Author(s): Cairns, Johannes; Frickel, Jens; Jalasvuori, Matti; Hiltunen, Teppo; Becks, Lutz

Title: Genomic evolution of bacterial populations under co-selection by antibiotics and phage

Year: 2017

Version:

Please cite the original version:

Cairns, J., Frickel, J., Jalasvuori, M., Hiltunen, T., & Becks, L. (2017). Genomic evolution of bacterial populations under co-selection by antibiotics and phage. Molecular Ecology, 26(7), 1848-1859. https://doi.org/10.1111/mec.13950

Received Date : 11-Aug-2016

Revised Date : 06-Oct-2016

Accepted Date : 28-Nov-2016

Article type : Special Issue

Genomic evolution of bacterial populations under co-selection by antibiotics and phage

Johannes Cairns[1], Jens Frickel[2], Matti Jalasvuori[3], Teppo Hiltunen[1] and Lutz Becks[2*]

[1]Department of Food and Environmental Sciences / Microbiology and Biotechnology

P.O. Box 65, 00014 University of Helsinki, FINLAND

[2]Department of Evolutionary Ecology / Community Dynamics Group, Max Planck Institute for Evolutionary Biology, August Thienemann Street 2, Plön 24306, Germany

[3]Department of Biological and Environmental Science / Centre of Excellence in Biological Interactions, P.O. Box 35, 40014 University of Jyväskylä, FINLAND

[*]**Corresponding author**: lbecks@evolbio.mpg.de

Running title: Genomics of antibiotics & phage co-selection

Keywords: experimental evolution, sublethal antibiotic concentrations, antibiotic resistance, phage resistance, *Pseudomonas fluorescens*, phage Φ2

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/mec.13950

Abstract

Bacteria live in dynamic systems where selection pressures can alter rapidly, forcing adaptation to the prevailing conditions. In particular, bacteriophages and antibiotics of anthropogenic origin are major bacterial stressors in many environments. We previously observed that populations of the bacterium *Pseudomonas fluorescens* SBW25 exposed to the lytic bacteriophage SBW25Φ2 and a non-inhibitive concentration of the antibiotic streptomycin (co-selection) achieved higher levels of phage resistance compared to populations exposed to the phage alone. In addition, the phage became extinct under co-selection while remaining present in the phage alone environment. Further, phenotypic tests indicated that these observations might be associated with increased mutation rate under co-selection. In this study, we examined the genetic causes behind these phenotypes by whole-genome sequencing clones isolated from the end of the experiments. We were able to identify genetic factors likely responsible for streptomycin resistance, phage resistance and hypermutable (mutator) phenotypes. This constitutes genomic evidence in support of the observation that while the presence of phage did not affect antibiotic resistance, the presence of antibiotic affected phage resistance. We had previously hypothesized an association between mutators and elevated levels of phage resistance under co-selection. However, our evidence regarding the mechanism was inconclusive, since although with phage mutators were only found under co-selection, additional genomic evidence was lacking and phage resistance was also observed in non-mutators under co-selection. More generally, our study provides novel insights into evolution between univariate and multivariate selection (here two stressors), as well as the potential role of hypermutability in natural communities.

Introduction

Microbial communities are dynamic systems, which are often exposed to different biotic and abiotic stressors. Bacterial populations have been shown to be well adapted to their local conditions (Belotte *et al.* 2003; Martiny *et al.* 2006; Zumsteg *et al.* 2013; Koskella & Vos 2015; Kraemer & Kassen 2015) and are able to adapt rapidly to novel conditions (Beaumont *et al.* 2009; Brooks *et al.* 2011; Ketola & Hiltunen 2014). Among the most prominent examples of local adaptation in microbial communities are the evolution of antibiotic resistance and adaptation to the interaction with bacteriophages. Adaptation to antibiotics has been observed in natural communities and in laboratory studies, both for clinically relevant concentrations and for below minimum inhibitory concentrations (sub-MIC) (Hermsen *et al.* 2012; Andersson & Hughes 2014; Laehnemann *et al.* 2014). Similarly, previous work has shown that bacterial populations evolve resistance against their local phage populations in time and space (Buckling & Rainey 2002; Brockhurst *et al.* 2007; Koskella & Brockhurst 2014).

Exposing (microbial) populations to the same environmental stressor often leads to the same phenotypic adaptation. For example, all replicated bacterial populations of the species *Pseudomonas fluorescens* (strain SBW25) that coevolved with a dsDNA lytic phage (Φ2) showed phage resistance combined with decreased competitive fitness when grown in the absence of phage (Scanlan *et al.* 2015). Similarly, most experimental populations of the bacterial species *Escherichia coli* (strain MC4100) exposed to different antibiotics (doxycycline, ciprofloxacin, erythromycin, and amikacin) evolved high levels of resistance, growing at rates similar to drug-free environments, within approx. 170 generations (Hegreness *et al.* 2008). The repeatability of evolution in these and other studies with bacterial populations (co)evolving in the presence of phage or antibiotics has allowed

identifying several genes involved in resistance against these two stressors as well as other associated mutations (Davies & Davies 2010; Labrie *et al.* 2010; Andersson & Hughes 2012). For example, mutations in genes associated with lipopolysaccharides (LPS), cell envelope biogenesis and the outer membrane have been previously associated with altered LPS length and resistance phenotypes of *Pseudomonas fluorescens* SBW25 against the phage Φ2 (Scanlan *et al.* 2015).

Co-selection by two or more stressors potentially modifies selection for resistance to antibiotics or phage, and thus local adaptation. This has previously been shown phenotypically for bacterial populations exposed to antibiotics and phages (Escobar-Páramo *et al.* 2012; Knezevic *et al.* 2013; Coulter *et al.* 2014), phage and protozoan predation (Friman & Buckling 2013; Zhang *et al.* 2014; Örmälä-Odegrip *et al.* 2015), phage and resource limitation (Harrison *et al.* 2013; Lopez Pascua *et al.* 2014; Gómez *et al.* 2015), or antibiotics and heavy metals (Seiler & Berendonk 2012; Gullberg *et al.* 2014; Zhou *et al.* 2015). Results are equivocal, as co-selection has been found to amplify selection strength and increase rates of adaptation (eventually resulting in diversification into different lineages (Seiler & Berendonk 2012; Gullberg *et al.* 2014)), as well as to decrease rates of adaptation (Zhang & Buckling 2012) or to alter the mode of evolutionary (Friman *et al.* 2016) or coevolutionary dynamics (Lopez Pascua *et al.* 2014). One reason for these different outcomes is that various, non-exclusive population genetic and ecological mechanisms could drive adaptive changes. For example, potential evolutionary and energetic constraints can lead to trade-offs and lower the efficacy of selection (Torres-Barceló *et al.* 2014; Örmälä-Odegrip *et al.* 2015). Similarly, clonal interference, different directions of selection and reduced population sizes can alter the evolutionary potential and trajectory of populations (see e.g. Hughes & Andersson 2015).

