The Evolutionary Ecology of Antibiotic Resistance in an Insect Model

Submitted by Elitsa Georgieva Penkova

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I certify that all material in this dissertation which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

Abstract

The fitness consequences of antibiotic resistance are commonly quantified *in vitro*, and fitness estimated from growth rate measurements or direct competition experiments in drug-free media, overlooking environmental factors within a live infection which may also affect bacterial fitness, such as colonisation rate, transmission potential, or the presence of other interacting microbes.

This dissertation aimed to assess the effects of both *in vitro* and *in vivo* conditions on bacterial fitness post-acquiring spontaneous resistance mutation and validate earlier studies in nutrient rich media or other laboratory systems in live infections. To achieve this, I utilised the larvae of the diamondback moth, *Plutella xylostella*, and its natural gut symbiont, the opportunistic pathogen *Enterobacter cloacae*. This experimental system provided novel means of exploring multiple components of fitness in the presence and absence of antibiotics including within-host competitive ability and between-host transmissibility.

First, I generated and characterised a collection of *E. cloacae* mutants, selected on either cefotaxime, nalidixic acid, or rifampicin and explored the pleiotropic fitness costs associated with resistance. Fitness parameters were quantified *in vivo* and compared to measurements *in vitro*. Results suggest that bacterial competitiveness can be environment-dependent, with costs generally enhanced *in vivo*, highlighting that *in vitro* measurements may be an unreliable basis for antimicrobial resistance management.

Next, I explored the sociality of antibiotic resistance as a potential mechanism explaining the co-occurrence of sensitive and resistant strains under varying

demographic and environmental conditions. The relative fitness of the susceptible strain was measured in broth and biofilms *in vitro* but also *in vivo*. Cooperative protection in mixed genotype infections allowed the persistence of susceptible bacteria under selection, conferring fitness benefits when susceptible bacteria were rare. Frequency-dependent fitness suggests stable maintenance of both genotypes, despite resistance costs. Overall these results emphasise the value of exploring diverse fitness components of microbes and illustrate how insect models can provide valuable systems for testing and refining hypotheses on the fitness consequences of resistance mutations.

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Author’s Declaration

I declare that the work and data presented in this dissertation is my own, carried out under the supervision of Prof. Ben Raymond, with the exception of the microscopy image presented as Figure 6, for this image Prof. Raymond assisted with dissection and photography.

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Abbreviations

AIC	-	Akaike Information Criterion
ANOVA	-	Analysis of Variance
CEF	-	Cefotaxime - resistant mutant
CFU	-	Colony Forming Units
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylenediamine Tetraacetic acid
EUCAST	-	European Committee on Antimicrobial Susceptibility Testing
ESBL	-	Extended Spectrum *β*-Lactamases
ESKAPE	-	An acronym for the following pathogens, with increasing cases of multidrug resistance: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acitenobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.
GLM	-	Generalised Linear Model
LB	-	Luria-Bertani broth
MIC	-	Minimum Inhibitory Concentration
NAL	-	Nalidixic acid - resistant mutant
PCR	-	Polymerase Chain Reaction
RIF	-	Rifampicin - resistant mutant
RPM	-	Rotations per minute
WT	-	Wildtype

General Introduction

The aim of the clinical use of antibiotics is to inhibit or kill bacteria by exerting selective toxicity, which disrupts critical prokaryote-specific cellular structures and processes (Schneider and Sahl, 2010, Kohanski *et al.*, 2010, Webber *et al.*, 2015, Monserrat-Martinez *et al.*, 2019). The toxicity of antibiotic compounds is derived from their natural origin, as microbial metabolites synthesised for use in the antagonistic microbial warfare for space and resources in soil and other environments (O'Brien and Wright, 2011, Peterson and Kaur, 2018).

Today, a broad range of antibiotic compounds are produced at a large scale by chemical synthesis or fermentation; these are based on natural products that may have been altered by chemically changing their pharmacokinetic properties or target range (Landecker, 2016). Antibiotic resistance emerges when bacteria acquire genes or alleles which allow them to escape the toxicity of antibiotic compounds at concentrations that were previously effective against them (Blair *et al.*, 2015).

The extensive production and use of antibiotics over decades have generated strong directional selection for resistance, although other drivers also exist (eg. biocides, heavy metal toxins, non-antibiotic pharmaceuticals) (Shallcross and Davies, 2014, Singer *et al.*, 2016, Chokshi *et al.*, 2019, Wang *et al.*, 2022). Resistance has emerged in a range of important community-acquired and nosocomial pathogens, including in *Enterobacteriaceae* spp, *Enterococcus* species, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, and *Neisseria gonorrhoeae* (Logan and Weinstein, 2017, Aliberti *et al.*, 2019, Mancuso *et al.*, 2021, Lin *et al.*, 2021, Merker *et al.*, 2022).

Antibiotic-resistant pathogens are one of the major challenges in modern health care. Their emergence and spread compromise treatment efforts and are associated with increases in morbidity and mortality worldwide (Frieri *et al.*, 2017). Recent surveillance data analysis revealed that in 2019 alone, 1.27 million patient deaths globally were a consequence of antibiotic-resistant infections, with the highest burden in low-income regions (Murray *et al.*, 2022).

In response to this ongoing crisis, a number of action plans have been proposed, including increased hygiene measures, restrictions on antibiotic use, and antibiotic cycling (Bal *et al.*, 2010, Melnyk *et al.*, 2015, Conlon-Bingham *et al.*, 2019). The idea of 'cycling' is to apply the crop rotation concept to antibiotic use (Kollef *et al.*, 1997). Cycling aims to escalate the heterogeneity of selection by using certain antibiotics, or antibiotic classes, for pre-defined time periods, which are then withdrawn from general use and replaced with other drug types/classes and so on, until the original drugs are circulated back into use to start a new cycle (Bal *et al.*, 2010). By minimising selection for resistant bacteria, their prevalence should in theory decline (Magee, 2005), although the theoretical and empirical support for this strategy has been mixed (Bonhoeffer *et al.*, 1997, review eg. Raymond, 2019).

Drug restrictions and cycling strategies rely on the assumption that in the absence of selection resistance mechanisms impose a fitness cost (Levin *et al.*, 1997, Bonhoeffer *et al.*, 1997, Andersson and Levin, 1999). This can result from impaired cellular functions due to mutations in the antibiotic targets, such as in DNA gyrase and RNA polymerase, or because resistance may bring a metabolic cost, for example, through increased expression of antibiotic-degrading enzymes (Andersson and Hughes, 2010).

Fitness disadvantages in resistant mutants are often observed in experimental work *in vitro*, when measuring fitness parameters, such as growth rate or competitiveness of resistant and sensitive strains in antibiotic-free environments (Binet and Maurelli, 2005, Norström *et al.*, 2007, Pranting and Andersson, 2011). Fitness measurements *in vivo* are less common (Melnyk *et al.*, 2015, Manktelow *et al.*, 2020) and costs do not always correspond to those observed *in vitro* (Johnson *et al.*, 2005, Hall *et al.*, 2015).

Outside a laboratory setting, empirical evidence reiterating the value of fitness costs comes from data on cefixime resistance dynamics in *N. gonorrhoeae* (Fingerhuth *et al.*, 2016, Whittles *et al.*, 2017). Surveillance data revealed that the frequency of resistance to cefixime increased with rates of antibiotic use and declined after cefixime was suspended, ultimately leading to the extinction of cefixime-resistant *N. gonorrhoeae*, presumably via competitive exclusion or reduced transmission. However, this case might be the exception rather than the norm, as empirical trends show that for many species and resistance mechanisms, stable co-existence of resistant and sensitive strains is frequently observed (Colijn *et al.*, 2010, Blanquart, 2019, Naylor *et al.*, 2018, Krieger *et al.*, 2020). Even when restrictions on antibiotic prescriptions do lead to declines in the frequency of resistance, the complete eradication of resistant strains is unlikely (Salyers and Amábile-Cuevas, 1997, Andersson, 2003, Johnsen *et al.*, 2011).

In the absence of selection, resistance frequencies in hospitals will be primarily driven down by two processes: the cost of the resistance mechanism and patient turnover (Bonhoeffer *et al.*, 1997, Levin, 2001, Whittles *et al.*, 2017). However, resistance mechanisms are diverse, and so are their effects on fitness (Buckling *et al.*, 2009). Fitness costs may also vary between

environmental contexts, adding further complications to studying their effects *in vitro* (MacLean *et al.*, 2010, Trindade *et al.*, 2012, Hall *et al.*, 2015, Durão *et al.*, 2015, Cardoso *et al.*, 2019). Furthermore, in addition to bacterial competitiveness, the success of a resistant genotype will also depend on its transmission potential (Andersson and Hughes, 2017), which cannot be assessed *in vitro*. A greater understanding of the fitness advantages of one strain over the other across different environmental contexts and the ecological conditions allowing co-existence between resistant and sensitive strains in response to varying strength of antibiotic selection, can inform antimicrobial stewardship (Levin *et al.*, 1997, Austin *et al.*, 1997).

With this project, first, I aimed to quantify the effects of *in vivo* conditions on the fitness cost of resistance in the absence of selection in terms of within-host competitiveness and between-host transmissibility of spontaneously-acquired resistance mechanisms in *Enterobacter cloacae* and compare how measurements *in vivo* correspond to those *in vitro*. Chromosomally-mediated resistance mechanisms for this bacterium and other members of the *Enterobacteriacae* family are common and clinically relevant (eg. constitutive expression of AmpC *β*-lactamases) (Seeberg *et al.*, 1983, Kaneko *et al.*, 2005, Guérin *et al.*, 2015).

