Old Herborn University Seminar Monograph 34: The Biological Empire of the Bacteriophage.
Editors: Peter J. Heidt, John Bienenstock, Colin Hill, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 39-55 (2022).

MYCOBACTERIOPHAGES: DIVERSITY, DYNAMICS, AND THERAPEUTIC POTENTIAL

GRAHAM F. HATFULL

Department of Biological Sciences, University of Pittsburgh,
Pittsburgh PA 15260, USA

SUMMARY

Mycobacteriophages are viruses that infect *Mycobacterium* hosts, including the pathogens *Mycobacterium tuberculosis* and *Mycobacterium abscessus*, as well as environmental strains such as *Mycobacterium smegmatis*. A large collection of phages isolated on *M. smegmatis* – of which more than 2,000 have been completely sequenced and annotated – provides a high-resolution view of viral diversity, origins, and the mechanisms of viral evolution. This diversity underlies extensive variations in phage life cycles, gene expression and its regulation, and is driven by the highly dynamic relationship between bacteria and their viral invaders. Many *Mycobacterium* infections in humans – especially those caused by multidrug-resistant tuberculosis or non-tuberculous mycobacteria (NTM) strains such as *M. abscessus* – are very challenging to treat with currently available antibiotic regimens, and mycobacteriophages present a plausible option for patients with no further traditional treatment options. A series of case studies using phages to treat a variety of NTM infections provides insights into the considerable opportunities as well as the challenging limitations of this therapeutic strategy.

INTRODUCTION

The bacteriophage population is vast, highly dynamic, and old (*Hendrix*, 2002). It is perhaps not surprising then that it is highly diverse genetically, shaped by billions of years of variation, selection, and pervasive genetic exchange (*Hatfull* and *Hendrix*, 2011). Bacteria are under constant attack by lytically growing viruses, and must evolve resistance to survive, while the phages must co-evolve to infect sensitive bacterial hosts (*Hampton* et al., 2020). These bacterial-phage dynamics have dominated microbial evolution for perhaps three billion years and have strongly influenced nearly all aspects of bacterial and bacteriophage genomes (*Bernheim* and *Sorek*, 2020).

To characterize bacteriophages, it is not uncommon to focus on one or a relatively small number of bacterial hosts, which simplifies the technical approaches, and it is expected that general principles learned this way will be applicable to phages of other bacterial hosts (*Hatfull*, 2015a). Different bacterial systems offer various attributes, and several years ago we chose to isolate and study phages of *Mycobacterium smegmatis*, a non-pathogenic relative of the human pathogens *Mycobacterium tuberculosis* and *Mycobacterium abscessus* (among others) (*Hatfull*, 2006). There are three key attributes favouring the development of this system. First, mycobacteriophages

(viruses of *Mycobacterium*) can offer insights into viral diversity, evolution, and origins, and represents a notable departure from well-studied model organisms such as *Escherichia coli*. Secondly, mycobacteriophages are a source of broadly applicable genetic tools, and can thus advance the genetic analysis of important pathogens such as *M. tuberculosis*, which grows very slowly and for many years was genetically intractable (*Jacobs*, 2000). Third, mycobacteriophages have potential utilities clinically for both diagnostic and therapeutic applications. Bacteriophage discovery and genomics have also proven to constitute a terrific platform for advancing science education, and course-based implementations of mycobacteriophage isolation and characterization have strongly impacted the phage science while inspiring many fledgling student researchers (*Hatfull*, 2015b). Here, I will discuss some of the key advances in our understanding of the diversity, dynamics, and therapeutic potential of mycobacteriophages.

MYCOBACTERIOPHAGE DIVERSITY

Mycobacteriophage isolation and genomics

Phages of *Mycobacterium* hosts were first isolated over 60 years ago, motivated in part by finding potential tools for typing of clinical isolates of *Mycobacterium*, particularly *M. tuberculosis* (*Snider* et al., 1984; *Good* and *Mastro*, 1989; *Cater* and *Redmond*, 1961; *Murohashi* et al., 1963). However, the faster growing and non-pathogenic *M. smegmatis* was shown to be a useful host for phage isolation, and several phages were characterized by host range and electron microscopy (*Takeya* et al., 1959a, 1959b, 1961). These early studies in the pre-genomic era showed that there was considerable variation in host range and morphotypes, and while a multitude of other features were described, their evolutionary relationships remained elusive (*Mizuguchi*, 1984).

The first mycobacteriophage with a completely sequenced genome was that of phage L5 (*Hatfull* and *Sarkis*, 1993), a temperate double-stranded DNA tailed phage of *M. smegmatis* isolated in Japan (*Doke*, 1960). This fuelled a series of further studies to characterize the phage, but also prompted a deeper dive into comparative mycobacteriophage genomics. Over the next decade a dozen more mycobacteriophages were sequenced (*Pedulla* et al., 2003), revealing that the overall diversity was indeed substantial and that many more phages would need to be characterized to provide a higher resolution genomic view. This was a major impetus for the development of integrated research-education programs that served the dual purposes of expanding the repertoire of sequenced phage genomes, while also providing authentic discovery-rich research experiences for novice scientists, including high school and undergraduate students (*Hanauer* et al., 2006). Initially, the Phage Hunters Integrating Research and Education (PHIRE) program was established to run locally at the University of Pittsburgh (*Hatfull* et al., 2006) but was subsequently expanded nationally in the US (but with some international participants) as the Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) (*Hanauer* et al., 2017, *Jordan* et al., 2014); both programs are funded by the Howard Hughes Medical Institution. The SEA-PHAGES

