle (adapted from Technology Networks)

A single lytic cycle can produce a dozen to hundreds of phage progeny (Zhang *et al.*, 2022). Lytic phages direct host metabolism towards nucleotide biosynthesis by upregulating host-encoded nucleotide metabolism genes upon infection. Also, there is synthesis of organosulfur compounds such as sulfur-rich amino acids which are degraded for energy and increase phage production (Kieft *et al.*, 2021). Another potential effect of the lytic phage infection is the starvation response in the host to drive carbon through non-glycolytic pathways and promote dNTP biosynthesis.

Lytic infection impacts the microbial community by lysing its members.

3.3.2.3 *The life cycle of lysogenic phages*

In the lysogenic replication cycle (see Figure 3), the phage also attaches to a susceptible host bacterium and introduces its genome into the host cell cytoplasm. However, upon introduction, the phage genome is integrated into the circular bacterial chromosome (this is called a prophage) or more rarely, exists as an extrachromosomal plasmid within the bacterial cell. In both cases, the phage genome is replicated and passed on to daughter bacterial cells without killing them. Bacteria containing an un-induced prophage are termed lysogens. Prophages can convert back to a lytic replication cycle and kill their host, most often in response to changing environmental conditions (see also section 3.3.2.4). A phage using the lysogenic replication cycle is referred to as a temperate phage.

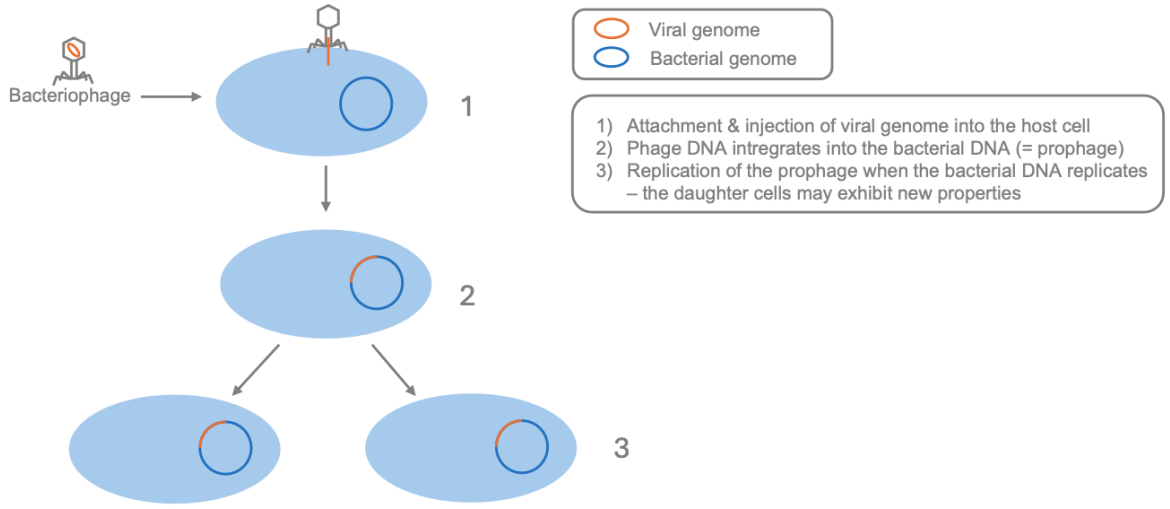

Figure 3. Stages of the bacteriophage lysogenic cycle (adapted from Technology Networks)

Bacteria infected by a lysogenic phage are often protected from the superinfection of a phage of the same genotype or homologs (Zhang *et al.*, 2022). This immunity provides survival benefits to the hosts. Morphological changes in the bacterial membrane block superinfection by blocking adsorption and preventing the injection of DNA. Different phages can still infect the host (poly-lysogeny), but this will often lead to lysis of the host.

Lysogeny allows the prophage and host to coexist. The temperate phages can modify the host genome through integration and excision, a phenomenon known as active lysogeny. Integrated genomes of lysogenic phages or prophages may provide benefits to the host for survival in adverse environmental conditions, such as under oxidative, acidic, osmotic, antibiotic stresses and as reaction upon environmental conditions, for example, by formation of biofilms (Liu *et al.*, 2015). Prophage genes are among the most highly upregulated genes during biofilm development e.g. in *E. coli*. Imprecise excision may allow genes from bacteria to transfer to other bacteria and therefore influence bacterial evolution. Indeed, prophage induction potentially facilitates horizontal gene transfer (HGT) among bacteria and phages (see section 3.3.3).

3.3.2.4 Transition between the lysogenic and the lytic life cycle

Lysogenic phages can convert to a lytic cycle. Such conversion frequently occurs as a result of environmental stress (e.g. ultraviolet (UV) light, low nutrition, pH) (Zhang et al., 2022). For example, phosphorus is an important element as phages require this for the replication of their genome. Phosphorus deficiency would therefore hinder the lytic replication phase. Environmental availability of, for example, organic carbon and inorganic nitrogen can indirectly affect the lytic production of phages by controlling the host metabolism. As such, low availability of carbon and nitrogen will lead to lysogeny. Also, salinity and aeration are important factors that affect bacterial growth and therefore the lysogeny/lytic status. For salinity, the impact is not fully clear. Some reports mention that high salinity results in a lytic life cycle. However, other studies report the opposite. In anaerobic conditions the appearance of lysogeny is high. UV has varying effects on prophage induction, depending on the environment. Regarding temperature, studies have found that phages predominantly underwent a lytic cycle at a higher temperature. However, seasonal variation in the phage life cycle may be influenced by more factors than just temperature. Several studies indicate that copper induces prophages to enter the lytic cycle, whereas no effect has been observed with zinc. Environmental pollutants such as aliphatic hydrocarbons, chlorinated aromatic compounds and others, promoted the induction of prophages.

For several bacteriophages (e.g. phages of *Bacillus subtilis* SBβ family such as phiT3), it has been described that they encode highly specific signalizing peptides, the so-called 'arbitrium communication system'. This system plays a role in promoting either a lytic or a lysogenic life cycle and in deciding the bacterial host cell's fate (Figure 4).

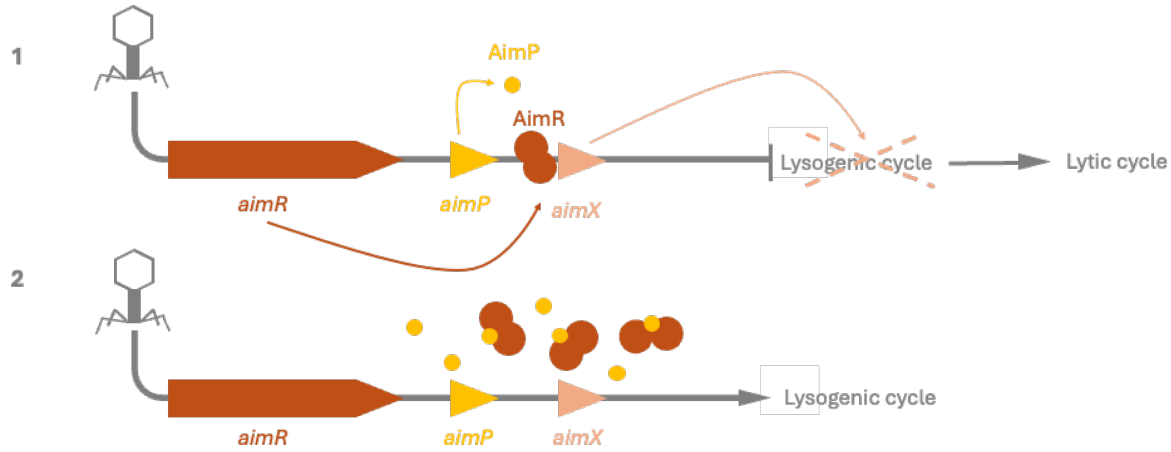

Figure 4. Mechanism of the arbitrium system in phages of the SBβ family (adapted from Brady *et al.*, 2021)

After phage infection, AimR is being expressed and binds to the operator site promoting expression of the *aimX* sRNA thereby promoting the lytic cycle. After AimP accumulates above the threshold levels, it binds to AimR thereby disrupting its binding to the operator site promoting expression of the aimX and reducing expression of aimX, leading to lysogeny.

The arbitrium system is composed of three genes: *aimP*, which encodes the arbitrium peptide AimP, *aimR,* encoding a transcription factor that recognises and binds to AimP, and *aimX*, which exerts a negative regulatory effect on lysogeny, thus promoting lysis. In brief, in the initial stages of infection, when the number of active phages is low, the presence of the arbitrium peptide AimP in the bacterial cell is low; AimR activates *aimX* expression, which maintains the lytic cycle of the phage (Figure 4.1). During phage replication, AimP is released from the bacterial cell into the surrounding medium. AimP is then internalized by the host bacteria, increasing the intracellular concentration of mature AimP peptide until it reaches the threshold level required to bind to its cognate AimR receptor. Due to the binding of AimP, AimR can no longer activate *aimX* transcription. A switch to the lysogenic cycle occurs (Figure 4.2) in order to prevent the killing of the entire bacterial population (Erez *et al.*, 2017; Stokar-Avihail *et al.*, 2019) (Gallego del Sol *et al.*, 2022)

Different phages encode homologues of this communication system with different peptides which suggests that phages use a sequence-specific molecular language.

3.3.2.5 The non-lytic phage life cycle

Non-lytic phages replicate in bacteria by permanent slow release of progeny by extrusion through the cell membrane without lysis of the host bacterium (or budding). Examples include filamentous phages such as the single-stranded (ss) DNA *Inoviridae* Enterobacteria phage, and the M13 *Pseudomonas* phage Pf3 (Drulis-Kawa *et al.*, 2015; Mäntynen *et al.*, 2021). Depending on the phage, the genome may either integrate into the host genome or remain in the cytoplasm. The physical dimensions of the filamentous virion force the particle assembly to take place at the cell membrane where the mature particles are secreted through the membrane. Yet, differences in the assembly mechanism and non-lytic progeny release may exist (Mäntynen *et al.*, 2021).

In addition to filamentous phages, *Mycoplasma*-infecting dsDNA plasmaviruses (pleomorphic virus morphotype resembling membrane vesicles) are released through the cell membrane without lysing the cell in a budding-like process (i.e. phage L2 infecting *Acholeplasma laidlawii*). Similarly, non-lethal chronic infection has been observed in archaeal pleomorphic viruses, which are released without cell lysis. However, the detailed mechanisms of these systems are lacking (Mäntynen *et al.*, 2021).

Non-lytic phages have gained interest in the field of biotechnology for several reasons. For example, the long, thin filamentous phages have proven valuable due to their small genome size and unique chronic life cycle (Mäntynen *et al.*, 2021). However, some applications are limited by the high length-to-diameter ratio of filamentous Ff phages (Sattar *et al.*, 2015). Also, non-lytic phage infection does not have the undesirable consequences of a lytic phage infection, i.e. rapid release of toxins during lysis and subsequent inflammatory response (Drulis-Kawa *et al.*, 2015).

Non-lytic infection has also been described in a so-called pseudo-lysogenic phage infection. In the pseudo-lysogenic cycle, the phage nucleic acid neither forms a stable long-term connection

(lysogeny), nor induces lytic response after infecting the host. Instead, the phage nucleic acid stays inside the host cell in an inactive form (Colavecchio *et al.*, 2017; Hyman and Abedon, 2010). The phage develops this type of interaction in order to avoid starvation and elimination from the environment when the nutrients are limited, and bacterial hosts are lacking.

Under unfavourable growth conditions, some phages can adopt a pseudolysogeny state where their genome does not degrade but instead exists as a plasmid within the cytoplasm and during bacterial cell division becomes incorporated into only one daughter cell (Colavecchio *et al.*, 2017).

3.3.3 Transfer of genetic information

In their interaction with bacteria, bacteriophages may play an important role in the transfer of genetic information from one bacterium to another. This process is called transduction. After infection of a bacterial host, phages assemble and occasionally remove a portion of the host's bacterial DNA. Later, when one of these phages infects a new host cell, this piece of bacterial DNA may be incorporated into the genome of the new bacterial host, thereby altering its genome and that of the bacterial progeny. The amount of host DNA that is picked-up by a phage will depend on the space in the phage head (Campbell, 2003).

Several types of transduction have been described. In the case of **"generalised" transduction**, phages can pick up random pieces of the bacterial genome. Generalised transduction has been described for lytic as well as lysogenic phages. The frequency of generalised transduction by lytic bacteriophages is roughly estimated to occur in approximately 1 out of every 10^5 to 10^7 phage offspring, or 10^{-5} to 10^{-7} phages per plaque-forming unit. In lysogenic phages, frequencies are lower, typically ranging from 10^{-6} to 10^{-8}-10^{-9} phages per plaque-forming unit (Birge, 2006; Chen *et al.*, 2018; Goh, 2016; see e.g. Schneider, 2021).

In lysogenic phages, also **"specialised" transduction** can occur. Specialized transduction poses a higher risk compared to generalised transduction because it involves the transfer of specific bacterial genes located near the integration site of the phage genome. This can lead to the transfer of virulence factors or antibiotic resistance genes between bacteria, potentially contributing to e.g. the spread of antibiotic resistance. In contrast, generalised transduction involves the transfer of random bacterial DNA, which is less likely to include harmful genes. Not all lysogenic phages display specialised transduction. Indeed, lysogenic phages without a fixed insertion site can potentially carry any part of the genome.

Transduction, and in particular specialised transduction, allows bacteria to obtain new genetic traits and transforms non-pathogenic bacteria into pathogenic ones by transferring genes coding for antibiotic resistance and bacterial toxin, or genes affecting bacterial colonization, adhesion, invasion and transmission, resistance to serum and phagocytes (reviewed by e.g. Wagner and Waldor (2002) and Verheust *et al.* (2010)). Clear examples include the passage of the *Vibrio cholerae* toxin gene ctxABT by its lysogenic bacteriophage CTXΦ between *V. cholerae* strains lacking the toxin gene, thus converting them into highly virulent strains (Fan and Kan, 2015) and the use of filamentous Pf phages by multi-drug resistant *Pseudomonas aeruginosa* to evade the immune system and bacterial clearance resulting in more severe and longer lasting infections (Sweere *et al.*, 2019). Consequently, it can contribute to increasing the virulence of bacteria (Fortier and Sekulovic, 2013).

"Lateral" transduction occurs when the integrated prophage starts replicating DNA before prophage excision, thereby resulting in the replication of large segments of adjacent bacterial DNA (Chen *et al.*, 2018). These long segments are then packaged into virions and can be horizontally transferred to other bacteria, thereby causing the transfer of genetic material at frequencies 1000 times greater than those seen with generalised or specialised transduction (Chiang *et al.*, 2019).

Interestingly, a specific group of bacteriophages, namely those with a so-called non-redundant genome cannot perform transduction. They do not contain repetitive sequences or duplicate genes but have fixed start and end points on the genome, which are recognized by the genome packaging machinery (e.g. DNA terminases). Only unit lengths of the phage DNA are packaged into the new virions, without bacterial DNA. A typical example are phages with 'direct terminal repeats' at the ends, such as coliphage T7 (Jensen *et al.* (2020); pers. comm. Ceyssens PJ). Also, phage T4 cannot perform transduction unless several mutations in a specific region of the T4 genome occur which then allow a very low level of transduction (Wilson *et al.*, 1979; Young *et al.*, 1982).

3.4 Coevolutionary dynamics of phage-bacteria interaction

In nature, bacteria and phages are in a continuous coevolutionary process that drives the ecological and evolutionary processes in microbial communities. This process involves bacteria to develop phage resistance through bacteria defense systems under phage selection pressure, and conversely, phages developing counter adaptations against bacterial antiphage mechanisms. This chapter provides an overview of the bacteriophage defense mechanisms and the response of phages.

3.4.1 Bacteriophage resistance

Bacteriophage or phage resistance implies that bacteria become resistant to infection by or multiplication of a bacteriophage. Resistance can occur at almost every stage of the phage infection cycle and there is a wide variety of mechanisms by which bacteria can limit phage binding, infection, replication and assembly (Figure 5).

Below, several common resistance mechanisms are presented. A more extensive overview of phage resistance mechanisms is provided by review articles such as those of Samson *et al.* (2013), Kowalska *et al.* (2020a), Egido *et al.* (2022) and Ali *et al.* (2023).

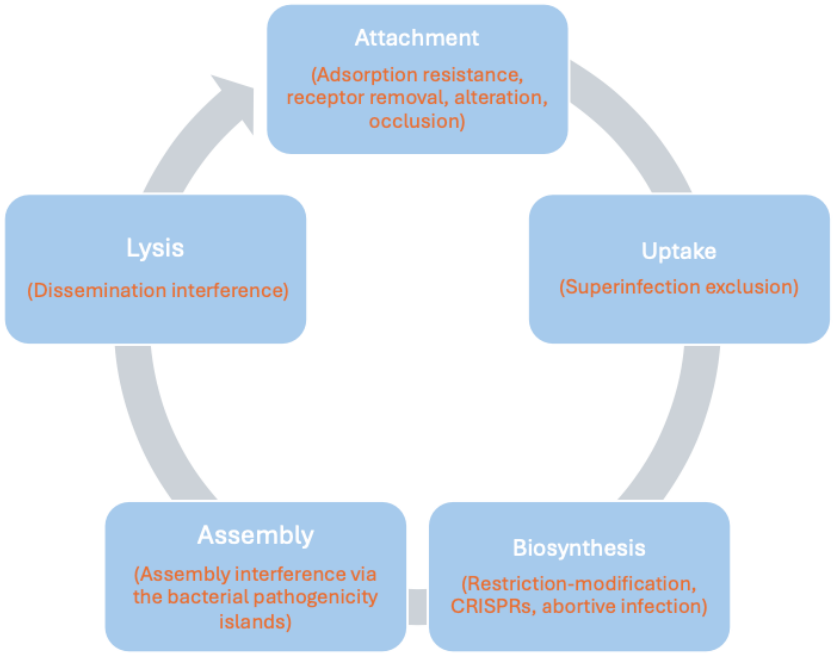

Figure 5. Stages of phages infection and corresponding examples of resistance mechanisms at each stage (from Moller *et al.*, 2019)

3.4.1.1 Receptor adaptations

One of the primary strategies used by bacteria to achieve phage resistance is receptor alteration. As a result of random mutations or phenotypical variations in bacteria that result in receptor adaptations, phage adsorption will be reduced. Mutations in the genes that encode receptor binding proteins and other proteins involved in host recognition have been noted to occur quite frequently (Guo *et al.*, 2014).

Additionally, bacteria may express certain receptors only under specific circumstances, referred to as stochastic expression (e.g. during a specific growth phase), thereby limiting the timeframe in which phages can adsorb.

3.4.1.2 DNA injection blocking

Bacteria can prevent phage DNA injection, even when the phage has successfully attached to the surface receptors. This is particularly relevant for bacteria that contain prophages. Hereby, the prophages prevent DNA injection by a mechanism called superinfection exclusion (Sie) system (Kalatzis *et al.*, 2018). It is important to mention that phages themselves may also benefit from superinfection exclusion, as a superinfecting phage will compete with the prophage for the available resources in the bacterial cell.

3.4.1.3 Cleavage of the invading phage genome

Even when a phage genome manages to enter the cell, there is still a multitude of intracellular antiviral barriers to overcome. These barriers are summarized under the term restriction-modification (R-M) systems and include a variety of mechanisms (Murray, 2002; Roberts *et al.*, 2003; Tock and Dryden, 2005). Frequently, a methyltransferase (MTase) modifies a particular nucleic acid sequence inside a cell to protect the host DNA. Any foreign DNA that has not already been protected by the same alteration and contains a particular recognition site is destroyed by the restriction endonuclease (REase) (Labrie *et al.*, 2010). The more restriction sites a phage genome contains, the more prone it is to this endonuclease restriction (Moineau *et al.*, 1993; Roberts *et al.*, 2005).

Phages with fewer restriction sites in their genome are less prone to DNA cleavage and thus have a selective advantage (Bickle and Krüger, 1993; Krüger and Bickle, 1983; Tock and Dryden, 2005). In addition, DNA topology, including the orientation of restriction recognition sites and the distance between them, can influence R-M targeting. Indeed, some restriction enzymes have to recognize two sequences in a specific orientation in order to be able to cleave the DNA.

Another way how bacteria can cleave the phage genome is via the ‘clustered regularly interspaced short palindromic repeats’ (CRISPR)-Cas immune system (Sorek *et al.*, 2008). Hereby, the bacteria incorporate phage genetic material into CRISPRs as spacers, i.e. short stretches of non-repetitive DNA. Upon recognition of specific spacers, the bacterial CRISPR-Cas system cleaves the DNA in a sequence specific manner. CRISPR–Cas systems are present in approximately 40% of sequenced bacterial genomes and display considerable diversity with regards to their composition and mode of action (Makarova *et al.*, 2020).

3.4.1.4 Induction of bacterial cell suicide

Bacterial suicide can be initiated by so-called abortive infection (Abi) systems and is also a frequently employed phage resistance technique. Abortive infection (Abi) systems inhibit various steps of the phage replication cycle, but in contrast to the mechanisms described above, they also induce death of the bacterial cell before the phage can complete its replication cycle. Such suicide allows the remaining non-infected bacterial population to escape phage infection. Abi systems typically consist of a single protein or protein complex, the latter for example being a toxin-antitoxin (TA) system. A TA system comprises a pair of genes, usually transcribed from the same operon; the first one codes for a toxin, causing ’altruistic cell suicide’ in the infected host and the second one codes for a neutralizing antitoxin, rendering the toxin ineffective during normal bacterial growth. However, when the bacterial cell encounters stress, the less stable antitoxin is degraded after which the toxin can induce cell death or dormancy (reviewed by Samson *et al.*, 2013).

Abi systems have been studied predominantly in lactococci (e.g. ABiD1) and have also been identified in some Gram-negative species, such as *Escherichia coli* (*lit* system; lit protease is activated when gp23, the major head protein of phage T4, is expressed in the cell).