The second aim of the first chapter was to explore the value of insects as *in vivo* model systems for the study of antibiotic resistance ecology and evolution. Insects are more accessible, easy to rear, with short generation time, and importantly, reduce the need for using mammals in research (Ramarao *et al.*, 2012, Browne and Kavanagh, 2013, Tsai *et al.*, 2016).

Galleria mellonella, or the greater wax moth larvae, is a popular choice for work on host-pathogen interactions (Glavis-Bloom *et al.*, 2012, Mukherjee *et al.*, 2013, Dinh *et al.*, 2021), as well as in the assessment of both the toxicity and efficacy of novel antimicrobial agents (Piatek *et al.*, 2021). The advantages of working with *G. mellonella* larvae include their low cost, ease of use (Fedhila *et al.*, 2010, Tsai *et al.*, 2016, Kavanagh and Sheehan, 2018), and importantly, their ability to remain responsive at 37°C, allowing for any temperature-dependent virulence factor of infectious causing agents in humans to remain active (Kavanagh and Reeves, 2004, Junqueira, 2012, Kavanagh and Sheehan, 2018).

However, compared to other insects, *G. mellonella* has been argued to have a weaker cellular based immunity and reduced antimicrobial peptide expression (Jarosz, 1993, Ehlers *et al.*, 1997, Ono and Yoshiga, 2019). Considering the abundant, albeit circumstantial, evidence indicating that host immune challenges are important determinants for shaping fitness costs of resistant mutants (Strauss *et al.*, 2008, Collins *et al.*, 2010, Trindade *et al.*, 2012, Hall *et al.*, 2015, Durão *et al.*, 2015), the suitability of an insect model with weaker immunity for studying the cost of antibiotic resistance is questionable.

To address this, in addition to *G. mellonella,* here I used a second host, the diamondback moth, *Plutella xylostella*. While *P. xylostella* cannot be reared at 37°C, it is an established gut infection model for *E. cloacae* (Somerville *et al.*, 2019, Matthews *et al.*, 2019). In addition, *P. xylostella* has high fecundity, quick generation turnover, and can be reared aseptically on artificial diet (Manktelow *et al.*, 2020). These factors increase the value of *P. xylostella* as an alternative host by providing opportunities to assess relative fitness in ways beyond simple

competitive fitness, including in the context of gut colonisation, and horizontal transmission of infection.

The symbiotic relationship of *E. cloacae* and *P. xylostella* allows the study of fitness costs of an intestinal commensal and opportunistic Gram-negative microbe belonging to the family *Enterobacteriaceae* (Bhar *et al.*, 2021). Opportunistic pathogens which may persist as harmless commensals are an underappreciated reservoir of resistance mechanisms and add further complications, as resistance may emerge during treatment for other infections (Marshall *et al.*, 2009, Raymond, 2019, Blanquart, 2019). Opportunistic *Enterobacteriaceae*, in particular, account for the majority of nosocomial infections and are especially problematic in terms of multidrug resistance emergence (Livermore *et al.*, 2011)

The final aim of this dissertation was to explore the sociality of antibiotic resistance, as a potential mechanism for co-existence of sensitive and resistant strains (Frost *et al.*, 2018, Amanatidou *et al.*, 2019, Bottery *et al.*, 2021). Again, in contrast to previous studies, the aim here was to test hypotheses *in vivo* and *in vitro* and to validate earlier laboratory results in live infections.

In terms of sociality, antibiotic resistance mechanisms can broadly be categorised as selfish (private) or cooperative (public) (Bottery *et al.*, 2016). Examples of selfish mechanisms include target modification, increased antibiotic export, or decreased cell permeability (Blair *et al.*, 2015, Li *et al.*, 2015). These mechanisms are private in the sense that they confer a benefit for the individual bacterium harbouring them. In contrast, cooperative resistance mechanisms bring group-level benefits. Examples include enzyme secretion of antibiotic-degrading enzymes, such as *β*-lactamases, and intracellular

detoxification (eg. to aminoglycosides) (Dugatkin *et al.*, 2005, Perlin *et al.*, 2009, Yurtsev *et al.*, 2013, Medaney *et al.*, 2015, Bottery *et al.*, 2016, Sorg *et al.*, 2016).

Previous studies have shown that when *β*-lactam detoxification allows susceptible cells to grow in the presence of resistant genotypes, the extent of social protection and thus exploitation varies with spatial structure, bacterial physiology and environmental conditions (Medaney *et al.*, 2016, Amanatidou *et al.*, 2019). Detoxification by cells in a planktonic state in well-mixed environments *in vitro* can be efficient, albeit over a narrow antibiotic range (Medaney *et al.*, 2015). In contrast, detoxification can be very limited in simple structured environments *in vitro* (agar plates) (Medaney *et al.*, 2015), unless bacteria are introduced as an established biofilm, where social protection is significant (Amanatidou *et al.*, 2019). Increased sociality in biofilms is due to a combination of efficient *β*-lactamase activity and differences in bacterial physiology of sessile cells living in densely populated structures with some level of segregation (Amanatidou, 2017). Up to 80% of bacterial growth in nature is estimated to occur in biofilms (Hall-Stoodley *et al.*, 2004, Flemming and Wuertz, 2019). However, the significance of biofilm formation in host infections in terms of antibiotic detoxification is unclear.

In the second chapter, I investigated the demographic and environmental conditions which allow the survival and persistence of sensitive *E. cloacae* cells in the presence of a *β*-lactam resistant mutant, constitutively expressing AmpC *β*-lactamases, characterised in the previous chapter. Cooperative protection was assessed in cells either in a planktonic state in broth; as sessile biofilms on agar plates; and *in vivo* using the larvae of the diamondback moth, *P. xylostella*.

Fitness of susceptible cells was measured over a range of antibiotic concentrations and with varying initial population proportions.

Given that in natural settings, bacteria exists within diverse ecological contexts, to gain a greater understanding of how the complex patterns of evolutionary dynamics are generated, we need to take into account the various indirect ecological factors at play, in addition to directional selection (Blanquart, 2019, Kolter *et al.*, 2022). Including the heterogeneities between and within host populations, along with the interplay between microbes at the population level.

Chapter 1: Genotyping and quantifying competitive fitness and transmission costs of antibiotic resistance in *Enterobacter cloacae*

Introduction

In response to increasing poor health outcomes resulting from emerging antibiotic- and multidrug-resistant infections, a number of antibiotic stewardship strategies have been put forward and implemented with varying success (Ohl and Luther, 2011). Commonly enforced interventions include rationalising antibiotic usage and drug cycling (Raymond, 2019). These rely on exerting negative selection pressure through restricted use of drugs or drug classes to control the frequency of resistance, provided resistance mechanisms carry a substantial fitness cost retained in the absence of selection (REX-Consortium, 2013).

Antibiotic resistance acquisition can occur via diverse genetic mechanisms. Some examples include horizontal gene transfer of novel genes, metabolic adaptations, target modification, or export (Munita and Arias, 2016). Spontaneous mutations provide another means of acquired resistance (Seeberg *et al.*, 1983). As antibiotics target essential cellular functions (eg. cell-wall biogenesis, DNA replication, transcription, or translation), spontaneous resistance-conferring mutations typically occur within the genes encoding for these functions (Andersson and Hughes, 2010, Durão *et al.*, 2018). Target site mutations may be advantageous under selection, however, in the absence of antibiotics, these often result in some level of physiological impairment, or a fitness cost (Fajardo *et al.*, 2009, Andersson and Hughes, 2010).

Resistance mutations may also occur in regulatory genes, such as *ampR* or *marR,* which can regulate the expression of multiple genes. For instance, in *Pseudomonas aeruginosa*, *ampR* positively regulates the levels of expression of *ampC*, *rh1R,* and *lasB* and negatively regulates the expression of *lasR*, *lasA*, *lasI*, and *poxB*, resulting in increased levels of resistance but also altered virulence and fitness during an infection event (Kong *et al.*, 2005). Epistatic and pleiotropic effects of acquired resistance are not uncommon (Trindade *et al.*, 2009, Hall and MacLean, 2011, Miskinyte and Gordo, 2013, Vogwill and MacLean, 2015, Durao *et al.*, 2016) and typically prompt an increase in fitness costs in the absence of selection (Reynolds, 2000, Enne *et al.*, 2004, Gagneux, 2006, Melnyk *et al.*, 2015).

The presence of costs due to antibiotic resistance acquisition predicts a decrease in the frequency of resistant cells, outcompeted by susceptible wildtype strains once a drug is withdrawn from clinical use for a time period (MacLean and Buckling, 2009, Andersson and Hughes, 2011, Hall *et al.*, 2015). However, the success of this approach is highly conditional. First, the cost of the resistance mechanisms should be substantial, and resistant genotypes eliminated rapidly prior the potential occurrence of compensatory mutations which may reduce these costs (Whittles *et al.*, 2017, Cardoso *et al.*, 2019). In addition, within a hospital context, the success of drug cycling will also be impacted by the rate of immigration of susceptible genotypes through patient turnover; selective pressures from related drugs and other antimicrobials (cross-resistance); and the acquisition of genetically linked resistance mechanisms which may affect different antimicrobials (Szybalski and Bryson, 1952, Levin, 2002, Cantón and Ruiz-Garbajosa, 2011, Birgy *et al.*, 2016, Raymond, 2019).

