

# Temperate phages both mediate and drive adaptive evolution in pathogen biofilms

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Temperate phages drive genomic diversification in bacterial pathogens. Phage-derived sequences are more common in pathogenic than nonpathogenic taxa and are associated with changes in pathogen virulence. High abundance and mobilization of temperate phages within hosts suggests that temperate phages could promote within-host evolution of bacterial pathogens. However, their role in pathogen evolution has not been experimentally tested. We experimentally evolved replicate populations of *Pseudomonas aeruginosa* with or without a community of three temperate phages active in cystic fibrosis (CF) lung infections, including the transposable phage,  $\phi 4$ , which is closely related to phage D3112. Populations grew as free-floating biofilms in artificial sputum medium, mimicking sputum of CF lungs where *P. aeruginosa* is an important pathogen and undergoes evolutionary adaptation and diversification during chronic infection. Although bacterial populations adapted to the biofilm environment in both treatments, population genomic analysis revealed that phages altered both the trajectory and mode of evolution. Populations evolving with phages exhibited a greater degree of parallel evolution and faster selective sweeps than populations without phages. Phage  $\phi 4$  integrated randomly into the bacterial chromosome, but integrations into motility-associated genes and regulators of quorum sensing systems essential for virulence were selected in parallel, strongly suggesting that these insertional inactivation mutations were adaptive. Temperate phages, and in particular transposable phages, are therefore likely to facilitate adaptive evolution of bacterial pathogens within hosts.

*Pseudomonas aeruginosa* | cystic fibrosis | mobile genetic element | experimental evolution | bacteriophage

Comparative genomics suggests that temperate phages play an important role in the evolution and genomic diversification of bacterial pathogens (1). Bacterial genomes often contain a range of intact and remnant prophage elements (1–3), and ecologically important bacterial traits are believed to be phage-derived (e.g., phage-derived bacteriocins) (4). Phage-related sequences are observed more frequently in pathogenic than nonpathogenic strains (5), and prophage acquisition can be associated with changes in pathogen virulence (6, 7). Prophages can directly contribute accessory gene functions (1, 8) or disrupt bacterial genes by insertional inactivation. Of particular note are the transposable class of temperate phages (also known as mutator phages), including D3112 of *Pseudomonas aeruginosa* (9, 10), which integrate throughout the chromosome disrupting existing genes and increasing the supply of mutations available to selection. Recent reports of high rates of phage mobilization within hosts (11) and high temperate phage abundance in humans (12), including at sites of chronic infection where phage particles have been observed to exceed bacterial host densities by 10- to 100-fold (13), suggests that temperate phages could play an important role in driving within-host evolution of bacterial pathogens. However, experimental tests of the hypothesis that temperate phages contribute to rapid evolutionary adaptation of pathogenic bacteria are lacking.

*P. aeruginosa* is an important opportunistic pathogen and the major cause of chronic lung infection leading to morbidity and mortality in cystic fibrosis (CF) patients (14). Populations of *P. aeruginosa* in the CF lung grow as microcolony biofilms suspended within lung sputum and undergo extensive genetic diversification (15–17) and rapid evolutionary adaptation (18, 19) to this host environment. Characteristic bacterial adaptations to life in the CF lung and the transition to chronicity include the evolution of mucoidy, altered metabolism, loss of motility, quorum sensing defects, and resistance to antibiotics (18, 20). Despite detailed knowledge of the targets of selection, we still have only a very limited understanding of the causes of selection driving the evolution of these phenotypes. Phages are known to be present in the CF lung, have been cultured from lung sputa (21, 22), and have been detected at high abundance using culture-independent molecular approaches (13). Moreover, prophages are a common feature of *P. aeruginosa* sequenced genomes (23), and lysogenic conversion has been linked to the evolution of key clinical phenotypes (e.g., mucoidy) (24, 25). Therefore, it is likely that temperate phages may both impose selection on *P. aeruginosa* in the CF lung and contribute to pathogen adaptation to this host environment.

