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Reflections

Broadscale phage therapy is unlikely to select for widespread evolution of bacterial resistance to virus infection

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Abstract

Multi-drug resistant bacterial pathogens are alarmingly on the rise, signaling that the golden age of antibiotics may be over. Phage therapy is a classic approach that often employs strictly lytic bacteriophages (bacteria-specific viruses that kill cells) to combat infections. Recent success in using phages in patient treatment stimulates greater interest in phage therapy among Western physicians. But there is concern that widespread use of phage therapy would eventually lead to global spread of phage-resistant bacteria and widespread failure of the approach. Here, we argue that various mechanisms of horizontal genetic transfer (HGT) have largely contributed to broad acquisition of antibiotic resistance in bacterial populations and species, whereas similar evolution of broad resistance to therapeutic phages is unlikely. The tendency for phages to infect only particular bacterial genotypes limits their broad use in therapy, in turn reducing the likelihood that bacteria could acquire beneficial resistance genes from distant relatives via HGT. We additionally consider whether HGT of clustered regularly interspaced short palindromic repeats (CRISPR) immunity would thwart generalized use of phages in therapy, and argue that phage-specific CRISPR spacer regions from one taxon are unlikely to provide adaptive value if horizontally-transferred to other taxa. For these reasons, we conclude that broadscale phage therapy efforts are unlikely to produce widespread selection for evolution of bacterial resistance.

Key words: antibiotic resistance; bacteriophage; CRISPR; horizontal genetic transfer; fitness trade-off.

The development of antibiotics in the mid-20th century showed promise to combat every bacterial pathogen that had ever plagued humanity. However, owing to the penchant for bacteria to evolve antibiotic resistance, as well as declining interest from the pharmaceutical industry in developing new antibiotics (Fair and Tor 2014), there is justifiable worry that we are reaching the end of the antibiotic era. Indeed, at least 35,000 and perhaps as many as 160,000 people die each year in the USA from bacterial pathogens that have become resistant to antibiotic treatment (Burnham, Olsen and Kollef 2019; CDC 2013, 2019); yearly deaths are expected to rise to ten million globally by 2050 (Review on Antimicrobial Resistance 2016).

Phage therapy (Chan et al. 2018; Kortright et al. 2019; Sulakvelidze and Morris 2015; Turner 2019) is one of several approaches (Pieren and Tigges 2012; Worthington and Melander 2013) being developed to extend our capacity to control bacterial infections. Originally developed by Felix d'Herelle (d'Herelle 1917; Kortright et al. 2019) in the early 20th century, phage therapy typically utilizes strictly lytic bacteriophages (bacteria-specific viruses that kill cells) as natural enemies of bacteria to fight infectious pathogens. With the development of broad-spectrum antibiotics, Western microbiologists abandoned phage therapy in the mid-century. However, through most of the 20th century Eastern European microbiologists made

progress toward harnessing phages to control bacterial infections (Abedon 2017; Housby and Mann 2009), and Western medicine shows recent renewed interest in phage therapy.

Early on in phage research, bacteria were found capable of evolving resistance to therapeutic phages (Summers 2012), although the underlying mechanisms were poorly known. The breadth of bacterial mechanisms of phage resistance are reviewed elsewhere (Hyman and Abedon 2010; Labrie, Samson, and Moineau 2010; Seed 2015; van Houte, Buckling, and Westra 2016). The mechanisms include reductions in phage adsorption (binding to cells), such as loss of phage receptor molecules on hosts, and physical barriers (e.g. capsules) that shield receptors, as well as post-adsorption resistance mechanisms, such as restriction endonucleases that broadly target various phage types, uptake blocks that prevent phage genomes from reaching the cytoplasm and clustered regularly interspaced short palindromic repeats (CRISPR) immunity, which targets phages containing nucleotide sequences that the bacterium has already encountered (Hyman and Abedon 2010). In contrast, abortive infections are phage-exclusion mechanisms that prevent phage infection, but the bacterial host dies too; here, anti-phage intracellular proteins target one or more key steps in phage multiplication, such as transcription, protein synthesis, maturation and host cell lysis (Labrie, Samson, and Moineau 2010).