How fitness costs of resistance are measured is another challenge, as costs can be highly variable under different environmental contexts, influenced by nutrient availability, host immune defence challenges, and cell-to-cell interactions (MacLean *et al.*, 2010, Trindade *et al.*, 2012, Hall *et al.*, 2015, Durão *et al.*, 2015, Cardoso *et al.*, 2019). These factors are important for the spread of resistance within a bacterial population, yet they are often overlooked as relative fitness is typically assessed as growth rate or competitive ability *in vitro* (Vogwill and MacLean, 2015).

While costs *in vitro* have been argued to result in concomitant reductions in live infections (Vogwill and MacLean, 2015), the experimental evidence remains mixed and limited. Several studies show that costs under negative selection *in vitro* can exhibit similar selection dynamics *in vivo* (Björkman *et al.*, 1998, Nilsson *et al.*, 2004, Marcusson *et al.*, 2009). However, others argue that this association is not always strong and can exhibit context-dependent variability (Bjorkman *et al.*, 2000, Lindgren *et al.*, 2005, Johnson *et al.*, 2005).

In addition, *in vivo* studies on resistance costs are predominantly conducted with murine models (Giraud *et al.*, 2003, Lindgren *et al.*, 2005, Luo *et al.*, 2005, Rozen *et al.*, 2007, Marcusson *et al.*, 2009, Kunz *et al.*, 2012, Fernández *et al.*, 2012, Rifat *et al.*, 2017, Cardoso *et al.*, 2019), which are ethically fraught, costly and labour intensive. Furthermore, in the specific context of murine gut infection studies, streptomycin treatment is typically employed to improve colonisation by competing pathogens, which impacts the host's natural ability to resist an infection (Croswell *et al.*, 2009, Leatham-Jensen *et al.*, 2012, Cardoso *et al.*, 2019).

Finally, there are fitness components beyond competitiveness in a mixed-strain infection, which are also likely to impact the success of a resistant pathogen within a live infection, such as its transmission potential (Lipsitch and Samore, 2002). Assessing both the competitive ability and transmission is important to understand how selection dynamics of resistance frequencies will be directed by processes across multiple scales, as the dynamics at one level can shape dynamics at the other over evolutionary time (Childs *et al.*, 2019). This is especially important for non-obligate pathogens, where colonisation and shedding/transmission may occur without necessarily causing disease at the same time (Blanquart, 2019, Raymond, 2019).

Here, I will test hypotheses on how *in vivo* processes affect the magnitude of fitness costs of spontaneously-evolved resistance mechanisms. More specifically, I will measure within-host competitive ability of resistant strains in pair-wise competition with their susceptible ancestor in the absence of antibiotic pressure; in addition, I will also assess the transmission potential of each genotype, including the ancestor, in terms of population growth within a single-strain infection, the rate of infectious propagule shedding, and direct host-to-host transmission.

A second aim of this chapter is to explore the practicality of insects as alternative models for studying the cost of antibiotic resistance *in vivo*. Insect models are higher-throughput systems with shorter generation times which speed up the progress of experimental work and advance the ‘Replace, Reduce and Refine’, or the 3Rs policy, by significantly reducing the need for experimental testing on mammals (Ramarao *et al.*, 2012, Browne and Kavanagh, 2013, Tsai *et al.*, 2016).

The appeal of using insects as alternative model systems, despite lacking the adaptive immunity of vertebrates, stems from the similarities shared between insect and mammal innate immune responses (Kavanagh and Reeves, 2004). For instance, invertebrate haemocytes act similarly in both function and structure to mammalian neutrophils - with both cellular responses involved in phagocytoses or the process of encapsulating and killing or neutralising microbes and other foreign particles (Choi *et al.*, 2002).

Mammalian and insect humoral responses are also highly homologous, with both using similar pathogen recognition receptors and signalling cascades to detect and 'tag' the surfaces of pathogens (Royet *et al.*, 2005, Bergin *et al.*, 2005). For instance, detected pathogen-associated molecular patterns via Toll receptors result in the activation of signalling pathways, which trigger the secretion of immune proteins, such as antimicrobial peptides, lectins, and other proteins, into the circulating system, working to eradicate the microbial infection (Tassanakajon *et al.*, 2018).

These conserved features have enabled the utilization of insects as *in vivo* model systems of microbial infection, or antimicrobial toxicity and efficacy, with results showing similar trends to those obtained with mammalian *in vivo* models (Piatek *et al.*, 2020). Typical examples of insect study systems in the literature include *Drosophila melanogaster*, *Bombyx mori*, and *G. mellonella* (Kavanagh and Reeves, 2004, Lionakis and Kontoyiannis, 2005, Fuchs and Mylonakis, 2006, Apidianakis and Rahme, 2011, Meng *et al.*, 2017, Mikulak *et al.*, 2018).

Here, I studied spontaneous resistance mutations in housekeeping genes, where pleiotropic effects on fitness are likely to be elevated (Reynolds, 2000, Enne *et al.*, 2004, Gagneux, 2006, Melnyk *et al.*, 2015), with resistance

acquisition based on metabolic upregulation of *β*-lactamase production. I examined the difference in relative fitness of the same antibiotic-resistant *E. cloacae* isolates across a total of five competition assays against their susceptible ancestor in the absence of selection. These include the traditionally favoured *in vitro* assays in broth and *in vivo* competition assays via injections in the heamplymph of *G. mellonella* but also in *P. xylostella*. In addition, *in vivo* experimental studies examine the fitness effects of antibiotic resistance in *E. cloacae* by conducting experiments in its natural environment - the gut of *P. xylostella*, and by quantifying between-host fitness parameters: population size within single-strain live infections, rates of bacterial shedding in frass and comparative rates of host-to-host transmission.

Methods

Bacterial isolates

The ancestor wildtype strain *E. cloacae* (WT) is a commensal symbiont and an opportunistic pathogen, originally isolated at the University of Oxford from the gut of a diamondback moth larvae in culture (*P. xylostella*) (Matthews *et al.*, 2019). This strain is the susceptible ancestor used to generate all other resistant mutant genotypes used in this work (see Isolation of Mutant Strains).

Isolation of Mutant Strains

To isolate mutants with spontaneous chromosomal mutations for antibiotic resistance, an adapted protocol of the Fluctuation Test assay was applied, originally developed to detect mutations in bacteria when induced by a chemical (Bridges, 1980).

In brief, approximately 100 cells per 10 µl of a fresh overnight WT culture were inoculated to 1 ml of antibiotic-free 2% Luria-Bertani (LB) broth and left at 30°C, 180 rotations per minute (rpm) shaking for 24 hours, with ten independent replicates per target antibiotic, as follows: cefotaxime (4 µg/ml), nalidixic acid (64 µg/ml), rifampicin (50 µg/ml). After the incubation period, each replicate was centrifuged at 13,000 g for 4 minutes, the supernatant removed, and the pellet re-suspended in 100 µl (0.85%) saline. The total biomass of each replicate was spread onto a 2% LB agar plate, supplemented with an antibiotic, as listed above. Plates were left at 30°C for 48 hours. Mutant colonies growing on the plates after the incubation period were then subcultured every 24 hours for three days on LB agar, supplemented with a selective antibiotic as listed above, in order to produce clonal cultures with stable resistance. Selected mutants were then stored in glycerol stocks at -80°C for further study.

Characterisation of antibiotic-resistant mutants

Minimum inhibitory concentration. All selected resistant isolates were tested for a change in their antibiotic sensitivity by determining their minimum inhibitory concentration (MIC), or the lowest concentration to prevent the growth of bacteria. MIC was assessed following EUCAST recommendations for micro-broth dilution susceptibility testing (EUCAST, 2003). In brief, two-fold dilution series of the appropriate antibiotic concentrations were prepared in 96-well plates, in triplicates, and inoculated with approximately 100 cells from an overnight culture grown under selection. Plates were sealed and left at 30°C, 180 rpm, for 24 hours. The lowest concentration of antibiotic, which failed to produce any turbidity, was recorded as the MIC.

PCR & sequencing of bacterial isolates. Primer sets for amplifying *ampD* and *ampR* genes in cefotaxime-resistant mutants were based on work by Hilty *et al.* (2013). All other primer sets (Table 1) were designed in Geneious 10.2.6, based on: 1) optimum length for the length of a primer between 16 and 25 base pairs (bp); 2) melting temperature (TM) - within 50-62°C; GC content - acceptable between 40-60%; 3) at the 3' end, the primer could end with either GG, GC, CG, or CC: 4) at the 5' end, a primer may begin with either A or T; 5) a primer set may cover a sequence of around 1000 base pairs (bp) when a longer sequence cover was necessary (*acrB* and *rpoB*), multiple primer sets were selected, as shown in Table 1.

Table 1. Primer set sequences were selected for the amplification and sequencing of antibiotic target sites for all selected antibiotic-resistant mutants.