## Significance

During chronic infection, bacterial pathogens undergo rapid evolutionary adaptation and extensive genetic diversification affecting patient symptoms and treatment outcomes. Temperate phages are common in pathogen genomes, and phage particles can reach high abundance in human infections, but their role in pathogen evolution is unclear. Using experimental evolution and population genomics, we show that temperate phages found in human infections accelerated pathogen evolution by increasing the supply of beneficial mutations and imposing strong selection on bacterial populations. Notably, phages accelerated the loss of clinically important virulence-related bacterial traits, including motility and quorum sensing. Temperate phages are likely therefore to facilitate rapid evolution of bacterial pathogens and contribute to their adaptation to the host environment and clinical treatments.

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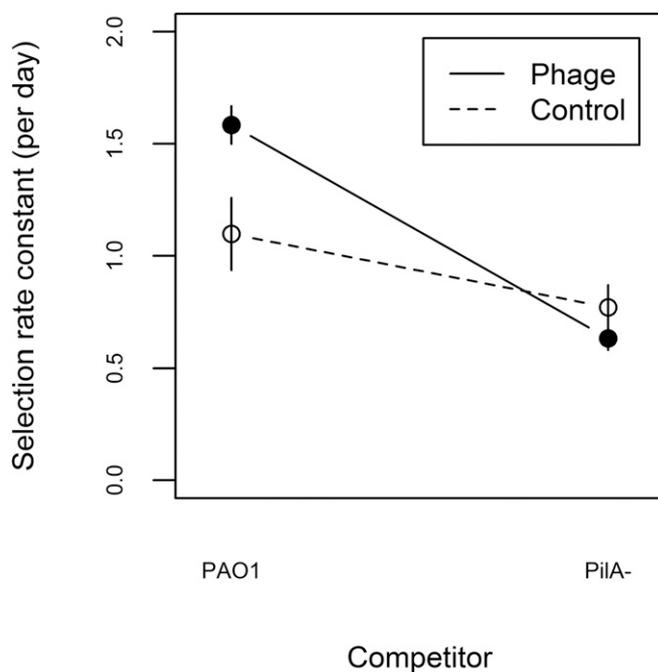
We used experimental evolution to directly test how temperate phages affect *P. aeruginosa* adaptation in artificial sputum medium (ASM), an in vitro environment that recapitulates key physiochemical and biofilm growth properties of CF lung sputum (26). Specifically, we propagated six replicate populations of *P. aeruginosa* PAO1 in the presence vs. absence of an assemblage of three temperate phages (LES $\phi$ 2,  $\phi$ 3, and  $\phi$ 4) for ~240 bacterial generations. These temperate phages naturally coexist as prophages in the genome of the *P. aeruginosa* Liverpool epidemic strain (LESB58) (27), the dominant clone infecting the UK CF population (28), and contribute to its competitiveness in vivo (27, 29–31). Whereas  $\phi$ 2 and  $\phi$ 3 are insertion site specific,  $\phi$ 4 is closely related to D3112, which is known to insert randomly throughout the *P. aeruginosa* chromosome (9, 10) and may therefore play an important role in facilitating the evolutionary adaptation of *P. aeruginosa* by increasing mutational supply. All phages display high rates of lytic activity in chronic CF lung infections (13), including being induced into the lytic cycle by clinically relevant antibiotics (21).

## Results and Discussion

In the experimental populations, phages had no effect on bacterial densities (Fig. S1A) despite evidence of ongoing phage lysis in all replicate populations of the phage treatment (Fig. S1B). At the end of the experiment, free virions of all phages were detected in four of six populations, whereas in the other two populations, only  $\phi$ 3 and  $\phi$ 4 virions were detected (Fig. S1C). We observed high rates of lysogeny [i.e., integration of prophage(s) into the bacterial chromosome] in five of six populations, but the phages differed in their propensity to form lysogens: lysogens of the transposable phage  $\phi$ 4 approached fixation in five of six populations, whereas lysogenization of bacteria by the other phages was less common, and, where observed, was typically as a polylysogen in combination with  $\phi$ 4 (Fig. S2). Thus, lysogeny, and indeed polylysogeny, was rapidly established in our experimental populations; moreover, lysogeny appears to have been essential for the long-term maintenance of phages in populations.