3.4.2 Phage defense mechanisms to avoid resistance of bacteria

In response to the above-mentioned resistance mechanisms, phages have co-evolved resulting in specific defence strategies to counteract the phage resistance mechanisms of bacteria (reviewed by e.g. Samson *et al.*, 2013). Below, representative examples of counteracting strategies are described.

3.4.2.1 Adapting to new receptors

Tailed phages can modify their receptor-binding site to acquire novel receptor tropism. Furthermore, phages have been shown to evolve to recognize an altered receptor structure.

When access to bacteria is hindered by a physical block, such as presence of exopolysaccharide (EPS) compounds, phages can gain access to a receptor by hydrolysing the barrier. EPS-degrading enzymes may be obtained via genetic transfer from one phage to another.

To circumvent stochastic expression (i.e. random fluctuations in gene expression levels within a cell population, leading to variability in protein levels which can affect cell behaviour and function), some phages can shuffle their receptor-binding proteins (RBPs) through gene mutation resulting in multiple RBP variants of differing specificity, leading to an increased probability of a phage infecting its host and, potentially, an expansion of host range.

3.4.2.2 Avoiding DNA cleavage

To circumvent DNA cleavage by the wide variety of R–M systems, phages have developed an array of anti-restriction strategies. These include passive and active mechanisms. In case of passive evasion, the phage is protected because of the speed whereby its DNA is modified by host methyltransferase (MTase), i.e. before recognition by the host restriction endonucleases (REase). Although it has been described that some bacterial restriction endonucleases can also recognize and cleave modified DNA. Another passive way how phages can avoid DNA cleavage by some restriction endonucleases is by incorporating modified bases in their genome. Finally, Kowalska *et al.* (2020a) highlight that very often phages do not have recognition sites for endonucleases and thereby are not prone to DNA cleavage.

Active mechanisms include masking phage DNA or structurally mimicking host DNA by specific phage proteins that are co-injected into the bacterial host cell. By doing so, the proteins interfere with the activity of the R-M system. Additionally, phages can encode modification genes. These modification genes protect the phage genome against the activity of the bacterial restriction endonucleases, for example through methylation (Krüger and Bickle, 1983; Wilson and Murray, 1991). Finally, phages can actively hamper the DNA modification process induced by the host, thereby accelerating protection of the phage genomes, for example by encoding anti-restriction proteins or proteins that modify the structural conformation of a specific restriction endonuclease (King and Murray, 1995; Zabeau *et al.*, 1980). The latter however has been described to initiate an abortive-infection system for the bacterial cell (Penner *et al.*, 1995).

To circumvent DNA cleavage by the CRISPR-Cas system, phages have also developed a multitude of defense mechanisms (reviewed in Malone *et al.*, 2021; Samson *et al.*, 2013). These include but are not limited to interference through very specific single-nucleotide substitutions in or around the protospacer regions of the phage genome, by deleting protospacer regions from the phage genome, by expressing anti-CRISPR (Acr) genes, by DNA modifications, by formation of a proteinaceous nucleus-like structure for phage replication, and/or by expressing phage-encoded CRISPR-Cas complexes that interfere with the host antiviral defense system.

3.4.2.3 Escape abortive-infection mechanisms

Phages have developed several countermeasures to avoid that the bacterial cells commit suicide via abortive infection systems (see 3.4.1.4). Indeed, some phages have a specific mutation that, upon infection of a bacterial cell, hinders the suicide response (Penner *et al.*, 1995). One example is a mutation in specific phages that hinders depolarization of the bacterial membrane upon infection, and consequently interferes with the bacterial Abi response (Slavcev and Hayes, 2003; Snyder, 1995). Another example is a mutation in T4 gp23 hampering cleavage by the *E. coli* lit protease (reviewed by Lopatina *et al.*, 2020; Samson *et al.*, 2013). Also, by inhibiting the protease that breaks down the antitoxin or by producing their own antitoxin counterpart, phages can avoid the effects of TA pathways (Sberro *et al.*, 2013).

3.4.2.4 Destruction of biofilms

Phages can express specific depolymerase or endolysin enzymes as components of proteins with tail spikes or fibres or release such enzymes in a soluble form after the lysis of infected bacteria. The enzymes act on the biofilm layers produced by some bacteria and help the phages to reach the cell surface of the bacterial cells (Liu *et al.*, 2022; Sutherland *et al.*, 2004).

3.4.3 Implications of phage resistance for successful phage application

Even though phage resistance has been proven to emerge swiftly *in vitro*, research on humans has revealed both the presence (Zhvania *et al.*, 2017) and absence (Khawaldeh *et al.*, 2011) of phage resistance *in vivo.* Therefore, it is unclear whether such resistance could impair the potency of phages in clinical or agricultural applications, or whether uncontrolled application of phages in the environment could reduce efficacy of phages in other settings. In that perspective, it has been recommended to use phage cocktails to broaden the range of activity and reduce the likelihood of developing bacterial resistance. Interestingly, Koskella *et al.* (2012) showed that bacterial host populations evolved specific resistance *in vitro* to phages, regardless of whether they were in single or multiple-phage environments, and that hosts evolving with multiple phages were no more resistant to novel phages than those evolving with a single phage. However, hosts from multiple-phage environments paid a higher cost, in terms of population growth in the absence of the phages, for their evolved specific resistances than those from single-phage environments. The latter has also been highlighted by several other authors (Bohannan and Lenski, 2000; Gómez and Buckling, 2011; Hagens *et al.*, 2004; Hall *et al.*, 2012; Inal, 2003). Additionally, to reduce the likelihood of developing resistance, also the combined use of phages and antibiotics has been recommended (Escobar-Páramo *et al.*, 2012; Zhang and Buckling, 2012).

3.5 Stability of phages outside the bacterial host

The stability of phages outside their host is highly variable and depends on the nature of the phage itself (Verheust *et al.*, 2010). Also, the surrounding environmental conditions, such as pH, ionic strength, temperature, soil structure, adsorption properties, or sunlight are important.

Information on bacteriophage ecology and stability is limited, but detailed information is available for cyanophages. Cyanophages are viruses that infect cyanobacteria or blue-green algae. Harmful algal blooms (HABs) caused by cyanobacteria negatively affect ecosystems and drinking water resources through the production of potent toxins. Cyanophages are suggested to provide a highly specific control strategy of HABs with minimal impacts on non-target species and propagation in the environment (see e.g., Grasso *et al.*, 2022; Jassim and Limoges, 2013; Suttle, 2002).

Grasso *et al.* (2022) provide a detailed review of cyanophage life cycle, diversity, and factors influencing infectivity. Major loss processes for cyanophages (in aquatic environments) are destruction by solar radiation (ultraviolet B or UV-B radiation), attachment to particles, attachment to host cells and consumption by protozoan grazers (Suttle, 2002).

For cyanophages, destruction by solar radiation in near-surface waters is the most significant and can result in the destruction of the entire infectious viral community in a matter of hours. DNA is damaged by formation of pyrimidine dimers. Nevertheless, photo reactivation (repair of damaged DNA by photolyase) can restore infectivity to a significant portion of the damaged viruses. Destruction rates of cyanophages exposed to solar radiation are similar to those measures for marine bacteriophages (Grasso *et al.*, 2022; Suttle, 2002). Iriarte *et al.* (2007) conducted a field study to investigate bacteriophage persistence on tomato leaves. Sunlight UV had a dramatic effect on phage viability. Phage solutions sprayed at 6 a.m. on tomato leaves dropped 6 log units in the following 10 hours and completely disappeared by 4 p.m. The effect of temperature on phage viability is variable. Some phage types survive for 10 days at 15, 28 and 32 °C, whereas exposure to 15 and 32 °C is detrimental for other phage types. Desiccation caused only a slight reduction in phage populations after 60 days, whereas fluorescent light eliminated phages within 2 weeks. Formulated phage (phage mixed with skimmed milk) reduced the deleterious effects of the studied environmental factors.

Several studies found that cyanophage populations increased with a seasonal increase in water temperatures and that their stability tended to be consistent with the stability of cyanobacteria at water temperatures up to 50 °C. More specifically, at temperatures up to 40 °C, 85% of cyanophages remained virulent, while at 45 °C only 55% of cyanophages remained virulent, and at or above 50 °C, less than 0.001% of cyanophages remained virulent (Grasso *et al.*, 2022). Cyanophages can be generally stored for a month or more at 4 °C with little loss of titre. In seawater samples (with particulate material > 0.2 µm removed) cyanophage titres were stable for several months when stored at 4 °C. Titres of more than 10^5 cyanophages per mL were measured in 0.2 µm filtered samples stored a year or more. At 40 °C titres were stable for one hour or more, while other

thermosensitive strains were reported to be unable to form plaques at 35 °C. At temperatures above 50 °C infectivity is rapidly lost (Suttle, 2002). However, it must also be noted that both thermotolerant and thermosensitive cyanophage strains have been identified (Grasso *et al.*, 2022). Yahya *et al.* (1993) evaluated the survival of two bacteriophages which have been shown to adsorb poorly to soils, i.e. the *Escherichia coli* phages MS-2 and PRD-1 which infect *Salmonella* Typhimurium. Samples of ground water from various regions of North America were inoculated with the test phages and incubated at temperatures near the ambient ground water temperature of the collection site. At 7 °C no significant inactivation of the phages occurred over a period of 80 days. At higher temperatures (10 to 23 °C) PRD-1 was far more resistant to inactivation than MS-2, persisting for periods of 7 to 10 times longer in most water samples.

Cyanophage infectivity may also depend on the concentration of cations (Mg^{2+}, Mn^{2+}, Na^{+}) as availability of cations favour phage stability (Mg^{2+} plays a role in maintaining capsid integrity). This is optimal for some phages at 1 mM or above. At concentrations of 0.1 mM infectivity is lost. However, this is not universal; some cyanophages have no cation requirement to maintain infectivity (Suttle *et al*., 2001).

Most contacts between phages and host cells are thought to result in adsorption. Cyanophage studies have shown that after 1 hour incubation with host cells 86-88% of the phages is adsorbed. Adsorption rates can be affected by environmental factors or the physiological status of the host. A cofactor (cation) may be required for adsorption. Light can also affect rates of cyanophage adsorption; adsorption occurs faster in light than in dark (Suttle, 2002).

In cyanophages, phosphorus limitation results in decreased burst size and infectivity and an increase in latent period and a life cycle shift toward the lysogenic pathway. Limited information available for marine cyanophages indicates that a shift to lysogenic life stages also may occur when environmental nitrogen concentrations are limited; limited availability of nitrogen may also reduce adsorption and/or overall abundance (Grasso *et al.*, 2022).

Whitworth *et al.* (2020) studied the stability of enveloped bacteriophage phi 6, a member of the family Cystoviridae. Phi 6 has been used as a surrogate for several pathogenic enveloped viruses (e.g. Ebola virus, influenza virus, SARS-CoV, Venezuelan equine encephalitis virus), for example in studies addressing viral persistence. The authors showed that phi 6 suspended in a body fluid simulant persisted over 76 days on stainless steel and up to 77 days on plastic. In general, Phi 6 persisted better at low absolute humidity (3.0 g/m^3) than at higher absolute humidity (14.4 g/m^3).

Shiga-toxin-encoding phages have been shown to survive and remain infectious in soil for over a month and can survive osmotic changes, chlorination, and heat treatments better than their bacterial hosts (Reviewed in Verheust *et al.*, 2010).

Some phages, such as T1, can survive on laboratory surfaces for years and are capable of forming stable aerosols. They remain infectious and are difficult to eliminate (Birge, 2006). They may not be inactivated by water treatment processes, food purification, and pasteurization. This tolerance to environmental conditions helps phages to survive during periods of low host abundance and may give them time to find another suitable host (Naureen *et al.*, 2020).

Of particular relevance is the impact of the gastrointestinal tract which may significantly impact the success of orally administered phages. For example, the very low pH of the gastric environment of most mammals significantly reduces phage titre and proliferation. Also, body temperature of mammals may impact phage penetration and consequent proliferation, as may gastrointestinal enzymes, such as pepsin in gastric fluid, and amylase, lipase and protease in pancreatic fluid, which result in degradation of phages (Nobrega et al., 2016). On the other hand, high stability of phages after release in the environment in view of a specific agricultural, therapeutic or other application may raise concerns about unwanted persistence (see also section 6.1.5).

3.6 The role of bacteriophages in nature

Bacteriophages are the most abundant biological entities on the planet. Overall, they are approximately 10 times more abundant than bacteria, with a total amount of about 10^{30} - 10^{32} phage particles on earth (Comeau *et al*., 2008; Moineau, 2013; Naureen *et al*., 2020; Verheust *et al*., 2010). Table 1 shows several examples of the presence of phages in specific environments.

It has been shown that phages are not limited to a specific habitat but can move from one environment or biome (soil and seawater) to another by infecting alternative hosts. This is exemplified by the fact that phages isolated from terrestrial environments can also propagate on marine microbes (Sano *et al.*, 2004).

It is generally accepted that bacteriophages have an important role in constant regulation in the environment thereby contributing to the environment's diversity, richness, abundance, evolution, and microbial communities in a particular habitat. Below, representative examples of the role of phages in specific habitats are described. It remains outside the scope of the current study to discuss the role of phages in nature in greater detail.

Table 1. Presence of bacteriophages in natural environments

Environment	Estimated amount of phages	Estimated phage-bacterium ratio	Reference
Ocean waters	± 4×10^{30} (overall)	15 to 1	Batinovic *et al.* (2019)
Dry soil	± 10^9 per gram of dry soil Varies depending on the soil type	- 1 to 10 or 100 (desert and agricultural soil) - 1000 to 1 (Antarctic soil)	Batinovic *et al.* (2019)
Human body	< 10^{12} (overall) Varies depending on the tissue type; highest abundance in the gut; also ubiquitous in/on skin, oral cavity, lungs, intestines, and urinary tract	At least 10 to 1 (1 to 1 in guts of infants)	Batinovic *et al.* (2019), Fernández *et al.* (2021)
Human faeces	± 10^{12} virus-like particles (including phages) per gram	-	Batinovic *et al.* (2019)
Biological wastewater treatment plant	±10^7 -10^9 VLP per ml	-	Runa *et al.* (2021)

3.6.1 Maintaining equilibrium of microbiota

Bacteriophages play an important role in maintaining the equilibrium of microbiota in various habitats. Examples are marine and terrestrial environments and the human gut. For example, phages can contribute to ocean communities by regulating bacterial levels, as it is thought that they lyse 20 - 40% of ocean bacteria every day. They are important players in the energy and nutrient cycles through the lysis of host microbial cells. Lysis of heterotrophic (i.e. an organism that requires organic substrates to get its carbon for growth and development) and autotrophic (i.e. an organism that can produce its own food using light, water, carbon dioxide, or other chemicals) bacteria contributes to nutrient availability in marine ecosystems by what is known as the 'viral shunt' of the 'microbial loop', which is exploited by other heterotrophs (Brown *et al.*, 2022; López-Pérez *et al.*, 2017)).

Terrestrial microbiota are generally more complex and require phages to use different dynamics for success. Generalised soil microbiota are hypothesized to undergo community temporal shift through the 'Seed-Bank' phenomenon. Here, bacteria can survive low water conditions, while other members lie dormant and are only detected after rainfall, and their phages likely cycle accordingly. This spatiotemporal segregation of the community could influence the phage-bacterial interactions where lysogeny protects phages from decay, as observed in hyperarid deserts soils. Higher amounts of lysogeny were also observed in chromium contaminated soils by inferring lysogeny from the increased presence of integrase genes in viromes and the observation that more phages could be induced from contaminated soils (Brown *et al.*, 2022).

In the human body, phages play a significant role in shaping the composition of the bacterial communities within various bodily compartments. Phage composition varies significantly between people, with the relative abundance of certain types of phages between healthy and diseased individuals differing. Gut dysbiosis is characterized by low species diversity and high frequency of extracellular lytic phages, but also an increase in temperate phages. The underlying phage interactions during dysbiosis have been poorly characterized, but low bacterial diversity will drive low phage diversity, suggesting phages will adapt strategies that do not require host abundance, possibly favouring a temperate life cycle (Batinovic *et al.*, 2019; Brown *et al.*, 2022). Application of phages to

treat diseases caused by bacterial dysbiosis shows potential. This latter application would be comparable to the use of probiotics or prebiotics since bacteriophages can indirectly improve the growth of beneficial bacteria in the gastrointestinal tract by removing undesirable competitors (Fernández *et al.*, 2021).

While phage-induced lysis and selection of the bacterial host is one way in which phages influence microbiota, another is their ability to encode auxiliary metabolic genes (AMGs), redirecting and augmenting the hosts' metabolism, providing a benefit for phage-infected bacteria over non-infected bacteria within a given microbial community. These AMGs may supplement rapidly degrading proteins, contribute proteins at rate-limiting steps or induce a state of starvation to increase the hosts' metabolic pathways (Brown *et al.*, 2022). Aquatic environments contain the most extensively studied AMGs to date. *Synechococcus* cyanobacterial host and phage pairs isolated from coastal and estuarine waters contain AMGs involved in photosynthesis (e.g. *psbA, petF, cepT, hli*), carbon metabolism (e.g. *talC, cp12*), phosphate stress conditions (e.g. *pstS, phoA*) and cell metabolism (e.g. *cobS, mazG, phoH*), indicating the ability of phages to enhance the growth of their host (Brown *et al.*, 2022). Analysis of organochloride-contaminated soil revealed AMGs associated with pesticide degradation (e.g. *norD, norQ, cysD, cysH*) in addition to carbon, phosphorus, and nitrogen metabolism. The role of AMGs within the human gut is largely unknown. Recent mining efforts of 44 human gut metagenomes revealed carbohydrate-active enzymes, peptidases, carbon- and nitrogen-utilization AMGs, reflective of site-specific metabolic processes (Brown *et al.*, 2022).

3.6.2 Interaction with the eukaryotic host cell

Although generally considered as prokaryote-specific viruses, bacteriophages can also interact with eukaryotic cells such as immune cells (in view of phagocytosis, immune responses), endothelial cells, mammary epithelial cells and neuroblastoma cells (Chatterjee and Duerkop, 2018; Dabrowska *et al.*, 2005; Podlacha *et al.*, 2021; Van Belleghem *et al.*, 2018). Contemporary assumptions include that human cells well tolerate phages and that phage particles do not directly affect human health (Champagne-Jorgensen *et al.*, 2023). Phages can penetrate epithelial cell layers and spread throughout sterile regions of the human body, including blood, lymph, organs, including brain tissue (Nguyen *et al.*, 2017). It is estimated that 31 billion bacteriophage particles pass the epithelial cells of the gut into the human body every day.

Interaction with epithelial cells has been described to occur in different ways. For example, phages have been described to interact with specific receptors showing similarity to polysaccharides present on bacterial hosts. Nguyen *et al.* (2017) also described that phages can enter and cross epithelial cell layers by a non-specific transcytosis mechanism involving the Golgi apparatus. Curiously, cellular uptake of phage virions was first observed in 'professional phagocytes', such as macrophages or granulocytes. For this reason, it was historically referred to as 'phagocytosis'. The modern definition of phagocytosis, however, identifies this process as a type of endocytosis within a larger repertoire of endocytic pathways, such as macropinocytosis, clathrin-mediated endocytosis, and caveolar endocytosis (Miernikiewicz and Dąbrowska, 2022). It is important to note that during endocytosis intracellular pH changes may occur; as already mentioned in the previous chapter low pH values may affect phage viability (see also chapter 3.5).

The ability of bacteriophages to penetrate and/or translocate in eukaryotic cells provides valuable information on the pharmacokinetic aspects of bacteriophage treatment, which is vital in the context of using bacteriophages as an alternative antimicrobial therapy. It also emphasizes the possibility of using bacteriophages as vectors in drug and gene delivery systems, including therapies targeted towards the brain tissue, gastrointestinal tract, and lung via systemic or local delivery (Putra and Lyrawati, 2020).

However, there is compelling evidence that phages do in fact directly influence mammalian cell activities. Phages appear to share a double-edged sword relationship with their human host.

Phages provoke immune responses via both intracellular and extracellular signals, activating multiple arms of the innate and adaptive immune systems (Champagne-Jorgensen *et al.*, 2023). Interactions between phage and eukaryotic cells can be beneficial for the cell. Several examples have been described in review articles by Chatterjee and Duerkop (2018) as well as Bodner et al. (2021). These include but are not limited to the assistance of phages in killing bacterial pathogens present in the cell, the contribution in a non-host-derived layer of immunity at mucosae, opsonization of bacteria subsequently resulting in increased phagocytosis by e.g. macrophages, minor suppression of (LPS-induced) inflammation, hampering metastasis of cancer cells, and a direct

stimulation of immune cells. For the latter, results should be interpretated carefully, as many experiments have been performed with phage lysates containing remnants of lysed bacteria (e.g., LPS, cytosolic proteins, or membrane particles) or fragments of the host bacterial cell wall adhered to phage tails, which may have induced the response of the immune cells rather than a specific component of the phage itself. However, the uptake of phages into the cell activates conserved viral detection receptors and initial immune responses to phages are generally proinflammatory (cytokine secretion) (Champagne-Jorgensen, et al 2023). Highly purified T4 phages do not activate DNA-mediated inflammatory pathways in mammalian cells but do trigger protein phosphorylation cascades that promote cellular growth and survival (Bichet *et al.*, 2023).

Even though phages can enter eukaryotic cells, infection of eukaryotic cells seems unlikely since elements of the phage tail structure only bind to specific receptors on the surfaces of their target bacteria, and because of major differences between eukaryotes and prokaryotes regarding key intracellular machinery that are essential for translation and replication (Van Belleghem *et al.*, 2018).

3.7 Risk considerations for wild type bacteriophages

Taking their biological properties into account, phages are promising tools for many applications in agriculture, healthcare, and industry (see section 4). However, some of their characteristics may also involve specific risks. This section will briefly focus on the risk considerations that should be considered when applying wildtype bacteriophages. Section 6 focusses on the additional risk considerations for genetically modified phages.

