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 non-pathogenic taxa, and are associated with changes in pathogen virulence. High abundance and mobilisation 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, ϕ4, 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. While 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 ϕ4, 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

INTRODUCTION

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 phage (also known as mutator phage), including D3112 of *P. 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.

Pseudomonas 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 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.

We used experimental evolution to directly test how temperate phages affect *P. aeruginosa* adaptation in artificial sputum

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.

Reserved for Publication Footnotes

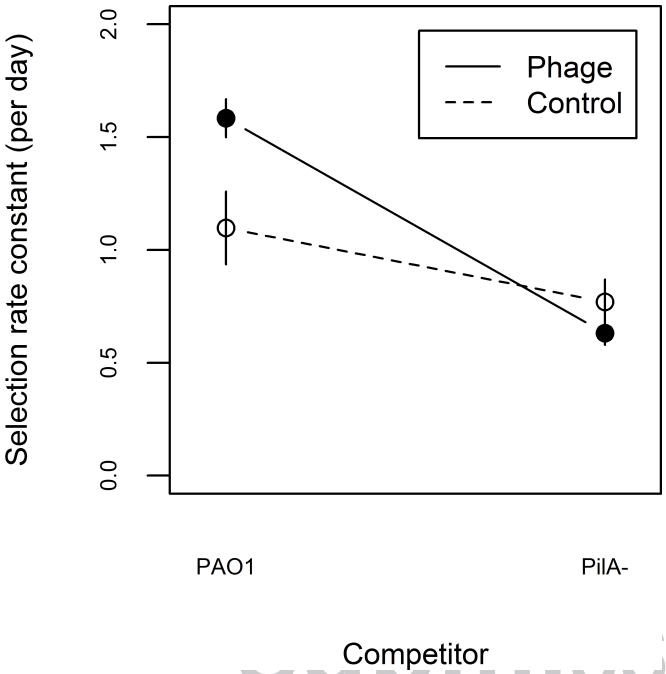

Fig. 1. | Fitness response to selection in populations evolving with and without phages. Data points represent the mean ± 1 S.E. fitness calculated as selection rate for populations evolved with (filled symbols) or without (open symbols) phages in competition against either ancestral PAO1, or an isogenic phage-resistant competitor, PAO1Δ*pilA*.

medium (ASM), an *in vitro* environment that recapitulates key physiochemical and biofilm growth properties of CF lung sputum (26). Specifically, we propagated 6 replicate populations of *P. aeruginosa* PAO1 in the presence versus absence of an assemblage of 3 temperate phages (LESϕ2, ϕ3 and ϕ4) for approximately 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, 30). Whereas ϕ2 and ϕ3 are insertion site specific, ϕ4 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 on-going 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 4/6 populations, whereas in the other 2 populations only ϕ3 and ϕ4 virions were detected (Fig S1c). We observed high rates of lysogeny (i.e., integration of prophage(s) into the bacterial chromosome) in 5/6 populations but the phages differed in their propensity to form lysogens: lysogens of the transposable phage ϕ4 approached fixation in 5/6 populations, whereas lysogenisation of bacteria by the other phages was less common, and, where observed, was typically as a polylysogen in combination with ϕ4 (Fig S2). Thus lysogeny, and indeed polylysogeny, was rapidly established in our experimental populations, and 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), we also performed competitions against a phage-resistant PAO1Δ*pilA* (all of the temperate phages used here infect via the type IV pilus (32)). We observed that evolved populations from both treatments were fitter relative both to PAO1 and PAO1Δ*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 PAO1Δ*pilA* (Fig 1; treatment × competitor interaction: $F_{1,20}$ = 8.54, MSE = 0.59, $p < 0.01$; simple effect of treatment against competitor PAO1: $F_{1,10}$ = 7.12, MSE = 0.71, $p < 0.025$; simple effect of treatment against competitor PAO1Δ*pilA*: $F_{1,10}$ = 1.53, MSE = 0.06, $p = 0.24$). 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 single nucleotide polymorphisms (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 phage-free populations and 17 to 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 1004 bp sized 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 ≥2 replicate populations per treatment (Fig 2, 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 ± 0.016 s.e., without phages 0.024 ± 0.007 s.e., $p < 0.05$ 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) regulators *lasR* and *mvfR* were each observed in 5/6 replicate phage-treated populations, compared to only 1/6 replicate phage-free populations. In addition, 3/6 populations evolving with phages versus 1/6 evolving without phages contained mutations in *fha1*, which encodes an FHA domain protein that post-translationally activates type VI secretion (33). 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 ± 3.2 s.e.m / 40) compared to the control treatment (mean allele frequency = 22.67 ± 3 s.e.m. / 40), suggesting that selective sweeps in the phage treatment were closer to fixation (discussed in more detail in Supplementary Materials).