Hypermutability can play an important role in bacterial populations and communities adapting to stressors by increasing rates of adaptation. Hypermutability has been described for bacterial populations exposed to antibiotic stress (Ren *et al.* 1999; Nagel *et al.* 2011; Andersson & Hughes 2012) or in the presence of phage (Pal *et al.* 2007; Morgan *et al.* 2010) and is thought to provide a (short-term) advantage for populations adapting rapidly to a new stressor or environment as many mutations can be sampled over a short timescale (Taddei *et al.* 1997). Hypermutability has also been shown to be important in co-selective environments with phage and antibiotics by outweighing associated costs of antibiotic resistance (Tazzyman & Hall 2015).

Whereas co-selection by two or more stressors has been studied on the phenotype level, adaptive changes have rarely been followed on the genomic level and compared to environments with only one stressor present. Based on the above-mentioned population genetic and ecological mechanisms, we expect, however, to find differences between populations evolving with one or two stressors. We would, for example, expect to see a smaller number of mutations at high frequencies (or fixed) in the co-selection environment due to clonal interference and lower efficacy of selection. Furthermore, changes in directional selection could result in unique sets of mutations in the co-selection environment that are not found in single stressor environments. Another prediction is that co-selection could lead to disruptive selection and that the same sets of genes are found as in populations exposed to only one of the stressors, but not within the same genotypes. Stronger selection could also drive population sizes to low numbers, affecting mutation supply, drift and/or fixation times and probability. Finally, these predictions might change when hypermutability evolves.

In a recent study, we tested adaptation of populations of the bacterium *Pseudomonas fluorescens* SBW25 (Rainey & Bailey 1996) grown with and without the antibiotic streptomycin at sub-MIC levels (approx. 1% or 10% of MIC) and the lytic bacteriophage SBWΦ2 (Buckling & Rainey 2002) in a factorial design (Cairns *et al.* 2016). Specifically, we asked whether and how phenotypic adaptation differed between populations exposed to a single or two stressors simultaneously and explored the role of hypermutability in this context. We found that populations evolved resistance against phage and streptomycin over the course of the experiment. Interestingly, in a 66-day experiment, we found that sub-MICs of streptomycin increased the rate of phage resistance evolution, and in a separate 12-day experiment with higher replication, that streptomycin and the phage interactively enhanced the evolution of streptomycin resistance. One result of increased phage resistance in the presence of streptomycin was the extinction of phage by half-way through the experiment. We still consider these environments as co-selected, as the major adaptations against the stressors occurred early on during the experiment. Furthermore, the elevated levels of phage resistance compared to the phage only environment are even more startling when considering that the phage had gone extinct from these populations early on. Our analysis at the phenotype level showed also that experimental streptomycin concentrations increased bacterial mutation rate in some populations, suggesting that higher phage resistance and antibiotic resistance when exposed to two stressors in combination might have been driven by hypermutability. Here we now present results from whole-genome resequencing of ten clones from each population from the end of the 66-day experiment. This approach enabled us to identify genes potentially involved in adaptation to the different stressors in isolation and in combination. We found several potential streptomycin and phage resistance mutations as well as only a few differences in the genomic changes in populations exposed to one or two stressors.

Materials and methods

Evolutionary experiment

As the bacterial host, we used *Pseudomonas fluorescens* SBW25 (Rainey & Bailey 1996), and as the viral parasite, the lytic bacteriophage SBWΦ2 (Buckling & Rainey 2002). We followed standard protocols for microcosm experiments with bacteria-phage systems (Rainey & Travisano 1998; Buckling & Rainey 2002; Brockhurst *et al.* 2007; Hiltunen & Becks 2014). A detailed description of the experimental methods and results are presented in Cairns *et al.* (2016). To test the effect of sub-MICs of streptomycin on phage resistance evolution, we conducted a 66-day microcosm experiment, representing approx. 380 *P. fluorescens* generations. We had a full factorial experimental design where bacterial populations adapted to antibiotics (0, 0.2, 2 μg ml^{-1}) in the absence or presence of phages. All treatments were started from a single colony of *P. fluorescens* and replicated three times in 25 ml glass vials containing 6 ml KB (5% nutrient concentration). Every 48 hours, 1% (60 μl) of each culture was transferred to a new vial containing fresh KB.

After the microcosm experiment, we isolated 20 bacterial clones from each population by diluting the samples and plating them on proteose peptone yeast extract (PPY) culture medium (20 g proteose peptone and 2.5 g yeast extract in 1 l deionized H_2O). We tested the minimum inhibiting concentration (MIC) of streptomycin and phage resistance for every clone following the methodology described in detail in Cairns *et al.* (2016). Briefly, to determine phage resistance, each clone was cultured overnight in PPY medium in a 96 well plate that was pin replicated on a PPY plate containing a top soft agar layer with the ancestral phage at high density. Phage resistance or sensitivity was determined after culturing overnight as the presence or absence of bacterial grown, respectively. To determine the MIC of streptomycin, a 100 μl inoculum from an overnight culture (PPY medium) was spread-

plated on a PPY agar plate, followed by placing of an MIC test strip on the plate. The MIC value was read from the test strip after overnight culture. All clones were stored at –80 °C for sequencing.

Whole-genome resequencing and analysis

We randomly selected 10 out of the 20 clones used in the resistance assays and isolated DNA from overnight cultures using standard methods (DNeasy Blood & Tissue Kit, Qiagen). Paired-end libraries were prepared using Illumina Nexterra XT sequence reads obtained by high-throughput sequencing (Illumina Nextseq 500 high output). Read quality was assessed using FastQC 0.11.3 (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc, last accessed Apr 2016), after trimming (5 bp at the 5′-end and 10 bp at the 3′-end). The reads were aligned to the *P. fluorescens* SBW25 reference genome (assembly: ASM922v1) with BWA 0.7.12 (Li & Durbin 2009). Detailed information about the proportion of mapped reads, genome coverage and insert size is provided for a representative subset of the data (30/180 samples) in Table S2. Picard 1.138 (http://picard.sourceforge.net, last accessed Apr 2016) was used to mark duplicates and insert read groups, and SAMtools/BCFtools 1.2 (Li 2011) was used to index BAM files. We used GATK (McKenna *et al.* 2010) for indel realignment, SNP and indel discovery (for haploid genome) and genotyping across all 10 clones per replicate population simultaneously according to GATK Best Practices recommendations (DePristo *et al.* 2011; Van der Auwera *et al.* 2013), using hard filtering with custom parameters (min. depth of coverage: 100; min. Phred-scaled *p*-value: 50). Read-depth based detection of copy number variation (CNV) was performed using CNVnator 0.3.2 (Abyzov *et al.* 2011) with a bin size of 500 bp.