Target gene	**Selective antibiotic**	**Primer names**	**Primer Sequences**	**Tm (°C)**
gyrA	Nalidixic acid	EcGyrA1734_Fw EcGyrA_2627_Rv	Fw: 5'-CGCATACCGTCTTTGTCAGA-3' Rv: 5'-TGCGAGAGAAATTACACCGG-3'	55.3
parC	Nalidixic acid	EcParC_907_Fw EcParC_2088_Rv	Fw: 5'-CAGAATCGCCTGAAGCTGAT-3' Rv: 5'-GCCAAGTTCAAGAAATCCG-3'	53.5
marR	Nalidixic acid	Fw_marR_1365 Rv_marR_2311	Fw: 5'-ATCATTATGGCAAAGACG-3' Rv: 5'-TTTTGAGTAACCTGAACGC-3'	57.5
acrB_1	Nalidixic acid	Fw_acrB_684 Rv_acrB_1740	Fw: 5'-TCTGCATCCTACCG-3' Rv: 5'-ACTGGTCATCTTCTCG-3'	49

acrB_2	Nalidixic acid	Fw_acrB_1521 Rv_acrB_2508	Fw: 5’-TGACAAACGCATCTATCG-3’ Rv: 5’-TATTTGCGGCTTCACG-3’	61
rpoB_1	Rifampicin	16S_Fw_71 16S_Rv_1307	Fw: 5'-AGGATTTTGGTAAACGTCC-3' Rv: 5'-ATGTCGTCTTTGCTCAGG-3'	53
rpoB_2	Rifampicin	16S_Fw_1030 16S_Fw_2472	Fw: 5'-ATCGAAACGCTGTTCACC-3' Rv: 5'-TTCGAAGTTGTAACCGTTCC-3'	54
rpoB_3	Rifampicin	16S_Fw_2315 16S_Rv_3326	Fw: 5'-AGAACACCTGTATCAACC-3' Rv: 5'-AGTACGATATCTACCGGC-3'	52
rpoB_4	Rifampicin	16S_Fw_2773 16S_Rv_4063	Fw: 5'-AAAGACTCTTCTCTGCGC-3' Rv: 5'-TTACTCGTCTTCCAGTTCG-3'	54
ampR	Cefotaxime	EcAmpR_74F EcAmpR_1112R	Fw: 5’-TGTGCCTGACAAACGGTTAA-3’ Rv: 5’-AGCGGTAAAGGGGTCTTCTA-3’	54
ampD	Cefotaxime	ampD_F ampD_R	Fw: 5’-TATTAATACGTTCCAGAAGC-3’ Rv: 5’-CATGGTAAACAACGTCATGT-3’	56

I extracted DNA for PCR amplification using a boilate method. A fresh colony from an overnight plate was diluted in 100 µg Tris EDTA (10 mM), then frozen at -80°C for 15 minutes. Samples were then heated at 95°C for 10 minutes and returned to -80°C. After 15 minutes, samples are taken out to thaw on the bench, then centrifuged at 6000 g for 5 minutes. 70 µl of the supernatant is transferred to a clean tube and stored at -20°C until needed.

PCR reactions were prepared using the HotStarTaq Plus Master Mix Kit (Qiagen). A single reaction was prepared, as follows: 12.5 µl HotStarTaq Plus

DNA polymerase; 1.25 µl of each forward and reverse primers at 25 nM; 7 µl RNase-free water, 2 µl coral load concentrate, 1 µl DNA template. Reaction mixtures were activated at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, melting temperature as required (per Table 1) for 30 seconds, and extension at 72°C for 30 seconds. Amplification was completed with a final cycle of 72°C for 7 minutes.

PCRs were subjected to clean-up with the QIAquick PCR Purification Kit before being sequenced by Eurofins.

Final selection of resistant mutants for competition assays. Only fully characterised resistant mutants were considered for final selection. A preliminary study estimating the cost of resistance was carried out for all mutant isolates to calculate the relative fitness (w), both *in vitro* and within a live (gut) infection (*P. xylostella*).

In brief, competition mixes were prepared by diluting mutant isolates and the wildtype strain 1000-fold, then mixing them at a 1:1 ratio. From each mixture, 10 µl were used to inoculate 1 ml non-selective LB, six replicates per competition mixture, and left at 30°C, 180 rpm shaking, for 18 hours. To obtain initial and final proportions, inocula were plated on selective and non-selective media. The same competition mixtures were used to cover the surface of the diet for *P. xylostella* larvae.

P. xylostella eggs were sterilised 48 hours prior to competitions by washing in 10% Distel (manufactured by Tristel Solutions Limited, 2017) for 1 minute, followed up by two washes in sterile water for 10 seconds each. Larvae were left to hatch on a non-selective wheatgerm diet at 25°C. Post-incubation larvae were transferred to two 50 mm Petri dishes per competition mix, containing a

block of fresh non-selective diet inoculated with 150 µl of each competition mix, covering the entire surface of the diet. After 48 hours, 16 larvae per replicate were moved to a fresh microbe-free diet and allowed to feed together for 72 hours at 25°C. Prior to homogenisation larvae were surface sterilised (70% ethanol) and plated on selective and non-selective media to obtain final proportions. This protocol was later modified to allow an increased number of independent replicates.

Aseptic insect rearing

Diamondback moth populations originated from a cross of two diet-adapted populations, as described previously (population VLSS) (Zhou *et al.*, 2014), and stock populations are grown in controlled environment facilities at the University of Exeter. *P. xylostella* is aseptically reared at 25°C, ~50% humidity, and 8:12 light/dark cycles. The complete life cycle of the lab-reared parental stock is 20 days. From egg to pupa, *P. xylostella* larvae go through four stages of development (instars), taking approximately 14 days when adults begin to emerge. Once most larvae have pupated, they are moved to oviposition chambers. These are clear cubic Plexiglass containers with dimensions of 35x35x35cm. Prior to moving pupae, chambers are surface sterilised with 70% ethanol and left to fully dry in a Class 2 safety cabinet. Once adults begin to emerge, the chamber is supplied with autoclaved 10% sucrose solution for feeding the adults. Typically, 24 hours after emerging, adults have already begun mating, and the first cabbage-infused egg strip is introduced to the chamber. Eggs are collected every 24 hours for four days when the chamber is moved to -20°C for 48 hours, ending the cycle. Eggs are stored at 4°C in a humid environment for up to a week.

Egg sterilisation protocol. Eggs are surface sterilised by washing in 10% Distel, a high-level medical surface disinfectant for 1 minute, then rinsed twice in autoclaved water for 10 seconds. Loose eggs detached from the egg strips during the washing process are collected by filtering both the Distel solution and water through non-cellulose genesis filter paper (V. Mueller, USA). Collected loose eggs, along with the filter paper, are frozen at -20°C for 48 hours before being safely disposed of. Sterilised eggs are left for 48 hours at 25°C in a sealed, surface-sterilised with 70% ethanol deli pot, containing a sterile tissue, dampened with autoclaved water. Post-incubation, first instar larvae emerge and egg strips are moved onto the appropriate diet, either for stock rearing or as required by experimental protocols.

Wheatgerm diet. The VLSS population is adapted to feed on a wheatgerm-based diet (Hunter *et al.*, 1984), as described in Table 2. All ingredients, excluding the temperature-sensitive vitamins (Vanderzant and Ascorbic acid), are kept as a dry mix at 10°C.

To prepare the wheatgerm diet, 80 grams of dry mix ingredients are mixed with 400 ml water using a domestic handheld blender, and the mixture is autoclaved. Once the diet is cooled to approximately 50°C, temperature-sensitive vitamins are added to the diet after each being dissolved in 50 ml water and filter-sterilized (0.22 µm). The diet is well-mixed and poured into 90 mm Petri dishes, then left to dry in a Class 2 safety cabinet for 45 minutes.

In addition to the components described in Table 2, the diet for the parent population is also supplemented with 200 mg/ml streptomycin and 400 mg/ml chlortetracycline. Antibiotics are added along with temperature-sensitive vitamins. Antibiotics are not added to diet for larvae used in experimental work

unless explicitly specified. The wheatgerm diet is stored at 10°C for up to a month.

Table 2. Full list of ingredients, excluding antibiotics, used to prepare wheatgerm diet (*Temperature-sensitive vitamins).

Ingredients:	**Quantity (%)**
Inositol	0.2
Choline chloride	0.4
Cholesterol	1.258
Sodium propionate	2
Sorbic acid	2
Cellulose	2.15
Fructose	2.15
Wessons salts	8
Agar	12.8
Caesin	25
Wheatgerm	60
*Vanderzant	0.4
*Ascorbic acid	0.36

Validation of aseptic rearing protocol. To test the validity of the protocol for aseptic insect rearing, a PCR amplification assay was performed using the broad-range 16S rRNA gene primer set 27F (forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (reverse primer: 5'-GGTTACCTTGTTACGACTT-3') (Frank *et al.*, 2008). This primer set should detect the presence of any unculturable bacteria which may be present in the insect microbiome.

DNA was extracted from larvae at the 4th instar reared for 1) the stock (parental) population (microbe-free and fed on wheatgerm diet containing streptomycin and chlortetracycline); 2) larvae reared for an experiment as negative control (microbe-free and fed on wheatgerm diet containing no antibiotics); and 3) larvae reared for an experiment, infected with the wildtype *E. cloacae*, to serve as a positive control. Five larvae were used per treatment group. Each sample was ground using a pellet pestle in a microcentrifuge tube, treated with 180 µl tissue lysis buffer (ATL) and 10 µl proteinase K then incubated for 6 hours at 56°C, vortexing samples several times during incubation. Post incubation, DNA was extracted using the DNeasy Blood & Tissue Kits (Qiagen).

PCR reactions were prepared using the HotStarTaq Plus Master Mix Kit (Qiagen). A single reaction was prepared, as follows: 12.5 µl HotStarTaq Plus DNA Polymerase; 1.25 µl of each primer at 25nM; 7 µl RNase-free Water, 2 µl CoralLoad Concentrate, 1 µl DNA template. Reaction mixtures were incubated for 5 minutes at 95°C, followed by denaturation at 95°C for 30 seconds, melting temperature at 58°C for 30 seconds, and extension at 72°C for 30 seconds, repeated for 35 cycles, followed by a final cycle of 72°C for 7 minutes.