To determine the fitness response to selection, we competed each evolved population against the ancestral PAO1 in ASM, and because lysogens may have higher fitness simply due to phage-mediated killing of susceptible competitors (29, 31, 32), we also performed competitions against a phage-resistant PAO1 $\Delta$ *pilA*, an isogenic knockout mutant strain lacking the gene encoding the type IV pilus protein PilA [all of the temperate phages used here infect via the type IV pilus (33)]. We observed that evolved populations from both treatments were fitter relative both to PAO1 and PAO1 $\Delta$ *pilA* [Fig. 1; one-sample *t* test (alt = 0), all significant at an  $\alpha$  level of 0.0125]. Populations evolved with phages had higher fitness than populations evolved without phages relative to PAO1, but this fitness advantage of evolving with phages was lost when competing against PAO1 $\Delta$ *pilA* (Fig. 1; treatment  $\times$  competitor interaction:  $F_{1,20} = 8.54$ ,  $P < 0.01$ ; simple effect of treatment against competitor PAO1:  $F_{1,10} = 7.12$ ,  $P < 0.025$ ; and simple effect of treatment against competitor PAO1 $\Delta$ *pilA*:  $F_{1,10} = 1.53$ ,  $P = 0.24$ ). Together these data confirm that populations in both treatments adapted to the sputum-like environment and that lysogenised hosts had enhanced competitiveness against phage-susceptible competitors.

To determine the genetic basis of the observed evolutionary adaptation, we performed whole genome sequencing on population samples containing 40 random clones pooled per population from the end of the experiment. All populations contained SNPs and small insertions or deletions (indels), and all replicate populations that had evolved with phages contained integrated prophages. At the genome-wide scale, populations evolved with or without phages did not differ in abundance or frequency of SNPs and indels (excluding insertions caused by prophage integrations) and both groups had high variance of polymorphic sites: between 16 and 173 among the