Variant effect prediction was performed with Ensembl Bacteria release 31 (http://bacteria.ensembl.org/, last accessed Jun 2016) using the methodology described in McLaren *et al.* (2010). The *Pseudomonas* Genome Database (Winsor *et al.* 2016) and the STRING database v10.0 (Szklarczyk *et al.* 2015) were used for functional gene assignment (through information provided by GO term annotation and gene annotation search functions) and functional enrichment analysis, respectively. Graphics for visual depiction of variant data were created in R 3.3.0 (R Core Team 2015).

Statistical analysis

All statistical analyses were performed in R 3.3.0. We used a two-way permutational analysis of variance (PERMANOVA; Zapala and Schork (2006)) to test whether treatments (presence / absence of phage; 0, 0.2 or 2 μg ml^{-1} streptomycin) affected the types of mutations. Each clone was scored by the presence (1) or absence (0) of a non-synonymous mutation in each gene, and this data was used to calculate mutation frequency per population and the Euclidian distance between populations (Excoffier *et al.* 1992). Before performing PERMANOVA, its assumption of homogeneity of multivariate dispersions within treatments was tested with the betadisper function in the vegan package (Oksanen *et al*. 2016) that uses the PERMDISP2 procedure as described previously (Anderson 2006). The adonis function in the vegan package was then used to test the probability that the observed distances could arise by chance by comparing them with random permutations of the raw data (Anderson 2001). In addition, ANOVA and an unequal variances *t*-test was used to compare genotypes, populations or treatments in a number of genomic features, with the presence of a variant, mutation or mutated gene scored as 1 and the absence as 0. The statistical probabilities of *t*-tests performed to compare mutator and non-mutator genotypes, for which multiple comparisons were performed, were adjusted by sequential Bonferroni correction.

Results

Genome-wide variant profiles

We studied the genomic adaptations to phage, streptomycin at sub-MIC levels and the combination of the two stressors in three replicated bacterial populations per treatment. Single nucleotide polymorphisms (SNPs) and short insertions and deletions (indels) were the dominating variants observed. Larger duplications (copy number variations, CNVs) were less often found and almost exclusively in phage treatments with fewer duplications with increasing streptomycin concentrations (Fig. 1).

We used all variants (SNPs, indels) observed in the populations to test for the predictions for differences in changes on a genome wide scale in populations evolving with one or two stressors. Overall, we found that the number of variants was significantly increased in clones that evolved hypermutability (t_{51} = –15.99, p = 8.30 × 10^{-21}, Fig. 2), but we did not find differences in the overall number of variants between the treatments (two-way ANOVA: $F_{5, 12}$ = 0.586, p = 0.711). Examining the frequency of clones carrying variants within a population, we found interesting differences between the treatments but independent of the individual or combined stressors (Fig. 3). In control populations and populations exposed to only phage, most variants were found at low frequencies, whereas we observed variants at high frequency or fixed in all populations that were exposed to streptomycin independent of the presence of the phage, suggesting positive selection for those variants. Comparing the sets of genes carrying variants (Figs. 1, S2), PERMANOVA could not be used to test for differences between the two and one stressor environments because of higher multivariate dispersion in the two compared to the one stressor environments (PERMDISP2 ANOVA: $F_{1,13}$ = 5.93, p = 0.030). This is likely explained by the high frequency of mutators in the two stressor environment, each possessing a large amount of unique variants (Fig. 4), causing a higher

average distance of populations from the group centroid compared to the one stressor environments (Fig. S1). However, distinct differences were not observed in the sets of variants between the two and one stressor environments, likely driven by the fact that the replicates within one treatment varied considerably (Fig. 4).

Phage and antibiotic resistance

We next compared phenotypic and genomic adaptations to phage and streptomycin across and within the different environments and linked both phenotypic resistances with previously established genetic determinants. Phenotypic phage resistance was observed under selection by the phage but was more frequently observed with sub-MIC levels of streptomycin compared to phage alone. Specifically, phage resistance phenotypes occurred in approx. 6% of clones in the no phage environments (5/90 across three populations), and only occurred in one of three replicate populations in the 0 μg ml^{-1} streptomycin with phage environment (in 7/10 genotypes) (Fig. 1). Notably, resistance was tested against the ancestral phage, so it is possible that the two populations in the phage alone environment sensitive to the ancestral phage still harbor resistance against contemporary phages. Furthermore, in the 0.2 and 2 μg ml^{-1} streptomycin with phage environments, all populations were dominated by phage resistance phenotypes, with only one genotype in one population (0.2 μg ml^{-1} streptomycin with phage) displaying a phage susceptible phenotype.

Streptomycin resistant phenotypes were only observed in the treatment with the higher sub-MIC level (2 μg ml^{-1}, approx. 10% MIC), occurring in 5/6 populations. Thus, streptomycin resistant phenotypes were observed independent of the presence of phage. We further observed differences in the phenotypic streptomycin resistance which can be divided into two groups: clones with an MIC of approx. 25 μg ml^{-1} (24.7 ± 8.6 ml^{-1}) and those with an MIC > 1024 μg ml^{-1} (Fig. 1).

To identify adaptive genomic changes in response to the antibiotics and/or phage, we focused on those mutations that have previously been described to provide resistance to the phage Φ2 in *P. fluorescens*or streptomycin resistance in bacteria. Populations that evolved in the presence of the phage had different sets of mutations, with the same genes being more likely to be mutated, compared to populations that evolved without the phage (PERMANOVA: $F_{1,12}$ = 2.26, p = 0.007). Similarly to previous findings (Scanlan *et al.* 2015), among those genes with non-synonymous variants that occurred only among genotypes with phage resistance phenotypes (n = 321), several are associated with LPS biosynthesis. Specifically, mutations in the closely located LPS biosynthesis associated genes PFLU1657, PFLU1660, PFLU1663 and PFLU1668 occurred in several genotypes across populations. Based on this and previous observations, all genes with non-synonymous variants across treatments associated with gene ontology (GO) terms related to LPS, cell envelope biogenesis and the outer membrane (Table S4) were identified (Table S5) and compared between phenotypes. Variants in these genes occurred in all genotypes with a phage resistance phenotype, and in approx. 6% of genotypes with a phage susceptible phenotype. The genes in which variants occurred differ between phage resistant and susceptible phenotypes, apart from one gene in common (PFLU3467, with a variant in one resistant and one susceptible genotype). LPS, cell envelope biogenesis and outer membrane genes with non-synonymous variants occurring in genotypes with phage resistant phenotypes in this study are shown in Figures 1, 4.