Competition assays

Selected mutant isolates competed against their susceptible ancestor under five antibiotic-free conditions. These were broadly categorised as ‘Traditional’ or ‘Naturalistic’ assays. The former included direct competition in broth (*in vitro*), and via injection directly into the haemolymph of *P. xylostella* and *Galleria melonella*. In contrast, ‘naturalistic’ assays measured fitness in two distinct ways: 1) as within-host competitive ability to form a persistent gut infection through inoculated diet and 2) as between-host transmission of infection from infected to sterile larval hosts.

All competition mixes were prepared by combining 1000-fold dilutions of a selected mutant isolate with the ancestral strain at a 1:1 ratio, with the exception of *G. melonella* injection assay, where undiluted inocula were required.

Traditional approaches

In vitro. Competition inoculum of 10 µl was added to 1 ml antibiotic-free LB in 24-well plates, with six independent replicates, leaving uninoculated wells between as negative controls. Plates were sealed and left at 30°C/180 rpm for 18 hours. To obtain initial ratios, each inoculum was plated on both selective and antibiotic-free media. An *in vitro* assay was repeated alongside each *in vivo* experiment.

***In vivo* injection assays (*P. xylostella* & *G. melonella*).** *P. xylostella* eggs, sterilised as described previously, were left to hatch and develop on a sterile non-selective wheatgerm diet at 25°C. Once larvae reached 4th instar, 15 individuals of similar size were selected per treatment (competition mix) and placed on ice for 15 minutes, then injected with 2 µl of a competition inoculum,

using 10 µl Hamilton syringes. Saline was used as a negative control. Injections were administered into the haemolymph from the rear end. Larvae were left in 90 mm Petri dishes with no diet at 25°C for 24 hours. Post incubation, all larvae were individually homogenised in a bead beater (Qiagen TissueLyser II) at 30Hz per second for 4 minutes with a lysing ball and 150 µl saline (0.85% w/v NaCl), then plated on both LB agar and LB agar supplemented with the appropriate antibiotic and left at 30°C overnight.

G. mellonella, sold as WaxWorms, were purchased by Livefood UK Ltd, Somerset. Competition inocula of WT (susceptible ancestor) and each resistant strain (CEF, NAL, RIF) were prepared at a 1:1 ratio after washing cells twice by centrifuging 1 ml of culture at 13,000 g for 1 minute and replacing 700 µl supernatant with sterile 0.85% saline. Larvae were injected with 10 µl of each inoculum, with 24 larvae per treatment, including a control, where sterile 0.85% saline was injected instead. Larvae were incubated at 37°C for 24 hours and then processed as above, except homogenization used 1 ml of saline.

Naturalistic approaches (*P. xylostella* only)

Gut infection. To measure the relative fitness (W) of resistant mutants as their ability to establish a persistent gut infection, just prior to hatching (24 hours after sterilization), eggs were placed on sterile diet and allowed to feed for 48 hours. Larvae were then transferred to twelve 50 mm Petri dishes per treatment, containing diet inoculated with 150 µl of a competition mix. After 24 hours of incubation at 25°C, 48 larvae per treatment were transferred to bio-assay trays, where larvae were placed in individual wells containing sterile non-selective insect diet. Once larvae had reached their final (4^{th}) instar, they were individually homogenized in a bead beater (Qiagen TissueLyser II) at 30Hz per

second for 4 minutes with 500 µl (0.85%) saline, and a lysing ball then plated on both LB and LB supplemented with the appropriate antibiotic.

Between-host fitness: bacterial shedding from frass

Between-host fitness was assessed in two distinct sets of controlled experiments. First bacterial shedding from frass was measured, where fitness was measured as the number of infectious propagules shed from hosts carrying single-strain infections. The number of bacteria per mg larvae weight was also measured. Second, we measured comparative rates of horizontal transmission, where fitness was measured as the ability of resistant strains to establish secondary host gut colonisation relative to susceptible strains within shared experimental chambers.

Both between-host fitness experiments (bacterial shedding through frass and horizontal transmission) were conducted in parallel, using the same starting stock of larval (primary) hosts, infected with single-strain genotypes, as follows:

Eggs sterilised as previously described were incubated for 24 hours at 25°C, then cut into approximately 1 cm^2 squares. A non-selective diet was prepared by covering the surface of each diet block with 150 µl with a single strain at 1000-fold dilution: either the susceptible ancestor, a resistant mutant strain, or 0.85% saline - not in pairs. The diet was then left to dry for 45 minutes in a Class 2 safety cabinet. Egg strips were then left to hatch on the inoculated diet. The plates containing diet and egg strips were sealed with parafilm to ensure enough moisture was retained and incubated for 24 hours. After incubation the parafilm and egg strips were removed from the Petri dishes and larvae left back at 25°C until they reached early 4^{th} instar.

To measure the shedding of infectious propagules from single-strain infections, I estimated the number of bacteria shed through frass from infected hosts. In addition, I measured the number of bacteria per mg of larval weight.

To establish whether there was a difference in the rate at which selected mutants and their susceptible ancestor (WT) were shed by infected hosts, 6 larvae from each treatment (infected with either WT, CEF, NAL, RIF, or saline) were weighted to control for size then transferred to fresh non-selective sterile wheatgerm diet and incubated at 25°C for 24 hours. Post incubation, larvae were weighted again, surface sterilised in 70% ethanol and homogenised in pairs in a bead beater (Qiagen TissueLyser II) at 30Hz per second for 4 minutes in 300 µl (0.85%) saline and a lysing ball. After removing the larvae, the surface of each plate where the infected larvae had been feeding was washed with 1 ml 0.85% saline solution and plated. Each treatment contained ten replicates.

Between host fitness: horizontal transmission

From the same (primary) larval stock carrying a single-strain infection of either WT, CEF, NAL, or RIF, 8 early 4th instar larvae from each treatment were selected and again weighted to control for size, then transferred in sets at a 1:1 ratio (8 hosts infected with a mutant strain and 8 hosts infected with the susceptible ancestor) to 90 mm Petri dishes containing aseptically reared, uninfected *P. xylostella* larvae, hatched 24 hours prior to set-up (2nd generation). Each set had four replicates. Infected and uninfected larvae were allowed to interact for 48 hours when the late 4th instar larvae were removed, surface sterilised homogenised in a bead beater (Qiagen TissueLyser II) at 30Hz per second for 4 minutes in 300 µl (0.85%) saline and a lysing ball and plated. The 2nd generation of larvae was left to reach early 4th instar in the same

90 mm Petri dishes, when 32 larvae per plate, or 128 larvae per treatment were surface sterilised with 70% ethanol, homogenised in pairs in a bead beater (Qiagen TissueLyser II) at 30Hz per second for 4 minutes in 300 µl (0.85%) saline and a lysing ball, and plated on both LB agar and LB supplemented with the appropriate antibiotic - a nested experimental design.

Data analysis

Total initial and final cell densities were calculated using total colony counts on LB agar, and resistant colony counts on selective agar plates. Where the number of colonies on selective media exceeded the total colony count on LB media, the cell densities for the susceptible (WT) were assumed to be zero. In addition, to account for pipetting error, the number of colonies on selective media within 10% of the total cell densities were assumed to be a mono-culture of resistant cells.

The relative (Darwinian) fitness for resistant mutants was calculated using the ratio of the Malthusian parameters (W). W estimates the rate of change in the densities of the two competing strains. It is important to note that this method relies on estimating initial bacterial densities, which are directly estimated in Traditional assays, and indirectly estimated for the initial infection bottleneck in 'Naturalistic' assays, although previous analyses have shown that measurements of relative fitness are insensitive to the estimates of the bottleneck here (Zhou *et al.*, 2020, van Leeuwen *et al.*, 2015).

Relative fitness was analysed using generalised linear models (GLM), direct horizontal transmission was analysed in a mixed-effect model with replicate as a random factor. Akaike Information Criterion (AIC), along with the effect of

model assumptions were used to determine the best model fit. All analyses were performed in R v1.4.1106 (R Core Team, 2021).

Test of association was used to determine whether a certain genotype was more likely than others to colonise the gut of *P. xylostella* larvae, data obtained from the 'Gut Infection' assay.

Results

Isolation of mutant isolates

Spontaneous mutant isolates were selected from colonies growing on LB agar supplemented with either 4 µg/ml cefotaxime; 32 µg/ml; 64 µg/ml nalidixic acid, or 50 µg/ml rifampicin after 48 hours of incubation.

Characterisation of mutants

Six resistant mutants from each antibiotic treatment were selected for sequencing. Cefotaxime-resistant mutants were sequenced at two potential target genes - *ampD* and *ampR*; nalidixic acid-resistant mutants were sequenced at *gyrA*, *parC*, *marR*, and *acrB*; 16S rRNA was sequenced in rifampicin-resistant mutants. Sequences of resistant mutants were aligned and analysed against the ancestral strain sequences as positive control.

A variation of chromosomal changes was observed in the *ampC* transcriptional regulator *ampR* in cefotaxime resistance mutants, as described in Table 3, while no changes were observed in the alignments of *ampD*.