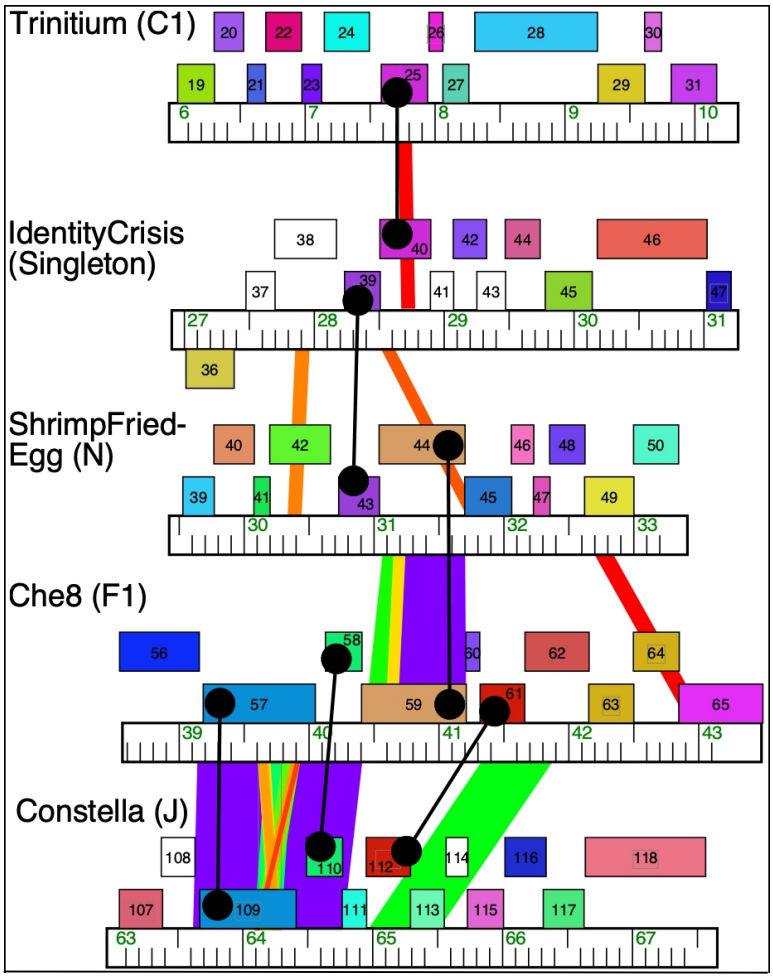

Figure 1: Genetic mosaicism in mycobacteriophage genomes.
Short segments of five phage genomes are shown, with their phage name and cluster/subcluster/singleton designations in parentheses. Genes are shown as coloured boxes either above or below the genome rulers, indicated rightwards and leftwards transcription, respectively, with gene numbers within the boxes. Genes in the same family are coloured similarly, and dumbbell lines are shown linking homologues in different genomes that share amino acid sequence similarity. Nucleotide sequence similarity is shown as colour-spectrum shading between the genomes, with violet being the most similar, and red being the least similar above a threshold BlastN value of 10^{-4}. Note that Trinitium *25* and IdentityCrisis *40* are related (50% amino acid identity) but are situated in different genomic contexts with different genes to their left and to their right. Che8 (F1) and Constella (J) have several genes in common in this region and note the segment of internal repeats reflected in the nucleotide similarity between Che8 *57* and Constella *109*.

program is large with over 170 participating institutions, and over 5,500 students each year. As a consequence, a very large collection of over 20,000 individual phages isolated from environmental samples has developed, of which over 4,000 are completely sequenced and annotated (*Hatfull*, 2020). Over 10,000 of these phages were isolated using *M. smegmatis*, of which over 2,000 are completely sequenced; the other phages were isolated using other bacteria within the phylum Actinobacteria and will not be

discussed in detail here. The complete collection is described at https://phagesdb.org.

Mycobacteriophage comparative genomics

How best to present the mycobacteriophage diversity? Early on in the genomic era it became apparent that groups of evidently related phages emerged, and that they shared little or no sequence similarity to phages outside their group (*Pedulla* et al., 2003, *Hatfull* et al., 2006, 2010). A system was therefore introduced in which sequence-related phages are grouped into 'clusters' (designated Cluster A, B, C etc.). Phages with no close relatives are designated as 'singletons'. When the collection was small, this classification was simple, because related phages were usually very similar at the nucleotide sequence level over large spans of their genomes, whereas phages in different clusters were completely different at the sequence level, often sharing few if any genes with shared amino acid sequences (*Hatfull* et al., 2010).

As the phage collection grew, a key architectural feature emerged, in that phage genomes are pervasively mosaic; that is, they are composed of genome segments – often single genes – that are exchanged among the phages, presumably by illegitimate or non-sequence-directed recombination (*Hendrix* et al., 1999; *Pedulla* et al., 2003). Thus, individual genes – or variants of genes – may be found in different genomes but in different contexts, flanked by different and unrelated genes (Figure 1). As a consequence, the expectation is that with deeper sampling of the phage population, the boundaries between clusters would become less clear, and that a continuum of diversity would emerge, albeit with unequal representation caused by isolation biases and differences in abundance of different phage types (*Pope* et al., 2015). This was indeed observed and warranted revision of the parameters used for cluster assignations. Currently, a value of 35% pairwise shared gene context is used for cluster inclusion, although this is essentially arbitrary, and does not reflect any underlying biological process (*Pope* et al., 2017). In fact, it is not hard to find pairwise shared gene content values straddling the 35% threshold value (*Mavrich* and *Hatfull*, 2017). A further finding is that there is often considerable intra-cluster diversity, and sometimes this appears sufficiently structured as to warrant division into 'subclusters' (i.e. Subcluster A1, A2, A3, within the broader Cluster A phages) (*Hatfull* et al., 2010).

Currently, the >2000 sequenced mycobacteriophages are grouped into 31 clusters and seven singletons, and a network phylogeny is shown in Figure 2. The largest by far is Cluster A with 722 members grouped into 20 subclusters, but several (Clusters U, V, X, Y, Z, AA, AB, AC, AD, AE) have five or fewer members (https://phagesdb.org/), together with the seven singletons. The sampling would thus seem incomplete, and there are likely more and different phages to be recovered from environmental samples, but the overall heterogeneity means that these are isolated with decreasing frequency. Clusters/subclusters often have common features and behaviours, including lifestyles and host ranges (*Mavrich* and *Hatfull*, 2017). For example, over 50% of the cluster/singleton set are predominantly temperate, and have genomic components such as repressor genes, integrases, or partitioning cassettes. However, it is not uncommon for lytic phages to be isolated that group within a temperate cluster but have lost their repressor gene and can no longer form lysogens (*Ford* et al., 1998). A simple explanation is that obligatorily lytic

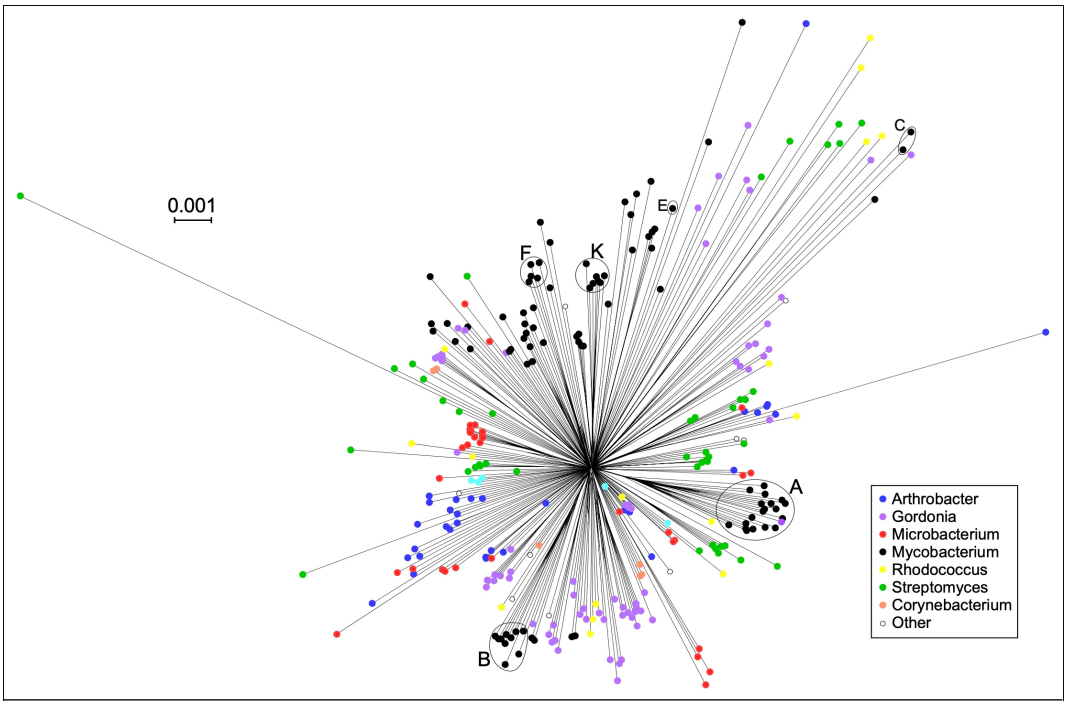

Figure 2: A network phylogeny of actinobacteriophages.
A randomly chosen phage from each subcluster and non-subclustered cluster together with the singletons were compared by their gene contents and the relationships displayed using SplitsTree (*Huson*, 1998). Each phage node is indicated by a coloured circle indicating the genus of the bacterial host used for isolation. Clusters containing 100 or more individual phages are circled and the cluster indicated. (Reproduced with permission from *Hatfull*, 2020).

phages form larger and clearer plaques and thus are easier to identify and to choose for purification during the isolation process. Cluster/subcluster designation can also correlate with host range, as demonstrated by the abilities of these phages to infect either *M. tuberculosis*, or other strains of *M. smegmatis* (*Jacobs-Sera* et al., 2012; *Guerrero-Bustamante* et al., 2021).

