

Fitness Tradeoffs of Antibiotic Resistance in Extraintestinal Pathogenic *Escherichia coli*

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Abstract

Evolutionary trade-offs occur when selection on one trait has detrimental effects on other traits. In pathogenic microbes, it has been hypothesized that antibiotic resistance trades off with fitness in the absence of antibiotic. Although studies of single resistance mutations support this hypothesis, it is unclear whether trade-offs are maintained over time, due to compensatory evolution and broader effects of genetic background. Here, we leverage natural variation in 39 extraintestinal clinical isolates of *Escherichia coli* to assess trade-offs between growth rates and resistance to fluoroquinolone and cephalosporin antibiotics. Whole-genome sequencing identifies a broad range of clinically relevant resistance determinants in these strains. We find evidence for a negative correlation between growth rate and antibiotic resistance, consistent with a persistent trade-off between resistance and growth. However, this relationship is sometimes weak and depends on the environment in which growth rates are measured. Using in vitro selection experiments, we find that compensatory evolution in one environment does not guarantee compensation in other environments. Thus, even in the face of compensatory evolution and other genetic background effects, resistance may be broadly costly, supporting the use of drug restriction protocols to limit the spread of resistance. Furthermore, our study demonstrates the power of using natural variation to study evolutionary trade-offs in microbes.

Key words: antibiotic resistance, trade-off, whole-genome sequencing, compensatory evolution, epistasis.

Introduction

Trade-offs occur when an improvement in one trait is accompanied by deterioration in another. Evolutionary biologists have had a long-standing interest in trade-offs, dating back at least to Darwin, who recognized that directional selection on a given trait would often have unforeseen, and frequently detrimental, effects on other traits (Darwin 1859; Agrawal et al. 2010). The negative correlations that result from trade-offs are thought to be important drivers of both intra- and interspecific diversity (Schluter 1996; Clark et al. 2007; Ferenci 2016), and may limit the extent and pace of adaptation (Futuyma and Moreno 1988; Agrawal and Stinchcombe 2009).

Fitness costs associated with antimicrobial resistance (AMR) constitute a trade-off of particular importance to human

health. It is often assumed that AMR is accompanied by reduced fitness in the absence of antibiotic—AMR pathogens may suffer from reduced competitive ability (Melnik et al. 2015; Vogwill and MacLean 2015), growth rates (Bagel et al. 1999), and/or virulence (Alonso et al. 2004; Olivares et al. 2012). This assumption underlies public health efforts to reduce the prevalence of resistance by restriction of antibiotic use (Enne 2010): costly resistance mutations should decrease in frequency in the absence of drug. Consistent with this assumption, laboratory studies on the fitness effects of individual AMR elements (either chromosomal mutations or mobile elements) typically find costs, although some resistance mutations appear to be cost-free (Andersson 2006; Melnik et al. 2014; Hughes and Andersson 2015; Vogwill and MacLean 2015). In extreme cases, such as the antifungal drug amphotericin B,

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resistance is so costly as to prevent emergence of clinically relevant resistance (Vincent et al. 2013).

Nonetheless, while individual resistance elements may impose reduced fitness, these costs can be mitigated by genetic background (reviewed in Wong 2017). In some cases, segregating polymorphism can mitigate the costs of resistance. For example, fitness of the quinolone resistant *gyrA* C257T mutation in *Campylobacter jejuni* can vary drastically depending on background. On one background, this mutation is costly and is outcompeted by susceptible genotypes in a chicken model of infection. On an alternative genetic background, however, resistance is cost-free, and in fact the resistant mutant outcompetes susceptible strains (Luo et al. 2005). Similarly, background-specific costs have been documented for other resistance mutations and for plasmid-mediated resistance (Björkholm et al. 2001; Humphrey et al. 2012).

Even when a mutation is initially costly on a given background, fitness can be readily recovered in the lab by serially passaging resistant populations in drug-free media for dozens to hundreds of generations (Bouma and Lenski 1988; Bjorkman et al. 2000; Dionisio et al. 2005; Kugelberg et al. 2005). Importantly, at least some degree of resistance is typically maintained following serial passage, indicating that fitness recovery is not due to simple reversion of the original resistance mutation. Thus, mutations elsewhere in the genome—referred to as “compensatory mutations”—can mitigate the costs of resistance, and may be either presegregating, or arise following the acquisition of resistance.

Given considerations of genetic background and compensatory evolution, it is still unclear whether AMR is accompanied by costs “in the wild”—that is, among microbes found in clinical, environmental, and/or agricultural settings. Although compensation is easily attainable in the lab, data are sparse concerning its importance in clinical settings: there are strong data to support compensation for costs of rifampicin resistance in *Mycobacterium tuberculosis* (Brandis et al. 2012; Cohen et al. 2015; Coscolla et al. 2015), but not in other systems. The question thus remains as to whether AMR trades off with other fitness components in natural populations.

In studying trade-offs associated with resistance, microbiologists have focused on the costs imposed by specific AMR mutations. An alternative approach has been widely used in animal and plant systems, and to a lesser extent in microbes (reviewed in Stearns 1989; Agrawal and Stinchcombe 2009; Ferenci 2016). The underlying premise is straightforward. Two traits, such as offspring number and offspring size, may both be under directional selection for more extreme values. If there is a trade-off between these traits—perhaps because resources are limiting—then we expect to observe a negative correlation between them; offspring may be large or numerous, but they cannot be both. Trade-offs are widespread in natural populations, for example, between survival and reproduction (Clutton-Brock et al. 1983), between insect performance on alternate host plants (Via and Hawthorne 2002),

and between senescence and rapid reproduction (Partridge 1987).

Negative trait correlations have been used to infer trade-offs in a number of cases in microbes (Ferenci 2016). Most relevantly, Phan and Ferenci (2013) found a negative correlation between competitive fitness and resistance to two antimicrobials (the antibiotic chloramphenicol and the detergent SDS) among natural isolates of *Escherichia coli* derived from a range of animal and primarily healthy human sources. They were able to attribute this tradeoff, at least in part, to natural variation in membrane permeability, which governs the entry of both the antimicrobials and nutrients into the cell. However, to our knowledge, there has not been a natural-variation based study of the trade-offs of clinically relevant AMR in microbes.

Here, we address trade-offs between AMR and growth rates in clinical isolates of *E. coli*. In keeping with the assumptions of trade-off theory, we expect both resistance and growth rates to be under directional selection, at least some of the time: resistance should be selected for when antibiotics are present, and growth rates should be selected for during infection and under nutrient-rich environmental conditions. As such, if there is a trade-off between resistance and growth, we predict a negative correlation between these two traits. We go on to investigate mechanisms underlying the inferred trade-offs, and suggest that mitigation of the costs of resistance may be limited by the environmental specificity of compensatory mutations.