3.7.1 Transfer of undesirable genetic sequences between bacteria

Bacteriophages can transfer genetic information between bacteria by a process called transduction (see section 3.3.3). By doing so, they can change the characteristics of the bacteria they infect. If transferred genetic information consists of harmful sequences, bacteriophages can contribute to the generation of more virulent bacteria. Examples of hazardous characteristics that can be transferred include but are not limited to phage-encoded toxins, and virulence factors affecting bacterial colonization, adhesion, invasion and transmission, resistance to serum and phagocytes, antibiotic resistance. Giant phages may bring along additional genes that influence for example gene expression/regulation of their bacterial host, but also may interfere with bacterial defence mechanisms. For a more detailed overview, the reader is referred to review articles of Wagner and Waldor (2002), Verheust *et al.* (2010) and Iyer *et al.* (2021).

Taken the above into account, it is generally accepted that only strictly lytic phages should be used for phage applications (Carlton *et al.*, 2005). Although lysogenic phages may not be selected for application, they may become activated during propagation of lytic phages in bacterial host cells as most bacteria do harbour several prophages.

Laboratory assays (Waddell *et al.*, 2009) as well as bioinformatic tools (Kazimierczak *et al.*, 2019; Klumpp *et al.*, 2008; Kokkari *et al.*, 2018) have proven useful to determine whether a bacteriophage has a strictly lytic life cycle. Two bioinformatics tools were identified that specifically focus on predicting the phage life cycle, i.e. PHACTS using information based on proteomics (McNair *et al.*, 2012), and PhageAI using information based on nucleotide sequences (Tynecki *et al.*, 2020). Other tools are available that predict phage characteristics, identify conserved regions and/or compare phage relatedness but do not specifically refer to predicting the phage life cycle (non-limitative overview in Table 2). Also more general sequencing tools can be helpful (Reviewed by Ho *et al.*, 2023; reviewed by Schackart *et al.*, 2023). They are highly valuable in determining whether virulence genes are present in the phage. While bioinformatics can reveal whether a virulence gene is associated with phage sequences, it cannot reveal whether the gene is part of a prophage capable of transducing it or capable of influencing its expression. For that, mutational analyses can additionally be performed.

Still, the role that phages play in transduction of antibiotic resistance genes remains controversial since bioinformatic tools may overestimate the presence of antibiotic resistance genes in phages (Enault *et al.*, 2017). Furthermore, *in vivo* studies by Allen *et al.* (2011) suggested that while prophages were induced in the swine gut, this did not result in HGT of antibiotic resistance genes from the prophages to bacteria in the swine gut. On the other hand, in a modelling study by Volkova *et al.* (2014) with *Escherichia coli*, generalised transduction delivered an antimicrobial resistance

gene to eight *E. coli* bacteria per hour in a population of 10^8 *E. coli* bacteria per litre. Generalised transduction is thought to occur 1000 times less frequent than conjugation (see 3.3.3). It remains to be determined what frequency of transduction is safe. In that perspective, it has been questioned during a recent FDA meeting on the science and regulation of bacteriophage therapy whether in order to be safe there could be a limit for transduction in line with frequencies observed in nature or whether it should be zero (FDA, 2021).

Table 2. Non-limitative overview of bioinformatics tools to predict phage sequences and characteristics

Bioinformatics tool	Functionality	Link	Reference
Phamerator	Sort phage genes into phamilies (or phage families) of related sequences using pairwise amino acid sequence comparisons of predicted gene products; identify conserved regions	https://phamerator.org	Cresawn *et al.* (2011)
Phage Classification Tool Set or PHACTS	Predict life cycle characteristics based on proteome	N.a.	McNair *et al.* (2012)
PhageTerm	Determine DNA termini and phage packaging mechanisms using NGS data and relying on the detection of biases in the number of reads, which are observable at natural DNA termini compared with the rest of the phage genome	https://sourceforge.net/projects/phageterm/	Garneau *et al.* (2017)
PhageAI	Predict life cycle characteristics based on nucleotide sequences	https://phage.ai	Tynecki *et al.* (2020)
PhaGAA	Predict phage characteristics based on gene and protein analysis	http://phage.xialab.info/	Wu *et al.* (2023)
Pharokka	Predict phage characteristics based on gene and protein analysis	https://github.com/gbouras13/pharokka	Bouras *et al.* (2022)
PhamClust	Sort phages based the amino acid sequence identity and inter-genome relatedness	N.a.	Gauthier and Hatfull (2023)

Finally, it is important to mention that not all phage-encoded genes are always transmissible. Indeed, during generalised transduction, phages can package and transmit any chromosomal locus. On the other hand, even for generalised transducing phages, specific interactions with chromosomal virulence genes have been described indicating that packaging and transmission is not fully at random. An example is the specific interaction of phage 80α with *Staphylococcus aureus* pathogenicity island SapI1 (Ruzin *et al.*, 2001).

3.7.2 Interference with bacterial homeostasis

In contrast to the rather broad activity of antibiotics, phages typically target specific species or strains. Still, applying phages may interfere with the bacterial homeostasis present in certain ecosystems. Potential interference is particularly relevant when administering large amounts of phages in the context of an agricultural or therapeutic application. Indeed, if phages kill specific populations of bacteria, this may theoretically result in a disruption of the homeostasis or a dysbiosis, which in turn can trigger disease (Aggarwala *et al.*, 2017; Carding *et al.*, 2017; De Sordi *et al.*, 2017; Shkoporov and Hill, 2019). In this perspective, bacteriophages have recently been hypothesized as a possible initiator of Parkinson's disease (Tetz *et al.*, 2018) although additional data are required to validate these findings. Also, in patients with Crohn's disease and type 1 and 2 diabetes, changes in the phagobiome resulted in changes in the microbiome, which subsequently affected the severity of disease symptoms (Ma *et al.*, 2018; Pérez-Brocal *et al.*, 2015; Wagner *et al.*, 2013).

Still, care is to be taken since the impact of phages is likely to depend on the environment where they would be applied. In soil, where phage to bacteria ratios are expected to be nearly 1 to 1 (Reyes *et al.*, 2010), applying (large amounts of) phages could disrupt stable ecological communities. In other environments, phages can outnumber bacteria suggesting that applying (large amounts of) phages is expected to have much less impact. For example, in marine environment phages are likely to outnumber bacteria by a factor 2.5 to 15 (Batinovic *et al.*, 2019; Maranger and Bird, 1995; Pourtois

et al., 2020), whereas in freshwater phage-to-bacteria ratios of 22.5 to1 have been described (Maranger and Bird, 1995).

Furthermore, when applying bacteriophages as an alternative antimicrobial agent, the aim of such treatments is to correct a disbalance in the microbial community. For example, in cystic fibrosis patients the abundant presence of *Pseudomonas aeruginosa*, as compared to healthy individuals, limits the presence and growth of beneficial bacteria and consequently is contributing to a microbial disbalance making the lungs more susceptible to e.g. inflammation (Coburn *et al.*, 2015). Bacteriophage therapy selectively targeting *P. aeruginosa* is likely to restore the normal balance and thus contribute to a healthy microbiota.

Finally, Canete (2018) highlighted that is seems unlikely that phages are able to completely eradicate a bacterial population. Phages can be used to decrease the number of pathogens to a level where the immune system is able to eliminate the infection, as shown in animal models. Also, in a risk assessment report of the Food Standards Australia New Zealand on the Listex P100 bacteriophage preparation it is noted that the numbers of phages applied in food-related applications are considered negligible as compared to naturally present phages and because of this, are unlikely to cause detrimental environmental impacts, especially when combined with good hygiene practices (FSANZ).

3.7.3 Release and/or presence of endotoxin and other bacterial components

Bacterial endotoxins have a high immunogenicity. Endotoxin is one of the most potent inducers of the inflammatory cytokine response in Gram-negative bacterial infections. Endotoxins are released upon bacterial lysis. As phages can kill bacteria within minutes, phage therapy can potentially result in a rapid and significant endotoxin release (reviewed in Liu *et al.*, 2021). There have only been a few studies regarding potential bacterial lysis-related effects (reviewed in Liu *et al.*, 2021). However, comprehensive data on the release of endotoxin and its effects are rarely reported and are inconsistent.

Additionally, endotoxins and other bacterial components could be present in phage preparations as a result of the production process. These bacterial components are typically overlooked. They include bacterial DNA, staphylococcal enterotoxin B (a potent bacterial superantigen), alpha hemolysin and other exotoxins, or lipoteichoic acid (an important cell wall polymer found in Gram-positive bacteria). Bacterial components and toxins such as endotoxin, which are typically difficult to purify from phage agents, have the potential to induce infusion-related reactions. These reactions include hypersensitivity and cytokine release syndrome. Symptoms can include flushing, alterations in heart rate and blood pressure, dyspnoea, bronchospasm, back pain, fever, urticaria, edema, nausea, and rash. Endotoxin release and infusion-related reactions can be difficult to distinguish, but the presence of these bacterial components should be quantified and documented in phage preparations nonetheless (reviewed in Liu *et al.*, 2021) and removed whenever possible (Kortright *et al.*, 2019). Currently there are three major strategies employed regarding the purification of phages and removal of endotoxins: purification involving Caesium chloride (CsCl), polyethylene glycol (PEG) and filtration (anion exchange) (reviewed in Liu *et al.*, 2021).

3.7.4 Change in host tropism

For phages of *Bordetella* spp., it has been described that they can switch tropism allowing certain phages to preferentially infect virulent *Bordetella* strains, whereas others rather infect avirulent *Bordetella* strains (Doulatov *et al.*, 2004; Liu *et al.*, 2002). Tropism switch is mediated by a class of mobile elements, called diversity-generating retroelements (DGRs), that diversify DNA sequences and the proteins they encode. As such, DGRs support phage adaptation to rapidly changing environmental conditions and help to broaden their host range. More recently, DGRs have also been identified in *Bacteroides* spp. (Hedžet *et al.*, 2021, 2022).

DGRs that support a change in tropism between genera have thus so far not been described.

3.7.5 Unwanted interactions at the level of the eukaryotic cell

Phages have also been described to have negative effects for eukaryotic cells. For example, bacteriophages may assist the infection with pathogenic bacteria. This has been described e.g. for a phage of *Ralstonia solanacearum*. The phage was shown to enhance the virulence of the bacteria

for tomato plants by attaching to the bacterial surface and then indirectly activating a variety of different virulence factors (Addy *et al.*, 2012). For *Neisseria meningitidis* it has been shown that its filamentous phage increases its host-cell colonization in epithelial cells by increasing binding of the bacteria and formation of linkers between the bacteria (Bille *et al.*, 2017).

Also, phages have been described to assist bacterial pathogens in biofilm formation and thus in the potential survival of bacteria in a harsh environment, evasion of the host's immune system, and promoting chronic infections (Chen and Wen, 2011; DeBardeleben *et al.*, 2014; Sutherland *et al.*, 2004). Supporting biofilm formation may be the result of bacterial lysis whereby extracellular bacterial DNA serves as adhesive required for biofilm formation as was described for *S. pneumoniae* (Vilain *et al.*, 2009). Alternatively, presence of filamentous bacteriophages in *P. aeruginosa* and *E. coli* contribute to biofilm formation by their shape and negative charge (Secor *et al.*, 2015). Biofilms in the presence of phages turned out to be thicker, denser, more rapidly formed and/or tighter than those formed in the absence of phages. The filamentous Pf phage acts as a structural element in biofilms of *P. aeruginosa*. The extracellular matrix produced by *P. aeruginosa* self-assembles into a liquid crystal through entropic interactions between polymers and filamentous Pf bacteriophages, increasing its ability to persist in pathologic host contexts, such as in cystic fibrosis.

Several studies describe a role of phages in HGT in eukaryotic cells. For example, the bacteriophage WO that naturally infects *Wolbachia*, an intracellular bacterium of arthropods, was shown to carry several arthropod genes (Bordenstein and Bordenstein, 2016; Bordenstein *et al.*, 2006). Gene transfer may have occurred from eukaryotic cells to phages via bacteria or directly upon entering of the phage into the eukaryotic cells. Vice versa, sequence analysis demonstrated the presence of genes that relate to specific bacteriophages in several eukaryotic cells, such as those of a nematode and woodland strawberries (Rosenwald *et al.*, 2014). Phages have also been implicated in the dissemination of bacterial aerolysin and lysozyme genes within eukaryotic hosts (Metcalf *et al.*, 2014; Moran *et al.*, 2012).

The precise mechanisms by which HGT between phages and eukaryotic cells occur remain to be unravelled. The ability of phages to enter eukaryotic cells, as described above and the presence of a nuclear localization signal in several phages may already partly explain how such HGT could occur. Whether HGT may result in the introduction of genes with potential hazardous characteristics has not yet been described.

3.7.6 Immunogenicity

In a literature review on the safety and toxicity of phage therapy, based on data from animal studies and clinical trials, Liu et al. (2021) found that at least in animal studies, phage induced bacterial lysis may cause an inflammatory response (IL-1β and IL-6) and that phage therapy may induce anti-phage antibody titres (IgM, IgG).

Both *in vitro* and *in vivo* studies confirm that phages also impact innate and adaptive immunity directly. However, results related to immune response instigated by phages are inconsistent and their role in phage therapy is also unclear. Mathematical models have been developed showing their potential importance in a phage therapeutic setting. Independent of the phage purification strategy, it is often difficult to attribute these immune responses purely to the phage. Phages themselves are immunogenic biological entities that can stimulate adaptive immune responses. Clinical studies in adults and children with acute bacterial diarrhoea, showed no detectable phages in the blood stream nor any increase in IgG, IgM, IgA, and sIgA; however, when administered via intraperitoneal injection, phages triggered increases in phage-specific IgG and IgM antibody titres.

Phage antibody production may therefore depend on the route of phage administration. In addition, the antibody production was also dependent on the phage type and application time. Currently, antibody production is thought to affect the efficacy of phage therapy; yet their role in the safety of phage therapy is unclear. Data regarding phage-induced immune responses, including inflammatory cytokine production and antibodies, are an underexplored area and are generally lacking (reviewed in Liu *et al.*, 2021).

3.7.7 Risk group classification of wild type phages

Data on risk group classification of phages are scarce. Verheust *et al.* (2010), however, highlight specific phage characteristics that can guide in assigning a biological risk profile. For example, presence of virulence, toxin and antibiotic resistance genes, a lysogenic or non-lytic life cycle and a

broad host range are factors that increase the risk of the phage. Also, the bacterial host may affect the risk profile. Indeed, taking the dependency of the phage on its host into account, phages specific for pathogenic bacteria have a higher risk than phages that replicate in non-pathogenic bacteria (Verheust *et al.*, 2010). Finally, the packaging capacity of a lysogenic or non-lytic phage may be considered thereby speculating that a higher packaging capacity allows for larger genetic elements to be included and potentially transferred.

In a presentation by the Belgian Section Biotechnology and Biosafety (SBB, Sciensano) the following guidelines were given (Pauwels, 2021). The risk assessment should be done on a case-by-case basis. Toxin genes, genes increasing (bacterial) virulence, the capacity to transfer genetic material, (unknown) host range, environmental stability, capacity to lysogenise must be considered. The risk group of well-characterized (i.e. fully sequenced) phages will depend on the risk group of their bacterial host. Non-characterized phages (e.g. isolated from the environment, sewage) must be considered risk group 2 until fully characterised and absence of hazardous inserts has been confirmed.

Chapter highlights

- ✓ *Bacteriophages are viruses that infect bacteria. They are ubiquitous in the environment.*
- ✓ *The stability of bacteriophages is influenced by factors such as pH, temperature, and sunlight.*
- ✓ *Bacteriophages generally have a narrow host range. Host recognition specificity is amongst other determined by receptor binding proteins (RBPs) in the phage's tail.*
- ✓ *The replication cycle can be lytic, non-lytic, or lysogenic. Lytic phages replicate within host bacteria, leading to cell lysis and the release of new phages. Lysogenic phages integrate their genome into the host, potentially influencing bacterial evolution. Lysogenic phages can switch to a lytic cycle under stress or specific signals. Non-lytic phages replicate without causing lysis and do not cause toxic effects what renders them interesting for biotechnology.*
- ✓ *Transduction is a process where genetic information is transferred. Distinction is made between generalized transduction (involving random packaging of bacterial DNA) and specialized transduction (involving transfer of specific bacterial genes, possibly increasing virulence).*
- ✓ *Bacteria and phages are involved in an 'arms race': bacteria have developed various mechanisms to resist phage infection. Likewise, phages have developed tactics to counter these.*
- ✓ *Bacteriophages can also penetrate eukaryotic cells, which is an important feature for antimicrobial therapy and drug delivery.*
- ✓ *There is a lack of data on the pathogenicity and classification of wild-type phages into risk groups.*

4 Application of bacteriophages in research, development and industry: a summary

About a century ago, bacteriophages were discovered and reported in the scientific literature. Phage therapy has been proven an asset to deal with pathogenic bacterial infections since the early 1920s, and has been practiced ever since, especially in the former Soviet Union and in Eastern Europe. The Western world, however, remained sceptical and resorted to the widespread use of antibiotics since the 1940s.

Now that antibiotic resistance among pathogenic bacteria has spread alarmingly and few novel antibiotic compounds are in the pipeline, renewed attention is being directed to the use of phages as antibacterial agents in medicine. Moreover, because of this renewed interest in phage therapy in the Western world, novel applications with phages are being pursued in human health, veterinary medicine, agriculture (e.g. production of food crops, livestock, aquaculture), food safety, waste water treatment, sanitation of environment and equipment (reviewed in Vandamme and Mortelmans, 2018).

Figure 6 gives an overview of the current phage applications. Distinction is made between applications that are in a developmental stage (wild type phages and GM phages), applications that are already commercially available for application in an R&D setting (wild type phages and GM phages) or for clinical or environmental use (currently limited to wild type phages), and finally applications that are subject of patent application (limited to GM phages) (see Supporting Information in Annex 1). Also, the reader is referred to “bacteriophage products”[4], a curated database offering an overview of phage products available on the market with applications in animal & human health, aquaculture, environmental control, food safety, plant health and industry.

A summary of four representative applications is described below.

[4] https://www.bacteriophage.news/phage-products/,

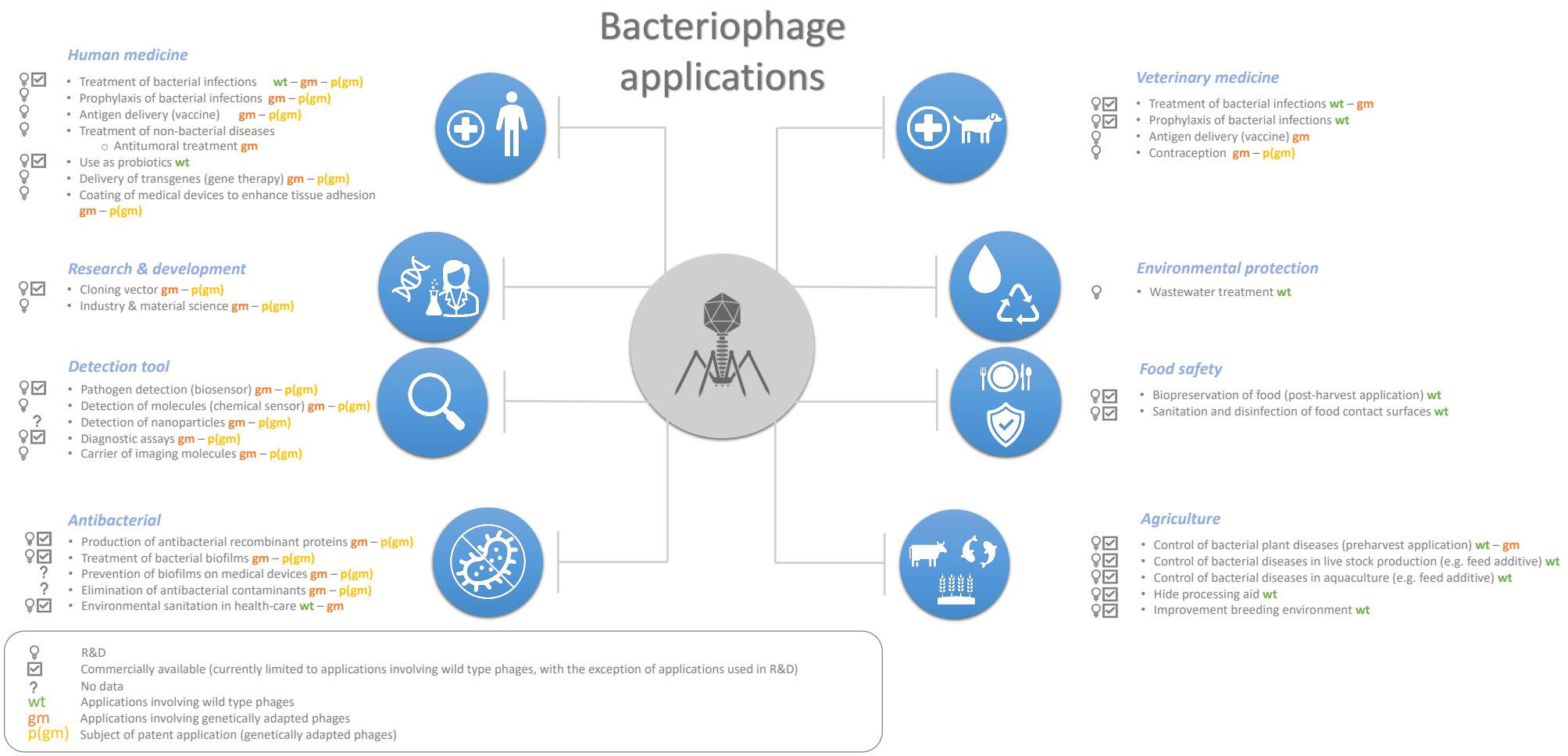

Figure 6. Overview of current phage applications

4.1 Food safety

4.1.1 Control of bacteria during agricultural production

4.1.1.1 Control of bacterial diseases in plants used for food production

Phage applications based on wild type phages are used in plants to control bacterial pathogens causing preharvest spoilage of plant products (e.g. fruits). Important causes of spoilage are *Pseudomonas syringae*, *Ralstonia solanacearum*, *Agrobacterium tumefaciens*, *Xanthomonas* spp., *Erwinia amylovora*, *Xylella fastidiosa*, *Dickeya* spp., and *Pectobacterium* spp. which all entail economic losses (Greer, 2005; Jagannathan *et al.*, 2022). However, extremely narrow host specificity of phages limits the market value of phage therapy products. Therefore, there are only few commercially available phage-based bactericidal products, all of which are wild type phages. Availability of these products is mostly limited to the US. The US Environmental Protection Agency (EPA) has permitted the use following phage products (reviewed in Jagannathan *et al.*, 2022);

- AgriPhage: prevents *Pseudomonas syringae* from infecting young tomato and pepper plants
- Agriphage-Citrus canker™: prevents *Xanthomonas citri* subsp. *citri* from causing citrus canker on grapefruit, orange, lime, lemon, tangelo, kumquat, tangerine, and orange
- Agriphage CMM™: prevents Clavibacter *michiganensis* from causing canker on tomato
- Agriphage-Fireblight™: prevents *Erwinia amylovora* from causing fire blight on apples and pear

Furthermore, the US FDA has given several single phages and phage cocktails the generally recognized as safe (GRAS) status.