MYCOBACTERIOPHAGE LIFE CYCLES

As noted above, temperate phages are common among mycobacteriophages. These temperate phages are capable of cycles of lytic growth, similar to obligatorily lytic phages, but differ in that they can also enter the state of lysogeny upon infection. Transcriptomic analyses of lytic growth show that there are predominantly two patterns of transcription (*Gentile* et al., 2019, *Dedrick* et al., 2013, 2017b, 2019b). Soon after infection, early lytic gene expression is observed, which continues for about 20-30 minutes, then late lytic expression begins and continues until lysis about three hours after infection. The early lytic genes typically include those that are not directly involved in virion structure and assembly, functionally defined genes for DNA metabolism and regulation, but also a multitude of genes of unknown function. These

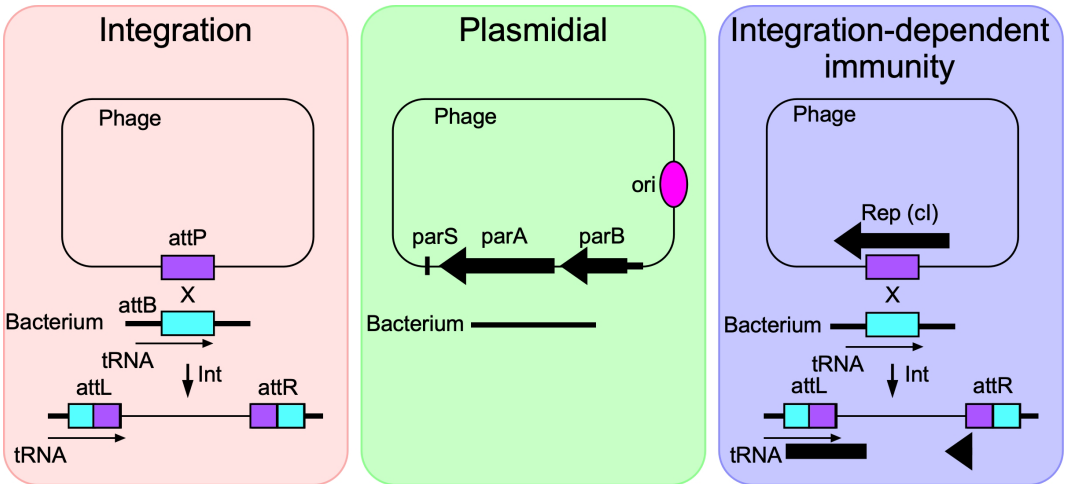

Figure 3: Systems for establishment of lysogeny and prophage maintenance.
When the linear viral DNA of a temperate phage is injected into the cell, the genome circularizes and either integrates ('Integration') or replicates as an extrachromosomal circle ('Plasmidial'). In the integration process, the phage-encoded integrase (Int) catalysed site-specific recombination between phage and bacterial attachment sites (*attP* and *attB* respectively) to form an integrated prophage flanked by attachment junctions *attL* and *attR*. The *attB* sites used by tyrosine-family integrases often overlap a host tRNA gene. Plasmidial phages do not integrate but replicate extrachromosomally from a phage origin of replication (ori) and encode partitioning systems (*parABS*) to prevent loss at cell division. Some phages use an unusual integration system ('Integration-dependent immunity') in which the *attP* site is located within the repressor gene (Rep, cI) of the temperate phage. Integration 'breaks' the repressor gene, separating its 5' and 3' parts in the prophage. The viral form of the repressor is inactive due to a C-terminal tag for proteolytic degradation, which is absent from the prophage-encoded repressor and can actively confer superinfection immunity.

genes are typically under the control of a single early lytic promoter, which is recognized by the host RNA polymerase, and in temperate phages is under control of the phage repressor (*Nesbit* et al., 1995, *Brown* et al., 1997). The late lytic genes are comprised of the virion structure and assembly genes, including those for DNA packaging and capsid and tail assembly. During late lytic growth, the lysis genes are also expressed, leading to cell rupture and the release of progeny particles (*Payne* et al., 2009; *Payne* and *Hatfull*, 2012).

For temperate phages, infection can result in either entry into lytic growth or the establishment of lysogeny. The frequencies with which these pathways are followed varies enormously for different phages and conditions, and lysogeny can result from as few as 5% of infections or as high as over 90% of infections. The molecular determinants of the decision outcomes are unclear for most mycobacteriophages, although they are well established for model systems such as phage lambda (*Ptashne*, 1987). Lysogeny itself, however, results from expression of the repressor protein and its down-regulation of the early lytic genes. Lysogens are characteristically immune to superinfection by the same phage, a phenomenon in which the prophage-expressed repressor down-regulates any newly-introduced genomes of the same phage (*Donnelly-Wu* et al., 1993). For most temperate mycobacteriophages, a prophage is established by site-specific

integration of the phage genome into the host chromosome (Figure 3), mediated by the phage-encoded integrase (*Lee* et al., 1991). However, some mycobacteriophages (all within Cluster A) do not integrate, and instead establish extrachromosomal 'plasmidial' prophages, which replicate at low copy number and encode partitioning systems to negate prophage loss at cell division (*Wetzel* et al., 2020) (Figure 3).

Mycobacteriophages use a variety of mechanisms for prophage integration and the establishment of lysogeny. Two main types of integration systems are prevalent, represented by the tyrosine-integrases (Int-Y) and serine-integrases (Int-S). Both catalyse site-specific recombination between a phage attachment site (*attP*) and a bacterial attachment site (*attB*) which share a short region of sequence identity (3-45 bp; the 'common core') within which strand exchange occurs. Recombination results in formation of an integrated prophage flanked by *attL* and *attR* sites (Figure 3). The integrase can also mediate excisive recombination by *attL* x *attR* exchange although this typically requires a phage-encoded Recombination Directionality Factor (RDF) (*Lewis* and *Hatfull*, 2001). Most Int-Y systems have common cores 25-45 bp long, and thus if the phage genome sequence is available, the *attB* site can usually be predicted by homology searching in the host chromosome. Interesting, these *attB* sites commonly overlap a host tRNA gene. In contrast, Int-S systems usually have shorter common cores (3-10 bp) and the *attB* sites cannot be readily predicted bioinformatically (*Kim* et al., 2003). Because of the overall diversity of the integration systems, at least 14 *attB* sites have been identified in *M. smegmatis* (*Hatfull*, 2022).