Materials and Methods

Strains and Growth Conditions

Thirty-nine extraintestinal pathogenic *E. coli* (ExPEC; Dale and Woodford 2015) isolates, collected as part of the CANWARD survey of antibiotic resistant pathogens in Canada (Lagace-Wiens et al. 2013; Zhanel et al. 2013), were obtained from the Zhanel laboratory at the University of Manitoba. These isolates were collected from patients at hospitals across Canada, from a variety of nongastrointestinal infection types, including urinary tract, blood, wound, and respiratory infections (supplementary table S1, Supplementary Material online). These isolates represent a wide range of quinolone and β -lactam sensitivities. Enterohemorrhagic (EHEC) strains of intestinal origin were excluded since antibiotic use is contraindicated for these infections due to an increased risk of haemolytic-uremic syndrome (Goldwater and Bettelheim 2012). Additional experiments were carried out using the *E. coli* laboratory strain K-12 (MG1655), as well as two *Cip*^R derivatives of MG1655, one carrying a *gyrA* D87G mutation, and the other carrying a *marR* R94C mutation (Wong and Seguin 2015).

Cultures were grown in lysogeny broth (LB; 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl), tryptic soy broth (TSB; 17 g/l tryptone, 3 g/l phytone, 5 g/l NaCl, 2.5 g/l K₂HPO₄,

2.5 g/l glucose), or M9 minimal media + glucose (M9; 1 g/l NH₄Cl, 3 g/l KH₂PO₄, 0.5 g/l NaCl, 6.8 g/l Na₂HPO₄; 15 mg/l CaCl₂ 15 mg/l, 0.5 g/l MgSO₄; 0.8% dextrose) overnight at 37°C with shaking at 150 rpm, unless otherwise indicated.

Phenotypic Assays

Antibiotic resistance was measured using minimum inhibitory concentration (MIC) assays (Andrews 2001). MICs were measured for four drugs: the quinolone ciprofloxacin, and the β -lactams ampicillin (a penicillin), meropenem (a carbapenem), and ceftazidime (a third generation cephalosporin). The use of these three β -lactams enabled us to distinguish between different types of β -lactamase, since only ESBLs will display high level resistance to ceftazidime, and only carbapenemases will confer resistance to meropenem. Overnight cultures of each strain were inoculated at a 1:100 dilution into 150 μ l LB, with a 2-fold dilution of ciprofloxacin, ampicillin, ceftazidime, and meropenem ranging from 32 000 ng/ μ l to 7.8 ng/ μ l, 1,024 μ g/ml to 0.25 μ g/ml, 128 μ g/ml to 0.25 μ g/ml, and 16 μ g/ml to 0.25 μ g/ml, respectively. Following 18 h of growth at 30°C with shaking at 150 rpm, optical density (OD) was measured at 600 nm. The MIC was defined as the concentration of antibiotic that visibly inhibited growth after overnight culture.

Growth curves were measured in LB, TSB, and M9 + glucose. Growth curves were collected from shaken 150 μ l cultures in 96-well plates at 37°C, inoculated at a 1:100 dilution from over-night cultures. OD₆₀₀ was measured every 37 min for 24 h, and lag phase, maximum growth rate, and density at stationary phase were estimated using GrowthCurves (Hall et al. 2014) with two replicates for each strain. We validated the use of growth curves to assess population density for a subset of strains (supplementary fig. S1, Supplementary Material online).

Whole-Genome Sequencing and Assembly

For each clinical isolate, a single colony was picked and grown overnight in drug-free LB. Genomic DNA was extracted using the One-4-All Genomic DNA Mini-prep Kit (Bio Basic) as described in the manufacturer's manual. Following DNA purification, DNA quantification was performed using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Library construction was carried out using the Nextera XT kit (Illumina). Libraries were then quantified by qPCR (KAPA Biosystems). Sequencing was carried out on the Illumina Miseq platform using paired-end, 300-bp reads. All raw sequence data have been uploaded to the NCBI short read archive (accession numbers SRP132562).

Quality control on the raw sequence files was performed using Trimmomatic-0.32 (Bolger et al. 2014). Fifteen bases from the beginning, and one base from the end, were removed from each read. In addition, reads were trimmed using a sliding window, with each read clipped once average

base-call quality score dropped <20 in a 4-bp window. Reads of fewer than 36 bp were also removed. Effects of quality control were visualized by FastQC, assuring that high quality data were used in further analyses (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc>).

Reference based alignment was carried out to identify single nucleotide polymorphisms (SNPs) in known resistance genes. *E. coli* K-12 (MG1655; NC_00913) was used as the reference genome. Assembly was performed using Bowtie2 v2.1.0 (Langmead et al. 2009), and SNPs were called using Samtools (Li et al. 2009). The quality of the alignment was assessed using Qualimap, version 2.0.1 (Garcia-Alcalde et al. 2012) and custom Perl scripts were used to filter SNPs (coverage >15, Phred-scale quality >20 and a frequency of 80% or higher).

De novo assemblies were constructed for all strains in order to conserve information regarding accessory genomes, which can be lost in a reference-based approach. The accessory genome contains strain-specific genes that are involved in processes like niche adaptation, specialization, and host-switching (Dobrindt and Hacker 2001; Didelot et al. 2009). VelvetOptimiser-2.2.5 was used to determine the optimal *k*-mer length for the data and de novo assemblies were constructed using Velvet-1.2.10 (Zerbino and Birney 2008). Contigs shorter than 200 bp were removed to improve the quality of the assemblies. QUAST-3.1 was used to assess the assemblies (Gurevich et al. 2013). Genomes were annotated using the toolkit provided by Rapid Annotation using Subsystem Technology (RAST) for batch submission (Brettin et al. 2015).