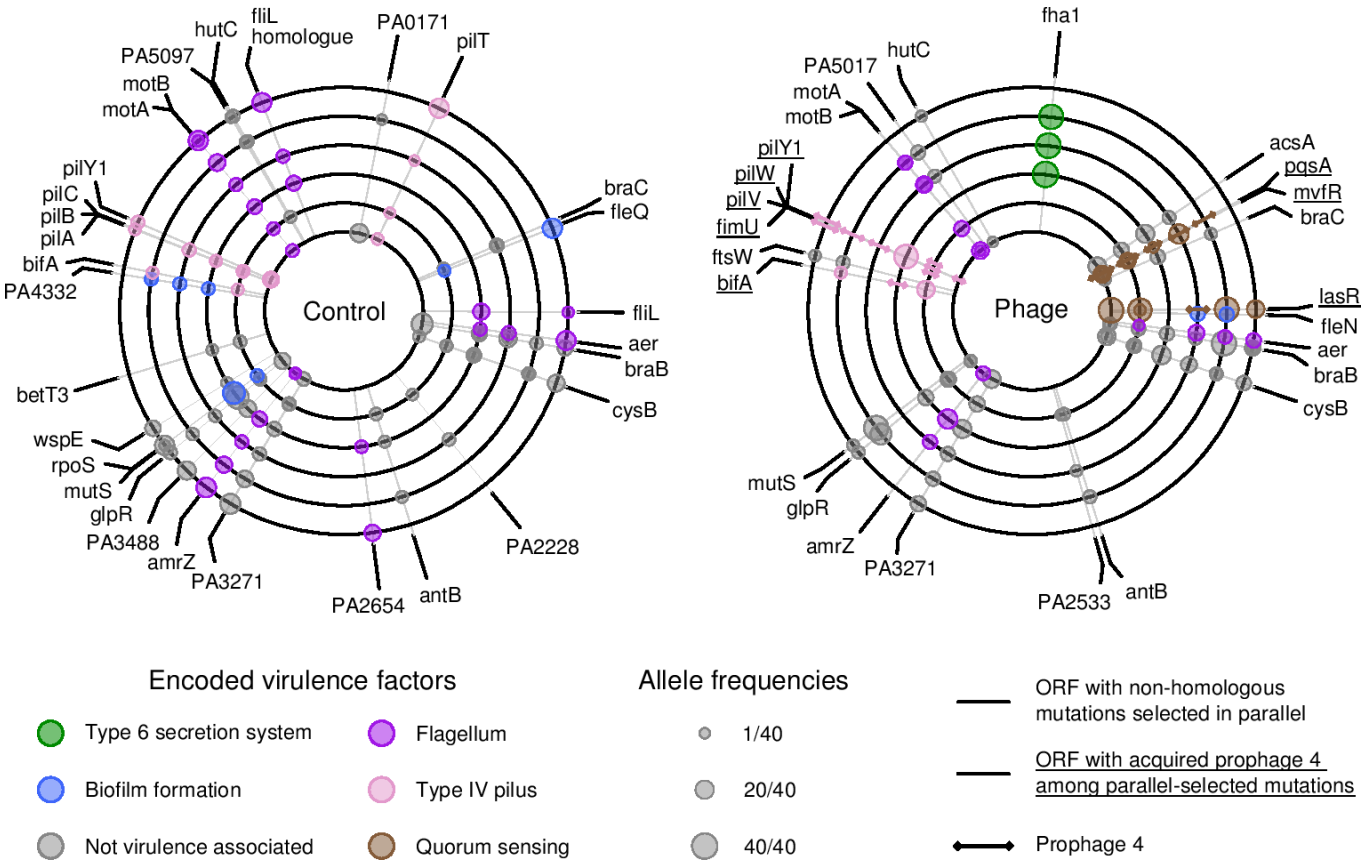

Fig. 2. | Genetic loci under positive selection as indicated by parallel genomic evolution in populations evolving with and without phages. Each concentric circle corresponds to a replicate population in either the control (without phages) or treatment group (with phages), as indicated. Positions around each concentric circle, starting at the 12 o'clock position and in a clockwise direction, correspond to positions around the published *P. aeruginosa* PAO1 single circular chromosome. The smaller circles around each concentric circle indicate the positions of variants in each replicate that were observed in an open reading frame under positive selection *i.e.*, mutated in parallel in at least one other replicate. Only variants in *fha1* were at precisely the same position and were likely to be homologous. Variants are also listed in Table S8.