A single base pair mutation was observed for three of the quinolone-resistant mutants isolated at 64 µg/ml, located at *gyrA*. All mutants isolated on the lower nalidixic acid dose (32 µg/ml) had wild-type sequences in *gyrA*, *parC*, *marR*, and *acrB*. Sequencing the 16S rRNA for rifampicin-resistant mutants showed a

variation in resistant genotypes, with each resulting in the same amino acid change (Phe) with different levels of rifampicin susceptibility.

Table 3. Characterisation of spontaneous mutants resistant to either cefotaxime (CTX), nalidixic acid (NAL), or rifampicin (RIF). Susceptible ancestor strain susceptibility for cefotaxime is 0.25 µg/ml, for nalidicix acid – 4 µg/ml, and rifampicin – 2 µg/ml. ***In bold*** – mutants selected for competition assays.

Mutant ID	**Selective antibiotic dose (µg/ml)**	**MIC (µg/ml)**	**Target gene**	**Mutation**	**Position of mutation (bp)**	**Amino acid change**	**Reference Genbank ID**
***cef_a**	**CTX 4**	**256**	**ampR**	**A to C**	**410**	**Asp to Ala**	AY789446
cef_b1	CTX 4	256	ampR	G to T	409	Asp to Tyr	
cef_b2	CTX 4	256	ampR	G to T	409	Asp to Tyr	
cef_ins	CTX 4	64	ampR	Insertion (25bp)	Begins at 370	+Asp, Leu, Gln, Leu, Ser, The, His, Asn	
***nal_a1**	**NAL 64**	**1024**	**gyrA**	**T to G**	**793**	**Val to Ala**	AWQ58471.1
nal_a2	NAL 64	1024	gyrA	T to G	793	Val to Ala	
nal_b	NAL 64	128	gyrA	T to G	793	Val to Ala	
***rif_a**	**RIF 50**	**1024**	**16S**	**T to G**	**436**	**Val to Phe**	QOR49167.1
rif_b1	RIF 50	128	16S	A to T	572	Ile to Phe	
rif_b2	RIF 50	128	16S	A to T	572	Ile to Phe	
rif_c1	RIF 50	256	16S	C to T	531	Ser to Phe	
rif_c2	RIF 50	256	16S	C to T	531	Ser to Phe	

Final selection of resistant mutants

Preliminary work allowed the estimation of the cost of resistance of mutant isolates in terms of relative fitness (w), based on 1:1 competition assays in the absence of selection, both *in vitro* and within a gut infection (*P. xylostella*). A single mutant genotype was selected per antibiotic type, with varying costs of resistance: no to low-cost for cefotaxime-resistance, moderate cost for quinolone-resistance, and high-cost for rifampicin-resistance. The aim was to investigate how this difference would hold across all competition assays. The final selected isolates were as follows: cef_a1, nal_a1 and rif_a, hereafter referred to as CEF, NAL and RIF, corresponding to the antibiotics they were selected on. These fully characterised mutants varied in their relative fitness (W) between genotype and environment (*in vitro* and *in vivo*), and overall produced consistent, repeatable data.

Validation of aseptic rearing

To confirm the sterility of larvae from the stock (parent) population and of larvae used in experimental work, DNA was extracted from 5 individuals from each aseptically reared population. DNA was also extracted from larvae infected with the wildtype *E. cloacae* strain as well as from a pure *E. cloacae* culture, included as positive controls. PCR with the broad-spectrum 16S rRNA gene confirmed the presence of *E. cloacae* from infected larvae and was not able to detect any unculturable bacteria from the parent and uninfected experimental population (Figure 1). The sterilisation technique and aseptic rearing conditions have successfully maintained bacteria-free populations of insects.

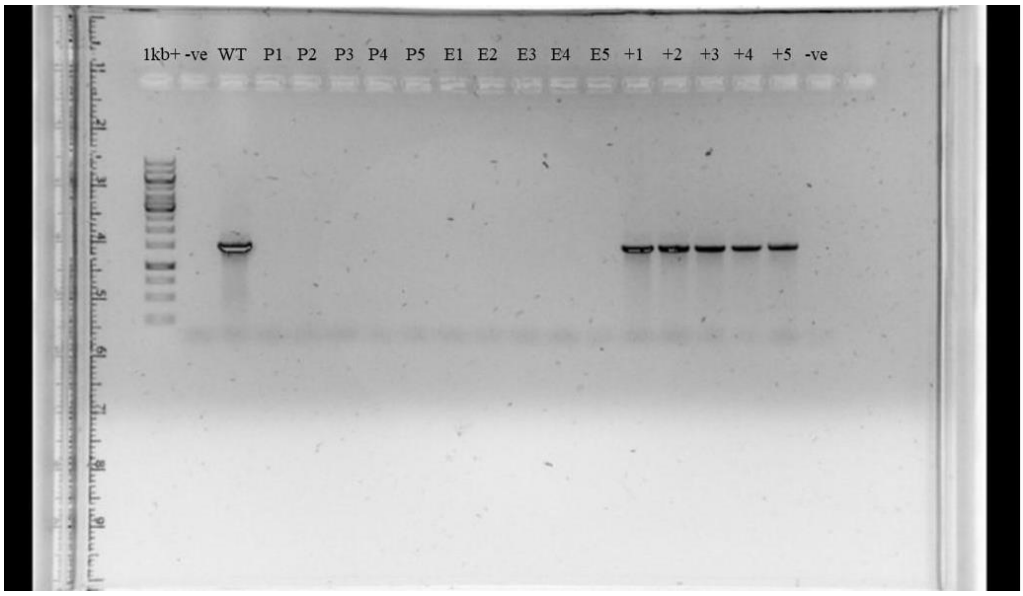


Figure 1. Gel electrophoresis of PCR of the broad-spectrum 16S rRNA, confirming larvae reared on selective diet for the parental population (P1 - P5) and uninfected larvae reared on non-selective diet for experimental work (E1-E5) are bacteria-free. Included are Thermo Scientific 1 kb GeneRuler 1 kb DNA Ladder (range 250 to 10,000 bp), negative control (-ve), positive control, including DNA extracted from the wildtype *E. cloacae* strain, as well as DNA from larvae infected with the same strain (+1 to +5).

Competition assays

Resistant mutants expressed differences in relative fitness (W) accross different environmental conditions, with a strong environment-genotype interaction (genotype-environment interaction, $F_{8, 371} = 12.31$, $p<0.001$) and effect of genotype (genotype, $F_{2,379} = 21.539$, $p<0.001$).

Relative fitness of the constitutive AmpC *β*-lactamase producing mutant (CEF) showed the smallest overall fitness cost but still had a fitness that was lower than that of the wild type since the confidence intervals for this parameter did not overlap with w = 1 (fitness mean = 0.93; 95% C.I. [0.892; 0.974]).

Higher fitness costs were observed for both quinolone- (NAL) (test for difference in fitness <1, fitness mean=0.86, 95% C.I. [0.819; 0.901]) and rifampicin-resistant (RIF) mutants (test for difference in fitness <1, fitness mean= 0.704, 95% C.I. [0.662; 0.756]).

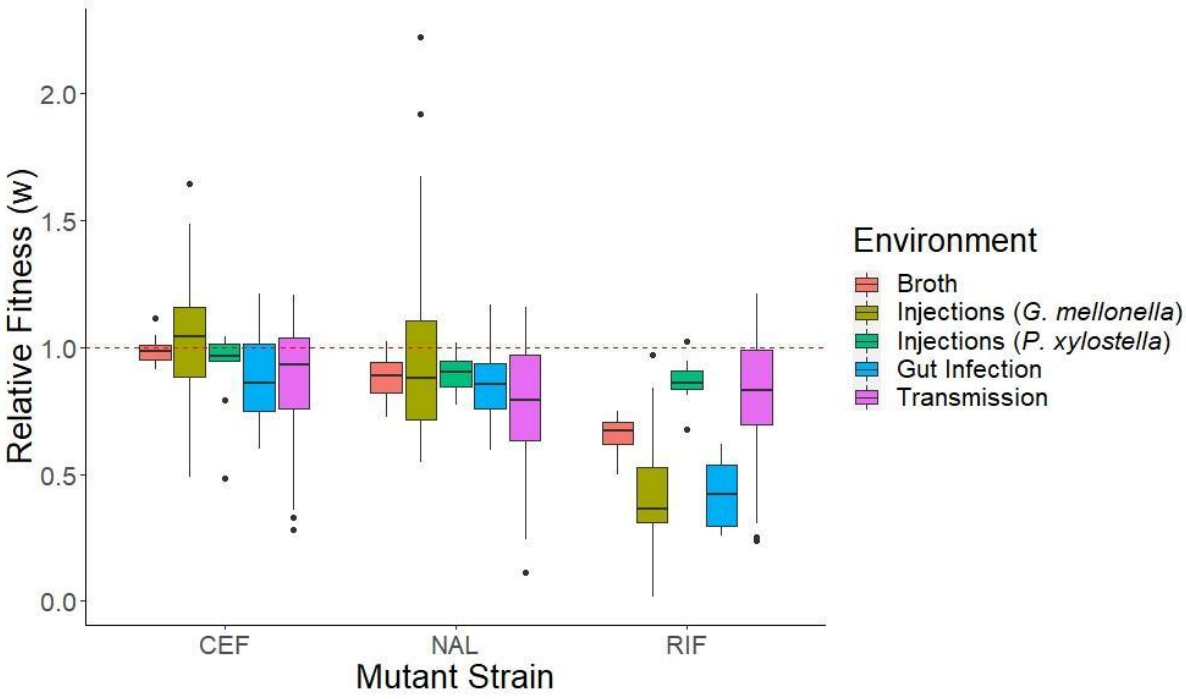


Figure 2. Relative fitness of *E. cloacae* mutants in pairwise competition assays against the wildtype ancestor *in vitro* (in LB) and *in vivo* infections. *G. mellonella* was suitable for injection assays only, while *P. xylostella* was used in all *in vivo* assays. 'Traditional approaches' include 'Broth' and 'Injections' assays, while 'Naturalistic approaches' include 'Gut infection' and 'Transmission'.