Some mycobacteriophages use an unusual system for lysogenic establishment referred to as 'integration-dependent immunity' (*Broussard* and *Hatfull*, 2013, *Broussard* et al., 2013) (Figure 3). The tell-tale feature of these is that the *attP* site is located within the repressor reading frame of the phage. This would seem counterintuitive, as integrative recombination will result in loss of the 3' part of the repressor gene, rendering it non-functional. In fact, the opposite is true. The viral form of the repressor carries a C-terminal SsrA-like tag that targets the protein for degradation by host proteases and is functionally incapable of conferring immunity to superinfection (*Broussard* et al., 2013). Integration results in loss of the degradation tag, stable protein is expressed, and immunity is established (Figure 3). Interestingly the Int-Y protein also carries a C-terminal degradation tag such that the stability of the integrase fundamentally determines the frequency of lysogenisation. Although these integration-dependent immunity systems were discovered in phages of *M. smegmatis*, they are quite prevalent among extant prophages of *Mycobacterium* genomes, especially those of *Mycobacterium abscessus* (*Dedrick* et al., 2021a).

MYCOBACTERIOPHAGE - HOST DYNAMICS

The determinants of phage host range and the dynamic interactions between bacteria and their phages are complex, and there is much to learn. *Mycobacterium* strains have been shown to carry restriction-modification systems (*Shankar* and *Tyagi*, 1993a, 1993b) and *M. tuberculosis* carries a CRISPR locus, although most mycobacterial strains are CRISPR-free (*He* et al.,

2012). Receptor variation is likely also important, although few mycobacteriophage receptors have been identified, other than the potential role of glycopeptidolipids in phage I3 infection (*Chen* et al., 2009). However, we note that a number of phage host range mutants have been reported with substitutions in tail spike proteins suggesting the direct role of phage-receptor interactions (*Jacobs-Sera* et al., 2012, *Dedrick* et al., 2019a, *Guerrero-Bustamante* et al., 2021). It is likely that there are a multitude of additional bacterial mechanisms influencing phage host ranges.

Prophage-mediated viral defence systems

Interestingly, temperate phages are directly involved in phage host range determination, because some prophages express defence genes that prevent productive infection of other unrelated phages (*Bondy-Denomy* et al., 2016). A multitude of such defence systems have been described in Cluster N phages (*Dedrick* et al., 2017a), but they are also present in other mycobacteriophages (*Gentile* et al., 2019). There are likely many yet to be discovered. Some of these may act by interference with DNA injection and viral exclusion similar to a related system in *E. coli* (*Cumby* et al., 2012, *Dedrick* et al., 2017a), whereas others appear to act after DNA injection has occurred and are mediated by abortive infection (*Dedrick* et al., 2017a). These defence systems are often quite specific for particular phages, and the specificities are unpredictable and need to be experimentally determined. In one notable case, an *M. smegmatis* lysogen carrying a Sbash prophage confers defence to infection by phage Crossroads (*Gentile* et al., 2019). Sbash (Subcluster I2) and Crossroads (Subcluster L2) are unrelated at the sequence level, and remarkably, the Sbash defence does not operate against any of the other eight Subcluster L2 phages tested, all of which are very closely related to Crossroads (*Gentile* et al., 2019). Discovery of such defence systems is only possible by making use of a very large collection of genomically-defined phages isolated on the same bacterial isolate (*M. smegmatis* mc^{2}155), and carefully determining their efficiencies of plating on a lysogenic strain relative to the non-lysogenic parent (*Gentile* et al., 2019).

Exclusion of superinfection in lytic growth

The competition in nature is not just between phages and their bacterial hosts, but also between lytically growing phages. When phages are growing lytically, there is strong selection for optimization of phage production, which can be threatened via superinfection by phage particles that seek to use microbial resources. It is thus no surprise that phages express genes in lytic growth that exclude superinfection by other phages (*Ko* and *Hatfull*, 2018). This can also be surprisingly specific and unpredictable bioinformatically. Experimentally identifying such functions, however, can also be challenging. One approach that has been informative is to use a surrogate assay that simply screens for phage genes that are toxic when expressed in the host (*Ko* and *Hatfull*, 2020). Some such genes act by interfering with the functions of essential host proteins that phages need for productive infection, and are thus toxic when expressed, although toxicity *per se* is irrelevant for phage growth. One good example is gp52 in phage Fruitloop (Subcluster F1). While gp52 is not required for Fruitloop lytic growth, it is expressed early in Fruitloop infection and interacts directly with the essential host

DivIVA (Wag31) protein (*Ko* and *Hatfull*, 2018). Fruitloop gp52 was discovered through a screen for proteins toxic to *M. smegmatis* and its interference with DivIVA specifically prevents infection by unrelated phage Rosebush (Subcluster B1).

A striking feature of phage genomes is the abundance of relatively small genes – leading to an average gene size that is only two-thirds of that of bacterial genes – most of which are of unknown function. What do all these genes do, and why are they present in phage genomes? Where they have been tested, many or most of these are expressed lytically but are not required for lytic growth (*Dedrick* et al., 2013). We suggest that most of these act to influence host-viral dynamics and to determine host range, by conferring defence against viral infection of lysogens, by excluding superinfection by other phages during lytic growth, or by acting in counter-defence against both host- and prophage-encoded defence systems. The specificity of these interactions complicates the untangling of this vast set of interactions, and thwarts simple bioinformatic or machine-learning strategies for predicting host range. But with many hundreds of thousands of phage genes of unknown function – just within the actinobacteriophage genomes – new approaches are needed to address these questions.

THERAPEUTIC POTENTIAL OF MYCOBACTERIOPHAGES

Many *Mycobacterium* pathogens cause diseases that are challenging to treat with antibiotics, including *M. tuberculosis* strains with multiple acquired resistances, and intrinsically resistant nontuberculous Mycobacterium (NTM) infections (*Nick* et al., 2021, *Mirzayev* et al., 2021). There is thus a need for new therapeutic approaches, and it is plausible that mycobacteriophages could contribute to the arsenal of anti-Mycobacterial 'drugs'. However, the opportunities and challenges are quite different for considering the therapeutic use of phages for NTM versus TB infections.

The first therapeutic use of mycobacteriophages

The first therapeutic use of mycobacteriophages was for a paediatric cystic fibrosis (CF) patient who had a disseminated *M. abscessus* infection following a bilateral lung transplant (*Dedrick* et al., 2019a). The infection could not be resolved with antibiotics, was life threatening, and the patient was at home on palliative care. The first challenge was to identify potentially useful phages, as few if any phages had been isolated on any *M. abscessus* strain and attempts at phage discovery using the specific patient strain (GD01) were not productive. A carefully chosen subset of *M. smegmatis* phages (based on the genomic relationships and host range information using *M. tuberculosis*) were then screened against the *M. abscessus* GD01 strain (*Dedrick* et al., 2019a). One lytic phage called Muddy infected GD01, while two others that infected (BPs and ZoeJ) were both temperate, and therefore needed to be engineered to inactivate the repressor gene (*Sampson* et al., 2009, *Dedrick* et al., 2019b). One of them (BPs) only infected *M. abscessus* GD01 at a reduced efficiency of plating, but a host range mutant (HRM) was isolated that efficiently infects the pathogenic strain. A three-phage cocktail was assembled, in the hope that these genomically diverse phages would infect through different

mechanisms such that resistance arising to one phage would not confer resistance to the others. The three phages were amplified, purified, and the cocktail was administered intravenously twice daily at a dose of 10^9 plaque forming units (PFU)/ml. The phages were provided as an adjunct to the ongoing antibiotic regimen. The patient showed substantial improvement, with resolution of an infected node in the liver, healing of the infected sternal transplant wound, and clearance of skin nodules (*Dedrick* et al., 2019a).