Indeed, bacteria have brought multiple mechanisms of resistance to bear against therapeutic phages, including altering cell-surface receptors utilized by phages, hindering phage access to these binding targets through greater extracellular matrix (Holst Sørensen et al. 2012), and producing competitive inhibitors (Oechslin 2018). Nevertheless, bacterial resistance to phage attack does not necessarily prevent the patient's recovery, as resistance frequently incurs fitness trade-offs for the bacteria, in particular reducing bacterial virulence against their human hosts (León and Bastías 2015; Nesper et al. 2000; Oechslin et al. 2017; Oechslin 2018). Interestingly, treating a bacterium with phages *ex vivo* has proven a reliable way to produce avirulent mutant bacteria, which can then be used to immunize against the wild type virulent bacteria (Capparelli et al. 2010).

Until recently, phages were chosen for therapy based solely on their ability to kill a target bacterium (Chan et al. 2018; Turner 2019), but in a new *cost-directed* phage therapy, phages are chosen so that the inevitable evolution of phage resistance will be particularly costly for the bacteria (Gurney et al. 2020; Kortright et al. 2019). Here, phages are chosen so that evolution of phage resistance renders the bacteria sensitive to traditional antibiotics (Chan et al. 2018; Schmidt 2019; Turner 2019). For example, a therapeutic phage that binds to the outermost protein of efflux pumps (protein complexes in some bacterial species that can actively remove various antibiotics from the cell) should be doubly effective as a treatment option. Here, the lytic phage kills susceptible bacteria, while also exerting selection pressure for evolution of phage resistance that causes a mutational change (or loss) of the protein, potentially driving a genetic trade-off that compromises efflux-pump function by impairing bacterial ability to remove antibiotics from the cell (Burmeister et al. 2020; Chan et al. 2016). This could involve a phage directly selecting for evolved pleiotropic changes in a receptor gene. Alternatively, a phage could indirectly cause resistance by selecting for a mutation in another gene that interacts with a receptor gene in a bacterial gene network (Kortright, Chan, and Turner 2020). Regardless of the underlying mechanistic details, such cost-directed phage therapy, synergistically combined with antibiotics, has shown both safety and efficacy in emergency treatment of patients with otherwise untreatable and sometimes chronic infections (Chan et al. 2018; Turner 2019). Other developments in phage therapy have also shown promise, including engineering phages in order to more effectively combat target bacteria, and treating infections using cocktails (mixtures) of several phages administered simultaneously (Dedrick et al. 2019; Gu et al. 2012; O'Flynn et al. 2004; Schooley et al. 2017).

Nevertheless, there is concern that widespread use of phage therapy would eventually lead to the problem seen today with traditional antibiotic therapy. That is, the global capacity for bacteria to evolve extensive phage resistance might cause massive failure of the therapeutic approach (Nilsson 2014; Nobrega et al. 2015; Ormälä and Jalasvuori 2013). This concern is a reminder that it is crucial to characterize phage candidates intended for therapy, to better understand how their interactions with target bacteria can fail due to existing resistance mechanisms, and via changes in evolved bacterial defenses caused by phage selection (Casey, van Sinderen, and Mahony 2018; Torres-Barcelo 2018).

One possibility is to avoid the potential widespread-failure problem by limiting phage therapy to a personalized-medicine approach, such as matching an individual phage or a cocktail to the patient's strain, and altering the phage(s) used in therapy if/when the target bacteria evolve resistance to treatment (Rohde et al. 2018). Of course, a personalized approach to phage therapy is necessarily more time-consuming than a widespread tactic, and one goal of phage therapy could be the efficient dissemination of broadly approved general treatments that could be administered to large numbers of individuals in patient populations. Here, we argue that bacterial evolution is unlikely to thwart phage therapy, even if it were implemented as a broad scale generalized approach (Ormälä and Jalasvuori 2013).

Bacterial pathogens have defeated antibiotic therapy largely through various mechanisms of horizontal genetic transfer (HGT), whereby they acquire resistance genes and mechanisms that originally evolved within other populations or species (Dahlberg and Chao 2003; Davies 1994; Knöppel et al. 2014; San Millan et al. 2015; van Hoek et al. 2011). Antibiotic therapy has enabled HGT because the vast majority of antibiotic compounds are broad-spectrum, with capability of targeting pathogens within both the Gram-positive and Gram-negative bacteria. Therefore, a given pathogen may acquire a resistance gene from potentially *any bacterium in any phylum* that has managed to evolve resistance (Fair and Tor 2014; Sulakvelidze and Morris 2015). With such a large range of potential donors of resistance, for any broad-spectrum antibiotic there is apparently a reasonable chance that some bacterial population or species in the world has evolved a low-cost means of resistance that can be transferred to other bacteria. Such low-cost resistance mechanisms include decreased drug accumulation, either by actively removing antibiotics via efflux or by reducing permeability of the cell to passive drug-entry, and provision of detoxification enzymes and novel altered targets (Dahlberg and Chao 2003; Davies 1994; Knöppel et al. 2014; San Millan et al. 2015; van Hoek et al. 2011).