Multisequence Alignment, Phylogenetic Inference, and Phylogenetic Correlations

Whole-genome alignment was carried out using our de novo assemblies of 39 clinical isolates, along with the genomes of *E. coli* K-12 (MG1655), *E. coli* O157, and the outgroup species *E. fergusonii* (GenBank accession numbers NC_00913, BA000007.2, and NC_011740.1, respectively). ProgressiveMauve-2.4.0 was used to align the genomes with iterative refinement using default settings (Darling et al. 2010). ProgressiveMauve is a multiple alignment tool that identifies locally collinear blocks (LCBs), each being a homologous region of sequence shared between genomes. LCBs shared among all 42 genomes were classified as the "core genome," whereas others were considered to be part of the "accessory genome." A core genome phylogeny was constructed using Bayesian phylogenetic inference as implemented by BEAST (Drummond et al. 2012), using a general time-reversible model with gamma correction (GTRGAMMA model). The Markov chain Monte Carlo (MCMC) chain was run for 20 million generations with sampling every 1,000 generations. The phylogenetic tree was visualized using

FigTree version 1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

BayesTrait 2.0 (Pagel et al. 2004) was used to infer phylogenetically informed correlations between phenotypic traits. The covarion model for trait evolution was enabled, and we used a variant of the continuous-time Markov model that allows for traits to vary their rates of evolution within and between branches. The MCMC chain consisted of 101,000 iterations, of which the first 10,000 were discarded as burn-in. The chain was sampled every 1,000 iterations, and 95% credible intervals for parameter estimates were calculated as the 2.5% and 97.5% quantiles. Inference of correlated evolution was carried out by running two chains, one allowing correlated evolution, and one disallowing correlated evolution. These two chains were compared using Bayes factors (BF), which indicate the degree of support for the correlated evolution model over the independent evolution model. Heuristically, $BF < 2$ indicates weak or no support for a correlation, BF of 2–6 indicate positive support, and BF of 6–10 indicate strong support (Kass and Raftery 1995).

Serotyping and Resistance Gene Prediction

Serotype prediction of the strains was carried out using SerotypeFinder 1.1, a publicly available web tool at the Center for Genomic Epidemiology (CGE) (Joensen et al. 2015). *E. coli* serotypes are defined based on the O and H antigens. The O antigen contains repeats of an oligosaccharide unit, and is part of the lipopolysaccharides present in the outer membrane of Gram-negative bacteria (Wang et al. 1998). The H antigen is the central, variable region of the flagellin protein of *E. coli* (Wang et al. 2003). Serotypes were characterized based on a threshold of 85% identity and minimum length of 60%.

ResFinder 2.1, also available at CGE, was used to identify genes associated with antibiotic resistance. Genes were identified based on a threshold of 98% identity and minimum length of 60% (Zankari et al. 2012). Mutations in chromosomal genes contributing to quinolone resistance (e.g., *gyrA*, *gyrB*, *parC*, and *marR*) were identified by manual inspection. Here, a custom Perl script was used to extract SNP calls from reference-based alignments, and multiple-alignments for each gene of interest were visualized using MEGA 6 (Tamura et al. 2013).

Laboratory Selection and Competitive Fitness Assays

Compensatory evolution experiments were carried out using 12 replicate populations of the *Cip^R* mutants *gyrA* D87G and *marR* R94C. Populations were grown in 200 μ l of drug-free liquid LB in a 96-well plate, shaking at 150 rpm at 30°C. Every 24 h, 2 μ l of culture were transferred to fresh media for a 1:100 dilution. Selection was carried out for 15 days, for a total of \sim 100 generations of selection.

Single colony isolates from each evolved population, as well as of the initial genotypes, were then subjected to competitive fitness assays against a Lac⁻ derivative of MG1655 (NCM520). Three to six replicate fitness assays were performed in each of LB, TSB, and M9 minimal media + glucose. Equal volumes of NCM520 and a given mutant genotype were inoculated at a 1:100 dilution in 200 μ l LB, followed by an overnight incubation at 30°C in LB with shaking. Relative frequencies of each genotype were estimated from counts of blue (*Cip^R*) and white (*Cip^S*) colonies on LB agar + IPTG + X-gal at the beginning and end of the competition experiment. Relative fitness (in units of per generation) was then calculated for each replicate using the following equation:

$$w = 1 + (\ln [\text{white}_{\text{final}}/\text{white}_{\text{initial}}] - \ln [\text{blue}_{\text{final}}/\text{blue}_{\text{initial}}]) / \# \text{ generations.}$$

Here, given the 1:100 dilution, the competition was allowed to proceed for \sim 6.6 generations.

Results and Discussion

Variation in Phenotypic Traits

We investigated tradeoffs associated with drug resistance using a collection of 39 clinical isolates of *Escherichia coli*. These ExPEC strains were obtained from a variety of nongastrointestinal infections, and fall into four broad classes of drug resistance: drug susceptible; extended-spectrum β -lactamase positive (ESBL, capable of degrading cephalosporins such as ceftazidime); ciprofloxacin-resistant (*Cip^R*) but ESBL-negative; and multidrug resistant *Cip^R*, ESBL-positive.

We measured a range of phenotypic characteristics in all 39 strains, and found substantial variation for most traits tested (fig. 1). Resistance to ciprofloxacin and to ceftazidime varied by 1,024- and 512-fold, respectively, as expected given that we intentionally sampled both susceptible and resistant isolates (fig. 1A and B). According to CLSI clinical resistance breakpoints (Clinical and Laboratory Standards Institute 2018), 13 isolates show only ciprofloxacin resistance ($MIC \geq 4 \mu\text{g/ml}$), 6 show only ceftazidime resistance ($MIC \geq 16 \mu\text{g/ml}$), and 11 show resistance to both drugs. Resistance to meropenem showed much less variation (fig. 1C), indicating that none of these strains is carbapenemase positive (CLSI breakpoint: 4 $\mu\text{g/ml}$). Variation was also evident for growth rate (a proxy for fitness) in three different laboratory media—lysogeny broth (LB), tryptic soy broth (TSB), and M9 minimal medium + glucose (fig. 1D–F).

Trade-Offs of Drug Resistance

Trade-off theory predicts negative correlations between traits under directional selection, if both traits cannot be simultaneously optimized. Consistent with this prediction, we find