Broadening the therapeutic use of mycobacteriophages

Can the therapeutic use of mycobacteriophages be extended to other patients with NTM infections? Addressing this has been facilitated by screening a large series (~250) of clinical isolates for phage susceptibility, learning about these profiles, and providing phages for additional compassionate-use cases where possible (*Dedrick* et al., 2021b, 2022). A key finding is that a substantial proportion (~40%) of the *M. abscessus* isolates have a smooth colony morphotype and no phages have been identified that efficiently infect *and* kill any of these strains. The other ~60% have a rough colony morphotype strains, with at least one potentially therapeutic phage identified for about 75% of these strains. This raises several additional questions. First, because the total number of potentially therapeutically useful different phages that have been identified is only around six to eight, where will additional phages come from? Second, how can we extend the proportion of rough strains that can be treated? Third, if strains are only sensitive to a single phage, can that phage be used without failure due to phage resistance? Fourth, how will phages be discovered with utility for smooth colony strains? Some preliminary answers are emerging for at least some of these questions.

In terms of identifying additional phages that could be used therapeutically, it is helpful to recognize that what is needed are phages that are genomically different to those already available. Such phages could be used in cocktails with extant phages to try and avoid resistance, or may have new tropisms (host preferences) that expand the proportion of rough strains that can be infected and killed. Several approaches to identifying more of these phages are plausible. First, although few phages have been identified using *M. abscessus* as a host for isolation, this warrants substantial additional effort, using a wider range of strains. Second, the current and new phages isolated on *M. smegmatis* may continue to be a fruitful source, especially through the isolation of HRMs that have acquired the ability to efficiently infect one or more of the *M. abscessus* isolates (*Dedrick* et al., 2021b). Finally, there is also the prospect of exploiting the prophages integrated into *M. abscessus* genomes (*Dedrick* et al., 2021a). Many of these appear to be intact and capable of lytic growth, and spontaneously induced particles are present in culture supernatants. The challenge then is to find another *M. abscessus* strain on which these phages can grow lytically and form plaques. An attractive feature of this approach is that ~85% of *M. abscessus* clinical isolates have one or more prophages integrated into their genomes, and some have as many as six prophages (*Dedrick* et al., 2021b). So, there is a wealth of such prophages which constitutes a large resource that can be exploited, and bioinformatic analyses show that these are distinct from the *M. smegmatis* phages (*Dedrick* et al., 2021a, 2021b). Pairwise screening has facilitated lytic growth of

Table 1: Summary of compassionate-use cases of phage treatment of Mycobacterium infections

Patient #	Strain	# phages	Resistance?[1]	Route[2]	Neut?[3]	Outcomes
1	*M. abscessus*	3	No	IV	Y/N/N	Favorable resolution for ~3 years
2	*M. abscessus*	1	No	IV	NT	Conversion to smear-negative
3	*M. abscessus*	2	NT	IV	N	Deceased, organ failure; not phage-related
4	*M. abscessus*	1	NT	IV	NT	Conversion to smear-negative
5	*M. abscessus*	1	No	IV	N	No substantial clinical improvement
6	*M. abscessus*	1	NT	IV	NT	No substantial clinical improvement
7	*M. abscessus*	1	No	IV	N	Conversion to culture negative (rough strain)
8	*M. abscessus*	1	No	IV/Neb	Y	Intermittently smear negative
9	*M. abscessus*	2	NT	IV	N	Favorable resolution
10	*M. abscessus*	2	NT	IV	NT	Conversion to culture/smear negative
11	*M. abscessus*	1	No	IV	Y	Intermittently smear negative
12	*M. abscessus*	3	Partial to one	IV/Neb	Y	Transient improvement only
13	*M. abscessus*	1	No	IV/Neb	N	FEV1 improvement; culture positive
14	*M. abscessus*	2	NT	IV	NT	No substantial clinical improvement
15	*M. abscessus*	2	No	IV	Y	Conversion to culture negative; transplant
16	*M. chelonae*	1	NT	IV	Y	Favorable resolution; negative biopsies
17	*M. avium*	1	NT	IV/Neb	N	FEV1 improvement; culture negative
18	*M. abscessus*	1	No	IV/Neb	Y	Ongoing
19	*M. abscessus*	2	No	IV/Neb	Y	No substantial clinical improvement
20	BCG	3	NT	IV	N	Improved clinically

[1]Resistance was determined by screening bacterial isolates recovered following the start of phage treatment. NT, not tested.
[2]The routes of administration were intravenous (IV) or nebulized (neb), or IV followed subsequently by additional of nebulization (IV/neb).
[3]Neutralization was determined *in vitro*. NT, not tested.

spontaneously induced prophages, and these can then be engineered to prevent lysogeny and potentially added to the therapeutic arsenal. These potential paths towards the expansion of the therapeutic phage bank should expand the number of rough strain morphotype infections that can be treated.

In the first treatment case, a cocktail of three phages was used because of concerns about phage resistance. However, subsequent observations have shown that phage resistance occurs relatively infrequently in *M. abscessus* strains, and resistance has not been encountered when used clinically, even in treatments where only a single phage was deployed (*Dedrick* et al., 2022, 2021b). Some phage-resistant *M. abscessus* mutants have been recovered *in vitro*, but it is not yet clear if these have compromised fitness for *in vivo* growth in the patient (*Dedrick* et al., 2021b).

A consecutive series of 20 compassionate use phage therapies for Mycobacterium infections

To date, a total of 20 case studies have been reported, a majority of which (11/20) show favourable microbiological or clinical indications (*Dedrick* et al., 2022) (Table 1). However, in four cases there was no evident clinical improvement, and in the remaining cases, treatment is either ongoing or incomplete. No adverse reactions were observed, and in several cases strongly favourable outcomes are reported, including resolution of a pulmonary infection in a CF patient (*Nick* et al., 2022), and resolution of a disseminated *Mycobacterium chelonae* cutaneous infection in a mildly immunosuppressed patient (*Little* et al., 2022). Together, these cases provide considerable encouragement for continued exploration of phage treatments for NTM infections, highlighting some opportunities and some limitations. For example, in 11 cases, only a single phage was used because it was the only phage to which the strain was sensitive. In these cases, however, phage resistance was not observed in any case nor was it the cause of treatment failure. Although this was unexpected, it bodes well for future treatments, moderating one of the major concerns in the general therapeutic use of phages (*Dedrick* et al., 2022) (Table 1). It is also noteworthy that these treatments are lengthier (years in some cases) than in phage therapies for other diseases, and these are not trivial interventions. But we know little about optimal dosage, routes of administration, or phage pharmacokinetics, and blinded clinical trials are needed to address these unknowns. Nonetheless, these case studies provide insights into safety and clinical and microbiological parameters to monitor during treatments that are invaluable for trial design and effective implementation (*Dedrick* et al., 2022, *Nick* et al., 2022).

Interestingly, clinical isolates of *M. tuberculosis* have limited genetic diversity and relatively minor variations in phage infections profiles (*Guerrero-Bustamante* et al., 2021). A set of five distinct phages, taking advantage of engineering and isolation of HRMs – as described above for NTMs – constitute a potential cocktail for a broad range of clinical isolates, although only some of these five might ultimately be needed. And subsets of these could be administered sequentially to negate impediments presented by neutralization. However, compassionate use cases of TB that are both suitable for phage treatment and meet regulatory requirements are rare, and initial clinical trials would seem warranted.