Populations evolved under subinhibitory streptomycin concentrations had different sets of mutations, with the same genes being more likely to be mutated, compared to those evolved without streptomycin (PERMANOVA: $F_{2,12}$ = 3.12, p = 0.001) (Fig. 4). All had non-synonymous mutations in genes previously associated with streptomycin resistance phenotypes (Table S6), in particular, *rpsL* (PFLU5532) encoding the ribosomal protein S12

associated with high levels of resistance (Barnard *et al.* 2010; Sun *et al.* 2010), and *gidB* (PFLU6128) encoding a 16S rRNA methyltransferase associated with low levels of resistance (Okamoto *et al.* 2007) (Fig. 4). Streptomycin sensitive genotypes do not contain mutations in these genes.

Other gene groups and genomic regions affected by variants

We found several other groups of genes affected by non-synonymous variants that are associated with particular functions, especially motility, nutrient transport, metabolic pathways and hypermutability (Figs. 1, 4). Genes affecting motility showed functional enrichment across treatments (*fliA* and *flhA* in Fig. 4), and genes related to nutrient transport were also frequently affected by variants (PFLU4745 and PFLU6047 encoding a putative magnesium transporter and phosphate transporter, respectively, in Fig. 4), although we were not able to identify a significant pattern within the different treatments. In addition, as described, a difference in the amount of large genomic duplications per genotype was observed between treatments, with more duplications in the presence of phage compared to without phage ($t_{121} = -5.29$, $p = 5.63 \times 10^{-7}$) (Fig. 1). The duplications occurred inconsistently throughout the genome, and because of their large size, ranging from tens of thousands to hundreds of thousands of base pairs, potential phenotypic effects could not be predicted.

Bacterial hypermutable (mutator) phenotypes have been frequently linked to mutations in *mutS* and *mutL*, whose products form the primary components of the methyl-directed mismatch repair (MMR) system. Such mutator phenotypes might play a key role in the adaptation of bacteria to stressful environments (Taddei *et al.* 1997; Oliver *et al.* 2002; Prunier & Leclercq 2005). Mutations in MMR genes occur in seven of the experimental

populations (three *mutL* mutation and three *mutS* mutation dominated populations and a single *mutL* mutated genotype in one population) across treatments, except for the 0.2µg ml^{-1} Sm without phage and the phage without Sm environments, amounting to approx. 29% (53/180) of all genotypes (Table S3). We observed mutators in 1 out of 9 populations in the single stressor (excluding the 2 µg ml^{-1} Sm without phage populations) and 3 out of 6 in the two stressor environment, but frequencies were not different (2-sample test for equality of proportions with continuity correction: $\chi^2 = 0.46$, df = 1, $p = 0.495$). Mutations in *mutS* and *mutL* were associated with an approx. 10-fold higher amount of SNPs and short indels (41.09 ± 16.08 variants per MMR mutant) compared with other genotypes (5.14 ± 1.76 variants) (Fig. 2). In addition, the proportion of SNPs and short indels present in only one genotype in a population was higher in MMR mutation containing populations (0.68 ± 0.18) compared with other populations (0.11 ± 0.12; $t_9 = -7.43$, $p = 1.99 \times 10^{-4}$). MMR mutant dominated populations exhibited high genetic divergence compared to each other and non-mutator populations (Fig. S1). The divergence is explained both by mutations in common genes across treatments and by mutations in unique genes under selection by the phage or subinhibitory streptomycin concentrations (Fig. S2).

Mutator alleles were associated with a number of potentially adaptive mutations. Among genotypes with phage resistance phenotypes, all non-mutators had a non-synonymous variant in only one phage resistance gene while mutators frequently had variants in two or three of these genes (1.24 ± 0.50 genes), significantly differing from non-mutators ($t_{32} = -14.22$, $p = 1.11 \times 10^{-14}$) (Fig. 4). There was considerable variety in within-population frequency of individual phage resistance associated mutations, ranging from a single to all genotypes in a population (Fig. 4). All phage resistant phenotypes from the no phage environments were mutators, suggesting that mutators might increase the supply rate of phage resistance

mutations. However, the number of potential phage resistance mutations between phage resistant populations that were dominated by mutators (3.67 ± 1.15 mutations/pop.) does not differ significantly from those that did not contain mutators (1.67 ± 1.15 mutations/pop.; t_4 = 2.78, p = 0.10). Mutator alleles showed no patter of co-occurrence with streptomycin resistance alleles, occurring both together and separately in multiple cases. Regarding the co-occurrence of mutator alleles with other potentially adaptive mutations, mutators contained a higher number of non-synonymous mutations (1.11 ± 0.87) in genes associated with motility functions compared to non-mutators (0.31 ± 0.47; t_{65} = –6.33, $p = 1.28 \times 10^{-7}$).

Discussion

Adaptation of bacterial populations to antibiotics and coevolving phage in isolation has been extensively studied. We previously noted that simultaneous exposure to antibiotic and phage increased the adaptation against both of the stressors (Cairns *et al.* 2016) despite the expectation that fitness costs associated with the resistant phenotypes, differences in selection, potential clonal interference and lower efficacy of selection would slow down or alter evolution. We here set out to investigate the genomic changes. Namely, we examined the effect of sub-MICs of streptomycin and the lytic phage Φ2, applied either individually or together, on the genomic evolution of *P. fluorescens*. Overall, we were able to link genomic changes to phenotypic observations (phage and streptomycin resistance, hypermutability) and we detected a few differences in changes on the genome-wide level between co-selected and selected environments.