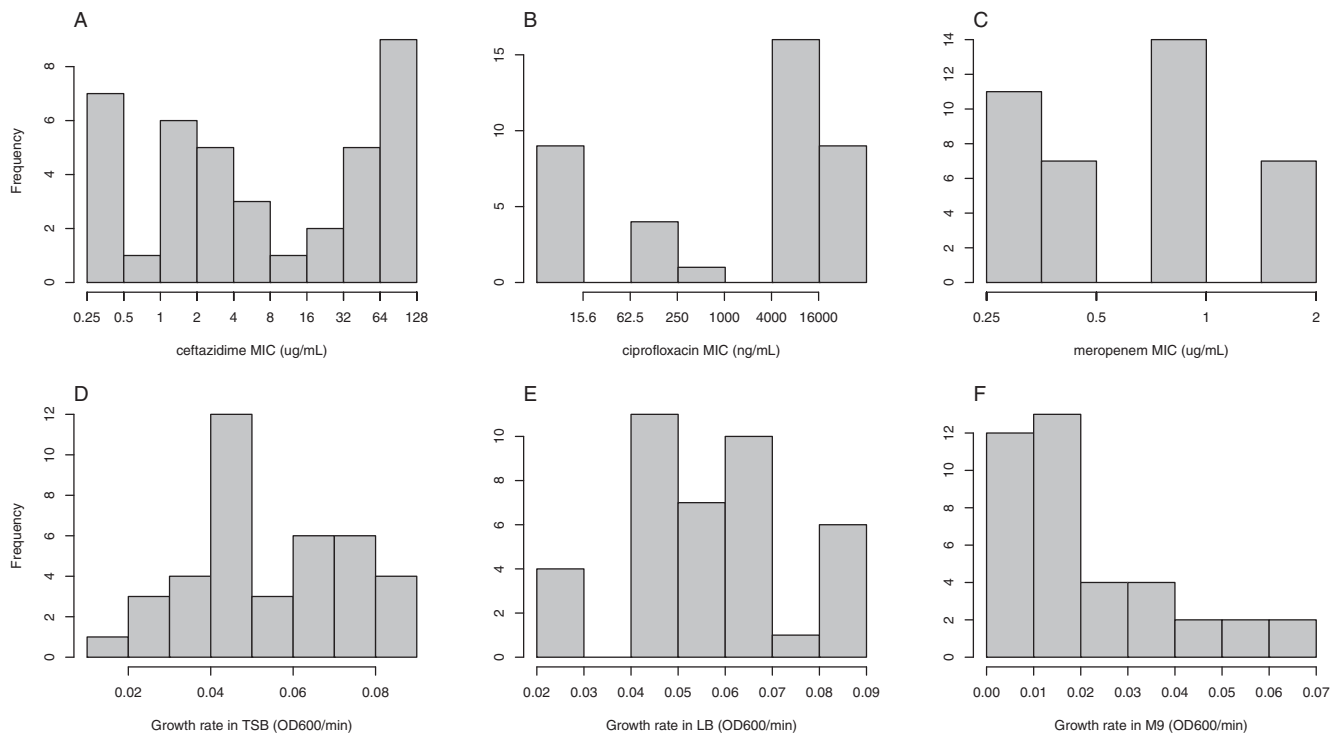


Fig. 1.—Variation in phenotypic traits among 39 clinical isolates of *Escherichia coli*. Substantial variation is evident for resistance to ceftazidime (A; CLSI resistance breakpoint: 16 $\mu\text{g}/\text{ml}$) and ciprofloxacin (B; CLSI resistance breakpoint: 4 $\mu\text{g}/\text{ml}$), but not for meropenem (C; CLSI resistance breakpoint: 4 $\mu\text{g}/\text{ml}$). Growth rates in TSB (D), LB (E), and M9 minimal medium + glucose (F) also show wide variation.

evidence for tradeoffs between drug resistance and growth rates in this set of clinical isolates. We find a strong negative correlation between level of ceftazidime resistance and growth rate in TSB ($P=0.005$, fig. 2A), and negative but nonsignificant relationships between other drug resistance and growth rate pairs (fig. 2B–F). In order to provide a more robust statistical analysis, we combined data from all three environments using a linear regression model, with environment and drug as main effects. We find negative relationships between drug resistance and growth rates, although the effect of ceftazidime resistance is nonsignificant (table 1). An observed reduction in growth rates in minimal medium is expected, since this defined medium represents a more nutrient-limited environment. Notably, interaction effects between drug resistance and growth environment were nonsignificant in all cases. This suggests that tradeoffs are broadly consistent across growth conditions, although they may be weak.

In phylogenetically structured data, correlations between traits may be spuriously strengthened or weakened by shared history (Felsenstein 1985; Velicer et al. 1999), since closely related species are expected to exhibit similarity solely due to ancestry. In order to correct for any potential bias due to underlying phylogeny, we carried out phylogenetically informed, Bayesian correlational analyses. Here, trait evolution is explicitly modeled on a phylogenetic tree, so that the appropriate null expectations can be generated. Following

whole-genome sequencing, a core-genome phylogeny was constructed under a general-time reversible model, and was used to infer phylogenetic-controlled correlations using BayesTraits (Pagel et al. 2004; see Materials and Methods for more details). We find strong evidence for trade-offs associated with antibiotic resistance (fig. 3). Resistances to both ceftazidime and ciprofloxacin are negatively associated with growth rate in TSB (fig. 3A; Bayes Factors of 6.78 and 9.18, respectively) and in LB (fig. 3B, BF = 4.17 and 3.40), although not in minimal medium (fig. 3C, BF = 0.05 and 0.10).

It is currently unclear whether phylogenetic or nonphylogenetic methods are more appropriate for measuring trait correlations in bacteria. Recombination is common in many bacterial species, including *E. coli*, such that different phylogenies underlie different regions of the genome. The presence of recombination is known to confound various phylogenetic approaches (Schierup and Hein 2000; Hedge and Wilson 2014). However, recombination tracts in *E. coli* are fairly short, with estimates of average length ranging from 50 to 550 bp (Touchon et al. 2009; Didelot et al. 2012). As such, large portions of the genome can in fact share the same phylogeny (Didelot and Wilson 2015; De Maio and Wilson 2017). Thus, whether recombination will in fact confound phylogenetically informed inferences of trait correlations is an open question. Moreover, nonphylogenetic methods are expected to yield spurious correlations when the data are phylogenetically