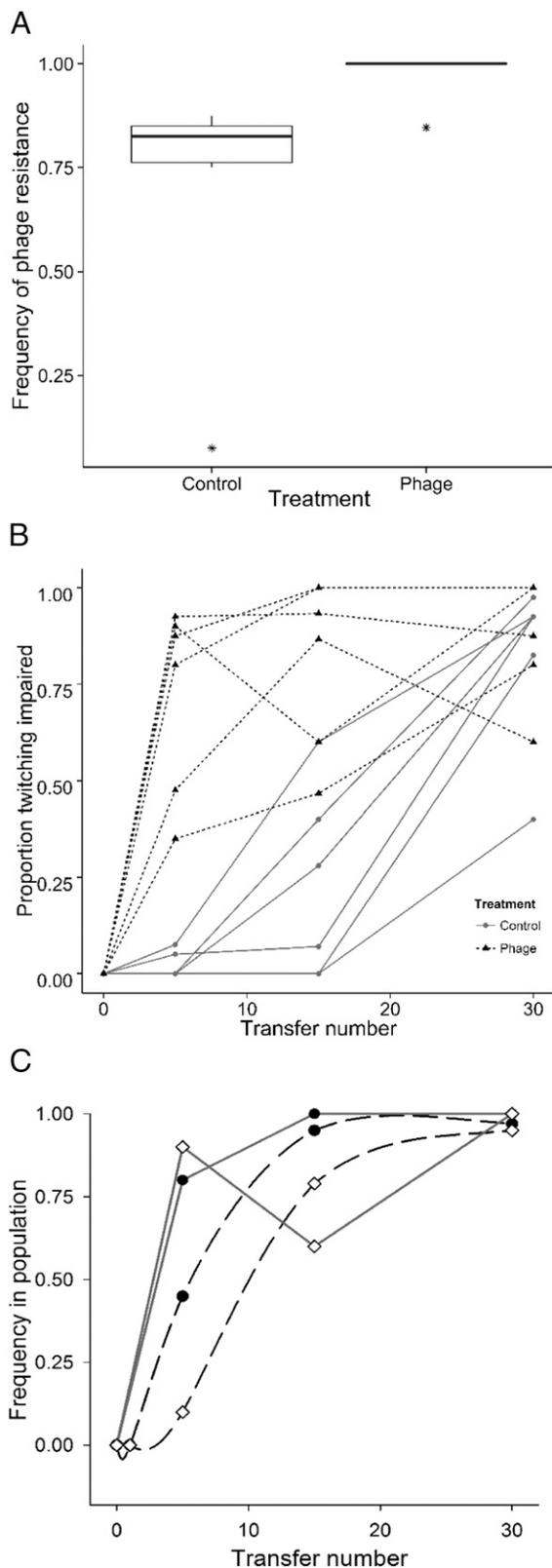


**Fig. 1.** Fitness response to selection in populations evolving with and without phages. Data points represent the mean  $\pm$  SE fitness calculated as selection rate for populations evolved with (filled symbols) or without (open symbols) phages in competition against either ancestral PAO1 or an isogenic phage-resistant competitor, PAO1 $\Delta$ *pilA*.

phage-free populations and 17–176 among the phage-containing populations (Table S1).

Parallel evolution at a particular locus, where independent mutations are observed more often than expected by chance, is strong evidence for positive selection. For example, in the absence of selection, the probability of observing a mutation in two populations at the conclusion of the experiment is only  $P = 0.003$ , and  $P = 0.0002$  if observed in three populations (for an average 1,004-bp protein-coding sequence in the PAO1 genome). Thus, to identify loci likely to have been under selection during experimental evolution, we concentrated our analyses on the subset of genes that had been targeted by mutations in at least two replicate populations per treatment (Fig. 2 and Tables S2 and S3). A greater degree of parallel evolution was observed in the presence of phages (measured as the probability of randomly drawing a pair of mutated genes from different populations, with phages  $0.056 \pm 0.016$  SE and without phages  $0.024 \pm 0.007$  SE,  $P < 0.05$  by bootstrap test). Some parallel targets of selection were shared among treatments, including genes involved in the type IV pilus motility, flagellar motility, biofilm formation, metabolism, and regulation, suggesting that these mutations were beneficial in the sputum-like environment per se. Interestingly, some loci were more likely to evolve in the presence of phages. In particular, mutations affecting the quorum sensing (QS) transcriptional regulators *lasR* and *mvfR* were each observed in five of six replicate phage-treated populations compared with only one of six replicate phage-free populations. In addition, three of six populations evolving with phages vs. one of six evolving without phages contained mutations in *pha1*, which encodes the forkhead-associated (FHA) domain protein that posttranslationally activates type VI secretion (34). A further indication of stronger selection due to phages is that parallel selected loci displayed higher allele frequencies in the phage treatment (mean allele frequency =  $34.33 \pm 3.2$  SEM/40) compared with the control treatment (mean allele frequency =  $22.67 \pm 3$  SEM/40), suggesting that selective sweeps in the phage





**Fig. 3.** The evolution of resistance to phages and pilus-dependent twitching motility traits. (A) Boxplot of phage resistance in end point populations. The thick horizontal line denotes the median frequency of isolates in a population resistant to one or more LES phages for each treatment. Asterisks denote outliers and narrow horizontal lines denote the upper and lower quartiles. (B) Frequency of bacterial isolates in each population through time displaying impaired twitching motility in the control (gray circles, solid line) and phage treatment (black triangles, dotted line). (C) Allele frequency dynamics of LES $\phi$ 4 integrated into *fimU*

associated loci. Across six phage-treated populations, 12 mutations of the type IV pilus-associated genes were detected in parallel, the majority ( $n = 9$ ) were caused by  $\phi$ 4 prophage integration, and half of them ( $n = 6/12$ ) occurred at high frequencies ( $\geq 10/40$  clones per population). However, in the absence of phages, although more mutations were detected in these genes in parallel ( $n = 16$ ), only 13% of these mutations occurred at high frequency (Table S3). Consistent with the phenotypic data (Fig. 3 B and C), this suggests that there was stronger positive selection for mutations disrupting type IV pilus-associated genes in the presence of phages, driving faster selective sweeps, compared with type IV pilus disrupting mutations occurring in the absence of phages. The exception to this pattern is replicate 3 of the phage treatment, where the impairment of twitching motility in 35 out of 40 isolates can be explained by a single frame-shift deletion variant in *pilY1* (PA4554; encodes a type IV pili biogenesis protein; Fig. 2). It is notable that a low frequency of lysogeny was observed in this population, unlike all other phage treatment replicates where lysogens approached fixation (Fig. S2).