One approach to limiting horizontal transfer of resistance factors is to use narrow-spectrum antibiotics. For a given narrow-spectrum antibiotic, the scope of potential donors of resistance is much narrower than for a broad-spectrum antibiotic (Melander, Zurawski, and Melander 2018). Nevertheless, these compounds can still target a breadth of closely related bacterial species and can even target a genus of bacteria (Melander, Zurawski, and Melander 2018).

In comparison to therapy through either broad- or narrow-spectrum antibiotics, bacteria should be less likely to acquire resistance to phages through HGT, at least from distantly related genotypes and species. This is because phages can be extremely narrow in the range of bacteria they can infect (Hyman and Abedon 2010). A phage's host range stems from the intimate association required between a phage and one or more protein receptors to which it binds, in contrast to antibiotics, which usually enter a cell by permeating the cellular membrane in a less discriminate way (Rakhuba et al. 2010). Thus, it is improbable that individual phages can be discovered or engineered to cover a very broad genotypic space even within one target bacterial species. It is even less likely that individual phages could serve as broad-spectrum killers of bacteria (Ho 2001; Nilsson 2014; Nobrega et al. 2015; Ormälä and Jalasvuori 2013).

Therefore, bacterial variants should be expected to evolve highly specific resistance genes or mechanisms for a given therapeutic phage, which should not be especially useful (and therefore not strongly positively selected) for horizontal spread to other bacteria. To the extent that phage resistance can be acquired by horizontal transfer, as seen in *Prochlorococcus*, the range of donors of resistance for a particular strain appear phylogenetically limited to close relatives (Avrani et al. 2011). With such limited access to useful resistance via HGT, evolution of phage resistance in target bacteria should be generally limited to mutations in existing chromosomal genes in the bacterium's genome (Koskella and Brockhurst 2014).

In the case of antibiotics, mutations in a bacterium's native genes are usually more costly than horizontally acquired resistance, such as those residing on extrachromosomal accessory elements, especially conjugative plasmids (Dahlberg and Chao 2003; Davies 1994; Knöppel et al. 2014; San Millan et al. 2015; van Hoek et al. 2011). This is because chromosomal resistance mutations frequently occur as molecular changes in the targets of antibiotics, and these targets are generally highly conserved structures that are central to bacterial metabolism, for example, the ribosomes and nucleic acid polymerases (Munita and Arias 2016). Changes in these target sites often come at a high cost by reducing bacterial growth rate (Chen et al. 2013; Cohan, King, and Zawadzki 1994; Gagneux et al. 2006; Johnsen et al. 2009; Melnyk, Wong, and Kassen 2015; Stickland et al. 2010). These *de novo* evolved resistance mechanisms, because of how intrinsically costly they tend to be, are in fact less likely to be the cause of widespread antibiotic resistance compared to resistance mechanisms that are horizontally obtained (Vogwill and MacLean 2015). Moreover, an altered target site in a highly conserved molecule, e.g. a ribosomal protein is unlikely to be compatible with the interacting molecules of a distantly related donor (e.g. the rest of the ribosome).

Likewise, resistance to phages through altering the target site may reduce a bacterium's fitness (Alseth et al. 2019; Burmeister et al. 2020). This is especially likely in the case of cost-directed phage therapy because the approach is designed to select for evolution of phage resistance that coincides with a trade-off causing lowered virulence and/or re-sensitization to antibiotics, which are both highly costly for the bacteria in clinically relevant environments.