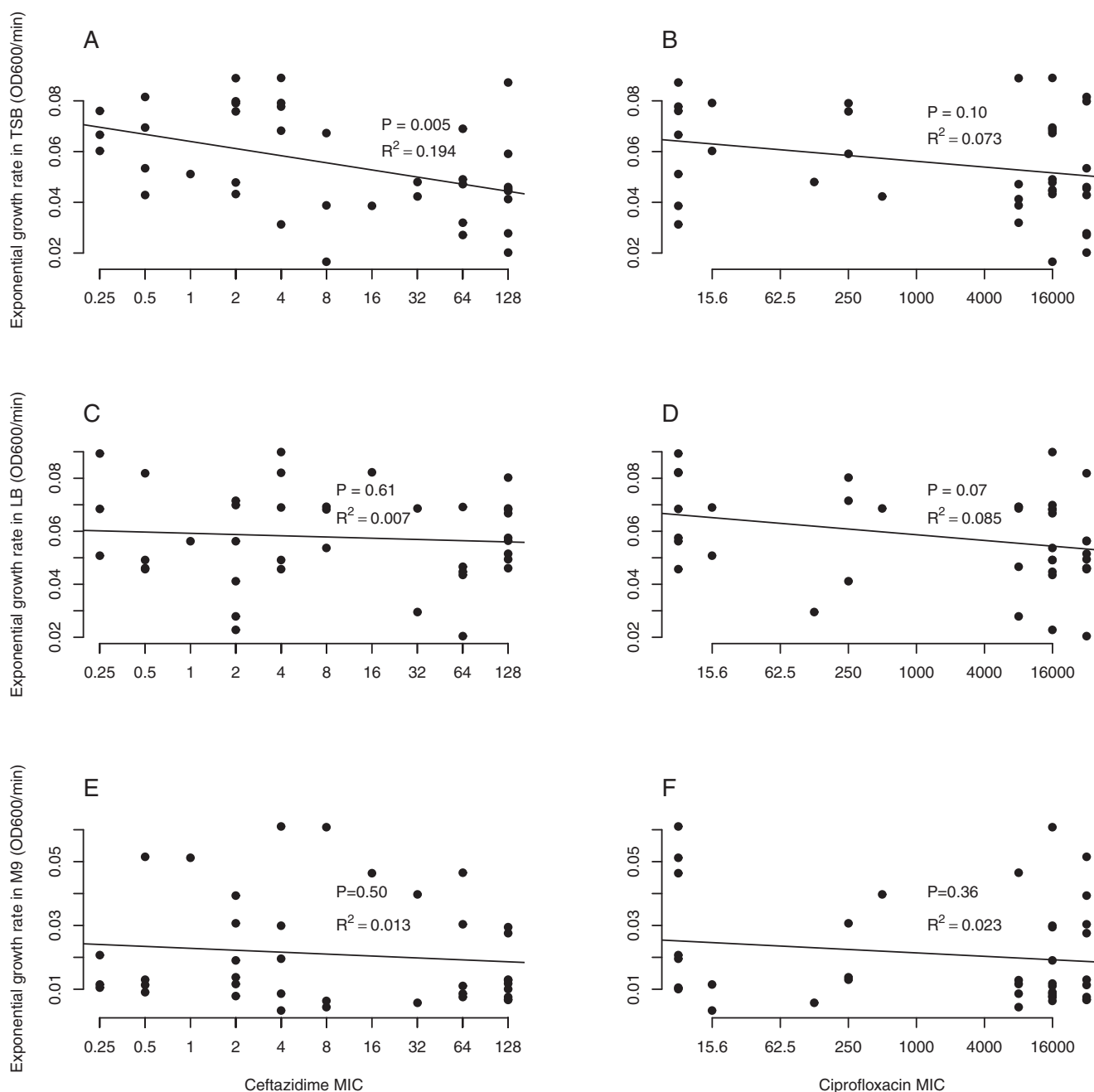


FIG. 2.—Correlations between growth rates and drug resistance. Growth rates were measured in two rich media, TSB (A and B) and LB (C and D), and in M9 minimal media + glucose (E and F). MIC assays were used to measure resistance toward ceftazidime (A, C, and E) and ciprofloxacin (B, D, and F). Linear regression (solid line) of growth rate on log₂(MIC) was used to assess the relationship between resistance and growth rate for each medium/antibiotic pair.

structured (Felsenstein 1985). Among the clinical strains studied here, negative correlations between growth rate and drug resistance are suggested by both phylogenetic and nonphylogenetic methods, albeit weakly in the latter case. We interpret this broad consistency between approaches as compelling evidence for a trade-off between resistance and fitness in the absence of drug.

Genetic Basis of Resistance

The most parsimonious explanation for a trade-off between resistance and growth rates is that resistance determinants are themselves costly. The major determinants of resistance to fluoroquinolones and to cephalosporins are well documented. High-level fluoroquinolone resistance is typically conferred by chromosomal mutations in genes encoding the

Table 1

Effects of Growth Environment and Drug Resistance on Growth Rates, As Estimated by Linear Regression

Factor	Estimate (SE)	T	P Value
Cip MIC	-7.4×10^{-4} (3.6×10^{-4})	-2.04	0.044
Cef MIC	-9.8×10^{-4} (5.5×10^{-4})	-1.80	0.075
Environment: min	-0.037 (0.004)	-9.28	1.55×10^{-15}
Environment: TSB	-0.003 (0.004)	-0.65	0.518

NOTE.—Interaction terms were nonsignificant in all cases (i.e., costs of resistance were similar across environments) and were dropped. Antibiotic resistance effects were included in the model as \log_2 -transformed MIC values. Bold indicates significance at $P < 0.05$.

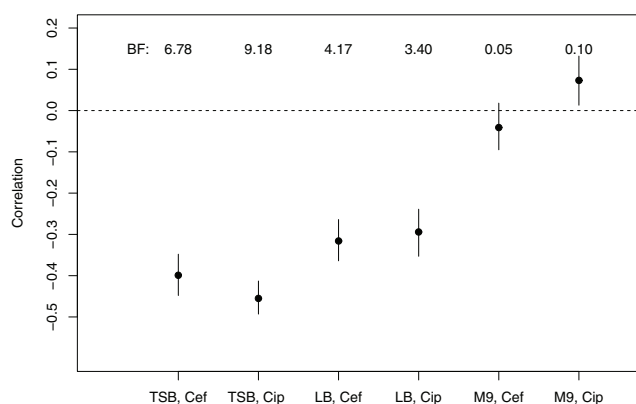


FIG. 3.—Phylogenetically controlled correlations between growth rates and drug resistance in three media (TSB, LB, M9 minimal medium + glucose) for two antibiotics (cefepime, ciprofloxacin). For each medium—drug pairing, the median estimate of the correlation coefficient is given, with 95% credible intervals. The Bayes Factor (BF) gives confidence in the correlation.

topoisomerases targeted by these drugs, with *gyrA* and *gyrB* encoding the subunits of DNA gyrase, and *parC* and *parE* encoding the subunits of Topoisomerase IV. Resistance can also be conferred by mutations affecting efflux and membrane permeability, typically in the transcriptional regulator *marR* or in the porin-encoding genes *ompF* and *ompC* (reviewed in Redgrave et al. 2014). ESBL-activity, by contrast, is conferred by plasmid-borne extended spectrum β -lactamases, with the CTX-M-14 and CTX-M-15 ESBL variants most prevalent (Canton et al. 2012).

We sequenced the genomes of all 39 clinical isolates to identify determinants of fluoroquinolone- and β -lactam-resistance. De novo assemblies were used for the detection of accessory genome elements, such as plasmids, that contribute to resistance. The average length of the de novo assemblies was 5.1 Mb, with an average coverage of 35 \times . Average N50, the size of the contig such that 50% of the genome assembly is contained in contigs of this length or larger (Lin et al. 2011), was 2,910,985 bp (supplementary table S1, Supplementary Material online). Chromosomal point mutations were called from reference-based assemblies using *E. coli* K-12 (MG1655) as a reference.