Our genomic data suggest that temperate phages promoted the loss of QS with positive selection of  $\phi$ 4 prophage integrations, SNPs, and indel mutations at the *mvfR* and *lasR* loci. Mutations to *lasR* lead to disruption of the acyl-homoserine-lactone (AHL) signaling system (37), whereas mutations to *mvfR* lead to disruption of the *Pseudomonas* quinolone signal (PQS) system (38), suggesting large-scale alterations to QS cell-cell signaling in populations evolving with phages. To test whether QS deficient bacteria have higher fitness in the presence of phages, we competed PAO1 against PAO1 $\Delta$ *lasR* in ASM with and without the temperate phages. There was no effect of phages on the fitness of PAO1 $\Delta$ *lasR* (Fig. S3; two-sample *t* test,  $t_{10} = -0.7989$ ,  $P = 0.44$ ), which was substantially fitter relative to PAO1 in both the presence [one-sample *t* test (alt = 0),  $t_5 = 5.0331$ ,  $P < 0.01$ ] and absence [one-sample *t* test (alt = 0),  $t_5 = 6.7065$ ,  $P = 0.001$ ] of phages. These data suggest that *lasR* mutations are beneficial in ASM per se. This interpretation is consistent with the observation that single populations in the phage-free treatment also acquired mutations in QS genes, but suggests that the rate of evolution at these loci was higher in the presence of phages. Second, we compared the rates of spontaneous phage lysis of  $\phi$ 4 lysogens constructed in both the PAO1 and PAO1 $\Delta$ *lasR* backgrounds: there was no significant difference in production of free phages in stationary phase cultures (median free phage density: PAO1,  $3.4 \times 10^8$  pfu/mL, PAO1 $\Delta$ *lasR*,  $3.3 \times 10^8$  pfu/mL; Mann-Whitney test;  $W = 92.0$ ,  $P = 0.345$ ). Thus, although direct interaction between temperate phages and bacterial QS has been reported in other systems, via QS induced lysis by phages (39) or QS mediated alteration of phage receptor expression by bacteria (40), this does not appear to be an important factor in our study. Phages may have simply increased the supply of large effect mutations available to natural selection, notably via  $\phi$ 4 prophage integrations into *mvfR* (Fig. 2). Alternatively, there may have been epistatic interactions between the fitness effects of QS mutations and other positively selected mutations, which strengthened selection for loss of QS in the presence of phages. Mutations in QS regulators are commonly observed to accumulate over time in CF chronic infection (41). Both AHL and PQS signaling are required for full virulence in *P. aeruginosa* (42), suggesting that temperate phage selection may accelerate the loss of virulence in chronic infections.

In summary, we showed that temperate phages enhanced parallel evolution in *P. aeruginosa* biofilms in a sputum-like environment. Our data suggest two ways in which this may have occurred: first, the transposable phage  $\phi$ 4 mediated adaptive evolution by

and *pilV* for populations P7 and P11, respectively, and loss of twitching motility in these populations. Closed black circles and open white diamonds represent populations P7 and P11, respectively, solid gray lines denote loss of twitching motility data, and dashed black lines denote allele frequency data.

increasing the supply of positively selected mutations via insertional inactivation of genes caused by prophage integrations, particularly in type IV pilus and QS associated genes. Second, we present evidence that temperate phage strengthened selection, particularly for mutations in type IV pilus associated genes, accelerating the evolutionary loss of type IV dependent pilus motility presumably to avoid superinfection and subsequent lysis by phages which infect via the type IV pilus. A recent transposon sequencing study of *P. aeruginosa* PA14 shows that mutations in type IV pilus associated genes increase fitness in the murine lung (43). Moreover, loss of both motility (44, 45) and QS (46, 47) functions are known to frequently evolve in *P. aeruginosa* chronic infections of the CF lung. Temperate phages, including those used here, can be present at very high densities in the CF lung [exceeding bacterial densities by orders of magnitude (13)], which taken together with our findings suggests that temperate phages could play an important role in CF lung infections by driving the evolution of these clinically important traits in *P. aeruginosa*. In addition, our data suggest that living in a sputum-like environment per se selects for mutations in genes associated with motility, biofilm formation, metabolism and regulation. Similar mutations observed in CF lung isolated *P. aeruginosa* are therefore likely to be at least partially explained simply as adaptations to selection imposed by the sputum environment, but could have implications for susceptibility to antibiotics (48) or host immune responses (49) as correlated responses (50). Experimental evolution in clinically relevant infection models has the potential to enhance our understanding of the causal links between sources of selection and the evolutionary responses of pathogens in infections (51), advancing our understanding of within host pathogen evolution and our ability to direct this for improved patient health.