A key difference between the populations evolving with, versus without phages is that a substantial fraction of ORFs under positive selection (8 of 26; figure 2) contained mutations caused by prophage integration in the phage-containing treatment. Prophage 2 and 3 were found exclusively at the intergenic sites homologous to their positions in the LESB58 chromosome from where they were isolated (4,629,220 bp and 4,103,724 bp in the PAO1 chromosome) and were therefore excluded from analyses. In contrast ϕ4 integrations were observed at 19 different positions across the 6 replicates, suggesting that ϕ4 integration provided an additional source of genetic diversity for selection to act upon. Although ϕ4 is closely related to D3112 and therefore likely to integrate randomly throughout the chromosome (27, 32, 34), we tested for hotspots of integration with an exhaustive search of sequence motifs using the MEME and MAST algorithms (35, 36) in the 1000 bp region around each of the integration sites. Only very weakly conserved motifs were identified (Table S4), suggesting that ϕ4 has the low integration specificity characteristic of transposable phages like D3112. Despite the very low integration specificity of ϕ4, all of the 19 integration sites were located within or adjacent to only 7 different operons, which is consistent with positive selection for integrations at those regions (Fig 2; Tables S5 and S8). The functions most commonly predicted to be disrupted by positively selected ϕ4 integration events were type IV pilus motility and QS. Thus transposable temperate phages like ϕ4 may commonly alter the mode of bacterial evolution by increasing the supply of mutations available to natural selection.

Because all of the temperate phages used here infect via the type IV pilus (32) and PAO1Δ*pilA* mutants showed higher fitness compared to PAO1 against lysogenized bacteria evolved in the phage treatment (Fig 1), we hypothesised that disruption of type IV pilus motility associated genes may have been selected to prevent superinfection and lysis of ϕ4 lysogens. Notably, while ϕ4 lysogens have strong superinfection immunity against ϕ4, they remain susceptible to infection and subsequent lysis by ϕ2 and ϕ3 (32), suggesting that loss of type IV pili could be beneficial in the presence of a diverse phage community. In support of this we observed higher rates of phage resistance in populations that evolved with versus without phages (Fig 3a; Mann–Whitney test; $W = 24.0$, $n_1 = n_2 = 6$, $p = 0.02$). Correspondingly, type IV pilus-dependent twitching motility was lost more rapidly in phage-containing populations than in phage-free populations, suggesting that the loss of type IV pilus function was more strongly selected in the presence of phages (Fig 3b). To determine whether loss of type IV pilus twitching motility phenotype was associated with ϕ4 integration, we tracked allele frequency dynamics in 2 replicate populations. Specific PCR primer sets (Table S6) were used to detect integrated ϕ4 prophage in *fimU* and *pilV*. In both cases there was a positive association between the allele frequency dynamics and the rise in frequency of twitching motility deficient mutants (Fig 3c). We next contrasted the allele frequencies of SNPs, indels and ϕ4 prophage-integration mutations occurring at type IV pilus associated loci. Across 6 phage-treated populations, 12 mutations of the type-IV pilus associated genes were detected in parallel, the majority (n=9) were caused by ϕ4 prophage-