The distribution of variant frequencies differed between the environments with clear signs of positive selection for streptomycin resistance (Fig. 3). We observed that streptomycin selected for high frequencies of (known) mutations providing streptomycin resistance in all

treatments with antibiotics at approx. 10% of MIC, suggesting that directional selection for streptomycin resistance alleles was not changed by the presence of the phage (Figs. 1, 3, 4). Signs of positive selection for phage resistance were only found in the co-selected environments, indicating that selection for phage resistance might have been facilitated in the presence of streptomycin. Furthermore, the total number of variants was not different when comparing treatments with the two stressors individually or in combination, although we observed a significant increase in variants in the presence of mutators. Also, we did not observe disruptive selection in the co-selected environments, as the same genotypes carried alleles for phage and streptomycin resistance. Thus our data suggest that neither clonal interference nor low selection efficacy altered the genomic evolution in the populations. Interestingly, large duplications of genomic regions were more often found in the presence of the phage, but the number of duplications decreased with increasing streptomycin concentrations. There was, however, no pattern shared across replicates and this observation warrants future investigation.

Examining the patterns of genes that are involved in streptomycin resistance, we found two associated genes and those were at high frequency in the 2 μg ml^{-1} streptomycin environments independent of the presence of the phage. We identified four genes associated with most instances of phage resistance but these were at lower frequencies compared to the streptomycin resistance alleles. Differences in these patterns for phage and streptomycin resistance are likely explained by variation in the population dynamics and differences in the mode of selection imposed by the coevolving phage and streptomycin. Selection by phage occurs typically via arms-race dynamics (short periods of directional selection) or negative frequency dependent selection in contrast to constant directional selection by the antibiotics. In addition, phage densities differed between treatments with different streptomycin

concentrations (see below). Thus, the time of isolation for sequencing might have influenced the variant patterns in terms of variants and their frequencies. We did not observe accelerated molecular evolution in the phage environments, as it has been described in this system before (Paterson *et al.* 2010), when comparing the total number of variants within populations (Fig. 2) of evolved and co-evolved populations (Fig. S1).

Surprisingly, phage resistance was not detected in two out of three populations in the phage alone environment. Since we tested phage resistance only against the ancestral phage, it is possible that prolonged coevolution in the phage alone environment might have resulted in the selection for bacterial genotypes no longer resistant against the ancestral phage but resistant against coevolved contemporary phages. Nevertheless, this appears to be unlikely due to the lack of phage resistance associated mutations in clones that evolved with the phage but were sensitive against the ancestral phage. It seems more likely that the frequency of phage resistance had decreased, potentially due to a decreased amount of phage, below the detection limit obtainable by sequencing 10 clones per population. Furthermore, it is possible that this decrease was prevented in the co-selective environments by the presence of other adaptive mutations in the same genetic background as phage resistance mutations.

We observed that the phage went extinct in the presence of streptomycin (Cairns *et al.* 2016), whereas streptomycin was present over the whole course of the experiment. As stated above, major adaptations against the phage occurred early during the experiment also with streptomycin, and we therefore consider these to be co-selected environments despite phage extinction later in the experiment. Interestingly, we still found phage resistance variants in the populations despite phage extinction, suggesting that these mutations were neutral or have only small fitness costs. Compensatory mutations or generally beneficial mutations are

common in phage and antibiotic resistant bacteria (Maisnier-Patin & Andersson 2004; Zhang & Watson 2009; MacLean *et al.* 2010). Based on the function, variants found here in genes associated with motility and nutrient transport might be compensatory (Gresham & Dunham 2014; Bailey & Bataillon 2016). We identified one potential generally beneficial mutation in a gene encoding a protein involved in magnesium transport (PFLU4745), as this was present in almost all populations (16 out 18) and typically at high frequencies. We further observed mutations in a large number of genes in those clones with mutator genotypes and streptomycin resistance, which could speculatively be associated with streptomycin resistance (genes associated with the structure or functioning of the ribosome, amino acid transport or polyamine transport that may affect the affinity and/or uptake of streptomycin (Holtje 1979; Taber *et al.* 1987) (Table S6). For both observations, further functional studies and/or longitudinal sequence data are required to understand the underlying functions.

Mutators played an important role in the amount of variants in our study. Under co-selection, we were able to identify mutator alleles in approx. 50% of the clones. In these, a higher number of mutations in phage resistance associated genes (LPS biosynthesis genes) were observed per genotype compared to non-mutators, but we also found elevated phage resistance and associated mutations in non-mutators under co-selection. This suggests that mutator genotypes alone are unlikely to explain the evolution of streptomycin and elevated phage resistance in the co-selected environments, although they have been found in previous studies to be associated with enhanced bacterial coevolution against phage, as well as phage extinction tendency (Pal *et al.* 2007; Morgan *et al.* 2010). There are several other potential mechanisms such as non-constitutive, transient mutability (Galhardo *et al.* 2007), differences in coevolutionary dynamics of bacteria-phage interaction in the presence of antibiotics (Buckling & Rainey 2002; Brockhurst *et al.* 2007; Hall *et al.* 2011), or epistatic interactions

between mutations conferring phage and streptomycin resistance (Vogwill *et al.* 2016). For the latter, the order of mutations is important, but this cannot be reconstructed from our end-point.

An interesting observation in our study is that mutators were present in a third of the populations but without a significant pattern. The common idea is that increased rates of adaptation through elevated mutation rates provide a short term advantage (Taddei *et al.* 1997). Bacterial populations adapted quickly to the presence of phage and antibiotics and the experimental conditions (Cairns *et al.* 2016) and increases in mutation rates were most likely costly after the initial adaptation, as most *de novo* mutations are neutral or deleterious. Under these conditions, we would have predicted selection against the mutators. Furthermore, antibiotic (Andersson & Hughes 2012, 2014; Tazzyman & Hall 2015) and phage (Pal *et al.* 2007) resistance mutations have been suggested to represent particularly important types of beneficial mutations driving indirect selection for hitchhiking mutator alleles. However, such hitchhiking mutators were unlikely in our study, as there was no clear association between treatments and mutators. Several factors might explain the presence of mutators across experimental treatments (and/or their common occurrence among natural isolates). It has recently been suggested that mutators might not be selected solely because of beneficial mutations but instead or also due to direct selection (Torres-Barceló *et al.* 2013). Specifically, MMR defective mutants can exhibit elevated sensitivity to environmental stressors, such as oxidative stress, ultraviolet light, or temperature (Oliver *et al.* 2002; Rodríguez-Rojas & Blázquez 2009), which may alter gene regulation and expression. In line with this, a *mutS* mutant strain of *Escherichia coli* was shown to have altered expression of genes involved in amino acid and carbohydrate transport/metabolism (Robbins-Manke *et al.* 2005), potentially providing a fitness advantage under certain environmental conditions. Alternatively or

additionally, the cost of hypermutability in terms of a high load of deleterious mutations might be alleviated by reductive evolution, that is, the deletion of redundant and/or dispensible functions in the genome (Rau *et al.* 2012), or by enhanced purging of deleterious mutations.