We found a wide range of known fluoroquinolone- and β -lactam-resistance elements in the genomes of these strains. Four groups of β -lactamase producing genes were identified using ResFinder (fig. 4 and supplementary table S1, Supplementary Material online): TEM (-1B, -1C, 116-like), OXA (-1 and 36-like), CTX-M (-14 and -15) and SHV (-2 and -12). Most of the ESBL-positive strains sequenced here carry CTX-M genes. The same strains harbor IncF-plasmids (supplementary table S1, Supplementary Material online), consistent with previous findings that CTX-M-14 and -15 are harbored on IncF plasmids (Carattoli 2009; Li et al. 2015). However, CTX-M genes were not found in three ESBL-positive isolates (pb27, pb31 and pb38), suggesting that the OXA and/or SHV β -lactamases carried by these strains are responsible for their resistance to cephalosporins. Plasmid carriage could well contribute to fitness reductions associated with ceftazidime resistance, as plasmid carriage often—although not always—confers a cost (reviewed in Vogwill and MacLean 2015; Wong 2017).

Among the Cip^R isolates sequenced here, the majority carry known resistance mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene. Out of 24 strains classified as Cip^R according to CLSI breakpoints (Clinical and Laboratory Standards Institute 2018), 16 carry two canonical QRDR mutations, S83L and D87N, and a 17th carries only the S83L mutation. All 17 of these strains also carry one or two mutations in the QRDR of *parC* (S80I and/or E84G and/or E84V). The fitness effects of fluoroquinolone resistance mutations have been measured in a variety of bacteria; most, but not all, target mutations are costly (Bagel et al. 1999; Kugelberg et al. 2005; Balsalobre and de la Campa 2008), and in some cases costs are dependent on genetic background (Luo et al. 2005).

Plasmid-mediated quinolone resistance determinants were also identified in our sample. Eight isolates carry predicted *aac(6′)Ib-cr* genes; this aminoglycoside-acetyltransferase variant has been shown to inactivate fluoroquinolones by acetylation (Robicsek et al. 2006). Six of these *aac(6′)Ib-cr*-bearing strains also carry chromosomal resistance mutations, whereas two do not. Finally, 5 isolates (pb12, pb14, pb19, pb28, and pb34) carry neither *gyrA* nor *parC* mutations, nor *aac(6′)Ib-cr* genes. *marR* mutations are also absent from these strains, and no plasmid-borne *quinolone resistance (qnr)* genes (Martínez-martínez et al. 1998) were predicted for these strains. These results suggest the presence of other, previously uncharacterized, resistance determinants.

Environmentally Variable Compensatory Evolution

At first blush, the available data suggest that costs associated with fluoroquinolone-resistance mutations, as well as with plasmid carriage, may well underlie fitness trade-offs associated with antibiotic resistance (figs. 2 and 3). Nonetheless, many studies have shown that the costs associated with

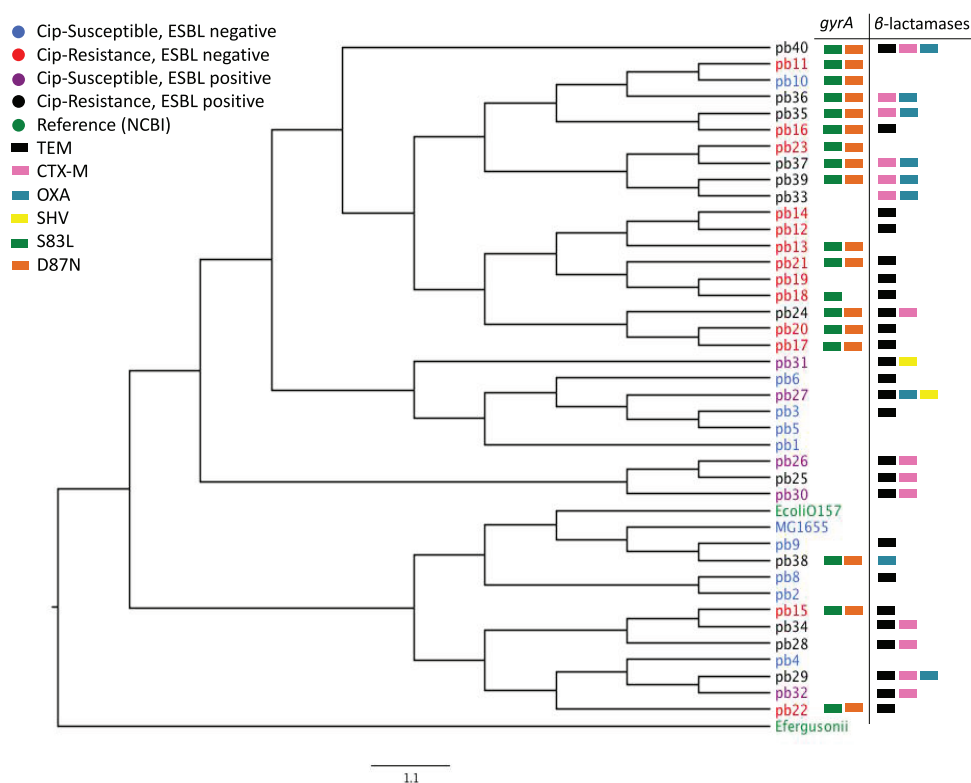


Fig. 4.—Phylogeny and drug resistance mechanisms for the sequenced clinical isolates. Presence/absence of gyrase mutations and beta-lactamases are given on the right-hand side of the figure. Strain names are color-coded according to their resistance profile (Cip^R or Cip^S, and ESBL-negative or positive).

resistance mutations can be easily and rapidly ameliorated via serial passage in antibiotic-free medium (Andersson 2006; Andersson and Hughes 2010; Wong 2017). Such compensatory evolution occurs in as little as a few dozen generations, and frequently leaves resistance intact. In the context of the current study, the apparent frequency and ease of compensatory evolution is problematic: if compensatory evolution were to occur so readily in clinical populations, then we would not expect to observe negative correlations between resistance and growth rates. In other words, why have the costs of resistance not been erased by compensatory evolution?

One possible answer to this question is that compensatory evolution may be environment specific (MacLean and Vogwill 2014): mutations conferring fitness benefits to the resistant mutant in one environment may be neutral or deleterious in other environments. As such, even if compensatory mutations have occurred in some clinically relevant setting (e.g., the blood stream or urinary tract), those mutations may not confer a benefit in other locales (e.g., the TSB, LB, and minimal medium used in this study). Previous findings on this topic are mixed: a few experiments suggest that mutations that are compensatory in one environment are also compensatory in other environments (Bjorkman et al. 2000; Nagaev et al. 2001). For example, fusidic acid resistance mutations in the EF-G gene in *S. aureus* are costly in both laboratory medium and in a rat model of infection, whereas compensatory

mutations evolved in the lab were also compensatory in rats. Interestingly, the reverse was also true: the most common compensatory mutation in rats, EF-G A67V, was also compensatory in the lab. The generality of this result remains unclear, however, as another common rat-compensatory mutation, EF-G V475L, did not provide a fitness increase in laboratory medium (Nagaev et al. 2001).