In Europe, a phage treatment (Biolyse™) against post-harvest spoilage of potatoes is available (reviewed in Alomari *et al.*, 2021).

4.1.1.2 Control of bacterial diseases in animals used for food production

In foods of animal origin, phage biocontrol strategies have been directed toward the control animal pathogens, including those posing a risk to human health such as *Salmonella* Enteritidis, *Salmonella* Typhimurium, *E. coli*, *Streptococcus faecium* and *Staphylococcus aureus* (Greer, 2005). Many of these bacteria compromise the yield and quality of foods derived from animals, and their shedding can have serious environmental and health consequences.

Phages have been evaluated under experimental conditions to control infections in fish, chickens, cattle, pigs, and lambs and to control pathogen shedding by asymptomatic carriers (Greer, 2005). Several phage products are commercially available as feed supplement for poultry or cattle, or as aerosol disinfectant for cattle such as Finalyse™ (available on the US market) and Bafacol® and Bafasal® (available on the European market) (Dec *et al.*, 2020). None of these commercial phage preparations involve genetically modified phages (Dec et al., 2020; Pizarro-Bauerle and Ando, 2020).

4.1.1.3 Control of bacterial diseases in aquaculture

Fish farms and hatcheries are likewise essential elements of the food industry and may deal with high losses due to bacterial diseases caused by *Aeromonas* spp., *Edwardsiella* spp., *Flavobacterium* spp., *Renibacterium* spp., *Streptococcus* spp., *Vibrio* spp., and *Yersinia* spp. These common fish pathogens either intrude the tissues and skin of aquatic food animals, causing spoilage by forming toxic chemical compounds like trimethylamine, ammonia, H_2S and indole (Jaglan *et al.*, 2022; Kowalska *et al.*, 2020b) or cause disease in fish. To treat bacterial diseases, antibiotics have been widely used. This overuse of antibiotics in aquaculture, for both disease prevention and promotion of fish growth, has resulted in the emergence of antibiotic-resistant food-borne pathogens and poses a threat to the aquatic environment (e.g. by accumulation in sediments and by disturbing the ecological balance of the aquatic environment). In addition, the presence of antimicrobial residues in aquaculture products is not desirable. As a result, several countries have made regulations for the control of antibiotic overuse (Jaglan *et al.*, 2022; Kowalska *et al.*, 2020a).

Phage applications have as advantage that they target specific pathogen species but do not harm natural and beneficial microflora of the aquatic environment or of the host organism. This was clearly

demonstrated by Silva *et al.*, 2016. In an experimental set-up they showed that phage therapy used to treat *Aeromonas salmonicida* infection in the Senegalese sole, reduced *Aeromonas* infections while treatment had no influence on natural bacterial communities of aquaculture water. In contrast, antibiotics, particularly those with a wide activity spectrum, altered the normal environment and host microflora (Kowalska *et al.*, 2020a).

Other studies investigated and applied phage therapy to mitigate the outbreak of diseases caused by, among others, *Lactococcus garviae*, *Aeromonos salmonicida*, *Aeromonas hydrophila*, *Vibrio anguillarum*, *Pseudomonas fluorescens*, *Streptococcus agalactiae* and *Flavobacterium columnare* Investigated application routes are intraperitoneal, oral, intramuscular, application by immersion or as feed additive (reviewed in Jaglan et al., 2022).

Bafador®, available in Europe, is an example of a commercially available phage cocktail for application in aquaculture. It contains 5 wild type bacteriophages against *Aeromonas* spp. and *Pseudomonas* spp. and is applied as feed additive. Next to its antibacterial action, the phage cocktail shows immunomodulatory activity (e.g. increased levels of immunoglobulins, protein, lysozyme and improved activity of spleen phagocytes) in at least rainbow trout (*Oncorhynchus mykiss*) and European eel (*Anguilla anguilla)* (reviewed in Kowalska *et al.*, 2020a) (Jaglan *et al.*, 2022).

4.1.2 Control of bacterial contamination in agricultural food products

A variety of food products are known to pose a risk to human health due to common bacterial contamination, which can result in serious illness and death. These foods include meats, seafood, dairy products, poultry, and vegetables, which are usually mass-produced through non-diversified farming, bulk co-packing, and multi-product transportation, resulting in an increased risk of contamination (Jagannathan *et al.*, 2022). Bacteriophages may be a practical option to increase food safety and diminish food waste. Indeed, several phage preparations for food have been approved for commercial use (Ge *et al.*, 2022; Jagannathan *et al.*, 2022; reviewed in Pérez Pulido *et al.*, 2016), including but not limited to:

- ListShield™ and Listex P100™ for *L. monocytogenes* (available in US, Canada, Australia, Switzerland)
- EcoShield™ for *E. coli*, (available in US, Canada and Israel)
- SalmoFresh™ for *Salmonella* (available in US, Canada and Israel)
- PhageGuard S™ and Salmonelex™ for Salmonella (available in the US, EU, Canada, China, Australia, New Zealand, Switzerland, Israel, Egypt and India)
- ShigaShield™ for *Shigella* spp. (available in US)

None of these products involve genetically modified phages. Various bacteriophages have been identified to infect *Yersinia enterocolitica*, a food-borne zoonotic pathogen of concern most commonly found on raw pork meat, but hitherto no specific phage product is commercially available (Leon-Velarde *et al.*, 2019). Phage preparations can be approved for application on foods or can be approved as part of surface decontamination protocols (Pulido et al, 2016). The latter includes phage preparations approved for treatment of food contact surfaces such as knives, chopping boards and other kitchen utensils, thereby reducing the risk of food contamination during processing with/on these surfaces. For example, ListShield™ and ListexP100™ have been approved as a processing aid in e.g. US, Canada, Australia and Switzerland (Dec *et al.*, 2020).

Even though several phage products have been commercialised in the context of food safety, several problems related to phage applications should be addressed before phage biocontrol can become more widely accepted. Table 3 provides an overview of the advantages and disadvantages of phage application in food safety. Additionally it is highlighted that undesirable phage contamination poses a serious problem in industrial fermentation processes (e.g. kill-off of industrially useful bacterial strains and starter culture used in large scale fermentation processes) (Vandamme and Mortelmans, 2018), and that there may be hesitancy among some consumers toward bacteriophage applications in food (Ge *et al.*, 2022).

Table 3. Advantages and disadvantages for developing a bacteriophage approach to control of foodborne bacteria (Greer, 2005)

Advantages	Disadvantages
Self-perpetuating	Limited host range
Selective modification of bacterial flora (specificity)	Phage-resistance in bacteria
Stable in foods and able to survive processing	Lower efficiency than conventional disinfection techniques (e.g. use of chemical compounds, UV, ozone) used on food products
Natural	Reduced phage titres in environments where they are to be applied
Ubiquitous and readily isolated	Requires large numbers of target bacteria
Cost-effective	Barriers in food environments
Ease of preparation and application	Transduction of undesirable characteristics
Nontoxic to eukaryotic cells	Lysogenic conversion (temperate phages)
No effect on food quality	Antigenicity (immune response, allergenicity)

4.2 Phage therapy in human and veterinary medicine

Unlike antibiotics or other chemical medicines, phages are natural organisms that can replicate in their host bacteria. In theory, a relatively small number of phages deposited at the site of infection are enough to treat a bacterial infection because of their replication and self-amplification. Once the pathogen is eliminated, phages no longer replicate and can be quickly cleared by the immune system or other mechanisms. Advantages of phages over antibiotics are multiple. For example, phages are highly specific to certain bacterial strains, targeting only those bacteria they can infect effectively. This specificity not only allows a highly targeted treatment, it also reduces the likelihood that resistance develops across a broad spectrum of bacteria. Furthermore, different phages use different mechanisms to infect and kill bacteria. If different types of phages are applied simultaneously, in a cocktail, the likelihood for the bacteria to develop resistance to all types of phages is low.

4.2.1 Treatment of bacterial diseases in humans

The use of phages in the treatment of bacterial diseases in humans is illustrated in a recent overview published by Pirnay *et al.* (2023). The overview presented the first hundred consecutive cases of personalized bacteriophage therapy of 'difficult-to-treat infections'. Difficult to treat infections included mainly those of the lower respiratory tract, skin, soft tissue, and bone. The study was facilitated by a Belgian Consortium (Queen Astrid Military hospital (QAHM), KU Leuven, Sciensano). For therapy, a combination of bacteriophages, individually selected bacteriophages and/or pre-adapted phages (i.e. grown in presence of recently isolated bacterial strains to obtain increased pathogen clearance) were used. None of the phages included were genetically modified. The selection of patients was largely based on clinical need, regulatory approval and the availability of well-characterized bacteriophages targeting the infecting bacteria. Most selected cases concerned personalized phage therapy after standard antibiotic treatment had failed. Personalized bacteriophage preparations were produced at the QAMH. Quality and safety of the bacteriophage preparation were verified by Sciensano (e.g. dose, bioburden, endotoxin levels, screening of phage genomes on genetic determinants known to confer to lysogen, toxicity, virulence, or antibiotic resistance). The treatment protocols were based on the experiences by the George Eliava Institute of Bacteriophages, Microbiology and Virology in Tbilisi (Georgia) and on the application instructions of the Ministries of Health and of Medical and Microbiology Industry of the former USSR. Clinical improvement was reported for 77.2% of the targeted infections and eradication of targeted bacteria

was observed in 61.3%. Eradication was 70% less probable when no concomitant antibiotics were used. As such, the authors recommend the use of personalized bacteriophages as additive to standard-of-care products (Pirnay *et al.*, 2023).

Phages may also find their application in dermatologic conditions such as acne, psoriasis, and atopic dermatitis. These conditions are characterized by a relative reduction in the abundance of phages on the skin and the overgrowth of the corresponding host bacteria or bacterial dysbiosis. Phage replacement, either topically or orally via phage cocktails may be an effective therapeutic strategy. A limited number of studies performed so far to assess phage-based therapies for the treatment of psoriasis, acne, and atopic dermatitis has been promising, both in murine models and human subjects (see e.g. Abo-elmaaty *et al.*, 2016; Natarelli *et al.*, 2023). Future research is necessary to assess the efficacy of phage replacement in large scale studies in addition to determining the optimal treatment vehicles, administration mechanisms, and dosing for particular purposes (Natarelli *et al.*, 2023).

4.2.2 Treatment of bacterial diseases in farm and companion animals

The agro-food industry plays a crucial role in the emergence of multidrug resistant (MDR) bacteria. In developed countries, livestock farming accounts for about 50 - 80% of total antibiotic use. Here, antibiotics are not only used for prophylaxis and treatment of foodborne pathogens such as *Salmonella*, *Campylobacter* and *E. coli* but also as feed additive for growth promotion (Wegener, 2003). Moreover, the highest rates of antibiotic resistance are detected against tetracycline, sulphonamide, and penicillin, antibiotics commonly used in the animal feed industry. Initiatives for replacing antibiotics as supplements result in part from current legal regulations in the European Union prohibiting the routine use of antibiotics in farm animals and limiting the chemical treatment of carcasses during processing (reviewed in Dec *et al.*, 2020). But also, in veterinary practices the use of antibiotics to treat companion animal infections has increased, contributing to the emergence of MDR bacteria in pets. These MDR bacteria can easily be transmitted to humans (Ferriol-González and Domingo-Calap, 2021).

As an alternative to antibiotics, phage therapy has been evaluated to treat livestock and companion animal infections, to control zoonotic transmission of diseases to humans and to reduce economic losses. Several successful applications in livestock intended for consumption have been reported and are commercially available. For example, phage therapy has been tested for treatment of mastitis and metritis in cattle, *Salmonella* and *E. coli* infections in pigs, and infections involving *Salmonella* spp., *E. coli*, *Campylobacter* spp., *Listeria* spp., and *Clostridium perfringens* in poultry (Ferriol-González and Domingo-Calap, 2021). Many experiments have dealt with combating infections caused by zoonotic microorganisms that pose a threat to human health, particularly pathogenic strains of *E. coli, Salmonella* spp., *Campylobacter* spp., and *Listeria* spp., which are foodborne pathogens (reviewed in Dec *et al.*, 2020).

Phage therapy has also been tested in companion animals e.g. to treat otitis, urinary tract infections, pyodermal infections, respiratory infections, and reproductive tract infections in dogs, urinary tract infections in cats, equine keratitis caused by *Pseudomonas aeruginosa* and *Salmonella* infections in pet reptiles such as bearded dragons (Ferriol-González and Domingo-Calap, 2021).

Several phage products are commercially available, e.g. Staphage Lysate (SPL)® for treatment of *Staphylococcus aureus* infections in dogs and Bronchophage for treatment of lower respiratory tract infections in horses[4].

4.2.3 Challenges of phage therapy

To become fully appreciated as a therapeutic option, several challenges still need to be addressed:

- **Obtaining sufficient titres of phages in targeted tissues**

  The optimal delivery route may be different for different phages and are likely dependent on phage virion morphology and potentially also the specific characteristics of the proteins making up the phage capsids (reviewed in Dąbrowska and Abedon, 2019). For example, intranasal administration of filamentous phages to mice is efficient in the delivery to the brain, while morphologically different spheroid forms of the same phages, are not able to penetrate brain tissue.

Also, depending on the route of administration, different parts of the body may be reached. Injections, and in particular intravenous injections, have been demonstrated to be the most effective routes of systematic phage delivery and are commonly used, especially in preclinical animal models. Intraperitoneal, subcutaneous, and intramuscular injection also result in phage presence in the blood; however, intraperitoneal injection is most effective since phages are detected sooner in the blood and at higher titres than after intramuscular or subcutaneous injection. Alternatively, phages may also be directly injected into infected tissues or applied topical to (damaged) skin or mucous membranes. This allows to bypass the systemic circulation, and reduce distribution obstacles and phage clearance from the blood (reviewed in Dąbrowska and Abedon, 2019). However, in many cases of topical delivery, phages are being delivered into biofilms, and phages need to distribute into this barrier to reach their bacterial target.

Oral administration of phages is the most convenient and accepted administration route by patients. Oral administration is ideal for targeting infections located in the gastrointestinal tract. However, phage delivery may be inefficient due to phage sensitivity to the extreme low pH values of stomach acid. However, acid neutralizers can be applied to improve phage passage through the stomach (reviewed in Dąbrowska and Abedon, 2019). Oral administration is not a consistently effective route to achieve high phage titres in blood, due to unreliable phage absorption from the gastrointestinal tract. Phage delivery to the lungs by inhalation has been demonstrated to be successful for the control of respiratory tract infections. Here, the effectiveness of phage therapy is found to be dose dependent (reviewed in Dąbrowska and Abedon, 2019). The accumulation of phages in the liver and spleen should generally be viewed as a form of phage clearance from the blood rather than phage distribution to the spleen or liver.

Bacteriophages have been demonstrated in animal brains and have also been used to successfully control intracerebral infections, despite a blood-brain barrier that is often challenging to drug distribution (reviewed in Dąbrowska and Abedon, 2019).

After distribution and absorption, phages may be cleared by several mechanisms such as but not limited to removal via excretion or destruction by enzymes present in the gastrointestinal tract and on mucosal surface. The level of clearance appears to be variable between individuals.

- **Overcoming the unsatisfactory results in larger clinical investigations**

  Such inconclusive results are in part due to the limited coverage and efficacy of natural phages (Meile et al., 2022). Issues to tackle include limited knowledge about the pharmacokinetics of the phage, the risk of immunogenic reactions of the host to the treatment, possible gene transfer to commensal bacteria, and rapid occurrence of resistance against the administered bacteriophages (Lakemeyer et al., 2018; Theuretzbacher and Piddock, 2019; Vázquez et al., 2022).

- **Finding a balance between the narrow host tropism and sufficient mode of action.**

  Indeed, since phages are exclusive to the bacterial species or even only subsets of strains of the particular species they infect (Kortright et al., 2019), cocktails of different phages must be used (Theuretzbacher and Piddock, 2019). This requires appropriate diagnostic tools and potentially the adaptation of cocktails, depending on the causative pathogenic bacterium (Theuretzbacher and Piddock, 2019). Phage therapy may be less effective against infected burn wounds, which are frequently colonized by many bacterial strains, since the applied phages may only target one or few of the pathogens present. This can be addressed by developing phage cocktails that are infectious against a variety of known pathogens, but the efficacy of this strategy depends on the type of infections that are being treated. Host specificity, a clear benefit of broad-spectrum antibiotics, has a considerable impact on the development and testing of treatments and also limits the potential of mass manufacturing and marketing (Ali *et al.*, 2023).

4.3 Environmental control

4.3.1 Prevention and destruction of biofilms

Bacterial biofilms are communities of bacteria that have attached to a surface and form an extracellular polymeric surrounding that renders them extremely difficult to eradicate and add to the pathogen's resistance to conventional antibiotics and common disinfectants. The persistence of these biofilms has presented a need for novel antibacterial agents. Bacteriophages and phage derived lytic proteins have shown potential as antibacterial agents against biofilms. For example, bacteriophages could be used to treat biofilms in aquaculture facilities. All bacterial fish pathogens can form biofilms given the proper environmental conditions. Indeed, *Vibrio* can form biofilms which are linked to its pathogenicity. The giant bacteriophage pVa-21 has been reported to naturally (without genetic modification) disperse biofilms formed by *Vibrio alginolyticus* (Kim *et al.*, 2019), but the number of studies conducted in aquaculture settings is limited (reviewed in Kowalska *et al.*, 2020a; Ramos-Vivas *et al.*, 2021).

Also in clinical surroundings, prevention or destruction of biofilms has gained interest for example in view of the microbial colonization on abiotic surfaces, such as those of urinary catheters, vascular access devices, endotracheal tubes, enteral feeding tubes and wound drains. A number of studies have reported promising outcomes (reviewed in Ali *et al.*, 2023).

4.3.2 Environmental sanitation in healthcare settings

Hospital surfaces are persistently contaminated by several, often drug-resistant, pathogens, most frequently including *Staphylococcus* spp. (including methicillin-resistant *Staphylococcus aureus*, MRSA), *Pseudomonas* spp., *Acinetobacter baumannii*, *Enterobacter* spp., *Klebsiella* spp., and *Citrobacter* spp. These pathogens are known to pose a high health-care burden due to their capacity to spread within hospitals via patients and staff, and to colonize environmental surfaces. Especially for carbapenem-resistant strains, treatment options are limited and infections often lead to high mortality (D'Accolti *et al.*, 2018; WHO, 2017). So far, sanitation of surfaces in healthcare settings relied on chemical-based sanitation using biocides, an approach with important limitations (e.g. temporary effect, high environmental impact, not targeted toward specific pathogens) and with the risk of selecting both disinfectant-resistant and antibiotic-resistant pathogens as major drawback (D'Accolti *et al.*, 2018).

D'Accolti *et al.* (2018) have studied the effect of combining phages and probiotic-based sanitation system consisting of an ecofriendly cleaning solution added with spores of probiotic *Bacillus* spp. (PCHS detergent). The *Bacillus* spores used have shown to inhibit bacteria by competitive antagonism and the production of bacteriocins, which exert an antibacterial effect. The sanitation system combines the rapid and specific action of bacteriophages with the stabilizing and general action of probiotics. Phages efficiently targeted all tested hospital bacteria (e.g. *E. coli*, *S. aureus*, *P. aeruginosa*), maintaining their full activity when added to the PCHS detergent. The combined use of phages and PCHS resulted in a rapid reduction (up to >90%) of the targeted pathogens, but also, due to the stabilizing effect of probiotics, the pathogens were maintained at low levels (>99%) up to 15 days post treatment, when the effect of the phages tends to diminish.

The study of (Chavignon *et al.*, 2022) delivers a proof-of-concept for the use of bacteriophages to eliminate environmental contamination with *Staphylococcus capitis* NRCS-A, a significant pathogen in neonates, from neonatal incubators.

4.3.3 Wastewater treatment

In the context of biological wastewater treatment (BWT), the presence of phages can alter the efficiency of the treatment process and influence the quality of the treated effluent by impacting bacterial communities present in the wastewater (Runa *et al.*, 2021). For example, phages can be used to eliminate harmful microorganisms including *E. coli* pathogenic strains, *Campylobacter* spp., *Vibrio cholera, Salmonella* spp., and various *Shigella* spp., as alternative to currently used disinfection methods such as filtration, chlorination, ozonation, ultraviolet radiation, and chemical coagulation (Shivaram *et al.*, 2023). Also, phages may be used to control detrimental sludge bulking, foaming, and biofilm formation caused bacteria, thereby improving sludge settling properties without impacting nutrient removal. Phages may provide a more sustainable and low-cost alternative to

chemical defoamers that are currently used. So far, studies on sludge properties have been limited to lab-scale systems with well-controlled conditions, with microbial communities usually having low diversity. Full-scale testing has not been yet reported but will be crucial for further development of this technology as a means of bacterial population control in BWT (Runa *et al.*, 2021; (Shivaram *et al.*, 2023).

Phages could also be used as monitoring tools and performance indicators, indicating the key functional bacteria of BWT systems (Runa *et al.*, 2021; (Shivaram *et al.*, 2023).