Bacterial systems are dynamic, often facing altering selection pressures due to evolution (Buckling & Rainey 2002; Hiltunen & Becks 2014)), ecology (Hibbing *et al.* 2010; Becks & Arndt 2013) or the interplay of these at one time scale (Harrington & Sanchez 2014; Hiltunen 2015; Friman *et al.* 2016) which, in turn, forces rapid, often continuous adaptation to prevailing (local) conditions. Understanding and predicting local adaptation of (microbial) populations remains a major challenge in biology, not only as a mere scientific curiosity but also in modern health care. Our study provides characterizations of the genetic factors and genome-wide changes caused by co-selection for phage and streptomycin resistance. We show that the genomic changes differed only little between the two and one stressor environments, as selection for phage resistance was affected by the antibiotic but selection for antibiotic resistance was not affected by the presence of the phage. Examining patterns of phage resistance on the genome level, we were able to identify several genes likely involved in phage resistance, and these were at higher frequencies in the presence of co-selection, although the underlying mechanism is still unclear.

Competing interests. We have no competing interests.

Author contributions. L.B. and T.H. designed the evolutionary experiment. J.C. and T.H. performed the experiment. J.F., J.C., and L.B. designed the analysis of sequence data. J.C. and J.F. performed the sequence analysis. All authors wrote the manuscript, gave final approval for publication, and accept accountability for the content and work performed.

Acknowledgements. We thank T. Niska, S. Suominen and T. Virolainen for help with collecting data for the evolutionary experiment, and CSC – IT Center for Science Ltd. for the allocation of computational resources. We thank S. Künzel and his team (Max Planck Institute for Evolutionary Biology) for DNA sequencing.

Funding. The study was funded by the Academy of Finland to T.H. (#106993), University of Helsinki grant to T.H. (#490152) and Finnish Cultural Foundation grant to J.C. (#160149), and supported by the Academy of Finland Centre of Excellence Program in Biological Interactions (#252411). L.B. thanks the German Research Foundation for funding (BE4135/3-1).

References

Data Accessibility

Raw sequence data has been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the BioProject accession number PRJNA354680. From down-stream analysis, Variant Call Format (VCF) files are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.62s22.

Figure legends

Fig. 1 Overview of phenotypic and genotypic results for replicate populations (n = 3) per treatment. The second column displays phenotypic results for streptomycin resistance, as an on/off trait (*dark* = resistant, *light* = sensitive) or minimum inhibitory concentration (MIC) value, and phage resistance (*dark* = resistant, *light* = sensitive). The third column shows genotypic results for the number of genes with non-synonymous mutations according to category, including phage resistance, streptomycin resistance and hypermutability associated genes, all other genes affecting motility (an enriched function) or with non-synonymous mutations in at least two experimental populations, and the number of large genomic duplications. The data is normalized from 0–1 within every subcolumn.

Fig. 2 Number of SNPs and short indels detected in experimental populations of *P. fluorescens* SBW25. Sub-bars indicate variants in non-coding regions (*black*), synonymous variants in coding sequences (*grey*), and non-synonymous variants in coding sequences (*white*). Sm = streptomycin; Φ2 = phage SBW25Φ2. Populations containing mutators (*mutL* or *mutS* mutants) are indicated by a triangle.

Fig. 3 Distribution of SNPs and short indels in evolving populations. The y-axis shows the number of variants in each category by frequency in the population (0.1 = 1 clone – 1 = 10 clones, decreasing grey scale). Variants from all three replicates per treatment are shown.

Fig. 4 Genomic evolution in experimental populations of *P. fluorescens* SBW25. All genes containing non-synonymous mutations in a minimum of three experimental populations (n = 18) are shown, representing cases of parallel evolution. In addition, those genes are indicated that were mutated at lower frequency but are associated with phage resistance or streptomycin resistance. Concentric circles correspond with replicate populations in the 0, 0.2 or 2 μg ml^{-1} streptomycin environments, indicated by different colors, either with (*right*) or

without (*left*) the phage SBW25Φ2. Positions around the circles correspond to positions around the *P. fluorescens* SBW25 reference genome, starting from 12 o'clock. Mutations are indicated by dots, with the size of the dot representing the proportion of mutated genotypes in each population (min. 10%, max. 100%).

References

Abyzov A, Urban AE, Snyder M, Gerstein M (2011) CNVnator: an approach to discover, genotype, and characterize typical and atypical CNVs from family and population genome sequencing. *Genome Research*, **21**, 974–984.

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, **26**, 32–46.

Anderson MJ (2006) Distance-based tests for homogeneity of multivariate dispersions. *Biometrics*, **62**, 245–253.

Andersson DI, Hughes D (2012) Evolution of antibiotic resistance at non-lethal drug concentrations. *Drug Resistance Updates*, **15**, 162–172.

Andersson DI, Hughes D (2014) Microbiological effects of sublethal levels of antibiotics. *Nature Reviews Microbiology*, **12**, 465–478.

Bailey SF, Bataillon T (2016) Can the experimental evolution programme help us elucidate the genetic basis of adaptation in nature? *Molecular Ecology*, **25**, 203–218.

Barnard AM, Simpson NJ, Lilley KS, Salmond GP (2010) Mutations in *rpsL* that confer streptomycin resistance show pleiotropic effects on virulence and the production of a carbapenem antibiotic in *Erwinia carotovora*. *Microbiology*, **156**, 1030–1039.

Beaumont HJ, Gallie J, Kost C, Ferguson GC, Rainey PB (2009) Experimental evolution of bet hedging. *Nature*, **462**, 90–93.

Becks L, Arndt H (2013) Different types of synchrony in chaotic and cyclic communities. *Nature Communications*, **4**, 1359.

Belotte D, Curien JB, Maclean RC, Bell G (2003) An experimental test of local adaptation in soil bacteria. *Evolution*, **57**, 27–36.

Brockhurst MA, Morgan AD, Fenton A, Buckling A (2007) Experimental coevolution with bacteria and phage. The *Pseudomonas fluorescens*—Φ2 model system. *Infection, Genetics and Evolution*, **7**, 547–552.

Brooks AN, Turkarslan S, Beer KD, Lo FY, Baliga NS. (2011) Adaptation of cells to new environments. *Wiley Interdisciplinary Reviews. Systems Biology and Medicine*, **3**, 544–561.