Nevertheless, one mode of phage resistance may possibly come at low cost and does not require genetic transfer from other organisms. This involves the widely distributed CRISPR immunity system, in which a CRISPR locus in a bacterium can obtain a 'spacer' DNA sequence from an infecting phage (Bondy-Denomy and Davidson 2014; Touchon et al. 2012). Incorporation of this spacer gives the CRISPR locus the capacity to kill any subsequent phage that contains that DNA sequence (Mojica et al. 2005). While the marginal cost of adding defense (i.e. the spacer) against one more phage is minimal (Vale et al. 2015), only about half of bacterial species have CRISPR loci and many bacterial pathogens lack this defense entirely (Karimi et al. 2018), suggesting that CRISPR-based immunity is not universally positively selected in bacteria. Perhaps this is due to the high fitness costs of expressing the Cas killer protein of the CRISPR system (Vale et al. 2015), evolution of CRISPR resistance in phages that makes the defense ineffective (Marraffini and Sontheimer 2010; Pawluk, Davidson, and Maxwell 2018), and selection against CRISPR immunity due to the benefits of acquiring mutualistic plasmids that would otherwise be targeted by these defense mechanisms (O'Meara and Nunney 2019). The latter is a reminder that CRISPR systems may be costly in certain environmental contexts, owing to the evolutionary ecology of bacteria interactions with infectious elements that can be positively versus negatively selected. Elsewhere, van Houte, Buckling, and Westra (2016) review the entirety of known defense strategies that have evolved to protect bacteria against phage attack, and highlight plausible short- and long-term costs associated with each of these mechanisms, depending on the ecological contexts faced by bacteria. For brevity, we next consider only CRISPR immunity, and whether this defense strategy could spread in bacteria to undermine broadly applied phage therapy.

The possibility exists for CRISPR-based phage resistance to transfer widely across bacteria, as has occurred for antibiotic resistance. CRISPR loci have been found on large plasmids that have moved across distantly related bacteria, spreading the CRISPR immunity system widely throughout the prokaryotes (Godde and Bickerton 2006). Moreover, CRISPR activity may promote HGT by preventing an infection from going to completion (killing the cell), thus allowing any host genes that were accidentally acquired previously by the phage to recombine into an infected cell. The CRISPR activity can even promote the transduction of a previous host's CRISPR system into an infected cell (Watson, Staals, and Fineran 2018). Because phages are known to occasionally yield transduction of adaptive genes across genera (Chen and Novick 2009), there is the possibility of wide transmission of CRISPR loci.

However, we argue that while the CRISPR-Cas system may be transportable across divergent bacteria, the phage-specific CRISPR spacer regions from one taxon are not likely to render any adaptive value when they land in another taxon. This expectation logically follows from the implicit assumption that phages specifically infecting different host species are sufficiently diverged that they are unlikely to share the same spacer sequences that dictate CRISPR immune recognition. Therefore, owing to the host specificity of phage, horizontal transfers of complete CRISPRs from distant relatives are unlikely to contain the phage-targeting DNA spacers that would upset therapeutic efforts.

Bacteria have evolved various mechanisms to thwart phage (Hyman and Abedon 2010; Labrie, Samson, and Moineau 2010; Seed 2015), presumably because both broad and narrow types of defenses (e.g. restriction enzymes, CRISPRs) are insufficient to protect bacteria against the diversity of phages encountered in nature, and due to the costs of the various mechanisms changing according to differing ecological contexts (van Houte, Buckling, and Westra 2016). Relatedly, it could be argued that administered phage therapy would necessarily occur in the presence of a potentially diverse and uncharacterized microbial community (the microbiome), where the possibility exists for

the target bacteria to be selected to resist both the therapeutic phage(s) and those residing within the patient. Would broadly-administered phage(s) combined with this unknown phage-community complexity change the selection exerted against bacteria, to cause widespread phage resistance? This prospect seems unlikely, given that mechanisms for pan-resistance to phages have not evolved in species of opportunistic bacterial pathogens, which are generally successful at infecting hosts such as humans that harbor species-diverse microbiomes.

Cost-directed phage therapy offers an efficient, effective and long-lasting approach to combating the growing challenges of antibiotic resistance. In addition, we note that all phages (unlike antibiotics) are biological entities capable of undergoing evolution by natural selection. Thus, widespread use of phage therapy should be less vulnerable to failure because phages themselves show an impressive capacity to coevolve strategies to counter bacterial defenses (Bondy-Denomy and Davidson 2014; Pawluk et al. 2018). Moreover, these various mechanisms that foster phage ability to persist often constitute novel discoveries occurring alongside increased characterization of the impressive and largely-undescribed phage biodiversity on earth (Seed et al. 2013). Relatedly, phages with naturally evolved counter-defense mechanisms might be particularly useful in phage therapy, or these defenses could be successfully engineered into phages which lack them. For the above reasons, we conclude that broadscale therapies harnessing cost-directed and other useful phages are unlikely to echo the unfortunate widespread evolution of resistance to antibiotics, which hampers current treatment efforts.

Conflict of interest: P.E.T. discloses a financial interest in Felix Biotechnology, a phage therapeutic company. Also, P.E.T. is an Advisor to Nextbiotics, Inc.

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