SUMMATION

The study of mycobacteriophages has contributed major advances to our understanding of phage diversity, phage biology including expression and regulation, host-virus dynamics, science education, and clinical utility for treating *Mycobacterium* diseases. However, there is still much to learn, to explore, and to discover. Many of the phages have not been investigated beyond genomic characterization and will give new insights when studied in detail. Collectively, there are hundreds of thousands of genes of unknown function and elucidating their roles will require new and higher-throughput strategies. We also know little about host range beyond the inclusion of infection profiles for the pathogens *M. tuberculosis* and *M. abscessus*. Our current understanding of mycobacteriophages is largely based on those isolated using *M. smegmatis* mc^2155 as a host, and it is likely that new and different types of phages would be discovered when using other mycobacterium hosts. We note that this is challenging for very slow growing strains and pathogens, but insights from prophages of sequenced NTM strains suggests there's an abundance of diverse phages awaiting discovery, many of which will add to the arsenal of therapeutic options.

ACKNOWLEDGEMENTS

I am extremely grateful to all members of my research group that have contributed to our understanding of mycobacteriophages over a span of many years. Many thanks to Bekah Dedrick, Dan Russell, and Debbie Jacobs-Sea for comments on the manuscript. This work was supported by funding from National Institutes of Health grant GM131729.

LITERATURE

Bernheim, A. and Sorek, R.: The pan-immune system of bacteria: antiviral defence as a community resource. Nat. Rev. Microbiol. 18, 113-119 (2020).

Bondy-Denomy, J., Qian, J., Westra, E.R., Buckling, A., Guttman, D.S., Davidson, A.R., and Maxwell, K.L.: Prophages mediate defense against phage infection through diverse mechanisms. ISME J. 10, 2854-2866 (2016).

Broussard, G.W. and Hatfull, G.F.: Evolution of genetic switch complexity. Bacteriophage 3, e24186 (2013).

Broussard, G.W., Oldfield, L.M., Villanueva, V.M., Lunt, B.L., Shine, E.E., and Hatfull, G.F.: Integration-dependent bacteriophage immunity provides insights into the evolution of genetic switches. Mol. Cell. 49, 237-248 (2013).

Brown, K.L., Sarkis, G.J., Wadsworth, C., and Hatfull, G.F.: Transcriptional silencing by the mycobacteriophage L5 repressor. EMBO J. 16, 5914-5921 (1997).

Cater, J.C. and Redmond, W.B.: Isolation of and studies on bacteriophage active against mycobacteria. Can. J. Microbiol. 7, 697-703.(1961).

Chen, J., Kriakov, J., Singh, A., Jacobs, W.R., Besra, G.S., and Bhatt, A.: Defects in glycopeptidolipid biosynthesis confer phage I3 resistance in Mycobacterium smegmatis. Microbiology 155, 4050-4057 (2009).

Cumby, N., Edwards, A.M., Davidson, A.R., and Maxwell, K.L.: The bacteriophage

HK97 gp15 moron element encodes a novel superinfection exclusion protein. J. Bacteriol. 194, 5012-5019 (2012).

Dedrick, R.M., Marinelli, L.J., Newton, G.L., Pogliano, K., Pogliano, J., and Hatfull, G.F.: Functional requirements for bacteriophage growth: gene essentiality and expression in mycobacteriophage Giles. Mol. Microbiol. 88: 577-589 (2013).

Dedrick, R.M., Jacobs-Sera, D., Bustamante, C.A., Garlena, R.A., Mavrich, T.N., Pope, W.H., Reyes, J.C., Russell, D.A., Adair, T., Alvey, R., Bonilla, J.A., Bricker, J.S., Brown, B.R., Byrnes, D., Cresawn, S.G., Davis, W.B., Dickson, L.A., Edgington, N.P., Findley, A.M., Golebiewska, U., Grose, J.H., Hayes, C.F., Hughes, L.E., Hutchison, K.W., Isern, S., Johnson, A.A., Kenna, M.A., Klyczek, K.K., Mageeney, C.M., Michael, S.F., Molloy, S.D., Montgomery, M.T., Neitzel, J., Page, S.T., Pizzorno, M.C., Poxleitner, M.K., Rinehart, C.A., Robinson, C.J., Rubin, M.R., Teyim, J.N., Vazquez, E., Ware, V.C., Washington, J., and Hatfull, G.F.: Prophage-mediated defence against viral attack and viral counter-defence. Nat. Microbiol. 2, 16251 (2017a).

Dedrick, R.M., Mavrich, T.N., Ng, W.L., and Hatfull, G.F.: Expression and evolutionary patterns of mycobacteriophage D29 and its temperate close relatives. BMC Microbiol. 17, 225 (2017b).

Dedrick, R., Guerrero Bustamante, C., Garlena, R.A., Russell, D.A., Ford, K., Harris, K., Gilmour, K.C., Soothill, J., Jacobs-Sera, D., Schooley, R.T., Hatfull, G.F., and Spencer, H.: Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant Mycobacterium abscessus. Nat. Med. 25, 730-733 (2019a).

Dedrick, R.M., Guerrero Bustamante, C.A., Garlena, R.A., Pinches, R.S., Cornely, K., and Hatfull, G.F.: Mycobacteriophage ZoeJ: A broad host-range close relative of mycobacteriophage TM4. Tuberculosis (Edinb.) 115, 14-23 (2019b).

Dedrick, R.M., Aull, H.G., Jacobs-Sera, D., Garlena, R.A., Russell, D.A., Smith, B.E., Mahalingam, V., Abad, L., Gauthier, C.H., and Hatfull, G.F.: The Prophage and Plasmid Mobilome as a Likely Driver of Mycobacterium abscessus Diversity. mBio. 12, e03441-20 (2021a).

Dedrick, R.M., Smith, B.E., Garlena, R.A., Russell, D.A., Aull, H.G., Mahalingam, V., Divens, A.M., Guerrero-Bustamante, C.A., Zack, K.M., Abad, L., Gauthier, C.H., Jacobs-Sera, D., and Hatfull, G.F.: Mycobacterium abscessus Strain Morphotype Determines Phage Susceptibility, the Repertoire of Therapeutically Useful Phages, and Phage Resistance. mBio. 12, e03431-20 (2021b).

Dedrick, R.M., B.E. Smith, M. Cristinziano, K.G. Freeman, D. Jacobs-Sera, Y. Belessis, A.W. Brown, K.A. Cohen, R.M. Davidson, D. van Duin, A. Gainey, C.B. Garcia, C.R.B. George, G. Haidar, W. Ip, J. Iredell, A. Khatami, J.S. Little, K. Malmivaara, B.J. McMullan, D.E. Michalik, A. Moscatelli, J.A. Nick, M.G. Tupayachi Ortiz, H.M. Polenakovik, P.D. Robinson, M. Skurnik, D.A. Solomon, J. Soothill, H. Spencer, P. Wark, A. Worth, R.T. Schooley, C.A. Benson, and G.F. Hatfull, (2022) Phage Therapy of Mycobacterium Infections: Compassionate-use of Phages in Twenty Patients with Drug-Resistant Mycobacterial Disease. Clin. Infect. Dis., ciac453, Epub ahead of print (2022).

Doke, S.: Studies on mycobacteriophages and lysogenic mycobacteria. J. Kumamoto Med. Soc. 34, 1360-1373 (1960).