4.4 Biological sensors

Phages can be used as biosensor for the detection of bacteria. Biosensors are structures with a component for biological recognition, a transducer, and an electronic system that amplifies, processes, and displays the signal, showing a promising alternative in bacterial detection. The most used receptors are antibodies, enzymes, and nucleic acids. However, bacteriophages appear as an interesting alternative in the field of rapid detection of bacteria (phages can be quickly amplified in the targeted live bacteria) as they present specific and efficient mechanisms to bind to bacteria in different environments. Biosensors are currently applied for the detection of pathogenic bacteria in clinical samples, food samples and wastewater (Cao *et al.*, 2016; Elois *et al.*, 2023; Sagona *et al.*, 2016; Shivaram *et al.*, 2023).

Chapter highlights

- ✓ *Interest in bacteriophages has grown due to rising antibiotic resistance in pathogenic bacteria, resulting in multidrug-resistant (MDR) bacteria. While some applications are still in the R&D phase, others are already commercially available the market. The availability of these applications differs notably between regions.*
- ✓ *Current uses of wild-type phages are diverse and including application in human health, veterinary medicine, agriculture (such as food crop production, livestock, and aquaculture), food safety, wastewater treatment, environmental and equipment sanitation, and bacterial detection.*

5 From wild type phage to genetically altered phage

5.1 Techniques to alter the genetic information of bacteriophages

This section provides a non-limitative overview of techniques that have been used to alter the genetic information of bacteriophages. Some of these techniques result in organisms that fulfil the criteria of genetically modified organisms in the scope of the European legislation. Others are induced and/or directed adaptations. It is not the scope of this report to discuss the applicability of the European legal framework for GMOs. Rather we present different types of techniques that result in an alteration of the genetic material. For a more extended overview, the reader is referred to review articles such as Chen *et al*. (2019), Lv *et al*. (2023) and Mahler et al. (2023).

5.1.1 Homologous recombination

One of the most widely used approaches for phage engineering is homologous recombination (Figure 7). This process also occurs in nature, when a heterologous segment of DNA is recombined with the phage genome at sites of homology within a bacterial host. To facilitate screening, it is possible to incorporate marker genes (e.g. luciferase gene, or bacterial host genes essential for phage replication but not essential for bacterial growth) that allow specific selection for mutated phages (e.g. bioluminescence) or to apply a subsequent counter-selection method using CRISPR-Cas to eliminate the wild type phages (Mahler *et al.*, 2023).

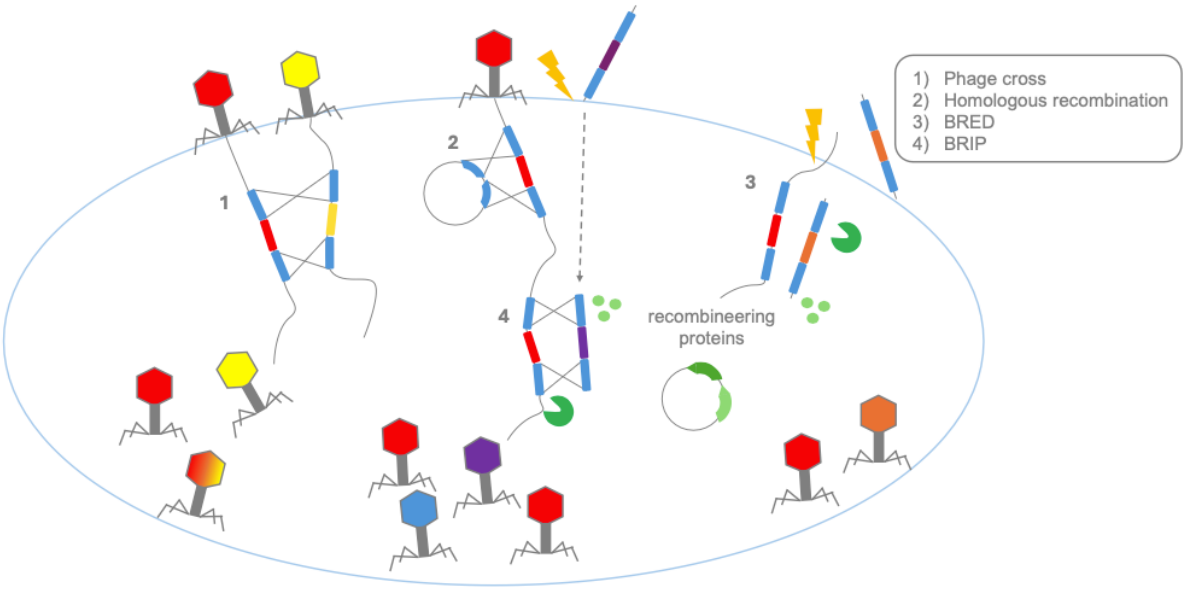

Figure 7. Different approaches for alteration of phages base on homologous recombination (adapted from Mahler *et al.*, 2023)

(1) phage crosses, (2) homologous recombination with a donor plasmid, (3) bacteriophage recombineering of phage DNA (BRED) makes use of recombineering proteins to enhance homologous recombination efficiency between electroporated phage DNA and a DNA substrate with the desired modification, (4) bacteriophage recombineering with infectious particles (BRIP)

Below, a summary of representative techniques of homologous recombination is provided.

5.1.1.1 Homologous recombination between 2 parent phages or classical phage cross

Host cells are co-infected with two parental phages, which have at least two selective markers (or phenotypes). The homologous recombination will occur between parental phage genomes (see Figure 7). The progeny phages are then screened for the desired phenotype(s), and the recombinants with appropriate phenotypes are then purified for further analysis. This approach was mainly used to exchange or combine the phenotypes of parental phages, but is unable to achieve specific modification of the targeted site in the phage genome, which limits its' use (Chen *et al.*, 2019).

5.1.1.2 Homologous recombination between a plasmid and a wild type phage genome

Homologous recombination between a donor plasmid and a wild type phage genome was developed to generate recombinant phages with gene replacements, deletions, or insertions. Several examples include insertion of reporter genes (e.g. coding for GFP, luciferase), whether or not in combination with suppression of lysozyme production (induced by a mutation in gene *e* or by inserting GFP encoding sequences into gene *e*) and/or fusion of GFP encoding sequences to the smaller outer capsid genes (*soc*) to enhance fluorescence (Namura *et al.*, 2008; Oda *et al.*, 2004; Tanji *et al.*, 2004). In a standard procedure, the plasmid containing a designed mutation flanked by homologous sequences of the phage is constructed and transformed into a host bacterium (by electroporation), which is then infected with the phage to be engineered (see Figure 7). The frequencies of recombination are quite low. Therefore, with this classical genetic strategy it is tedious and time-consuming to find the desired recombinants, unless there is a selection strategy for the recombinant phage (Chen *et al.*, 2019).

5.1.1.3 Bacteriophage Recombineering of Electroporated DNA (BRED)

BRED is probably the most efficient method for the construction of targeted bacteriophage mutants and can be used to delete, insert, and replace genes, as well as to create point mutations in phage genomes (Pires et al., 2016). This technique is based on homologous recombination but exploits a phage-encoded recombination system such as the Red system of phage lambda and RecE/RecT system of the Rac prophage to enhance the frequency of homologous recombination (see Figure 7).

RecE is a 5′–3′ exonuclease and RecT is a ssDNA-binding protein that promotes ssDNA annealing, strand transfer, and strand invasion *in vitro*. The RecE and RecT proteins accelerate the homologous recombination between the phage DNA and recombineering dsDNA which results in a generation of phage mutants carrying the desirable trait. This method allows to obtain modified phages at high frequencies (10 to 15%), thus enabling putative mutants to be screened by a small number of PCRs, without the requirement for further selection. As this technique requires highly competent bacterial hosts (Pires et al., 2016), the method is especially difficult to use in Gram-positive bacteria that exhibit low transformation efficiencies (Chen et al., 2019).

The lambda Red recombineering system consists primarily of three proteins: lambda exonuclease, which progressively digests the 5'-ended strand of a dsDNA end, beta protein, which binds to ssDNA and promotes strand annealing, and gamma protein, which binds to the bacterial RecBCD enzyme (Escherichia coli exonuclease) and inhibits its activities (Poteete, 2001). This method was initially developed for mycobacteriophages and has already been applied to modify mycobacteriophages for patient therapy (Marinelli et al., 2008; Payaslian et al., 2021). This technique is also generally applicable to phages of other hosts in which recombineering systems are available such as the T7 phage (Marinelli et al., 2008; Nobrega et al., 2016).

5.1.1.4 Bacteriophage recombineering with infectious particles (BRIP)

BRIP is a variation of BRED, in which the desired modification is provided by electroporation of a DNA substrate and subsequent infection of the cell with the phage rather than transferring the phage DNA into the cell by electroporation (Mahler et al., 2023) (see Figure 7).

5.1.1.5 CRISPR-Cas mediated genome engineering (based on homologous recombination)

As a gene-editing tool, CRISPR and the CRISPR-associated protein (Cas) have revolutionised biomedical research and have been applied in many organisms, including phages. CRISPR/Cas edits genes by precisely cutting DNA and then letting natural DNA repair processes to take over. Originally, CRISPR/Cas is the system that some bacterial species use as part of an antiviral mechanism.

Using this method, both donor DNA and CRISPR-Cas components (Cas9, crRNA (CRISPR RNA), and trans-activating crRNA (tracrRNA) are assembled in a single plasmid that is introduced into the bacterial host. The formed CRISPR-Cas9 complex specifically binds to the target site in the phage genome and creates a double-strand DNA break during phage infection. When a homologous donor sequence is provided, the DNA break can be repaired by recombination with the donor to generate mutants of interest.

Streptococcus pyogenes CRISPR-Cas is most often used for phage genome engineering (Chen et al., 2019). Also CRISPR-Cas of *Listeria monocytogenes* is used for engineering *Listeria* phages (Chen et al., 2019).

The cleavage efficacy of CRISPR-Cas9 complex depends on the selected crRNA. When the crRNA targeting site (protospacer sequence) in the phage genome is highly vulnerable to cleavage by Cas9 complex (high restriction spacer) only the recombinant phages can survive (non-recombinant DNA will be inactivated as a means for counter-selection). As such, all resultant progeny phages are recombinant mutants (Chen et al., 2019).

5.1.2 Rebooting phages using assembled phage genomic DNA

Engineered phages can be directly generated by transforming permissive bacterial host cells with naked full-length phage genomic DNA containing the desired mutations. Replication, transcription, and translation of genomic DNA in the host cells will lead to the assembly of infectious phages (Chen *et al.*, 2019).

Alternatively, the desired phage genome may be assembled from synthetic DNA fragments, e.g. by 'Gibson assembly' (i.e. a robust exonuclease-based method to assemble DNA without the need for restriction enzyme digestion or compatible restriction sites). The resulting genome is then introduced into an appropriate host bacterium for 'rebooting' or the production of viable phage particles from the genomic DNA.

This method requires high transformation efficiency of the host bacterium (reviewed in Mitsunaka *et al.*, 2022). *Escherichia coli* has been used to assemble, edit, and reboot a large panel of phages, to target Gram-negative bacteria, but no clinically relevant tailed phages have been rebooted and the methodology does not work for all phages (due to the toxic proteins encoded in the phage genome and subsequently expressed in *E. coli*, affecting the bacterial host) (Ipoutcha *et al.*, 2024).

Gram-positive bacteria such as *Listeria, Bacillus* and *Staphylococcus* usually exhibit low transformation efficiencies. However, Kilcher and Loessner (2019) have efficiently rebooted phages of Gram-positive bacteria using *Listeria monocytogenes* L-form bacteria as rebooting compartments. These L-form bacteria are cell wall-deficient bacteria, which, unlike its parent cells, can take up large DNA such as phage genome DNA. It was shown that L-form *Listeria* can be employed not only for rebooting of *Listeria* phages but also enable cross-genus rebooting of *Bacillus* and *Staphylococcus* phages (Kilcher and Loessner, 2019). Also *Salmonella* and *Pseudomonas* phages and mycophages (infecting *Mycobacterium* spp.) can be rebooted from *in vitro* genome assembly (Mitsunaka *et al.*, 2022).

5.1.3 Yeast-based assembly of phage genomes

Yeast-based or *in vitro* phage genome assembly methods have been developed to avoid the possible toxic effect of phage replication on the bacterial host (see 5.1.2). Since yeast cells have an efficient recombination system and phage genes are not toxic to yeast, phage genomes, either isolated from a modified phage or assembled *in vitro*, are electroporated into *Saccharomyces cerevisiae* with a linearized yeast artificial chromosome (YAC). Transformation associated recombination (TAR) allows recombination of multiple large DNA segments in yeast artificial chromosomes (YAC) and cloning. The assembled genomes can then be electroporated into a permissive bacterial host for rebooting (Figure 8).

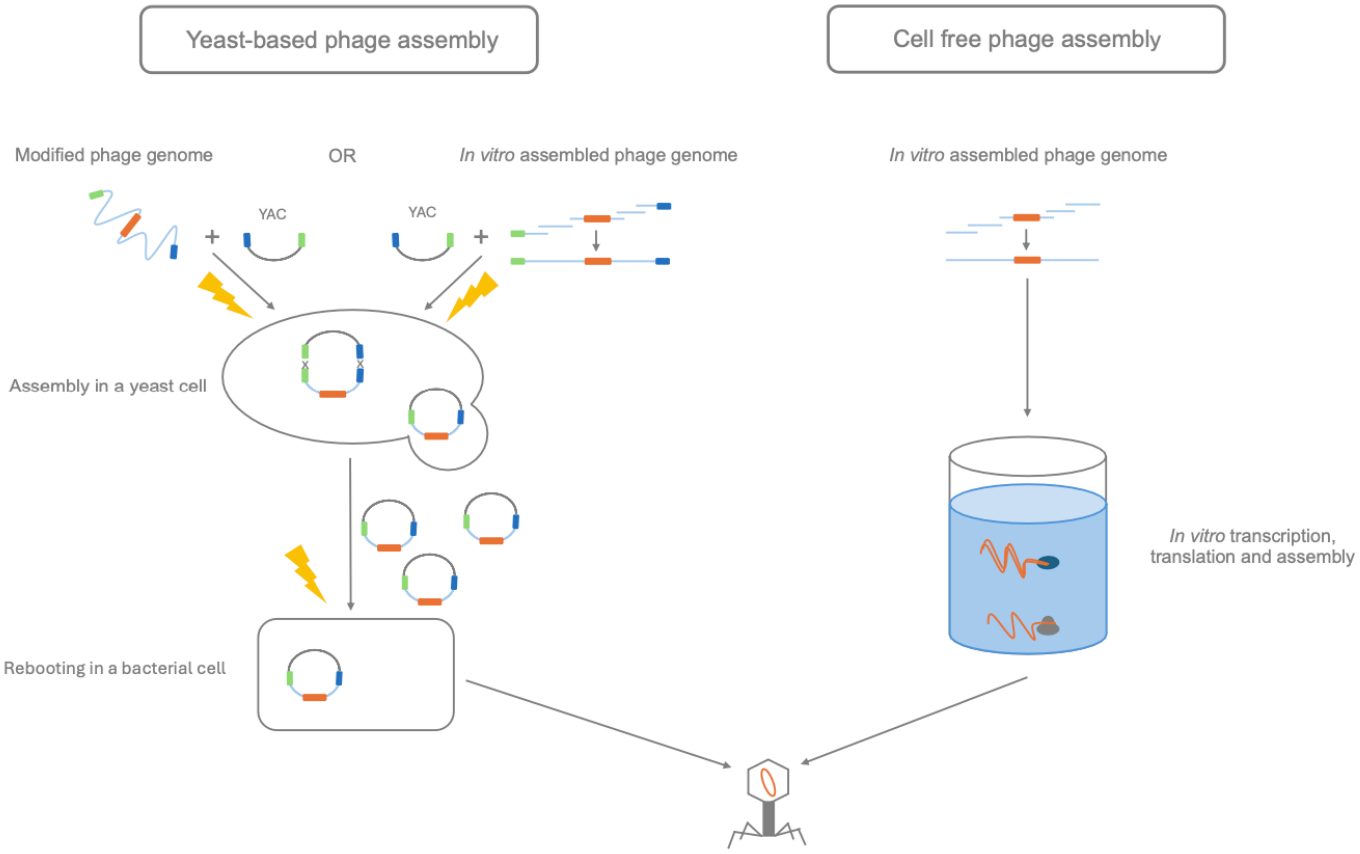

Figure 8. Yeast-based versus cell free phage assembly (adapted from Gibb *et al.*, 2021).

In yeast-based phage assembly (at the left), phage genomes (isolated from a modified phage or assembled in vitro) are electroporated into yeast with a linearized yeast artificial chromosome (YAC) containing hooks (blue and green boxes) to facilitate recombination. YACs containing the modified phage genomes can be electroporated into bacteria to produce recombinant phage particles. In cell-free phage assembly (at the right) phage particles can be produced from assembled recombinant phage genomes using cell-free transcription/translation systems.

5.1.4 Cell free transcription – translation systems

Cell-free transcription-translation (TXTL) systems can create phage or virus-like particles from DNA *in vitro,* in a single test tube*,* overcoming the need for a highly competent bacterial host (see Figure 8). The latter is indeed a bottleneck in the throughput and efficiency of *in vitro* and yeast-based systems for phage genetic engineering (see 5.1.2 and 5.1.3). Cell-free systems are available to successfully replicate, synthesise, and assemble infectious T7, phiX174, MS2 and even T4 phages (one of the largest coliphages with a 169 kbp genome) (Garenne and Noireaux, 2019; Pires *et al.*, 2016; Rustad *et al.*, 2018).

Rebooting of phages through a TXTL system may allow for the preparation of phage lysates on demand in hospitals, and/or for industrial mass production of phages (Mitsunaka *et al.*, 2022).

5.2 Purpose of altering the genetic information of bacteriophages

As mentioned above, wildtype bacteriophages may entail some risks that could hamper successful application. An important aim of altering the genetic information of phages is therefore to eliminate these undesired effects and improve the antibacterial activity of the phage. Furthermore, phages may be modified to insert a gene of interest after which the phages can be used to deliver or express the gene of interest in view of e.g. a therapeutic or diagnostic application (see Section 4). A summary of potential approaches and representative examples are provided in this section.

The reader is also referred to Figure 6 in Section 4 that provides an overview of the application of wildtype and genetically modified phages.

5.2.1 Improving antibacterial activity

5.2.1.1 Avoid phage resistance

As mentioned in Section 3.4.1, several mechanisms have been described by which bacteria can develop resistance to phages. By engineering phages, which involves modifying bacteriophages to enhance their effectiveness in targeting specific bacteria, it may be possible to reduce the likelihood of bacteria developing resistance mechanisms. For example, the RBPs of phages may be engineered in such a way that they recognize multiple receptors (Ando *et al.*, 2015; Dunne *et al.*, 2021; Kilcher and Loessner, 2019; Yosef *et al.*, 2017). To circumvent CRISPR-Cas interference and restriction-modification systems phages can be engineered by adding the cognate anti-CRISPR (acr) immunity genes (Li and Bondy-Denomy, 2021) or DNA methylases (Martel and Moineau, 2014).

By combining multiple mechanistically independent modifications, the likelihood of resistance development may be decreased (Meile *et al.*, 2022). While an approach with multiple mechanistically independent modifications holds great promise, it requires an in-depth molecular understanding of receptor–RBP interactions, which is frequently still lacking, and is thus not yet broadly applicable.

5.2.1.2 Increase antibacterial efficacy

By genetic engineering, phages can be created that contain heterologous, antimicrobial genes of interest that upon infection will lead to expression of specific antimicrobial products (reviewed by Meile *et al.*, 2022). Expressed products can then be released from the infected host (extracellular 'payloads' or genes of interest) and act on bystander cells or extracellular matrix in the vicinity of the producer. Other products may act inside the infected host (intracellular 'payloads' or genes of interest), thereby promoting enhanced killing or disruption of gene networks from within the cell. A representative overview of intra- and extracellular modifications is presented below in Table 4. For more extended information, we refer to review articles by e.g. Pires *et al.* (2016) and Meile *et al.* (2022).

Additionally, phages can be engineered in such a way that they will be endocytosed by eukaryotic cells to combat intracellular pathogens such as *Chlamydia trachomatis* (reviewed in Pires *et al.*, 2016). Even though this modification will not affect the intrinsic antibacterial activity of the phage, it will enable the phage to reach intracellular bacteria and thus will help the overall antibacterial activity.

Finally, phages can be engineered so that they specifically detect and remove pathogens. Such is useful in various areas including clinical, environmental microbial control and crop protection, specifically where the intensive use of other treatments has resulted in highly resistant bacterial strains.