In order to further test the environmental specificity of compensatory mutations, we evolved replicate populations of two Cip^R mutants of *E. coli*, *gyrA* D87G and *marR* R94C, in drug-free LB for 100 generations. Both genotypes initially suffered a cost in the absence of drug, with relative fitness estimates of 0.73 (SE: 0.035) and 0.88 (SE: 0.062), respectively. Following compensatory evolution, competitive fitness assays were carried out on a single clone isolated from each population. Substantial fitness gains were observed for populations evolved from the *gyrA* D87G ancestor (fig. 5A; one-tailed *t*-test $P=3.1 \times 10^{-9}$). Fitness increases in the *marR* R94C mutant were more modest, with a nonsignificant average increase in fitness ($P=0.17$). When fitness was measured in TSB, another rich medium, significant increases in fitness were observed for genotypes evolved from both *gyrA* D87G ($P=0.009$) and *marR* R94C ($P=0.013$). This increase in fitness is perhaps not surprising, given that both LB and TSB are rich media, and so the same mutations might confer an advantage under both conditions. In

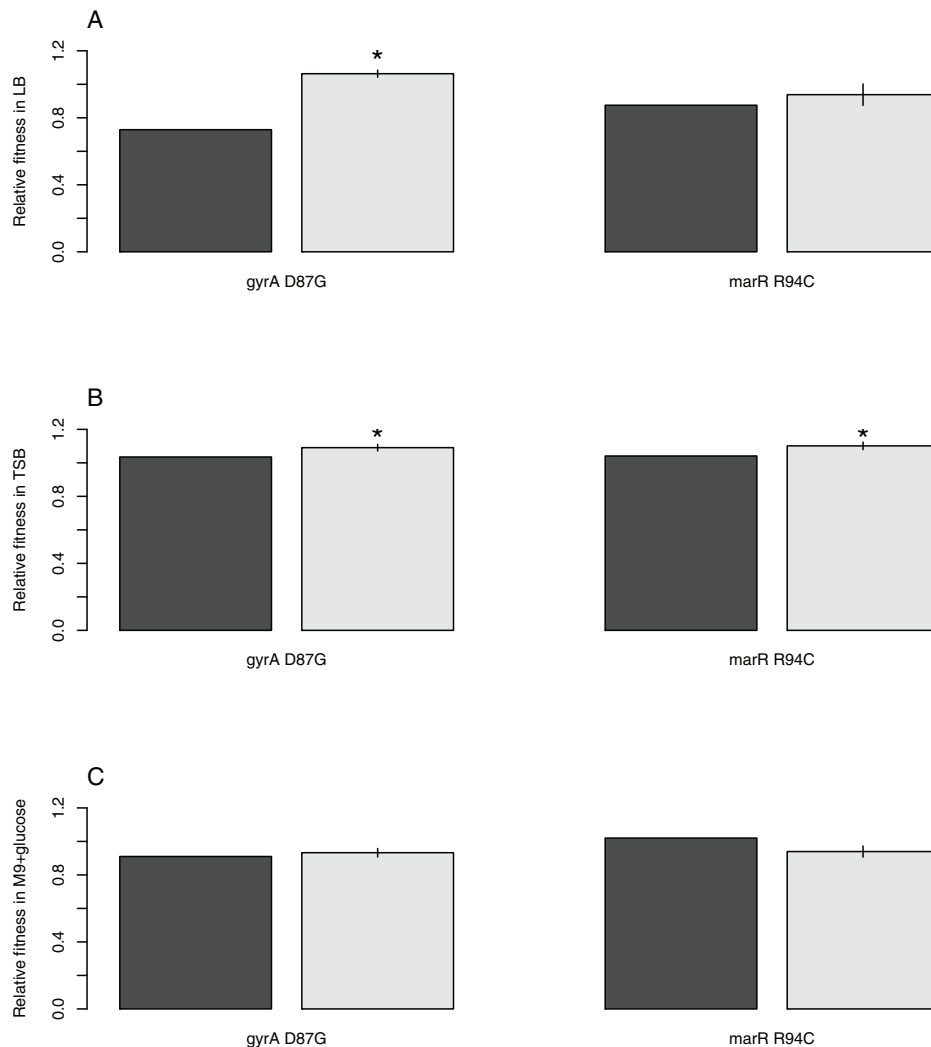


Fig. 5.—Direct and indirect responses to selection in the absence of antibiotic. Ancestral fitness is given by the black bar, and mean (\pm SE) fitness for 12 evolved populations is given in gray. Compensatory evolution in LB resulted in a significant increase in fitness in LB for *gyrA* D87G-derived populations (A), and for populations derived from both mutant ancestors in TSB (B). No increase in fitness was observed in M9 minimal medium + glucose (C), although neither resistance mutation imposed a significant decrease in fitness.

minimal medium + glucose, neither initial resistance mutation conferred a cost, with relative fitness estimates of 0.91 (SE: 0.08) and 1.02 (SE: 0.04). Genotypes that evolved in LB did not increase fitness in minimal medium for populations derived from either ancestor (fig. 6C; $P=0.19$ and $P=0.98$, respectively), but this may simply reflect the absence of initial fitness costs.

Overall, responses to selection (i.e., the change in fitness over the course of the selection experiment) were uncorrelated between TSB and LB, or between M9 minimal medium and LB (fig. 6). That is, the magnitude of the fitness gain in LB did not predict fitness changes in either alternative environment. This is true even in TSB (fig. 6A)—although fitness increased overall in TSB, the sizes of the fitness increases were uncorrelated. Correspondingly, ANOVA indicates significant effects of genotype, environment, and

genotype \times environment interactions on the response to selection (table 2).

We therefore propose that compensatory evolution may be limited by environmental context—whereas fitness gains may be achieved in specific settings, those improvements may be inconsistent in alternative environments (fig. 6). By contrast, the fitness costs imposed by resistance mutations do appear to be fairly (although not entirely) consistent across environments (MacLean and Vogwill 2014). Thus, we expect that resistance will sometimes trade off with fitness, even in the face of compensatory evolution.