Donnelly-Wu, M.K., Jacobs, Jr., W.R., and Hatfull, G.F.: Superinfection immunity of mycobacteriophage L5: applications for genetic transformation of mycobacteria. Mol. Microbiol. 7, 407-417 (1993).

Ford, M.E., Sarkis, G.J., Belanger, A.E., Hendrix, R.W., and Hatfull, G.F.: Genome structure of mycobacteriophage D29: implications for phage evolution. J. Mol. Biol. 279, 143-164 (1998).

Gentile, G.M., Wetzel, K.S., Dedrick, R.M., Montgomery, M.T., Garlena, R.A., Jacobs-Sera, D., and Hatfull, G.F.: More Evidence of Collusion: a New Prophage-Mediated Viral Defense System Encoded by

Mycobacteriophage Sbash. mBio. 10, e00196-19 (2019).
Good, R.C. and Mastro, T.D.: The modern mycobacteriology laboratory. How it can help the clinician. Clin. Chest Med. 10, 315-322 (1989).
Guerrero-Bustamante, C.A., Dedrick, R.M., Garlena, R.A., Russell, D.A., and Hatfull, G.F.: Toward a Phage Cocktail for Tuberculosis: Susceptibility and Tuberculocidal Action of Mycobacteriophages against Diverse Mycobacterium tuberculosis Strains. mBio 12, e00973-21 (2021).
Hampton, H.G., Watson, B.N.J., and Fineran, P.C.: The arms race between bacteria and their phage foes. Nature 577, 327-336 (2020).
Hanauer, D.I., Graham, M.J., Sea, P., Betancur, L., Bobrownicki, A., Cresawn, S.G., Garlena, R.A., Jacobs-Sera, D., Kaufmann, N., Pope, W.H., Russell, D.A., Jacobs, Jr., W.R., Sivanathan, V., Asai, D.J., and Hatfull, G.F.: An inclusive Research Education Community (iREC): Impact of the SEA-PHAGES program on research outcomes and student learning. Proc. Natl. Acad. Sci. USA 114, 13531-13536 (2017).
Hanauer, D.I., Jacobs-Sera, D., Pedulla, M.L., Cresawn, S.G., Hendrix, R.W., and Hatfull, G.F: Inquiry learning. Teaching scientific inquiry. Science 314, 1880-1881 (2006).
Hatfull, G.F.: Mycobacteriophages. In: The Bacteriophages (Ed.: Calendar, R.). Oxford University Press, New York, NY, 602-620 (2006).
Hatfull, G.F.: Dark Matter of the Biosphere: the Amazing World of Bacteriophage Diversity. J. Virol. 89, 8107-8110 (2015a).
Hatfull, G.F.: Innovations in Undergraduate Science Education: Going Viral. J Virol. 89, 8111-8113 (2015b).
Hatfull, G.F.: Actinobacteriophages: Genomics, Dynamics, and Applications. Annu. Rev. Virol. 7, 37-61 (2020).
Hatfull, G.F.: Mycobacteriophages: From Petri Dish to Patient. PLoS Pathog. 18, e1010602 (2022).
Hatfull, G.F. and Sarkis, G.J.: DNA sequence, structure and gene expression of mycobacteriophage L5: a phage system for mycobacterial genetics. Mol. Microbiol. 7, 395-405 (1993).
Hatfull, G.F. and Hendrix, R.W.: Bacteriophages and their Genomes. Curr. Opin. Virol. 1, 298-303 (2011).
Hatfull, G.F., Pedulla, M.L., Jacobs-Sera, D., Cichon, P.M., Foley, A., Ford, M.E., Gonda, R.M., Houtz, J.M., Hryckowian, A.J., Kelchner, V.A., Namburi, S., Pajcini, K.V., Popovich, M.G., Schleicher, D.T., Simanek, B.Z., Smith, A.L., Zdanowicz, G.M., Kumar, V., Peebles, C.L., Jacobs, Jr., W.R., Lawrence, J.G., and Hendrix, R.W.: Exploring the mycobacteriophage metaproteome: phage genomics as an educational platform. PLoS Genet. 2, e92 (2006).
Hatfull, G.F., Jacobs-Sera, D., Lawrence, J.G., Pope, W.H., Russell, D.A., Ko, C.C., Weber, R.J., Patel, M.C., Germane, K.L., Edgar, R.H., Hoyte, N.N., Bowman, C.A., Tantoco, A.T., Paladin, E.C., Myers, M.S., Smith, A.L., Grace, M.S., Pham, T.T., O'Brien, M.B., Vogelsberger, A.M., Hryckowian, A.J., Wynalek, J.L., Donis-Keller, H., Bogel, M.W., Peebles, C.L., Cresawn, S.G., and Hendrix, R.W.: Comparative Genomic Analysis of 60 Mycobacteriophage Genomes: Genome Clustering, Gene Acquisition, and Gene Size. J. Mol. Biol. 397, 119-143 (2010).
He, L., Fan, X., and Xie, J.: Comparative genomic structures of Mycobacterium CRISPR-Cas. J. Cell. Biochem. 113, 2464-2473 (2012).
Hendrix, R.W.: Bacteriophages: evolution of the majority. Theor. Popul. Biol. 61, 471-480 (2002).
Hendrix, R.W., Smith, M.C., Burns, R.N., Ford, M.E., and Hatfull, G.F.: Evolutionary relationships among diverse bacteriophages and prophages: all the world's a phage. Proc. Natl. Acad. Sci. USA 96, 2192-2197 (1999).
Huson, D.H.: SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics 14, 68-73 (1998).
Jacobs, Jr., W.R.: *Mycobacterium tuberculosis*: A Once Genetically Intractable Organism.

In: Molecular Genetics of the Mycobacteria. (Eds.: Hatfull, G.F. and Jacobs Jr., W.R.). ASM Press, Washington, DC, 1-16 (2000).

Jacobs-Sera, D., Marinelli, L.J., Bowman, C., Broussard, G.W., Guerrero Bustamante, C., Boyle, M.M., Petrova, Z.O., Dedrick, R.M., Pope, W.H., Science Education Alliance Phage Hunters Advancing Genomics And Evolutionary Science Sea-Phages Program; Modlin, R.L., Hendrix, R.W., and Hatfull, G.F.: On the nature of mycobacteriophage diversity and host preference. Virology 434, 187-201 (2012).

Jordan, T.C., Burnett, S.H., Carson, S., Caruso, S.M., Clase, K., DeJong, R.J., Dennehy, J.J., Denver, D.R. , Dunbar, D., Elgin, S.C., Findley, A.M., Gissendanner, C.R., Golebiewska, U.P., Guild, N., Hartzog, G.A., Grillo, W.H., Hollowell, G.P., Hughes, L.E., Johnson, A., King, R.A., Lewis, L.O., Li, W., Rosenzweig, F., Rubin, M.R., Saha, M.S., Sandoz, J., Shaffer, C.D., Taylor, B., Temple, L., Vazquez, E., Ware, V.C., Barker, L.P., Bradley, K.W., Jacobs-Sera, D., Pope, W.H., Russell, D.A., Cresawn, S.G., Lopatto, D., Bailey C.P., and Hatfull, G.F.: A broadly implementable research course in phage discovery and genomics for first-year undergraduate students. mBio. 5, e01051-13 (2014).

Kim, A.I., Ghosh, P., Aaron, M.A., Bibb, L.A., Jain, S., and Hatfull, G.F.: Mycobacteriophage Bxb1 integrates into the Mycobacterium smegmatis groEL1 gene. Mol. Microbiol. 50, 463-473 (2003).