Table 4. Representative examples of genetic engineering of phages to increase antibacterial activity

Aim of genetic modification	Payload / gene(s) of interest	Representative examples	References
Breach of biofilms	Enzyme that degrades a biofilm component or inhibits biofilm formation (e.g. depolymerases such as dispersin B (DspB))	Phage $T7_{DspB}$ in E. coli	Lu and Collins (2009)
Breach of pathogen capsules	Use of a depolymerase able to degrade the bacterial capsule of bacterial pathogens	Phage Y2 expressing a depolymerase able to degrade the bacterial capsule of the plant pathogen *Erwinia amylovora*	Born *et al.* (2017)
Collateral damage on neighbouring cells	Gene encoding for a product with damaging effect on bacteria other than those infected by the phage (including cross-species / cross-genus killing) (e.g. endolysin)	*Listeria*-specific endolysin Ply511 in *Listeria* phage PSA	Kilcher *et al.* (2018)
Disrupt (conserved) intracellular processes	Lethal agents to disrupt bacterial processes, thereby mediating bactericidal or bacterio- static effects	Phage M13 encoding antimicrobial peptides and protein toxin in *E. coli*	Krom *et al.* (2015)
Enhance antibiotic sensitivity	Gene products that modulate bacterial functions to render bacteria more susceptible to antibiotics	Phage λ expressing genes rpsL (streptomycin sensitivity) and gyrA (nalidixic acid sensitivity)	Edgar *et al.* (2012)
Enhance antibiotic sensitivity	CRISPR-Cas9 systems to sensitize/selectively kill antibiotic/multidrug resistant bacteria by targeting genes essential for antibiotic resistance genes	Phage lambda or M13 engineered to deliver CRISPR/Cas system	Sagona *et al.* (2016); Khambhati et al. (2023)
Neutralize virulence	Gene products that modulate bacterial functions to render bacteria less virulent	Phage encoding transcriptional repressor targeting the gene encoding Shiga toxin in *E. coli*	Hsu *et al.* (2020)
Suppress host SOS DNA repair system	Repressor of the SOS DNA repair system to enhance antibiotic-induced killing	Phage M13mp18 overexpressing lexA3 in *E. coli*	Lu and Collins (2009)
Confer sequence specific toxicity	RNA-guided CRISPR-Cas modules or their nuclease-deactivated derivatives to specifically remove disease-associated bacterial genetic elements Small regulatory RNA (sRNA)	Phagemids bearing sRNAs to knock down antibiotic resistance in *E. coli*	Libis *et al.* (2014)

5.2.1.3 Increase bacterial host tropism / extend host range

The host specificity of phages is evolutionarily refined, with most phages targeting one species and a varying number of strains within each species. This narrow specificity is an advantage in the sense that phages are not expected to disrupt commensal bacteria of the host. However, it also implies that each phage must be screened against each bacterial target to determine susceptibility prior to treatment. To circumvent this limitation, multiple phages can be mixed into a cocktail that will have a broader activity range.

Alternatively, phages can be genetically altered to change or expand their host range. A first approach is to modify the receptor binding proteins or domains. Indeed, host recognition specificity is conferred by the receptor binding domains that are found in either the tail-spike or tail fibre protein assemblies of the virions (Chen *et al.*, 2019; Gibb *et al.*, 2021). Several studies have shown that receptor-binding proteins can be exchanged between different types of phages to change host specificity (Chen *et al.*, 2019; Meile *et al.*, 2022). Even numbered T-phages such as T4 and T2 specifically recognize surface receptors on the bacterial host cells using the tip of their long tail fibers.

Replacing the long tail fiber genes of the *Enterobacteria* phage T2 with those from phage PP01, a virulent T2-type phage that infects *E. coli* strain O157:H7 with a high specificity, shifted the host range of T2 from *E. coli* K12 to *E. coli* O157:H7 (Yoichi *et al.*, 2005). Similarly, replacing the long tail fibre genes with those from phage IP008, a phage isolated from sewage effluent which can infect various *E. coli* strains, broadened the host range of the T2 phage (Mahichi *et al.*, 2009). Likewise, the tail fibre gene of *Pseudomonas* virus PaP1 was replaced by the one of phage JG004, a lipopolysaccharide specific broad-host-range phage of the *Myoviridae* phage family, which caused a change in host specificity (reviewed in Born *et al.*, 2017). Exchanging receptor-binding protein genes between more distant phages could even enable a synthetic coliphage to infect *Klebsiella* species (Ando *et al.*, 2015).

Dunne and colleagues (2019) performed a study whereby they were able to broaden the tropism of the *Listeria* phage PSA by applying targeted modification. First, they identified Gp15 as the PSA RBP and constructed a synthetic phage library featuring sequence randomized RBPs using bioinformatics. Subsequently, host range mutants were isolated and subsequently integrated into a synthetic, polyvalent phage which displayed an extended host range.

For *Siphoviridae*, which are double-stranded DNA phages with flexible tails, host range modification is particularly difficult, because their RBDs are an integral part of the baseplate, i.e. the phage adsorption and DNA delivery apparatus. As such, tail spikes are involved in multiple protein-protein interactions within the baseplate. Modification of tail spike sequences can affect the overall integrity of this macromolecular complex, ultimately impeding the formation of infectious virions (Dunne *et al.*, 2019).

The host range of filamentous phages can be expanded by incorporation of a heterologous receptor binding domain and fusion to capsid proteins (Heilpern and Waldor, 2003; Marzari *et al.*, 1997). Fd and IKe are two filamentous phages which infect their hosts by means of pili found on the host membrane: fd infects *E. coli* bearing F pili, whereas IKe infects *E. coli* bearing N or I pili. Fusion of the receptor-binding domain of IKe gene 3 protein (pIII) to the N terminus of the fd pIII expanded the host range of the fd phage. The modified fd phage can infect bacteria bearing either N or F pili (Marzari *et al.*, 1997).

Another approach is to affect the susceptibility of the phage to the bacterial restriction/modification system (see section 3.4.1). By engineering phages with genomes that do not contain restriction sites recognized by the bacterial host's restriction/modification systems, the host range of bacteriophages can be broadened (reviewed in Moller *et al.*, 2019; Verheust *et al.*, 2010).

5.2.2 Increase phage safety

5.2.2.1 Avoid lysogeny and transduction

As mentioned above, lysogenic phages are associated with a risk of specialised transduction and thus in the dissemination of hazardous gene elements such as antibiotic resistance genes. Therefore, lysogenic phages are not suitable for therapeutic purposes. On the other hand, genetic modification may allow adapting the characteristics of a lysogenic phage to become strictly lytic (reviewed in Gibb *et al.*, 2021). Modification may include partial or complete deletion of genes associated with lysogeny, a strategy also described as 'virulent conversion' (Meile *et al.*, 2022). Such genes include but are not limited to specific repressor genes as described for the ZoeJ phage of *Mycobacterium* (Dedrick *et al.*, 2019) or the cI repressor and integrase gene as described for a recombinant phage Δlys targeting *Clostridium difficile* (Selle *et al.*, 2020). Interestingly, although no lysogens were detected when studying the phage Δlys *in vitro*, the authors were able to detect lysogens in the faeces of mice treated with the recombinant phage. It is unclear whether other *C. difficile* genome encoded prophage genes are able to functionally complement those genes removed from the Δlys genomes (Selle *et al.*, 2020).

Another example concerns phage P1, a temperate phage; it can infect by both lytic and lysogenic cycles. To restrict the infection cycle exclusively to the lytic form, a vir mutation has been made by scientists creating the phage variant P1*vir*. The vir mutation prevents the phage from generating P1 lysogens among transductants, making the P1*vir* highly attractive as a vector (Thomason *et al.*, 2007).

Additionally, by targeted modification of terminases, the likelihood for generalised transduction can be reduced. Terminases are the key component of the DNA packaging machine in bacteriophages

and, in brief, consist of a DNA recognition subunit and an endonuclease/translocase subunit for translocation of phage DNA into empty capsids and cleavage. By modifying phage terminases, the ability of phages to perform a sequence-specific termination cleavage, or degrade host DNA early during infection is reduced (Kilcher and Loessner, 2019).

5.2.2.2 Avoid release of endotoxins

Like certain antibiotics, phages can cause rapid and massive bacterial lysis and the subsequent release of cell wall components (e.g., lipopolysaccharides of Gram-negative bacteria or endotoxins), which can induce adverse immune responses in the mammalian host. Lysis is mainly triggered by the holin-endolysin systems whereby holins form holes in the cell membrane, thereby letting endolysin cross the membrane and degrade the peptidoglycan layer of the cell wall. To avoid unwanted lysis, non-replicative or lysis-deficient phage mutants can be created in which genes responsible for host cell lysis (i.e. the holin-endolysin systems) can be removed (reviewed by Pires et al., 2016). For example, phages can be modified to express lethal but non-lytic proteins so that infection results in minimal endotoxin release, as was described for the MR13R phage (Hagens and Bläsi, 2003). The increase in endotoxin levels in the supernatants several hours upon *E. coli* infection with the modified phages was significantly lower than observed upon infection with a control phage. The authors did however report regrowth of the bacterial cells at later time points, indicating that phage-resistant *E. coli* emerged. A similar approach to create a lethal, non-lytic phage was described for a *P. aeruginosa* filamentous phage whereby an export protein gene in the phage genome was replaced with an endonuclease gene (Hagens *et al.*, 2004). The phage was tested in a mouse model and its application resulted in lower levels of inflammatory markers and a higher survival rate as compared to the original lytic phage.

Endotoxin levels in phage preparations can be limited by using a lipopolysaccharide (LPS)-free bacterial host for phage production. Several non-pathogenic *E. coli* strains with genetically modified LPS that does not trigger endotoxic responses in human cells are available (e.g. ClearColi® BL21(DE3)) (Mamat *et al.*, 2013). But also, non-pathogenic *Mycobacterium* species such as *Mycobacterium smegmatis* can be used, since *Mycobacteria* naturally do not have LPS in their cell wall. As such phages for intravenous treatment of *Mycobacterium abscessus* were successfully produced in non-pathogenic *Mycobacterium smegmatis* (Dedrick *et al.*, 2023).

5.2.2.3 Induce conditional replication

Of particular interest is the engineering of phages that replicate only under specific conditions, i.e. when externally adding the factor previously removed from the engineered phage. Two approaches to generate such phages are described below.

A first approach is based on synthetic transducing particles and involves a mutant T7 phage genome deprived of its packaging signal (Pac) and a plasmid DNA to complement it. Without the complementary plasmid, the phages are not able to replicate. Although this approach requires a deep understanding of phage packaging mechanisms, it allows phages to be easily converted into programmable transducing particles carrying genes of interest. However, the number of transducing particles produced upon complementation is completely dependent on the efficiency of electroporation. This is a major limitation of this strategy, especially for phage therapy, which needs high titre phage lysates (Mitsunaka *et al.*, 2022).

A second approach involved the deletion of virion gene(s) such as genes encoding major and minor capsids comprising the phage head, as described by Mitsunaka *et al.* (2022). Also here, the phages are not able to replicate if not provided with the complementary genes. The recombinant phages can be complemented by growing them in bacteria expressing the corresponding gene, resulting in high-titre phage lysates. In addition, by replacing a virion gene with ‘a gene of interest’, the recombinant phages can be customized for functions such as bacteria detection, increased killing efficiency, and toxin neutralization.

5.2.3 Improve phage presence

5.2.3.1 Increase phage presence in specific organs, tissues or bodily fluids

One of the concerns associated with the use of phages in the treatment of bacterial infections is the capacity of the human immune system to neutralize the phages due to their immunogenicity. Studies of the systemic inflammatory response in murine models show a decrease in the availability of active

phages in the circulation and in numerous tissues due to the action of phagocytes, antibodies, and the serum complement system (reviewed in Lv *et al.*, 2023). To avoid the problem of phage elimination by the host immune system, particularly by the reticuloendothelial system (RES), the serial passage technique can be used. In brief, phages are serially injected into mice to search for phage mutants capable of remaining in the circulatory system for longer times. This allows isolation of "long-circulating" mutants of the phage. For example, for *E. coli* two variants isolated after passaging phage λ through 10 selection cycles, had 16,000- and 13,000-fold higher capacities to evade RES clearance 24 h after intraperitoneal administration than that of the parental λ phage. Compared to the parental λ phage, both variant phages contained identical mutations in the major capsid protein E, consisting of the replacement of a glutamic acid with a lysine residue, and one of them had an additional mutation in the capsid D protein. This technique was also used to isolate long-circulating mutants of *Salmonella enterica* serovar Typhimurium phage P22 (reviewed in Pires *et al.*, 2016).

The potential for therapeutic phages to penetrate specific organs or tissues can be increased by molecular engineering of phage virions, specifically by the display of small peptides on phage surfaces that promote phage accumulation in these tissues as so-called specific molecular addresses (i.e. homing peptides specific to selected organs). Many peptides that can facilitate the delivery of nanoparticles to selected tissues have been identified using phage display libraries, i.e. pools of phages presenting short peptides on virions (reviewed in Dąbrowska and Abedon, 2019). Phage display involves the use of filamentous bacteriophages such as M13 to display foreign peptides or proteins on their surface (Figure 9). For this, a gene encoding a protein of interest is inserted into a phage coat protein gene, causing the phage to display the protein on the outside.

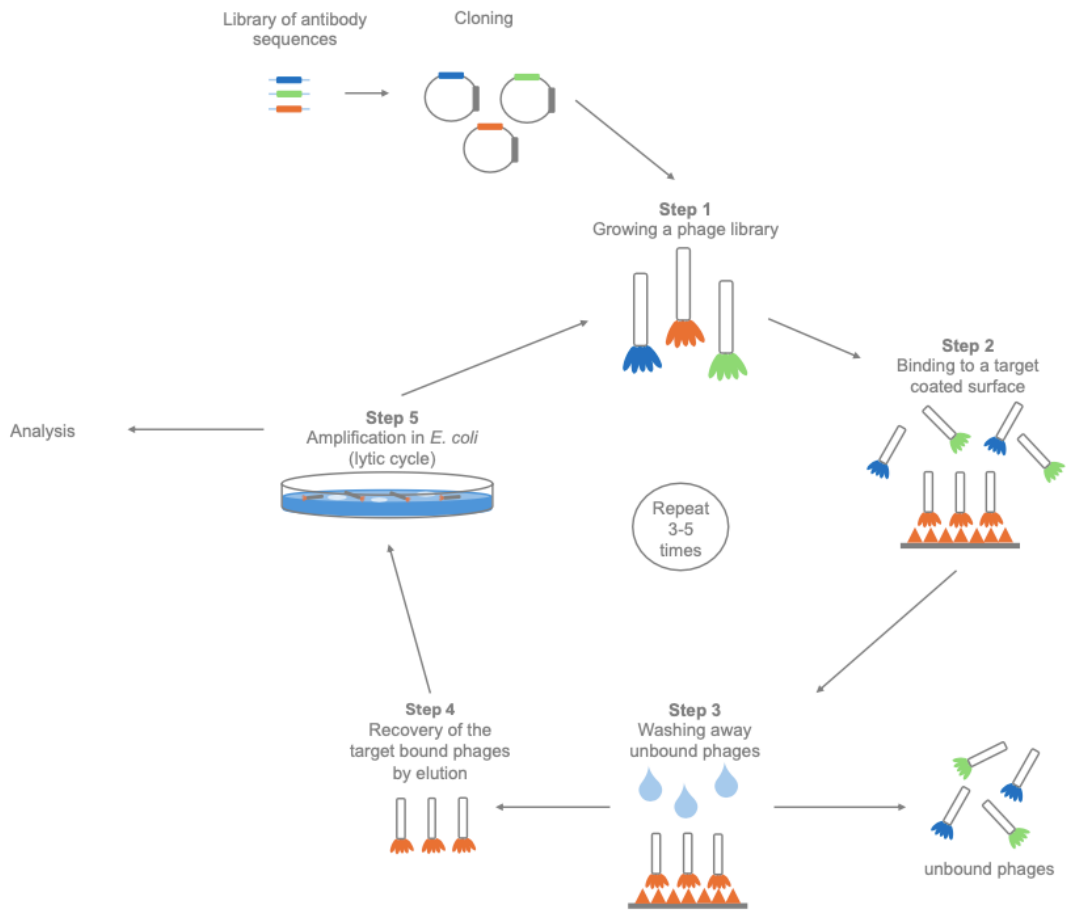

Figure 9. Phage display (Adapted from Yeoh *et al.*, 2022)

Phage display is a selection technique in which a library of variants of a peptide or protein is expressed on the outside of a phage virion, while the genetic material encoding each variant resides on the inside. This creates a physical linkage between each variant protein sequence and the DNA encoding it, which allows rapid partitioning based on binding affinity to a given target molecule (antibodies, enzymes, cell-surface receptors, etc.) by an in vitro selection process called panning.

5.2.3.2 Increase the stability of phages

As mentioned above, application of phages may be hampered due to inactivation by environmental conditions. A particular problem that is observed in human and veterinary medicine when a therapeutic phage preparation is orally administered, is the limited duration of an effective antibacterial titre of bacteriophages which is significantly linked to the sensitivity of bacteriophages to the gut environment. The harsh physicochemical conditions (e.g. acidity, temperature, presence of digestive enzymes) in the gut cause modifications to the phage's structural components and nucleic acids (Dec *et al.*, 2020; Nobrega *et al.*, 2016). One strategy to protect the phages is to use encapsulation, for example in natural biopolymer-derived matrices. Encapsulation resulted in increased survival of phages, however, the selection of the biopolymer is critical, and some important features must be met, such as the ability to be synthesised under mild environmental conditions, and to be easily tailored, non-toxic and environmentally friendly (e.g. not requiring the use of organic solvents). Another approach to protect phages against environmental conditions is by genetic engineering. For example, in the *Escherichia coli* phage T7 the *E. coli* PhoE signal peptide was genetically fused to its major capsid protein (10 A), enabling phospholipid attachment to the phage capsid. The stability of phages was analysed in simulated gastrointestinal tract conditions, demonstrating improved stability of the mutant phages with survival rates 10^2 to10^7 pfu mL^{-1} higher than wildtype phages. Such a "natural coating" may provide the phage with added protection against the acidic environment and other hostile conditions found in the gastrointestinal tract of mammals, potentially without affecting the phage's infection ability (Nobrega *et al.*, 2016).

5.2.4 Gene delivery in eukaryotic cells and tissues

Viral vectors are well known for their suitability to deliver genes of interest and modify eukaryotic host cells in such a way that they can exert new functionalities. However, as viral vectors originate from eukaryotic viruses, their use implies several risks such as immunogenicity (the body's immune system may mount an immune response against these vectors, potentially reducing their effectiveness), insertion of genetic material in the host genome in an unpredictable manner leading to disruption of normal gene function (insertional mutagenesis) or causing toxic effects in the host cell (David and Doherty, 2016; Wang and Shao, 2023).

Due to their bacterial host specificity, phages may offer advantages over viral vectors from eukaryotic viruses. Hereby, phages can be engineered in such a way that they target eukaryotic cells or tissues. An example of a phage that is highly suitable for gene delivery is the bacteriophage T4 (Zhu *et al.*, 2023). The main advantage of the T4 phage is its packaging capacity. The T4 artificial viral vector (or AVV) has a 120 × 86 nm capsid shell that can accommodate 171 kbp DNA. In comparison, adeno-associated viruses and lentiviruses engineered to deliver therapeutic DNA and RNA, have a 5 kbp and 10 kbp packaging capacity, respectively. T4 AVVs can thus efficiently deliver multiple copies of multiple and relatively large DNA molecules into cells. In one experimental configuration, T4 AVV was programmed with five different components: Cas9 genome editing nuclease, *Cre* recombinase, two gRNAs, donor DNA, and reporter plasmids. In another configuration, T4 AVV was programmed with a full-length human dystrophin gene (coding for a ~427 kDa dystrophin protein) fused in tandem with three reporter genes. The negatively charged T4 AAV capsids were coated with positively charged lipid molecules to mimic an envelope around these virus-like nanoparticles and to promote uptake by human cells. Moreover, a T4 CRISPR engineering strategy has been established, which facilitates the insertion of foreign DNA fragments into the phage genome.

Apart from the T4 bacteriophage, also other phages have been studied and some already been applied to deliver information to eukaryotic cells and tissues. Several representative applications are described below. In cases where phages are targeted to mammalian cells, it is important to mention that these phages are eventually degraded inside the host cell without being able to produce new infectious viral particles (Larocca *et al*., 2001). The latter is important in view of limiting potential risks to patient and environment.

5.2.4.1 Phage-based vaccine platforms

Phages have similar properties as mammalian viruses, and therefore can efficiently stimulate immune response. Therefore, they have the potential to be used as scaffolds to develop broadly applicable vaccine platforms. So far, many efforts have been focused on this topic, and many vaccine platforms have been tested experimentally using different phages (e.g. phages λ, T4, T7, MS2, Qβ)

(Chen *et al.*, 2019). Specific examples include a T7 bacteriophage vaccine against hepatitis B virus (Sagona *et al.*, 2016), and a T4 phage vaccine against anthrax (Sagona *et al.*, 2016)

The basic principle of using phages as antigen delivery vehicles involves assembly of the antigen on the phage capsid thereby forming a virus-like particle (VLP) expressing the antigen fused to the phage capsid protein. The antigens are presented on the capsid surface in a highly ordered and repetitive format, which is critical for activation of the innate immune response. Also, it facilitates the binding of IgM to the antigen epitopes (Chen *et al.*, 2019).

Furthermore, since phage capsids are usually composed of hundreds of capsid proteins, assembly of antigens on a phage capsid will result in highly localized epitope density. The highly localized epitope density was suggested to facilitate B cell activation through promoting cross-linking of the B cell receptors to antigens. Also, the highly localized epitope density on the surface of the VLPs allows efficient presentation by both class I and class II major histocompatibility complex (MHC) resulting in the activation of both CD4+ and CD8+ T cells. By genetic engineering, phages can additionally be modified to target and activate dendritic cells (DCs), for example by displaying a DC-specific targeting molecule (Chen *et al.*, 2019). DCs are of great interest to target, as they play a key role in connecting innate and adaptive immune responses.

Interestingly, due to their viral characters and their CpG containing genomic DNA, which is a ligand for Toll-like receptor 9, phages are able to stimulate the innate immune system and therefore potentially act as a natural adjuvant. The self-adjuvanting properties might elicit robust immune responses without administration of external adjuvants.

Despite the promising features of the phage as vaccine platform, there are still several hurdles to overcome before the technique can be applied commercially. A major challenge is that phages are not able to display antigens that require post-translation modifications, such as glycosylation, which are important for structural and conformational integrity of the protein (Chen *et al.*, 2019). Also, most phages do not display the antigen in a sufficiently high density to induce high titres of conformation-specific neutralizing antibodies. Additionally, pathogens may mutate certain key amino acids in the epitopes resulting in the evasion of the immune response raised by a peptide vaccine based on one or a few epitopes.

5.2.4.2 Phages as therapeutic agents in cancer

Phages are being studied in oncology for their potential in targeting tumoral tissues while sparing healthy cells. Promising research includes the use of filamentous phage fd388 to target breast tumours. Fd388 is applied as a biocompatible nanofiber genetically modified to bind to tumour-homing angiogenin and inhibit tumour angiogenesis (Ali et al., 2023).