Implications for the Control of AMR

Restriction protocols, whereby a drug or drug class is withheld in hopes of reducing the prevalence of resistance, have been

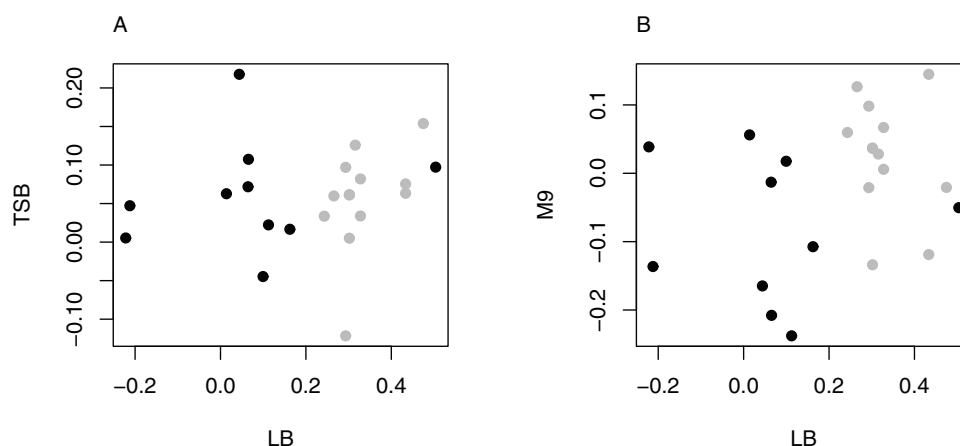


Fig. 6.—No correlation between direct and indirect responses to selection. Response to selection was measured as the difference in fitness between the ancestral and evolved genotypes, for genotypes derived from *gyrA* D87G (gray) or *marR* R77H (black). Correlations were nonsignificant between LB and TSB (A), and between LB and M9 minimal medium + glucose (B).

Table 2
Effects of Genotype and Environment on the Response to Selection

Factor	F	P Value
Genotype	21.05	2.33×10^{-5}
Environment	26.56	5.46×10^{-9}
Genotype \times environment	8.92	4.1×10^{-4}

NOTE.—Populations were evolved in drug-free LB for 100 generations, and fitness was measured in LB, TSB, and minimal medium.

moderately successful in both human and animal populations (Enne 2010). For example, ciprofloxacin resistance in *E. coli* dropped from 12–14% to 9% following restricted use of quinolones in Israel over a 7-month period (Gottesman et al. 2009). Similarly, wholesale restriction of veterinary antibiotic use in the Netherlands has been accompanied by widespread reductions in AMR in animal isolates (Speksnijder et al. 2015). The success of restriction protocols is broadly consistent with the assumption that AMR is costly; our data indicate that this assumption is largely well founded, with a negative association between resistance and growth rates in both phylogeny-independent (fig. 2 and table 1) and phylogenetically controlled (fig. 3) analyses.

There are nonetheless important exceptions to the success of restriction protocols, wherein drug limitation has not been followed by a drop in the prevalence of resistance. Restriction of clinical trimethoprim use in Sweden (Sundqvist et al. 2010), and of sulphonamide use in the United Kingdom (Enne et al. 2001), failed to reduce the prevalence of resistance to these two drugs. Furthermore, reduction of veterinary antibiotic use in the Netherlands has had no effect on the prevalence of the zoonotic pathogen MRSA ST398 (Dierikx et al. 2016), even though other AMR strains have dropped in frequency. There are a number of possible explanations for these failures of drug restriction, including coselection (whereby AMR elements are linked), the existence of AMR genotypes that do

not suffer a cost, and the influence of genetic background (Melnyk et al. 2014; Vogwill and MacLean 2015; Wong 2017).

Our findings suggest a role for genetic background in mitigating the costs of resistance. This is most clear in the case of quinolone resistance. Most of the Cip^R strains studied here carry the same *gyrA* S83L D87N haplotype, but the growth rates of these strains in TSB vary by >4-fold (supplementary table S1, Supplementary Material online). This variation in growth rates among strains bearing the same resistance allele may be due to epistasis, whereby the costs of resistance are dependent on genetic background. Alternatively, the variation in growth rates may be independent of resistance, such that Cip^S derivatives of these strains would show similar differences in growth rates. In either case, however, there are highly Cip^R genotypes that show similar growth rates to Cip^S genotypes. Assuming that growth rate is a reasonable proxy for fitness, these Cip^R genotypes would therefore be expected to persist in the absence of drug.

Natural Variation and Tradeoffs

Microbial studies of tradeoffs have largely focused on the effects of single mutations, such as AMR mutations (reviewed in Melnyk et al. 2014; Vogwill and MacLean 2015), or on experimentally evolved populations differing by a handful of mutations (Bohannan et al. 2002; Schick et al. 2015). In such studies, fitness tradeoffs can be attributed to specific mutations, guaranteeing that tradeoffs are in fact due to antagonistic pleiotropy rather than the presence of linked mutations that are deleterious in some environments but not others. Moreover, knowledge of the specific mutations underlying tradeoffs allows for greater mechanistic understanding.

There are nonetheless compelling reasons to adopt the complementary approach, that of measuring trait correlations among natural microbial isolates. Importantly, the fitness

effect of a mutation can vary depending on genetic background (Wong 2017)—a given mutation may be beneficial, neutral, or deleterious, depending on the presence of other mutations in the genome. Laboratory studies of trade-offs tend to be restricted to a single genetic background, which potentially limits the generalizability of their findings. Thus, by using natural variation to detect tradeoffs, we can take the effects of genetic background into account.

Although widespread in multicellular organisms, natural variation-based studies of trade-offs are relatively rare in microbes (reviewed in Ferenci 2016). Nonetheless, it is clear that trade-offs are widespread, and that they contribute to ecological specialization and to the maintenance of diversity. For example, Clark et al. (2015) measured a range of phenotypic traits among *Pseudomonas aeruginosa* isolates from an adult with cystic fibrosis (Clark et al. 2015). They found negative correlations between traits involved in virulence, and those involved in chronic infection, indicative of a trade-off between long-term survival and virulence (see also Workentine et al. 2013). Similarly, Phan et al. identified a trade-off between survival and nutritional competence, likely mediated by membrane permeability (Phan and Ferenci 2013).

Our finding of a negative relationship between growth rates and drug resistance (figs. 2 and 3; table 1) similarly suggests that *E. coli* must trade-off between growth and survival. However, this trade-off is not absolute, since no relationship between resistance and growth was observed in minimal media. The mechanistic underpinning of this relationship is unclear, although it may reflect slower growth in minimal medium; slow growth may be accompanied by reduced metabolic demands and a greater tolerance for reduced enzymatic function. More broadly, we highlight the importance of measuring a wide range of traits, in order to gain a more complete appreciation of the various trade-offs (or lack thereof) that may contribute to the maintenance of diversity.

Given the ease of bacterial genome sequencing, and the relative ease of phenotypic measurement in bacteria, there is much promise in using bacterial systems to measure, quantify, and understand trade-offs. Such efforts may in turn help to understand the considerable diversity of bacteria.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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