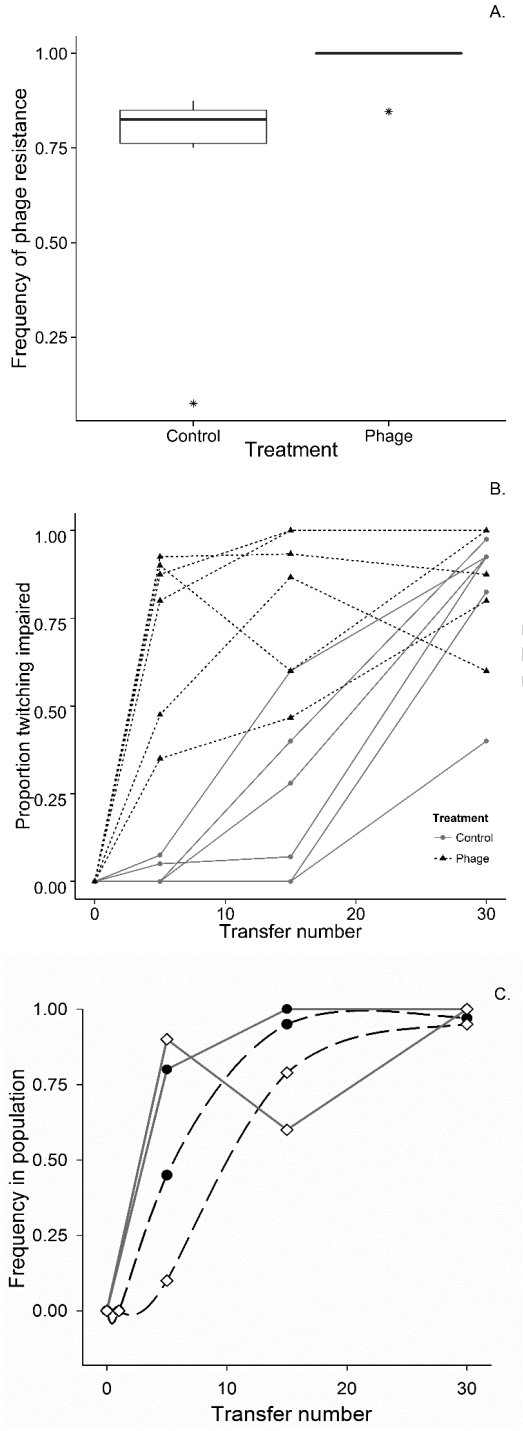

Fig. 3. | The evolution of resistance to phages and pilus-dependent twitching motility traits. (A.) Boxplot of phage resistance in endpoint populations. The thick horizontal line denotes the median frequency of isolates resistant to one or more LES phages in a population for each treatment. Asterisks and narrow horizontal lines denote outliers and the upper and lower quartiles, respectively. (B.) Frequency of bacterial isolates in each population through time displaying impaired twitching motility. (C.) Allele frequency dynamics of LESω4 integrated into *fimU* 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, solid grey lines twitching motility data, and dashed black lines allele frequency data.

integration, and half of them (n=6/12) occurred at high frequencies (≥10/40 clones per population). However, in the absence of phages, while 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. 3b, 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 to 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 isolates can be explained by a single frame-shift deletion variant at 35/40 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 ϕ4 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 PAO1Δ*lasR* in ASM with and without the temperate phages. There was no effect of phages on the fitness of PAO1Δ*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 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. Secondly, we compared the rates of spontaneous phage lysis of ϕ4 lysogens constructed in both the PAO1 and PAO1Δ*lasR* backgrounds: There was no significant difference in production of free phages in stationary phase cultures (median free phage density: PAO1, 3.4 x 10^8 p.f.u. per ml, PAO1Δ*lasR*, 3.3 x 10^8 p.f.u. per 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 ϕ4 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 have shown 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: Firstly, the transposable phage ϕ4 mediated adaptive evolution by 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. Secondly, 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 super-infection 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.

METHODS SUMMARY

Twelve replicate microcosms (30 ml glass universals containing 5 ml ASM) were inoculated with 5 x 10^7 cells *P. aeruginosa* strain PAO1 and grown as a biofilm (37 °C incubation with shaking at 60 r.p.m.). LES phages φ2, 3 and 4 were added to six microcosms after 24 hours 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 hours growth, biofilms were homogenized with an equal volume of Sputasol and the homogenate transferred (1:100) into fresh ASM. Transfers were repeated every 4 days, to a total of 30 transfers (approximately 240 bacterial generations). Every other transfer, bacterial and total free phage densities were enumerated, and every 5 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. European Nucleotide Archive accession number for the project is PRJEB9801. Polymorphisms were called from reads aligned to the published PAO1 genome treating each sample as having ploidy = 40, reflecting the number of pooled isolates. ϕ4 prophage insertion sites were estimated from the mapping locations of reads that mapped to the PAO1 chromosome and whose mate read mapped to the ϕ4 prophage sequence. The MEME software suite (35, 36) was used for motif analysis. First, the 20 most conserved motifs at ϕ4 prophage integration sites were selected using the MEME algorithm. Second, the entire host chromosome was searched for motif occurrences using the MAST algorithm (see supplementary materials 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. Open reading frame (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 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 "multi-diverse" signature of unlinked polymorphisms in the same ORFs. The method is implemented in the CheckLinkage option of BAGA (http://github.com/daveuu/baga) (54) and discussed in the Supplementary Materials.

Relative free phage abundances in endpoint populations were estimated separately for each phage using a qPCR assay of DNAse-treated supernatants, using primers targeted to each phage, and comparison to a set of standards of known concentration. Competitions were performed between endpoint populations against the ancestral PAO1 (labelled with a gentamicin resistance marker), or an isogenic LES phage-resistant competitor, PAO1Δ*pilA* (labelled 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 non-selective media. Fitness was calculated as the selection rate constant. Free phage densities were measured for LESφ4 lysogens in both PAO1 and PAO1Δ*lasR*. Ten independent lysogens were constructed in each host background (PAO1 and PAO1Δ*lasR)* and cultured in LB until stationary phase, and free phage densities in the supernatant quantified using a plaque assay. Full methods are included in the supplementary information.

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