Phages can also be modified to target Toll-like receptors (TLRs). *In vivo* studies in mice using the TLR antagonist 2-methoxyethoxy-8-oxo-9-(4-carboxybenzyl) adenine or 1V209 conjugated to phage Qβ showed a reduced tumour growth and prolonged survival of mice compared to those treated with free 1V209 or with a simple mixture of 1V209 and viral particles (Jung *et al.*, 2023). Whether phages conjugated to TLR agonists will be applicable in the clinic remains to be determined since non-specific side effects and poor pharmacokinetics may hinder actual application in humans.

Finally, the use of phages to deliver suicide genes or siRNA within cancer cells, and M13-derived bacteriophages engineered to display tumour-associated antigens is being evaluated (Foglizzo and Marchiò, 2021).

5.2.4.3 Phages in fertility control

Phages can be engineered to regulate fertility hormones in animals (e.g. mice and cats). Phage constructs have been developed to stimulate the production of gonadotropin-releasing hormone (GnRH)-specific antibodies which inactivate endogenous GnRH. Consequently, administration of these phages reduces the presence of gonadotropic hormones (e.g. testosterone), aiming to induce (temporary) infertility in animals (i.e. immunocontraception) (Johnson *et al.*, 2020; Samoylov *et al.*, 2015). Also phage constructs inducing neutralizing antibodies which target follicle-stimulating hormone (FSH), luteinizing hormone (LH) and their receptors (GnRHR, FSHR, and LHR), or sperm-specific antigens and zona pellucida (ZP) proteins participating in sperm-oocyte interactions and fertilization, have been evaluated in several animal models (e.g. rainbow trout, sheep, goats, bonnet monkeys, pigs) (reviewed in Samoylova *et al.*, 2017).

5.2.5 Reporting and imaging functions for diagnostic purposes

Reporting involves the use of reporter molecules or genes to monitor the activity or expression of a target molecule. Reporters can produce a measurable signal, such as fluorescence or luminescence, to indicate the presence or activity of the target molecule. Biosensors are structures with a component for biological recognition, a transducer, and an electronic system to amplify and display signals. Common receptors include antibodies, enzymes, and nucleic acids.

Phages are used as biosensor for the rapid detection of bacteria due to their specific and efficient binding mechanisms and their quick replication within in targeted bacteria. Biosensors are currently used to detect pathogenic bacteria in clinical, food and wastewater samples (Cao *et al.*, 2016; Elois *et al.*, 2023; Sagona *et al.*, 2016; Shivaram *et al.*, 2023).

Several assays are available such as lytic phage-based bioassays, reporter phage systems, and phage component assays, which will be highlighted briefly in this paragraph.

In lytic phage-based bioassays, phages infect host cells, causing bacterial lysis and release phage progeny. This progeny can be detected using immunoassays, plating, and molecular approaches like isothermal nucleic acid amplification, quantitative PCR, mass spectrometry and enzyme immunoassay (ELISA). Quantitative real-time PCR (qPCR) methods have been developed for detecting *Salmonella enterica* subtype Enteritidis in chicken, but also phage-based paper-stick biosensors to detect various foodborne pathogens in food matrices have been developed (reviewed in Elois et al., 2023). Detection can also involve cellular components released after bacterial lysis, such as β-galactosidase, adenosine triphosphate (ATP), and adenylate kinase.

Reporter phage systems involve engineered phages to carry reporter genes that are expressed upon infection of the target host producing a detectable signal. This can be a fluorescent (mainly luciferase, green fluorescent protein (GFP)), colorimetric, electrical, or luminescent signal (reviewed in Elois *et al.*, 2023; Schofield *et al.*, 2012). Insertion of reporter genes in combination with suppression of lysozyme production (induced by a mutation in gene *e* or by inserting a GFP encoding sequences into gene *e*) and/or fusion of GFP-encoding sequences to the smaller outer capsid genes (*soc)* may enhance fluorescence (see e.g. Namura *et al.*, 2008; Oda *et al.*, 2004; Tanji *et al.*, 2004). These systems offer rapid and sensitive detection of bacterial cells, with the advantage of only signalling when viable target cells are present. Compared to traditional culture-dependent methods, reported phage systems are faster and can omit lengthy enrichment steps (Cao *et al.*, 2016).

Currently, several commercial GM phage-based diagnostic kits are available for the clinical detection of human pathogens *Staphylococcus aureus, Yersinia pestis*, *Bacillus anthracis*, and *Mycobacterium tuberculosis* (Schofield *et al.*, 2012). Also, luciferase-based recombinant phage kits are commercially available for detection of *Listeria* in food samples (Sample6 DETECT/L^{TM}).

Next to methods using whole phages for detection also phage proteins, such as receptor binding proteins (RBP) or cell-binding domains of phage endolysins, may be used. RBPs assist in the capture and infection of the target bacteria. To identify cell capture, it is necessary to generate a signal, which is generated by the transducer, through flow cytometry, bioluminescence, or fluorescence. This type of assay is useful when bacterial cells have not lysed or released products (reviewed in Elois *et al.*, 2023).

Next to detection of bacteria, reporter phage systems can also be used to detect for cancer cells and viruses (Xu *et al.*, 2019). Moreover, phages are excellent carriers for imaging molecules and can as such be applied for *in vivo* fluorescence imaging of specific cancer cells (Foglizzo and Marchiò, 2021).

Besides biological sensors, phages may be applied as chemical sensors. As such M13 bacteriophages have been genetically engineered to detect drug contaminants such as hormone drugs (oestrogen), herbicides (e.g. atrazine, molinate), antibiotics, glucose and cancer cells (Kim *et al.*, 2020; Xu *et al.*, 2019).

5.2.6 Application in industry and material science

Engineered phages are being explored in material science and industry, serving as bionanofibres with a genetically tuneable surface chemistry and as building blocks for synthesis of nano and biomaterials (Cao *et al.*, 2016). Phages M13 and Fd are commonly used for these purposes.

Phages may enhance material stability in nanotechnology, improve magnetic binding in processes like high-gradient magnetic fishing (HGMF). Phages may be engineered to bind to elements such as gold, cobalt, and manganese for developing nanowires used in LiO_2 battery electrodes (Sagona *et al.*, 2016). Engineered phages are stable in acidic, basic, or organic solutions, allowing for organic functionalization of phages into nanofibers (Cao *et al.*, 2016).

Phages are also explored for constructing porous 3D structures as scaffold for electronic applications (Cao *et al.*, 2016; Pizarro-Bauerle and Ando, 2020).

Engineered phages are increasingly used to create biomaterials through phage display (see 5.2.3.1), enabling the development of multifunctional scaffolds for tissue construction. Phages can self-assemble into 2- and 3-D structures or be used in 3-D printing to create scaffolds or a matrix for cell growth. Filamentous phages, with multiple displayable proteins, allow for combining functional peptides to create versatile scaffolds that guide fibre attachment and support the growth of various cell types like fibroblasts, mesenchymal stem cells, and osteoblasts for tissue engineering applications (Cao *et al.*, 2016; Pizarro-Bauerle and Ando, 2020; reviewed in Gibb *et al.*, 2021).

5.3 Commonly used bacteriophages for genetic modification

An overview of commonly used genetically modified phages for various applications is given in Table 5.

Table 5. Examples of commonly used engineered bacteriophages and their applications (adapted from Sagona *et al.*, 2016).

Phage ID	Life cycle	Nucleic acid content (kb)	Host	Application
M13	Lysogenic	6,4	*E. coli*	Phage display, lethal delivery agent, engineered protein purification, nanomaterials, vaccinology, DNA sequencing
M13KE	Lysogenic	6,4	*E. coli*	Pathogen detection
T7	Lytic	40	*E. coli*	Phage display, gene therapy, biofilm control, vaccinology
MS2	Lytic	3,57	*E. coli*	Phage display, vaccinology
Lambda (λ)	Lysogenic	48,5	*E. coli*	Phage display, vaccinology, biocontrol
T4	Lytic	168	*E. coli*	Phage display, vaccinology, use as artificial viral vector
φA1122	Lytic	37,5	*Yersinia pestis*	Pathogen detection
A511	Lytic	134,5	*Listeria monocytogenes, Listeria ivanovii*	Pathogen detection
HK620	Lysogenic	38,1	*E. coli*	Pathogen detection
PBSPCA1 (or PBS1)	Lytic	No data	*Pseudomonas cannabina* pv. *alisalensis*	Detection of phytopathogens
fd	Lysogenic	6,4	*E. coli*	Phage display, nanodevice fabrication, bottom-up manufacturing, vaccinology, DNA sequencing
φX174	Lytic	5,38	*E. coli*	Model organism, positive control in DNA sequencing
Qβ	Lytic	4	*E. coli*	Vaccinology

Bacteriophage lambda (λ) has a long history of use as a cloning vector and has been used extensively as an expression vector. It has the capacity to clone up to 24 kb size of DNA which is larger than plasmids. Some lambda vectors have been designed to be readily converted into plasmids or phagemids. Phagemids are phage-derived vectors containing the replication origin of a plasmid. Phagemids usually encode no or only one kind of coat proteins. Other structural and functional proteins necessary to accomplish the life cycle of the phagemid are provided by a helper phage (Qi *et al.*, 2012). In addition, a variety of promoters and fusion proteins can be used in lambda to drive

expression of foreign genes. Screening lambda libraries with antibodies or ligands is a powerful way of identifying novel genes (Christensen, 2001).

The Ff class of filamentous phages, which includes strains such as fd and M13, infect *E. coli* cells displaying F pili. Ff virions are long and thin and contain a closed loop of single-stranded DNA. Because the phages readily accept inserts of foreign DNA and because they supply one strand of that DNA in an easily isolated form, vectors based on Ff phages have become the standard choice for sequencing DNA by the primed synthesis method and for oligonucleotide-directed mutagenesis (Smith, 1988). Filamentous phages such as fd and M13 are very stable under a variety of harsh conditions used for selection of phage binders including extreme pH, high temperature, presence of DNase, proteolytic enzymes or nonaqueous solution. Unique and other structural and genetic characteristics of filamentous phages make phage display an extremely powerful tool for bioengineering, for example, screening ligands, developing new drugs, designing vaccines, evolving molecules, diagnosing diseases, drawing the genetic maps, delivering targeted drugs or biosensing. Numerous proteins or peptides with high specificity and affinity have been isolated from phage display libraries using affinity selection (biopanning) and widely used in different fields (Qi *et al.*, 2012).

Chapter highlights

- ✓ *Different techniques are available for genetic alteration of phages, for an efficient selection of the desired recombinant phages and to increase transformation efficiencies of the bacterial host. Cell-free transcription-translation systems allow creation of recombinant phage like particles from DNA in vitro overcoming the need for a highly competent host.*
- ✓ *Bacteriophage lambda (λ) and filamentous phages fd and M13 are commonly used in biotechnology.*
- ✓ *In general, genetic modification of phages aims at mitigating or controlling risk factors associated with phages, enhancing intrinsic beneficial characteristics or introduction of new functionalities targeting eukaryotic cells, facilitating tissue (re) construction, detection of bacteria (biosensors) and compounds (chemosensors), and increasing stability of materials (bionanofibers).*
- ✓ *A broad range of applications using genetic modified phages are in the pipeline, yet commercially available applications based on genetically modified phages are limited to those used in R&D.*

6 Risk considerations for genetically modified bacteriophages

6.1 Impact of genetic modification on risk assessment, a literature perspective

When it comes to the risk assessment of genetically modified phages, information is limited. This section summarizes available scientific literature and other documentation. Hereby, it is taken into consideration that the adaptation can reduce a risk of a wildtype phage or can potentially increase a risk.

6.1.1 Removal of intrinsic hazardous characteristics

As mentioned in section 5.2, one of the aims to genetically adapt bacteriophages is to remove or minimize hazardous characteristics associated with wild type phages. When assessing the risk of the adapted phage, it thus needs to be determined what hazardous characteristics have been removed and how this affects the safety of the phage.

6.1.2 Potential risks of the modification(s) or gene(s) of interest

It remains outside the scope of the current document to discuss in detail all potential risks of the modification(s) or gene(s) of interest. However, when performing a risk assessment, it is to be determined whether they can increase or reduce the risk of the phage. For example, when host tropism of a phage is adapted in order to reach specific target cells in view of a therapeutic treatment, the broadening of the host tropism may result in an additional risk i.e. targeting cells that are not a natural host.

6.1.3 Transfer of genetic information from the genetically modified phage

Exchange of genetic information is an important biological property of phages, as already highlighted previously in the report. Thus, when introducing foreign genetic elements, this property holds a risk that (part of) the elements could be transferred to other phages or bacteria. Such transfer, in turn, may alter the characteristics of other phages and bacteria. However, as mentioned in section 3.3.3, the likelihood of gene transfer to occur depends greatly on the life cycle of the phage. By ensuring that the genetically modified phages maintain or obtain a strictly lytic cycle, the risk that genetic information is transferred is greatly reduced. Several bioinformatic tools have been developed that help to predict the phage life cycle (see section 3.7.1).

On the other hand, even if transfer of genetic information would occur, then it depends on the characteristics of the foreign genetic elements whether the transfer is hazardous. This is clearly illustrated by a risk assessment performed by the COGEM (CGM/030924-01, 2003). Briefly, COGEM issued a specific advice on the use of a genetically modified phage in R&D. Activities for which the advice was written involved a lambdoid recombinant phage (Φ24B::Kan) in which the verotoxin operon (vt2A) is inactivated due to the insertion of a kanamycin resistance gene into the verotoxin gene. The modification would prevent the production of verotoxin and the kanamycin resistance gene could be used to detect bacteria infected with bacteriophages in the intestinal flora. Since the activities were performed under contained use, limited recommendations were given in view of the environmental risk. However, it is worth noticing that reference is made to the genetic stability of modified phages. In brief, it is possible that recombinant bacteriophages may lose their insertion due to genetic instability. For example, the kanamycin resistance cassette may be cleaved from the bacteriophage by recombination, whereby an intact verotoxin operon can be formed. However, the recombined bacteriophage will have the same toxicity and same mechanism of action as the wild-type bacteriophage naturally occurring in ruminants (Van Donkersgoed *et al.*, 1999). Alternatively, the recombinant bacteriophage may exchange its kanamycin resistance cassette with a verotoxin operon of a wild type bacteriophage, resulting in the emergence of wild type bacteriophages that have lost the toxin gene and acquired the kanamycin resistance gene instead. The latter scenario

will only be possible when wild type bacteriophages are present, and a coinfection of a bacterial cell occurs with wild type and recombinant bacteriophages. As discussed previously, coinfection is unlikely to occur for lysogenic phages due to a phenomenon called superinfection inhibition, whereas lytic phages do not allow coinfection due to lysis of the infected host cell. Finally, the COGEM points out that even in the unlikely event of a breach of containment, the presence of kanamycin-resistant bacteriophages will not significantly contribute to the pool of kanamycin-resistant microbes already present in the gastrointestinal tract of humans and animals (Levy *et al.*, 1988; Nap *et al.*, 1992).

6.1.4 Resistance to intra- or extracellular genes of interest

When introducing a gene of interest that will increase the ability of a phage to exert a specific effect, there is a risk that the host develops resistance to this gene of interest. Whether resistance can develop and whether it impacts the risk of the phage, depends on what type of intra- or extracellular genes of interest have been inserted in the GM phage and on the characteristics of the phage. This is illustrated by a study of (Selle *et al.*, 2020). They created a lysogenic GM phage harbouring a CRISPR-Cas 3 system to treat a *Clostridium difficile* infection. When mice were treated with the GM phage, they showed a significantly reduced fecal *C. difficile* burden at 2 days post challenge infection as compared to non-treated mice or mice treated with the wildtype phage. However, by day 4 *C. difficile* numbers increased, and although the increase was less than in non-treated and wt-treated animals, it indicates a reduced efficacy of the GM phage. Similar results were obtained *in vitro*. The authors mentioned that the rebound in *C. difficile* numbers were most likely caused by lysogeny. Indeed, lysogens were demonstrated and subsequent genetic analysis revealed that in 30 of the 35 lysogen samples the spacer and one repeat from the CRISPR region were lost, explaining the reduced efficacy of the GM phages. By subsequently constructing a GM phage lacking key lysogeny genes, lysogeny occurred at a lower frequency and lysogens maintained the CRISPR spacer and repeats. However, the rebound in *C. difficile* numbers was not completely prevented indicating that other phage resistance mechanisms had occurred. Where the sole loss of a gene of interest during lysogeny does not perse create an additional risk, occurrence of other phage resistance mechanisms may raise a concern of creating a population of bacteria that is harder to treat. Using phage cocktails with different modes of action could be a suitable approach to reduce the impact of resistance against one single phage type.

6.1.5 Persistence in the environment

Application of phages implies a risk of (uncontrolled) release or persistence in the environment. On the one hand, genetic modification may help to reduce this risk by introducing characteristics that biologically contain the phages (see above). For example, in case GM phages that once administered to a patient are not shed or not shed as an infectious phage, (uncontrolled) release or persistence in the environment may no longer be a risk. Such an assessment was made for a GM phage administered in the context of a recent clinical trial (personal communication). Taking biological containment into account, the trial was considered a contained use study that did not require an environment risk assessment. Whether the above applies, will depend on e.g. the characteristics of the phage and the administration route, but also local regulatory viewpoints may influence the decision.

On the other hand, literature also describes modifications that aim at prolonging the presence of the phage in the host or the environment, as explained in previous sections. Whether prolonged presence of the GM phage is of greater risk than that of a non-GM phage again depends on the characteristics of the GM phage. As already mentioned before, lysogeny increases the likelihood of survival and thus lysogenic phages should not be applied. But even for lytic phages, Nair and Khairnar (2019) raises concerns thereby responding to a clinical trial with a GM phage described by (Dedrick *et al.*, 2019).

An ecological view on potential persistence of GM phages is provided by Gladstone *et al.* (2012). They focus on the concept of evolutionary biology and evolutionary stability. On the one hand, an adaptation or modification can help to ensure evolutionary stability when it provides a benefit to an individual organism carrying the (adapted or modified) gene. An example is a drug resistance gene in the presence of antibiotics, because beneficial traits are maintained by selection. On the other hand, evolutionary stability may also be ensured in case a gene is present that is beneficial for the population without the gene itself being a benefit for the individual organism carrying the gene. For example, a phage that produces a compound that facilitates infection of new hosts will benefit that

phage, provided the enhanced access to hosts is not outweighing the cost of production. If the compound is released as a free molecule into the environment, then all phages in the vicinity will also benefit because all phages have increased access to hosts. Yet within the group, the phages that do not produce the compound have the reproductive advantage because they avoid the metabolic cost of producing the compound. As a consequence, non-producers will over time outgrow the producers in the group even as the benefit to the group collapses, a phenomenon called 'tragedy of the commons'. All phages share equally in whatever group benefit remains, but only the non-producers have the individual benefit of avoiding production cost. In reality, costs and benefits will not be static and net fitness of a transgene (costs versus benefits) will depend on various conditions such as the environment and the level of gene expression. The highest likelihood for survival of a transgenic individual is when it can grow in a spatially structured environment whereby the benefit can only be experienced by them and not by non-transgenic individuals. Such a situation is however unlikely under natural circumstances taking the abundant presence of phages into account. Long-term survival of the transgenics can also be obtained by removing the benefits for the non-transgenics. For the example above, it could mean engineering the phage as such that the beneficial compound is not released and thus can only be used by the GM phage. The theoretical approach of the authors was confirmed in an experiment using a recombinant phage T7 that degraded the host K1 capsule and facilitated growth when plated on capsulated hosts. The genome carrying the endosialidase gene was favoured on a capsulated host if grown in physical isolation of control phages lacking the gene but was selected against otherwise.

The above shows that a careful risk assessment is to be performed on the gene of interest, its effect on the GM phage and non-GM phages and the environment in which the gene product is produced (Verheust *et al.*, 2010). Guidelines on how to perform such an assessment are not yet available.

6.2 Impact of genetic modification on the risk assessment, a regulatory perspective

The current section focusses on regulatory documents and guidance documents in which direct or indirect reference is made to application of bacteriophages. For (regulatory) guidance on the risk assessment of genetically modified (microorganisms) in general, the reader is referred to e.g. the European Directives 2009/41/EC related to the contained use of GMOs, 2001/18/EC on the deliberate release into the environment of genetically modified organisms, and 2000/54/EC related to the exposure of workers to biological agents.

In the FDA Guidance entitled '**Determining the Need for and Content of Environmental Assessments for Gene Therapies, Vectored Vaccines, and Related Recombinant Viral or Microbial Products**' (2015), it is written that an assessment of the environmental impact is required as part of an investigational new drug application (IND) or biologics license application (BLA) (FDA, 2015). Risk assessment parameters include the identification of substances and potential metabolites, degradants, or byproducts released into the environment, the identification and assessment of potential environmental effects, including magnitude of potential effects and likelihood to occur, as well as mitigation measures. On the other hand, the guideline refers to specific products that may be eligible for categorical exclusion under the Code of Federal Regulations 21 CFR 25.31. For these products, no environmental analysis needs to be submitted as the FDA has determined that these actions, individually or cumulatively, do not significantly affect the quality of the environment. For example, for INDs using gene therapies, vectored vaccines, and related recombinant viral or microbial products (GTVVs), the FDA considers that, in most cases, agency action on an IND for a clinical study will not significantly affect the quality of the environment because, in brief, these clinical trials are closely monitored and are limited to a designated study group. Therefore, such INDs are ordinarily categorically excluded from the requirement to submit an EA, unless extraordinary circumstances indicate that the specific proposed agency action may significantly affect the quality of the environment. Whether or not genetically modified phages are categorically excluded is not specified. During a recent meeting on the science and regulation of bacteriophage therapy, that was organised by the USDA Center for Biologics Evaluation and Research as well as the National Institute of Allergy and Infectious Diseases in 2021, it was highlighted that the fact that a phage is genetically modified does not necessarily mean that it cannot be eligible for categorical exclusion. The main reason for this statement is that genetically modified organisms are not recognized, at least in the realm of biologics, as a separate category as compared

to non-genetically modified organisms (Stibitz (FDA), personal communication). On the other hand, it is mentioned that further study on the risk benefit and the need for specific data remains to be discussed in close collaboration with each regulatory agency (Ousterout (Locus Biosciences), personal communication). No further indications were given on what (risk) data should be collected.