Buckling A, Rainey PB. 2002 Antagonistic coevolution between a bacterium and a bacteriophage. *Proceedings of the Royal Society B: Biological Sciences*, **269**, 931–936.

Cairns J, Becks L, Jalasvuori M, Hiltunen T (2016) Sublethal streptomycin concentrations and lytic bacteriophage together promote resistance evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences*. *Manuscript accepted for publication*.

Coulter LB, McLean RJ, Rohde RE, Aron GM (2014) Effect of bacteriophage infection in combination with tobramycin on the emergence of resistance in *Escherichia coli* and *Pseudomonas aeruginosa* biofilms. *Viruses*, **6**, 3778–3786.

Davies J, Davies D (2010) Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews*, **74**, 417–433.

DePristo MA, Banks E, Poplin R *et al*. (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nature Genetics*, **43**, 491–498.

Escobar-Páramo P, Gougat-Barbera C, Hochberg ME (2012) Evolutionary dynamics of separate and combined exposure of *Pseudomonas fluorescens* SBW25 to antibiotics and bacteriophage. *Evolutionary Applications*, **5**, 583–592.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

Friman VP, Buckling A (2013) Effects of predation on real-time host-parasite coevolutionary dynamics. *Ecology Letters*, **16**, 39–46.

Friman VP, Dupont A, Bass D, Murrell DJ, Bell T (2016) Relative importance of evolutionary dynamics depends on the composition of microbial predator-prey community. *The ISME Journal*, **10**, 1352–1362.

Galhardo RS, Hastings PJ, Rosenberg SM (2007) Mutation as a stress response and the regulation of evolvability. *Critical Reviews in Biochemistry and Molecular Biology*, **42**, 399–435.

Gómez P, Bennie J, Gaston KJ, Buckling A (2015) The impact of resource availability on bacterial resistance to phages in soil. *PLOS ONE*, **10**, e0123752.

Gresham D, Dunham MJ (2014) The enduring utility of continuous culturing in experimental evolution. *Genomics*, **104**, 399–405.

Gullberg E, Albrecht LM, Karlsson C, Sandegren L, Andersson DI (2014) Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. *mBio*, **5**, e01918-14.

Hall AR, Scanlan PD, Morgan AD, Buckling A (2011) Host-parasite coevolutionary arms races give way to fluctuating selection. *Ecology Letters*, **14**, 635–642.

Harrington KI, Sanchez A (2014) Eco-evolutionary dynamics of complex social strategies in microbial communities. *Communicative & Integrative Biology*, **7**, e28230.

Harrison E, Laine AL, Hietala M, Brockhurst MA (2013) Rapidly fluctuating environments constrain coevolutionary arms races by impeding selective sweeps. *Proceedings of the Royal Society B: Biological Sciences*, **280**, 20130937.

Hegreness M, Shoresh N, Damian D, Hartl D, Kishony R (2008) Accelerated evolution of resistance in multidrug environments. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 13977–13981.

Hermsen R, Deris JB, Hwa T (2012) On the rapidity of antibiotic resistance evolution facilitated by a concentration gradient. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 10775–10780.

Hibbing ME, Fuqua C, Parsek MR, Peterson SB (2010) Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology*, **8**, 15–25.

Hiltunen T, Ayan GB, Becks L (2015) Environmental fluctuations restrict eco-evolutionary dynamics in predator-prey system. *Proceedings of the Royal Society B: Biological Sciences*, **282**, 20150013.

Hiltunen T, Becks L (2014) Consumer co-evolution as an important component of the eco-evolutionary feedback. *Nature Communications*, **5**, 5226.

Höltje JV (1979) Induction of streptomycin uptake in resistant strains of *Escherichia coli*. *Antimicrobial Agents and Chemotheraphy*, **15**, 177–181.

Hughes D, Andersson DI (2015) Evolutionary consequences of drug resistance: shared principles across diverse targets and organisms. *Nature Reviews Genetics*, **16**, 459–471.

Ketola T, Hiltunen T (2014) Rapid evolutionary adaptation to elevated salt concentrations in pathogenic freshwater bacteria *Serratia marcescens*. *Ecology and Evolution*, **4**, 3901–3908.

Knezevic P, Curcin S, Aleksic V, Petrusic M, Vlaski L (2013) Phage-antibiotic synergism: a possible approach to combatting *Pseudomonas aeruginosa*. *Research in Microbiology*, **164**, 55–60.

Koskella B, Brockhurst MA (2014) Bacteria-phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *FEMS Microbiology Reviews*, **38**, 916–931.

Koskella B, Vos M (2015) Adaptation in natural microbial populations. *Annual Review of Ecology, Evolution, and Systematics*, **46**, 503–522.

Kraemer SA, Kassen R (2015) Patterns of local adaptation in space and time among soil bacteria. *The American Naturalist*, **185**, 317–331.

Labrie SJ, Samson JE, Moineau S (2010) Bacteriophage resistance mechanisms. *Nature Reviews Microbiology*, **8**, 317–327.

Laehnemann D, Peña-Miller R, Rosenstiel P *et al*. (2014) Genomics of rapid adaptation to antibiotics: convergent evolution and scalable sequence amplification. Genome Biology and Evolution, 6, 1287–1301.

Li H (2011) A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics*, **27**, 2987–2993.

Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, **25**, 1754–1760.

MacLean RC, Hall AR, Perron GG, Buckling A (2010) The population genetics of antibiotic resistance: integrating molecular mechanisms and treatment contexts. *Nature Reviews Genetics*, **11**, 405–414.

Maisnier-Patin S, Andersson DI (2004) Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. *Research in Microbiology*, **155**, 360–369.

Martiny JB, Bohannan BJ, Brown JH *et al*. (2006) Microbial biogeography: putting microorganisms on the map. *Nature Reviews Microbiology*, **4**, 102–112.

McKenna A, Hanna M, Banks E *et al*. (2010) The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research*, **20**, 1297–1303.

McLaren W, Pritchard B, Rios D *et al*. (2010) Deriving the consequences of genomic variants with the Ensembl API and SNP Effect Predictor. *Bioinformatics*, **26**, 2069–2070.

Morgan AD, Bonsall MB, Buckling A (2010) Impact of bacterial mutation rate on coevolutionary dynamics between bacteria and phages. *Evolution*, **64**, 2980–2987.

Nagel M, Reuter T, Jansen A, Szekat C, Bierbaum G (2011) Influence of ciprofloxacin and vancomycin on mutation rate and transposition of IS256 in *Staphylococcus aureus*. *International Journal of Medical Microbiology*, **301**, 229–236.