Ko, C.C. and Hatfull, G.F.: Mycobacteriophage Fruitloop gp52 inactivates Wag31 (DivIVA) to prevent heterotypic superinfection. Mol. Microbiol. 108, 443-460 (2018).

Ko, C.C. and Hatfull, G.F.: Identification of mycobacteriophage toxic genes reveals new features of mycobacterial physiology and morphology. Sci. Rep. 10, 14670 (2020).

Lee, M.H., Pascopella, L., Jacobs, Jr., W.R., and Hatfull, G.F.: Site-specific integration of mycobacteriophage L5: integration-proficient vectors for Mycobacterium smegmatis, Mycobacterium tuberculosis, and bacille Calmette-Guerin. Proc. Natl. Acad. Sci. USA 88, 3111-3115 (1991).

Lewis, J.A. and Hatfull, G.F.: Control of directionality in integrase-mediated recombination: examination of recombination directionality factors (RDFs) including Xis and Cox proteins. Nucleic Acids Res. 29, 2205-2216 (2001).

Little, J.S., Dedrick, R.M., Freeman, K.G., Cristinziano, M., Smith, B.E., Benson, C.A., Jhaveri, T.A., Baden, L.R., Solomon, D.A., and Hatfull, G.F.: Bacteriophage treatment of disseminated cutaneous Mycobacterium chelonae infection. Nat. Commun. 13, 2313 (2022).

Mavrich, T.N. and Hatfull, G.F.: Bacteriophage evolution differs by host, lifestyle and genome. Nat. Microbiol. 2, 17112 (2017).

Mirzayev, F., Viney, K., Linh, N.N., Gonzalez-Angulo, L., Gegia, M., Jaramillo, E., Zignol, M. and Kasaeva, T.: World Health Organization recommendations on the treatment of drug-resistant tuberculosis, 2020 update. Eur. Respir. J. 57, 2003300 (2021).

Mizuguchi, Y.: Mycobacteriophages. In: The Mycobacteria: A Sourcebook (Eds.: Kubica, G.P. and Wayne, L.G.). Marcel Dekker, Inc., New York, 641-662 (1984).

Murohashi, T., Tokunaga, T., Mizuguchi, Y., and Maruyama, Y.: Phage Typing of Slow-Growing Mycobacteria. Am. Rev. Respir. Dis. 88, 664-669 (1963).

Nesbit, C.E., Levin, M.E., Donnelly-Wu, M.K., and Hatfull, G.F.: Transcriptional regulation of repressor synthesis in mycobacteriophage L5. Mol. Microbiol. 17, 1045-1056 (1995).

Nick, J.A., Daley, C.L., Lenhart-Pendergrass, P.M., and Davidson, R.M.: Nontuberculous mycobacteria in cystic fibrosis. Curr. Opin. Pulm. Med. 27, 586-592 (2021).

Nick, J.A., Dedrick, R.M., Gray, A.L., Vladar, E.K., Smith, B.E., Freeman, K.G., Malcolm, K.C., Epperson, L.E., Hasan, N.A., Hendrix, J., Callahan, K., Walton, K., Vestal, B., Wheeler, E., Rysavy, N.M., Poch, K., Caceres, S., Lovell, V.K., Hisert,

K.B., de Moura, V.C., Chatterjee, D., De, P., Weakly, N., Martiniano, S.L., Lynch, D.A., Daley, C.L., Strong, M., Jia, F., Hatfull, G.F., and Davidson, R.M.: Host and pathogen response to bacteriophage engineered against Mycobacterium abscessus lung infection. Cell 185, 1860-1874 (2022).

Payne, K., Sun, Q., Sacchettini, J., and Hatfull, G.F.: Mycobacteriophage Lysin B is a novel mycolylarabinogalactan esterase. Mol. Microbiol. 73, 367-381 (2009).

Payne, K.M. and Hatfull, G.F.: Mycobacteriophage endolysins: diverse and modular enzymes with multiple catalytic activities. PLoS One 7, e34052 (2012).

Pedulla, M.L., Ford, M.E., Houtz, J.M., Karthikeyan, T., Wadsworth, C., Lewis, J.A., Jacobs-Sera, D., Falbo, J., Gross, J., Pannunzio, N.R., Brucker, W., Kumar, V., Kandasamy, J., Keenan, L., Bardarov, S., Kriakov, J., Lawrence, J.G., Jacobs, W.R., Hendrix, R.W., and Hatfull, G.F.: Origins of highly mosaic mycobacteriophage genomes. Cell 113: 171-182 (2003).

Pope, W.H., Bowman, C.A., Russell, D.A., Jacobs-Sera, D., Asai, D.J., Cresawn, S.G., Jacobs, W.R., Hendrix, R.W., Lawrence, J.G., Hatfull, G.F., Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science; Phage Hunters Integrating Research and Education; and Mycobacterial Genetics Course: Whole genome comparison of a large collection of mycobacteriophages reveals a continuum of phage genetic diversity. Elife 4, e06416 (2015).

Pope, W.H., Mavrich, T.N., Garlena, R.A., Guerrero-Bustamante, C.A., Jacobs-Sera, D., Montgomery, M.T., Russell, D.A., Warner, M.H., Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES); and Hatfull, G.F.: Bacteriophages of *Gordonia* spp. Display a Spectrum of Diversity and Genetic Relationships. mBio. 8, e01069-17 (2017).

Ptashne, M.: A Genetic Switch. Blackwel Science Ltd. and Cell Press, Oxford/Cambridge (1987).

Sampson, T., Broussard, G.W., Marinelli, L.J., Jacobs-Sera, D., Ray, M., Ko, C.C., Russell, D., Hendrix, R.W., and Hatfull, G.F.: Mycobacteriophages BPs, Angel and Halo: comparative genomics reveals a novel class of ultra-small mobile genetic elements. Microbiology 155, 2962-2977 (2009).

Shankar, S. and Tyagi, A.K.: MchAI and MchAII, two class-II restriction endonucleases from Mycobacterium chelonei. Gene 132, 119-123 (1993a).

Shankar, S. and Tyagi, A.K.: Purification and characterization of restriction endonuclease MgoI from Mycobacterium gordonae. Gene 131, 153-154 (1993b).

Snider, Jr., D.E., Jones, W.D., and Good, R.C.: The usefulness of phage typing Mycobacterium tuberculosis isolates. Am. Rev. Respir. Dis. 130, 1095-1099 (1984).

Takeya, K., Koike, M., Mori, R., and Toda, T.: Light and electron microscope studies of mycobacterium--mycobacteriophage interactions. III. Further studies on the ultrathin sections. J. Biophys. Biochem. Cytol. 11, 441-447 (1961).

Takeya, K., Koike, M., Mori, R., Yuda, Y., and Toda, T.: Light and electron microscope studies of Mycobacterium-mycobacteriophage interactions. II. Electron microscope studies. J. Bacteriol. 78, 313-319 (1959a).

Takeya, K., Mori, R., Nakashima, N., Koike, M., and Toda, T.: Light and electron microscope studies of Mycobacterium-mycobacteriophage interactions. I. Light microscope studies. J. Bacteriol. 78, 307-312 (1959b).

Wetzel, K.S., Aull, H.G., Zack, K.M., Garlena, R.A., and Hatfull, G.F.: Protein-Mediated and RNA-Based Origins of Replication of Extrachromosomal Mycobacterial Prophages. mBio. 11, e00385-20 (2020).