The European Medicine Agency **(EMA) 'Guideline on quality, safety and efficacy of veterinary medicinal products specifically designed for phage therapy'** (2023) aims to establish the regulatory/technical and scientific requirements applicable to veterinary medicinal products (VMPs) specifically designed for phage therapy and composed of bacteriophages. The guideline addresses the provisions as included in the Regulation (EU) 2021/805, which in turn amends Annex II to Regulation (EU) 2019/6. A summary of the (risk) considerations is provided in Table 6.

In view of the environmental risk assessment of bacteriophages in general, the EMA guideline specifically refers to **Regulation (EU) 2021/805** that describes in detail the dossier requirements for an application to obtain a marketing authorization for a veterinary medicinal product. In brief, the environmental risk assessment should be performed in two phases. The first phase shall always be performed and shall indicate the potential exposure of the environment to the product and the level of risk associated with any such exposure taking into account in particular the following items:

- the target animal species, and the proposed pattern of use;
- the method of administration, in particular the likely extent to which the product will enter directly into environmental systems;
- the possible excretion of the product, its active substances or relevant metabolites into the environment by treated animals; persistence in such excreta;
- the disposal of unused veterinary medicinal product or other waste product.

In the second phase, further specific investigation of the fate and effects of the product on particular ecosystems shall be conducted. This includes but is not necessarily limited to assessment of the extent of exposure of the environment to the product, and the available information about the physical/chemical, pharmacological and/or toxicological properties of the substance(s) concerned, including metabolites.

In the case of a veterinary medicinal product containing or consisting of genetically modified organisms, including genetically modified bacteriophages, potential adverse effects on human health and the environment, which may occur through gene transfer from GMOs to other organisms or arise from genetic modifications, shall be accurately assessed on a case-by-case basis. Therefore, the application shall be accompanied by the documents required under Article 2 and Part C of **Directive 2001/18/EC** on the deliberate release into the environment of genetically modified organisms.

Overall, the guideline remains cautious stating that due to the biological complexity and nascent nature of veterinary medicinal products specifically designed for phage therapy (none have yet been centrally authorized in the EU), the advice given in their guideline is general and does not enter into details. Therefore, developers would still need to seek case-by-case advice at the national or European level to guide product development, preferably as early as possible in the production process.

Table 6. Considerations for the (risk) assessment of phage therapy in the context of veterinary medicinal products

(based on EMA 'Guideline on quality, safety and efficacy of veterinary medicinal products specifically designed for phage therapy' (2023))

Topic	General (risk) considerations for non-GM phage therapy	Remarks	Additional considerations for GM phage therapy
Characterisation of phages	• Genetic (as per EMA Guideline - Annex II) • Phenotypic (in vitro microbiology methods as scientifically justified) • Host range • Absence of lysogenic activity • Potency for relevant bacterial pathogens • Adequate description of the source from which phages were isolated	-	• Type of modifications • Effects of modifications
Safety	• Pro-actively identify safety risks by applying a risk profiling methodology (as per quality risk management approaches in the Guideline) • MRL status for all pharmacologically active substances for food-producing animal species (as per Regulation (EC) No 470/2009) • Withdrawal period for food-producing animal species	If safety risks cannot be excluded, it may be possible to reduce such risks to acceptable levels by instating control/mitigation measures. Extrapolation between comparable strains of bacteriophages, or between target animal species, or different routes of administration may be possible based on representative/validated in vitro or in vivo parameters or in well justified cases, based on scientific justification for respective safety studies.	No additional requirements
Toxicity	No mechanism-based concern for toxicity in target animals or humans	-	No additional requirements
Resistance	Risk of developing/spreading resistance in the environment Related risks to humans associated with the use of the product	-	No additional requirements
Environmental risk	Environmental impact (as per IIIa.3A6.1 Regulation (EU) 2021/805)	-	Environmental impact (as per IIIa.3A6.2 Regulation (EU) 2021/805 and Directive 2001/18/EC)
Characterisation of production cells	No nucleic acid sequences coding for (1) toxins, (2) elements conferring antibiotic resistance, (3) prophages, and/or (4) any other genetic elements considered to be predictive for detrimental effects on safety or efficacy of the product	If freedom from these elements is not possible, it should be justified that this has no negative effects on the safety and efficacy of the bacteriophage product. An adequate threshold of the maximal amount of these elements in the host bacteria should be set.	No additional requirements

In a **Scientific Opinion** of **EFSA** on **'The use and mode of action of bacteriophages in food production'** (EFSA, 2009), the only environmental risk assessment property that was highlighted, was the risk of HGT of antibiotic resistances and of key virulence factors. Since bacteriophages are often reproduced in strains belonging to pathogenic species, the risk of transducing genetic determinants from production strains to wider bacterial communities in food or in the gastrointestinal tract should therefore be assessed, in particular for bacteriophages with potential for lysogeny (see also Section 3.3.3).

In the EFSA Scientific Opinion entitled '**Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use**' (EFSA, 2011), risk assessment criteria are provided in more general terms for products consisting of or containing GMMs capable of multiplication or of transferring genes (category 4 products). Such products may include bacteriophages although they are not specifically mentioned in the opinion. Guidance is mostly based in the European Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms and includes but is not limited to elements such as:

- Potential of the GMM to survive (persist) and proliferate in the receiving environments
- Possible interactions of the GMM with their abiotic and biotic environments
- Factors contributing to the degradation or stabilisation of recombinant DNA in relevant environments

In view of the risks related to horizontal transfer of sequences of concern, i.e. genes encoding harmful traits, the EFSA opinion document highlights that these are to be addressed in a risk assessment as described for the genetically modified microorganisms in general. With respect to methodology, the guidance does not report methods how to assess and quantitatively measure the potential gene transfer of sequences of concern present in GMM products. It does however mention that the use of bioinformatics analysis for measuring HGT potential of GM plants is equally applicable to genetically modified microorganisms.

In view of the ability of a product to persist in the receiving environments, various guidance documents and tools have been provided by EFSA, however, none of these tools specifically focus on (genetically modified) bacteriophages.

In a more recent **Scientific Opinion, EFSA** commented on the adequacy of the existing opinions and guidelines for the risk assessment of microorganisms obtained through synthetic biology for food and feed (EFSA Scientific Committee *et al.*, 2022). Therefore, existing guidelines for food and feed risk assessment from various domains were collected and evaluated for relevance for synthetic biology microorganisms. Second, the existing guidelines were challenged towards a selection of 15 cases representing products that could reach the market in the next decade, including a genetically modified bacteriophage. Finally, an overall gap analysis was done, based on the selected cases to identify gaps in the existing guidelines. Specifically, for the (environmental) risk of bacteriophages, an update of the guidance documents would be recommended to better address e.g. the possible formation of phages with transducing capabilities of genes coding for virulence factors and toxins, the influence on gut function, as well as the impact of HGT. Hereby, a strain-driven risk assessment approach is recommended. Also, it has been recommended to include the assessment of bacteriophages on microbiological homeostasis, in particular for the gut microbiome. Such assessment is relevant when applying phages via the gastrointestinal route, but also when applying phages on plants that could subsequently be consumed by animals or humans. The assessment could be performed by means of animal studies or human trials and subsequent analysis via an 'omics' approach. The omics approach has been shown to significantly contribute to the knowledge on the gut microbiome (Gacesa *et al.*, 2022). However, as there is still much unclarity about how a healthy microbiome should be composed, the application of omics data in view of a risk assessment remains limited.

It is worth mentioning that many commercial bacteriophage products in the United States are considered **GRAS** or **'Generally Recognized as Safe'**. The granting of GRAS status means the foods containing these bacteriophages are safe and not subjected to premarket approval by the FDA. Examples include but are not limited to ListShield™, SalmoFresh™ and CampyShield™. So far, the GRAS status has not been assigned to genetically modified bacteriophages. In the EU microorganisms intended for use in the food or feed chains similarly can obtain a qualified presumption of safety (QPS). The QPS provides a generic pre-assessment of the safety of microorganisms intended for use in the food or feed chains. The most recent QPS information at the time of writing this report can be found in the '**Update of the list of qualified presumption of safety (QPS) recommended microorganisms intentionally added to food or feed as notified to EFSA**' (EFSA Panel on Biological Hazards *et al.*, 2023). However, up till now (genetically modified) bacteriophages are excluded from the QPS assessment based on uncertainties such as the impossibility of allocating them to precise QPS taxonomic units (EFSA BIOHAZ Panel, 2009 and 2017), the frequent presence of genes encoding proteins that do not have counterparts in the databases, which hinders the understanding of their precise role (Hammerl *et al.*, 2012; Łobocka *et al.*, 2012) as well as the possibility of recombination between phages that might allow gene-shuffling

(Hammerl *et al.*, 2012), which might lead to subsequent changes of host ranges and their virulent/lysogenic life cycle (Vegge *et al.*, 2006).

Chapter highlights

- ✓ *Defining risk criteria for genetically modified phages poses challenges, but factors like the presence/absence of certain genes, genetic transfer ability, life cycle type, host range, and environmental stability are important considerations.*
- ✓ *Robust testing methods, including genome sequencing and bioinformatics analysis, are crucial for identifying genetic and phenotypic changes in phages and ensuring their stability. Additional investigations like mutational analyses and phenotypic assays can offer insights into the nature of phages and their potential to transfer harmful genetic material.*
- ✓ *The lack of regulatory frameworks for genetically modified phage products may hinder their widespread acceptance. Current regulations mainly target wildtype phages, overlooking the unique aspects of modified phages. There is uncertainty on how to conduct risk assessments for modified phages compared to wildtype phages.*

7 Discussion

Bacteriophages have emerged as a highly promising and widely applicable tool for both basic and applied research. This review of scientific literature identifies their applications across diverse sectors, including human and veterinary medicine, research and development, diagnostics, food safety, environmental protection, and agriculture. Similar as for the set-up of the literature review, the discussion will consecutively focus on topics related to wildtype bacteriophages and on those highlighted for genetically modified phages.

Wildtype phages

Despite the wide range of potential applications that have been identified for wildtype bacteriophages, only a limited set of phages has actually been used in R&D or developed into commercial products. There are several reasons for this, for example phages are highly specific in their host range, meaning that each phage can only infect certain types of bacteria. This specificity limits the range of applications for each individual phage. Also, the characterisation and understanding of phages can be time-consuming and resource-intensive, leading researchers to focus on a select group of phages for in-depth study. But most importantly, researchers must ensure that the phages chosen for their study or application do not pose unnecessary risks to human or animal health or the environment. **Guidance** on what factors to include **in the risk assessment** is available but remains limited. Avoiding lysogeny has been highlighted as an important factor in the risk assessment. Lytic phages are typically considered safer for therapeutic use because they immediately infect and destroy the host bacteria, leading to cell lysis and the release of new phages. In contrast, lysogenic phages replicate within the bacterial host without killing it. Also, the ability to transfer genetic information is different between lytic and lysogenic phages. As described above, lytic phages can take along any piece of bacterial DNA during their replication, potentially facilitating transfer of a broad range of genetic segments. However, they are much less likely to integrate the DNA into the genome of the subsequent bacterium as compared to lysogenic phages. Lysogenic phages on the other hand transfer specific genes and flanking DNA from the insertion site and can integrate their genetic material into the host bacterium's genome, potentially leading to the transfer of virulence factors or antibiotic resistance genes, thereby increasing bacterial virulence. Also, other factors can be included in the risk assessment, whereby Pauwels (2021) specifically advocates for a case-specific approach. Furthermore, Pauwels underscores the importance of fully characterizing phages, with full sequencing as a first step to be taken. For phages lacking characterization, such as those isolated from environmental or sewage sources, a default risk group 2 classification is recommended until comprehensive characterization is achieved and the absence of hazardous genetic elements is confirmed.

Apart from the hurdles in view of the risk assessment, also **societal aspects** play an important role in the so far limited application of wildtype phages. Public opinion on bacteriophage therapy varies. Some welcome bacteriophages as a promising alternative to traditional antibiotics in the face of rising antibiotic resistance (see e.g. McCammon *et al.*, 2023), while others express concerns about the safety, efficacy, and regulation of bacteriophage therapy (see e.g. Loc-Carrillo and Abedon, 2011). This debate is influenced by media coverage, scientific research, and personal experiences. As a result, phage preparations are available as over-the-counter medicine in some countries, while in other countries or for other applications, regulatory restrictions may impact their deployment. An important challenge for broadening the use of phage therapy is thus the global education of people on the importance of phages in various applications. However, for such education rigorous safety evaluations are indispensable which are up till now still limited to the lack of thorough risk assessment data and the so far small sets of efficacy and safety data in clinical trials.

Finally, the **legal framework** regulating the use of wildtype bacteriophages is highly limited and varies significantly from country to country. In some regions, bacteriophage therapy is considered an experimental treatment and is subject to specific regulations governing clinical trials and medical practice. In other areas, more established guidelines exist for using bacteriophages in healthcare settings. The absence of a tailored legal framework for wildtype bacteriophages, or the lack of consensus on the appropriate legal framework, can impede the commercialisation of bacteriophage applications. This issue is exemplified by the case of the bacteriophage Phageguard Listex^{TM}, a food

processing aid used to combat *Listeria* contamination and biofilms. Phageguard products have held USDA and FDA GRAS (Generally Recognized As Safe) status for over ten years. Additionally, Phageguard L and Phageguard S are listed by the Organics Material Review Institute (OMRI) and comply with the USDA's National Organic Program. Phageguard products are also commercially available in Canada, Australia, New Zealand, Switzerland, Israel, Chile, and, since 2023, Egypt[5]. Efforts to obtain approval for Listex™ as a 'non-decontaminating processing aid' on animal-derived ready-to-eat (RTE) food in Europe have been ongoing since 2006. In 2016, the European Food Safety Authority (EFSA) concluded that Listex™ is "safe for use" on RTE food (EFSA, 2016). However, the unclear legal situation in the EU hampers the application and dissemination of phage technology. The European Commission's Directorate-General for Health and Food Safety (SANTE) determined that this product should be regarded as a 'decontaminant,' requiring authorization under the applicable EU Regulation for biocides. Despite this procedural clarity and positive EFSA evaluation, some Member States objected to the authorization arguing that decontaminating products may mask poor hygiene practices in production facilities. This situation illustrates the complexities and challenges of navigating regulatory frameworks for bacteriophage applications, highlighting the need for clearer and more consistent regulations to facilitate the broader adoption and commercialization of phage technology.

GM bacteriophages

Although a wide variety of genetically modified phages are being explored in research and development, the current literature study did not identify genetically modified phages in the commercial phase (except for those used in an R&D setting, such as vectors to transduce cells *in vitro)*. As for the wildtype bacteriophages, similar factors are likely to hamper (commercial) application of genetically modified phages at this moment. The challenges faced for wildtype phages are equally pertinent to genetically modified phages but some additional challenges can be brought forward, in particular for the risk assessment.

When it comes to **risk assessment**, it is important to mention that the initial step in the evaluation of genetically modified organisms (including genetically modified phages) involves identifying the inherent characteristics of the parent organism. However, as highlighted in the first section of this discussion, guidance on the risk assessment of wildtype phages is still limited and frequently based on a case-by-case evaluation. Therefore, thorough assessment of the parent organism may be hampered. Additionally, it needs to be taken into account that a frequent aim of genetic modification is to **reduce** or tightly control the **potential risk factors** associated with the parent organism, such as removing lysogenic characteristics or engineering replication-deficient phages (Kilcher and Loessner, 2019). The introduction of such modifications is expected to mitigate any identified risks associated with the wildtype phage. However, only a limited number of testing methodologies is available so far that is capable of identifying genetic and phenotypic alterations in phages and that may help to understand stability and irreversibility of the genetic modifications. Key tools include but are not limited to genome sequencing, bioinformatics analysis, mutational analyses[6], and phenotypic assays. Furthermore, validation data and acceptance criteria for the test methodologies are frequently lacking, as is a full understanding of the parameters to be tested to fully understand the phage characteristics and the impact of genetic modification. Overcoming these challenges is crucial for enhancing risk assessment approaches for genetically modified phages. Alternatively, redirecting attention towards genetic modification of producer cells presents a promising strategy for mitigating phage-related risks. Insights from discussions with a representative from a phage-producing company highlight the efficacy of this approach. By leveraging CRISPR-Cas technology, harmful genes can be precisely targeted and eliminated from producer cells. Consequently, the risk of phages acquiring these detrimental genes during their production is substantially reduced. This proactive approach not only minimizes the potential hazards associated with genetically modified phages but also underscores the importance of upstream interventions in ensuring product safety.

Phages can also be modified to **enhance** their **intrinsic characteristics**. Such ‘gain-of-function’ research may involve, amongst other, broadening the phage's host range or tropism, increasing its virulence, improving its stability outside the host, or increasing its transmissibility. It is crucial to

[5] https://phageguard.com/knowledge-center

[6] In the context of this discussion referring to the use of various mutagenesis techniques to systematically study the structure and function of genes and/or proteins

thoroughly assess the impact of the gain-of-function, as it could significantly increase biosafety and/or biosecurity risks. Guidance on risk assessment in these cases, which will also apply for genetically modified bacteriophages, is available by e.g the Dutch Biosecurity Office via their Dual-Use Quickscan[7], the National Institute of Health[8], and the European Commission[9].

In addition to reducing or enhancing existing traits, the modification of phages may involve the **introduction** of entirely **new functionalities**, again posing potential concerns regarding the risks of the genetically modified phages. These concerns stem from the fact that these newly introduced capabilities are not inherent to the phage's natural characteristics. One of the primary concerns pertains to the possibility of unintended outcomes, such as the emergence of novel pathogens, the transfer of genetic material to unintended organisms, the development of resistance to phage therapy, or the unwanted presence of phages in the environment impacting biodiversity. Similar as mentioned for the wildtype phages, the risk assessment for the new functionalities is to be done on a case-by-case basis using methodologies as described above. So far, guidance on how to perform such risk assessment is scarce.

When it comes to the legislation, there is currently no **regulatory framework** to accommodate genetically modified phage products, which represents another significant hurdle in the widespread adoption of these technologies. As mentioned above, the limited regulatory guidance focuses on wildtype phages. This situation underscores the pressing need for tailored regulatory guidelines that comprehensively address the safety of genetically modified phages. Achieving this requires collaborative efforts among regulatory bodies, industry stakeholders, and academic researchers to develop robust frameworks for evaluating, assessing risks, and approving genetically modified phages. In addition to technical expertise, involving the public in proactive discussions can foster transparency and accountability in the regulatory process, building trust and confidence in the oversight of genetically modified phage technologies. By striking a balance between innovation and safety, these efforts can ensure that the benefits of genetically modified phages are realized while upholding rigorous safety and ethical standards.

[7] https://www.bureaubiosecurity.nl/en/dual-use
[8] https://osp.od.nih.gov/policies/biosafety-and-biosecurity-policy/
[9] https://trade.ec.europa.eu/consultations/documents/consul_183.pdf

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9 Annex 1 - Supporting information

The patent landscape involving GM phages

In order to have an idea of the current applications of genetically modified bacteriophages that are in the pipeline, a patent search was conducted with "Espacenet". The search provided several patent families in different systems. A patent family is a collection of patent applications covering the same or similar technical content. In total 723 patent families were identified based on the search string. **Table 1** gives an overview of the numbers for the systems with the highest number. For further analysis, those indicated at the European Patent Office were screened.

Table 1

Country	# Patent families
United States of America	282
China	264
World Intellectual Property Organisation (WIPO)	258
European Patent Office (EPO)	**200**
Japan	162
Australia	150
Canada	143
Total	723

Out of the 200, 3 highly similar patent families were eliminated as doubles. The abstracts of the retained 197 families were screened.

The major part (120) was deemed not relevant for the present study for one of the following reasons:

- Use of bacteriophages in molecular or biochemical research, e.g. phage display and genomic libraries are routine laboratory techniques, genetic elements of bacteriophages have been used in genetic constructs.
- Deployment of bacteriophages for production of polypeptides, proteins, and enzymes. These have been associated with several uses, e.g. antimicrobial, treatment of infections and diseases.
- (Recombinant DNA) methods to make bacteria resistant to bacteriophages, which can be of interest for fermentation processes.

In the retained patent families, different uses of genetically modified phages were claimed. **Table 2** indicates types of uses that have been claimed, with a relative portion of the number of patent families and examples.

Table 2

Claimed use	%	Examples
Medical	33	- Treatment or prophylaxis of bacterial infections, mucosal infections (such as *Helicobacter pylori* infections,) *Methylobacterium* spp., non-specified human blood bacteria - Therapeutical and/or prophylactic vaccine - Delivery of transgenes in a variety of gene therapy applications - Medical device having a coating of cell adhesion polypeptides to enhance endothelial cell adhesion onto the medical device - Contraception in animals
Research & Method development	31	- Method for preparing modified bacteriophages - Bacteriophage engineering methods - Methods to produce mutant bacteriophages with altered host range - Generation of recombinant genes in bacteriophages - Method for producing or propagating engineered bacteriophages - Effective purification of bacteriophage preparations - Methods for delivering a recombinant bacteriophage to endogenous bacterial cells - Method of covalently attaching bacteriophages to a surface
Detection tool	27	- Devices, methods, and systems for rapid detection of microorganisms in a sample or on a surface (some claims are species specific: MRSA, *Listeria* spp., *Salmonella*)

Claimed use	%	Examples
		- Methods of detecting a molecule-of-interest in a solution and methods of detecting cells producing a molecule-of-interest - Diagnostic and detection assays, including diagnosis of breast cancer and in the prognosis of triple-negative breast cancer - Detection of nanoparticles
Antibacterial	9	- Production of recombinant protein with antibacterial properties - Treatment of bacterial biofilm - Device with antibacterial surface - Elimination of antibacterial contaminants

With priority dates starting in 1980, most of the patent families in the first decade relate to applications which were not relevant for this study. From 2000, patents on applications as indicated in the above table are reported and since 2010 these outnumber the patents not relevant for this study.

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