Okamoto S, Tamaru A, Nakajima C *et al*. (2007) Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. *Molecular Microbiology*, **63**, 1096–1106.

Oksanen J, Blanchet GF, Kindt R *et al*. (2016) vegan: Community ecology package. R package version 2.3-5. URL: http://CRAN.R-project.org/package=vegan.

Oliver A, Baquero F, Blázquez J (2002) The mismatch repair system (*mutS*, *mutL* and *uvrD* genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. *Molecular Microbiology*, **43**, 1641–1650.

Pal C, Maciá MD, Oliver A, Schachar I, Buckling A (2007) Coevolution with viruses drives the evolution of bacterial mutation rates. *Nature*, **450**, 1079–1081.

Lopez Pascua L, Hall AR, Best A *et al*. (2014) Higher resources decrease fluctuating selection during host-parasite coevolution. *Ecology Letters*, **17**, 1380–1388.

Paterson S, Vogwill T, Buckling A *et al*. (2010) Antagonistic coevolution accelerates molecular evolution. *Nature*, **464**, 275–278.

Prunier AL, Leclercq R (2005) Role of *mutS* and *mutL* genes in hypermutability and recombination in *Staphylococcus aureus*. *Journal of Bacteriology*, **187**, 3455–3464.

R Core Team. 2015 R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-project.org/.

Rainey PB, Bailey MJ (1996) Physical and genetic map of the *Pseudomonas fluorescens* SBW25 chromosome. *Molecular Microbiology*, **19**, 521–533.

Rainey PB, Travisano M (1998) Adaptive radiation in a heterogeneous environment. *Nature*, **394**, 69–72.

Rau MH, Marvig RL, Ehrlich GD, Molin S, Jelsbak L (2012) Deletion and acquisition of genomic content during early stage adaptation of *Pseudomonas aeruginosa* to a human host environment. *Environmental Microbiology*, **14**, 2200–2211.

Ren L, Rahman MS, Humayun MZ (1999) *Escherichia coli* cells exposed to streptomycin display a mutator phenotype. *Journal of Bacteriology*, **181**, 1043–1044.

Robbins-Manke JL, Zdraveski ZZ, Marinus M, Essigmann JM (2005) Analysis of global gene expression and double-strand-break formation in DNA adenine methyltransferase- and mismatch repair-deficient *Escherichia coli*. *Journal of Bacteriology*, **187**, 7027–7037.

Rodríguez-Rojas A, Blázquez J (2009) The *Pseudomonas aeruginosa pfpI* gene plays an antimutator role and provides general stress protection. *Journal of Bacteriology*, **191**, 844–850.

Scanlan PD, Hall AR, Blackshields G *et al*. (2015) Coevolution with bacteriophages drives genome-wide host evolution and constrains the acquisition of abiotic-beneficial mutations. *Molecular Biology and Evolution*, **32**, 1425–1435.

Seiler C, Berendonk TU (2012) Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Frontiers in Microbiology*, **3**, 399.

Sun YJ, Luo JT, Wong SY, Lee AS (2010) Analysis of *rpsL* and *rrs* mutations in Beijing and non-Beijing streptomycin-resistant *Mycobacterium tuberculosis* isolates from Singapore. *Clinical Microbiology and Infection*, **16**, 287–289.

Szklarczyk D, Franceschini A, Wyder S *et al*. (2015) STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Research*, **43**, D447–452.

Taber HW, Mueller JP, Miller PF, Arrow AS (1987) Bacterial uptake of aminoglycoside antibiotics. *Microbiological Reviews*, **51**, 439–457.

Taddei F, Radman M, Maynard-Smith J *et al*. (1997) Role of mutator alleles in adaptive evolution. *Nature*, **387**, 700–702.

Tazzyman SJ, Hall AR (2015) Lytic phages obscure the cost of antibiotic resistance in *Escherichia coli*. *The ISME Journal*, **9**, 809–820.

Torres-Barceló C, Arias-Sánchez FI, Vasse M *et al*. (2014) A window of opportunity to control the bacterial pathogen *Pseudomonas aeruginosa* combining antibiotics and phages. *PLOS ONE*, **9**, e106628.

Torres-Barceló C, Cabot G, Oliver A, Buckling A, Maclean RC (2013) A trade-off between oxidative stress resistance and DNA repair plays a role in the evolution of elevated mutation rates in bacteria. *Proceedings of the Royal Society B: Biological Sciences*, **280**, 20130007.

Van der Auwera GA, Carneiro MO, Hartl C *et al*. (2013) From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. *Current Protocols in Bioinformatics*, **43**, 11.10.1–11.10.33.

Vogwill T, Kojadinovic M, MacLean RC (2016) Epistasis between antibiotic resistance mutations and genetic background shape the fitness effect of resistance across species of *Pseudomonas*. *Proceedings of the Royal Society B: Biological Sciences*, **283**, 20160151.

Winsor GL, Griffiths EJ, Lo R *et al*. (2016) Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the Pseudomonas genome database. *Nucleic Acids Research*, **44**, D646–653. URL: http://pseudomonas.com/.

Zapala MA, Schork NJ (2006) Multivariate regression analysis of distance matrices for testing associations between gene expression patterns and related variables. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 19430–19435.

Zhang J, Ormälä-Odegrip AM, Mappes J, Laakso J (2014) Top-down effects of a lytic bacteriophage and protozoa on bacteria in aqueous and biofilm phases. *Ecology and Evolution*, **4**, 4444–4453.

Zhang L, Watson LT (2009) Analysis of the fitness effect of compensatory mutations. *HFSP Journal*, **3**, 47–54.

Zhang QG, Buckling A (2012) Phages limit the evolution of bacterial antibiotic resistance in experimental microcosms. *Evolutionary Applications*, **5**, 575–582.

Zhou Y, Xu YB, Xu JX *et al*. (2015) Combined toxic effects of heavy metals and antibiotics on a *Pseudomonas fluorescens* strain ZY2 isolated from swine wastewater. *International Journal of Molecular Sciences*, **16**, 2839–2850.

Zumsteg A, Bååth E, Stierli B, Zeyer J, Frey B (2013) Bacterial and fungal community responses to reciprocal soil transfer along a temperature and soil moisture gradient in a glacier forefield. *Soil Biology and Biochemistry*, **61**,121–132.

Örmälä-Odegrip AM, Ojala V, Hiltunen T *et al*. (2015) Protist predation can select for bacteria with lowered susceptibility to infection by lytic phages. *BMC Evolutionary Biology*, **15**, 81.