## Materials and Methods

Twelve replicate microcosms (30-mL glass universals containing 5 mL ASM) were inoculated with  $5 \times 10^7$  cells *P. aeruginosa* strain PAO1 and grown as a biofilm [37 °C incubation with shaking at 60 rpm (Stuart SI500 16-mm orbital shaking incubator, Bibby Scientific Ltd.)]. LES phages  $\phi$ 2, 3, and 4 were added to six microcosms after 24 h of growth to a total multiplicity of infection of 0.1 (phage treatment), and the remaining six were designated phage-free controls. Phages were added only once, at the beginning of the experiment. After a further 72-h growth, biofilms were homogenized with an equal volume of Sputasol and the homogenate transferred (1:100) into fresh ASM. Transfers were repeated every 4 d, to a total of 30 transfers (~240 bacterial generations). Every other transfer, bacterial and total free phage densities were enumerated, and every five transfers, the frequency of prophage carriage in the phage treatment was estimated with a multiplex PCR assay using primers targeted to each of the LES phages. At transfers 5,

15, and 30, 40 isolates per population were screened for the type IV pilus-mediated twitching motility phenotype using the agar stab method.

At the end of the experiment, DNA was extracted from 40 isolates per population and pooled, and the pooled DNA sequenced on an Illumina HiSeq 2000. Polymorphisms were called from reads aligned to the published PAO1 genome treating each sample as having ploidy = 40, reflecting the number of pooled isolates.  $\phi$ 4 prophage insertion sites were estimated from the mapping locations of reads that mapped to the PAO1 chromosome and whose mate read mapped to the  $\phi$ 4 prophage sequence. The MEME software suite (35, 36) was used for motif analysis. First, the 20 most conserved motifs at  $\phi$ 4 prophage integration sites were selected using the MEME algorithm. Second, the entire host chromosome was searched for motif occurrences using the MAST algorithm (see *SI Materials and Methods* for details). Frequencies of each prophage integration site were estimated based on the number of read pairs split between prophage and reference chromosomes as a proportion of read pair depth in that region. ORF annotations from the published sequence were supplemented using the STRING v10 database (52). Counts of ORFs affected by mutations in more than one population per treatment were implemented using the BioPython library ver. 1.65 (53), and chromosome map plots were implemented using the svgwrite library ver. 1.1.3 in Python ver. 2.7.10. Included in the parallel selected loci were those exhibiting the “multidiverse” signature of unlinked polymorphisms in the same ORFs. The method is implemented in the CheckLinkage option of BAGA (<https://github.com/daveuu/baga>) (54) and discussed in *SI Materials and Methods*.

Relative free phage abundances in end point populations were estimated separately for each phage using a quantitative PCR (qPCR) assay of DNase-treated supernatants, using primers targeted to each phage and comparison with a set of standards of known concentration. Competitions were performed between end point populations against the ancestral PAO1 (labeled with a gentamicin resistance marker) or an isogenic LES phage-resistant competitor, PAO1 $\Delta$ *pilA* (labeled with a tetracycline resistance marker), in conditions identical to one transfer of the selection experiment. The density of each competitor was determined by plating onto antibiotic selective and nonselective media. Fitness was calculated as the selection rate constant. Free phage densities were measured for LES $\phi$ 4 lysogens in both PAO1 and PAO1 $\Delta$ *lasR*. Ten independent lysogens were constructed in each host background (PAO1 and PAO1 $\Delta$ *lasR*) and cultured in LB until the stationary phase, and free phage densities in the supernatant were quantified using a plaque assay. Full methods are included in *SI Materials and Methods*.

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