Despite AmpC *β*-lactamase production (CEF) having little impact on fitness overall, there was an elevated cost in naturalistic *in vivo* assays when compared to traditional *in vitro* and *in vivo* approaches ($t = 3.476$, $p > 0.001$). Grouping assays into traditional *vs.* naturalistic approaches did not result in a significant loss of variation ($F_{3,127} = 1.212$, $p = 0.308$); this pooling of treatments produced better statistical models, based on the reduction in AIC (simpler grouped model -43.912, df = 3; full model: -41.638, df = 6).

Quinolone resistance (NAL) imposed relatively consistent fitness costs in different environmental conditions. As before, grouping naturalistic infection *vs.* traditional approaches provided the minimal adequate model with no significant loss in variation relative to the full model with separate treatments ($F_{3, 124}$=1.478, p = 0.224) and AIC (simpler grouped model: 11.849, df=3; full model: 13.315, df=6), with costs elevated in naturalistic assays (t = -3.187, p > 0.002).

Rifampicin resistance exhibited large heterogeneity in fitness costs between environments ($F_{4,120}$ = 23.575, p > 0.001). To exclude the possibility that the observed heterogeneity in RIF fitness may be determined by temperature differences between assay protocols, a follow-up *in vitro* competition assay was carried out at 25°C (corresponding to temperatures used for *in vivo* assays protocols working with *P. xylostella*), 30°C (*in vitro* protocol) and 37°C (*G. melonella* injection assay).

There was a significant variation in fitness costs *in vitro* among the three temperature conditions (ANOVA, $F_{2,15}$ = 38.39, p < 0.001). However, in contrast to fitness assessed *in vivo*, here costs decreased at 37°C when compared to 25°C (Tukey HSD, p < 0.001, 95% C.I. = 0.044, 0.099) and 30°C (Tukey HSD, p < 0.001, 95% C.I. = 0.058, 0.112), while no variation was observed between 25°C and 30°C (p = 0.39).

Some important differences were observed between the two naturalistic assays. There was a higher rate of infection (100%) in transmission, where *P. xylostella* larvae remained exposed to bacteria for four days. In contrast, only 57% of *P. xylostella* larvae exposed to competition cultures for 48 hours only (gut infection assay) had a persistent infection. In addition, in the gut inoculation treatments infections often consisted of a single strain (either the wildtype or a resistant

mutant, see Table 4). Counts of clonal and mixed infections did not vary across genotypes (test of association, $X^2 = 4.3273$, df = 6, $p = 0.63$, Table 5). In contrast, co-infection was the predominant type of infection in transmission.

Table 4. Counts of present/absent persistent gut infections in *P. xylostella* larvae with either a single strain or a competition mixture.

Infection\strain	CEF	NAL	RIF
Absent	20	22	20
Wild type only	8	9	12
Mutant only	7	7	10
Co-infection	13	10	6

The frequency of the final proportion of resistant mutants for each competition assay further emphasises the tendency of a single strain infection to be predominantly observed in gut infection assays (0 = WT; 1=mutant), and especially for RIF, as shown in Figure 3 below.

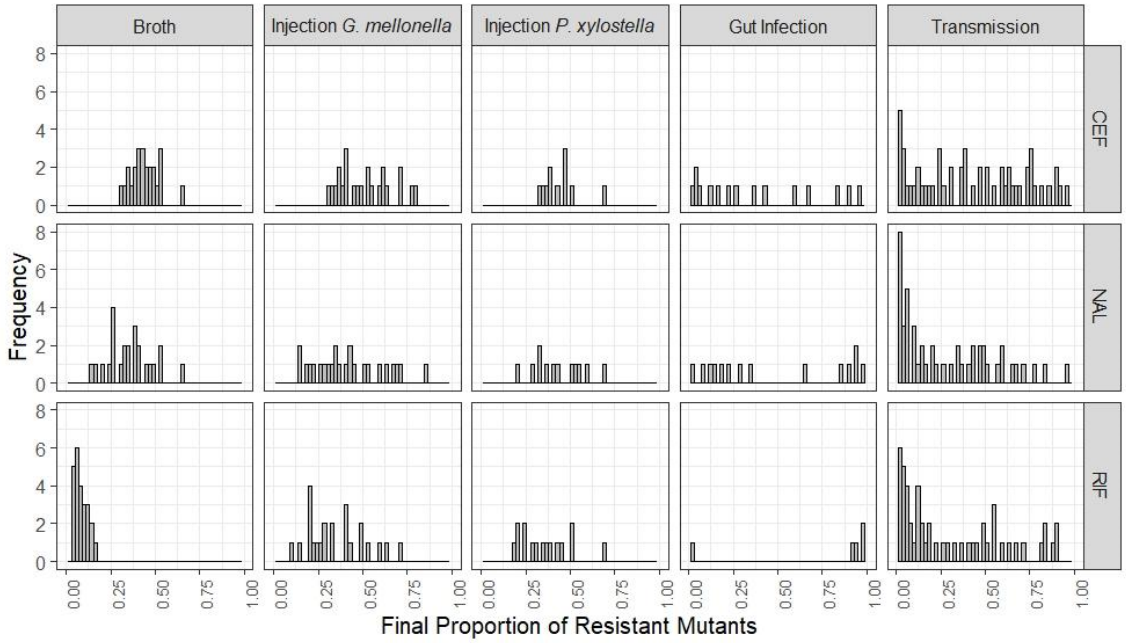


Figure 3. Frequency of the final proportions for each mutant genotype (CEF, NAL, RIF) per assay type (broth, injections in *G. mellonella* & *P. xylostella*, gut infection, transmission).

Between-host transmission

To understand how bacteria are shed by infected hosts and whether there was an equal chance of pathogen transmission, six larvae infected with a single strain type were transferred to a sterile plate and allowed to feed for 24 hours, ten replicates per strain. Post incubation, the load of bacteria shed through frass was measured along with total larval weight per replicate and total cell density within larval hosts. There were no strong differences found between these three parameters (CFU/frass, $F_{3, 36} = 0.188$, $p = 0.904$; larval weight, $F_{3, 36} = 0.982$, $p = 0.412$); density mean per larva, $F_{3, 36} = 0.77$, $p = 0.518$), as the amount of *E. cloacae* shed in frass increased with larval weight (Figure 4).

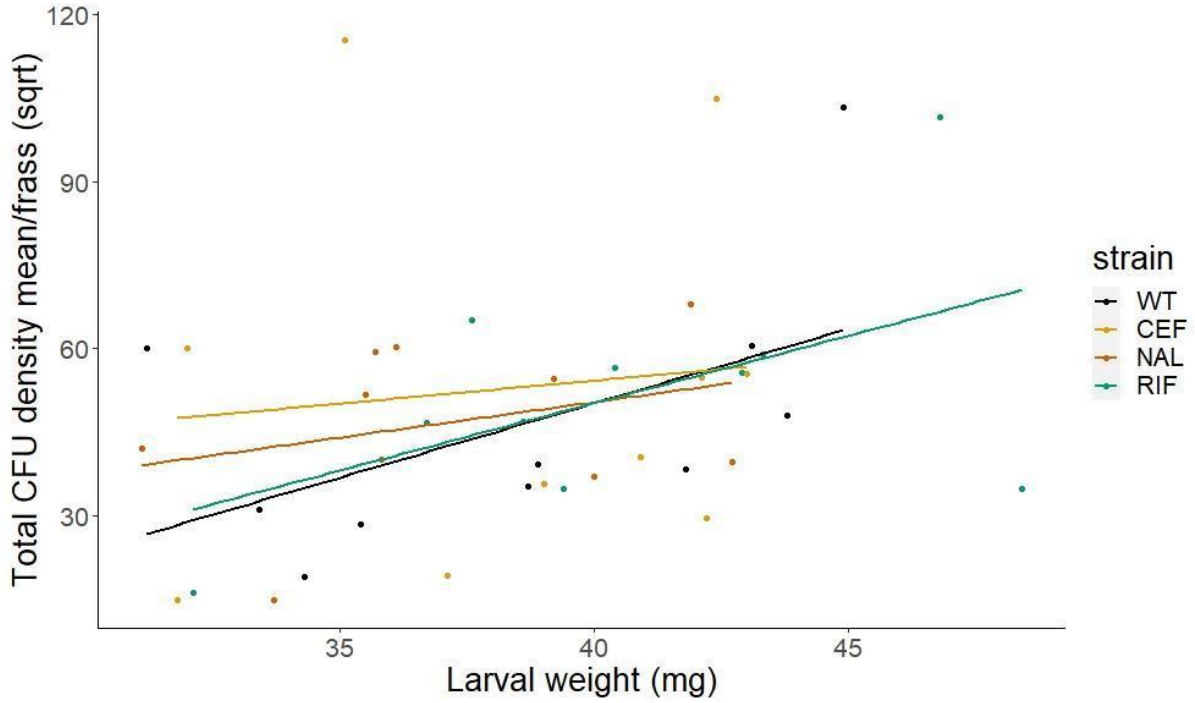


Figure 4. Mean cell density shed through the frass of *P. xylostella* larvae hosts, infected with a single-strain infection and plotted against larval weight (mg).

Fitness costs in horizontal host-to-host transmission were calculated as the relative fitness (w) of mutants retrieved from the guts of the secondary hosts post short-term interaction with paired primary hosts (carriers of a single-strain infection of either wildtype or mutant genotype and mixed at a 1:1 ratio). All three mutant genotypes exhibited reduced relative fitness in transmission when compared to the wild type as $w < 1$: (CEF: fitness mean = 0.875, 95% C.I. [0.819; 0.932]; NAL: fitness mean = 0.784, 95% C.I. [0.727; 0.840]; RIF: fitness mean = 0.818, 95% C.I. [0.761; 0.874].

Despite the large overall variability in fitness costs for RIF across all assays, when comparing relative fitness in transmission only, there was no difference between RIF and CEF (t = -1.413, p=0.159), while fitness in transmission for NAL was lower (t = -2.244, p = 0.026).

Discussion

How informative fitness costs of antibiotic resistance acquisition measured *in vitro* are in the context of naturalistic environments, and particularly for opportunistic commensals, is currently not well understood. Here, I present a valuable, tractable model system, an alternative to murine models, which provides more accessible means to examine the cost of resistance *in vivo* in colonising commensals at multiple levels with potential to inform antibiotic stewardship.

In this study, I explored the effects of resistance mutations in regulatory or housekeeping genes, conferring resistance to a *β*-lactam, a quinolone, or rifampicin. By comparing fitness costs between insect model systems in addition to prior studies, I provide a scope of both the value and limits of this system.

β-lactam resistance. *β*-lactam resistance associated with constitutive AmpC *β*-lactamase upregulation is commonly reported from clinical *Enterobacteriacae* (Kuga *et al.*, 2000, Kaneko *et al.*, 2005, Jacoby, 2009, Guérin *et al.*, 2015).

Typically AmpC upregulation results from mutations in *ampD*, and less commonly in *ampR* (Kopp *et al.*, 1993, Ehrhardt *et al.*, 1996, Hanson and Sanders, 1999, Kuga *et al.*, 2000, Kaneko *et al.*, 2005, Schmidtke and Hanson, 2006). Yet, all successfully characterised *β*-lactam-resistant isolates in this study carried chromosomal changes in *ampR* only. The selected *β*-lactam resistant isolate (CEF) carried a single base pair mutation, resulting in an amino acid change from Asp to Ala and a 1000-fold increase in MIC.

CEF showed no significant fitness disadvantage *in vitro* and in injection assays, regardless of insect species. This result is consistent with prior work *in vitro* on costs imposed by upregulation of AmpC *β*-lactamases and by the presence of plasmids encoding extended-spectrum *β*-lactamases (Hossain *et al.*, 2004, Dimitriu *et al.*, 2019). However, during gut colonisation and transmission fitness costs were present, suggesting traditional assays (i.e*., in vitro*, injection assays) might not be convincing models for a naturalistic infection, particularly in regards to colonising commensals.

Epidemiological studies on extended-spectrum *β*-lactamase-producing *Enterobacteriacae* (ESBL-PE) acquisition have shown that in the majority of healthy individuals who had acquired resistant strains during traveling to countries where the prevalence of ESBL-PE is high, de-colonisation typically occurred within 30 days (Arcilla *et al.*, 2017, ÖstholmBalkhed *et al.*, 2018), suggesting that in a natural gut infection and in the absence of selection *β* -lactam resistant commensals are likely to be at a disadvantage relative to susceptible genotypes.

Quinolone resistance. The selected quinolone-resistant isolate (NAL) carried a single base-pair mutation in *gyrA* (encoding for DNA gyrase) and had a 256-fold increase in MIC relative to wildtype. This enzyme is involved with DNA replication and transcription and is a well-known target for quinolones and fluoroquinolones, so is consistent with target site resistance (Champoux, 2001). No evidence was found for changes in *parC*, *acrB,* or mutations in *marR,* known as alternative sites for resistance to quinolones in the *Enterobacteriaceae* (Lindgren *et al.*, 2003, Andersson and Hughes, 2010, Machuca *et al.*, 2015).

All three spontaneous quinolone-resistant isolates carried the same single-base pair mutation in *gyrA* and lacked mutations in other target genes, similar to work by Ruiz *et al.* (1997), Piddock *et al.* (1998), and Giraud *et al.* (1999). Despite this, isolates varied in their susceptibility levels (either 1024µg/ml or 128µg/ml), suggesting there may be unaccounted differences in their genetic backgrounds.

Significant differences in susceptibility, despite identical *gyrA* mutations is consistent with findings in clinical isolates of *E. cloacae, C. freundii, and S. marcensens* (Deguchi *et al.*, 1997, Nishino *et al.*, 1997, Kim *et al.*, 1998). In *C. freundii* clones with genetically identical resistance mutations in quinolone resistance-determining regions, susceptibility variations were related to efflux mechanisms (Navia *et al.*, 1999). Here, efflux mutations were not determined, however, cell-to-cell variability in expression could be important (Pu *et al.*, 2016, Levin-Reisman *et al.*, 2017).

Resistance-conferring single-point mutations in *gyrA* are not unusual for Gram-negative organisms, including *N. gonorrhoeae, Klebsiella pneumonia, Salmonella enteritidis, S. typhimurium,* and *P. aeruginosa,* (Belland *et al.*, 1994, Kureishi *et al.*, 1994, Deguchi *et al.*, 1997, Fisher, 1997, Kotb *et al.*, 2019), although in clinical *Enterobacteriacae* isolates these are more commonly accompanied by mutations in other target genes (Minarini and Darini, 2012, Sekyere and Amoako, 2017, Kotb *et al.*, 2019).

The effects of quinolone resistance mutations on bacterial fitness are variable and can depend on the type of mutation(s), the genetic background, and/or environment, with resistant mutants exhibiting high, no, low, or even enhanced fitness *in vivo* (Giraud *et al.*, 2003, Lindgren *et al.*, 2005, Luo *et al.*, 2005, Kassen and Bataillon, 2006, Rozen *et al.*, 2007, Marcusson *et al.*, 2009, Kunz

et al., 2012, Ferreira *et al.*, 2018, Chang *et al.*, 2021). Unlike other *Enterobacteriacae* (Giraud *et al.*, 2003, Lindgren *et al.*, 2005, Luo *et al.*, 2005, Kassen and Bataillon, 2006, Rozen *et al.*, 2007, Marcusson *et al.*, 2009, Kunz *et al.*, 2012, Ferreira *et al.*, 2018, Chang *et al.*, 2021), however, there is limited information on the effect of antimicrobial resistance on fitness costs in *E. cloacae*. Here, no efflux mutations were confirmed, yet single-point mutation in *gyrA* showed a relatively consistent fitness burden relative to the wildtype across assays, with costs in gut colonisation and transmission being more pronounced.

Rifampicin resistance. A single base pair substitution was observed in the gene encoding for the *β*-subunit of RNA polymerase (*rpoB*), or the rifampicin binding pocket. Alterations in this target enzyme prevent rifampicin from binding properly but also typically result in disrupted gene expression and thus impaired fitness (Campbell *et al.*, 2001). Similar to previous studies (MacLean and Buckling, 2009, Miskinyte and Gordo, 2013, Vogwill *et al.*, 2016), fitness costs in the rifampicin-resistant mutant here exhibited variations with environmental conditions.

As competition assays were performed under different temperature conditions (i.e., 25°C for assays with *P. xylostella*, 30°C for assays *in vitro*, and 37°C for injection assay with *G. mellonella*), I hypothesised that temperature here might be a determining factor. Changes in temperature are commonly associated with shifts in gene expression in bacteria (Konkel and Tilly, 2000, Wurtzel *et al.*, 2012), and mutations in *rpoB* – a master gene expression regulator have previously been associated with changes in fitness in *P. aeruginosa* (Gifford *et al.*, 2016). Moreover, rifampicin resistance in *E. coli* can evolve under thermal

stress in the absence of selection (Rodríguez-Verdugo *et al.*, 2013). Thus, to exclude temperature as the sole determining factor for fitness differences in RIF, three additional *in vitro* competition assays were performed corresponding to each temperature condition used across fitness assays. However, there was no evidence linking variation in temperature to the observed differences in fitness.

High variability in fitness costs of rifampicin-resistant bacteria across different antibiotic-free environmental conditions is common (MacLean and Buckling, 2009, Miskinyte and Gordo, 2013, Vogwill *et al.*, 2016), yet why this occurs is not fully understood. Hall *et al.* (2011) explored the hypothesis that this phenomenon may stem from environmental differences leading to varying demand for RNA polymerase activity - the rifampicin target enzyme. This enzyme is involved in gene transcription into RNA and mutations in the *rpoB* gene, encoding for RNA polymerase, can affect several physiological processes, including growth and metabolism (Maughan *et al.*, 2004, Murakami, 2015).

Hall *et al.* (2011) experimentally manipulated demand for RNA polymerase activity in *P. aeruginosa* and observed significant decreases in fitness costs, corresponding to increasing concentrations of ribosomal inhibitors. To distinguish whether elevated fitness costs were due to a general effect of reduced growth, the authors further explored the effects of growth inhibition on fitness either through nutrient limitation or by growth-inhibiting antibiotics, but found no consistent effect on bacterial fitness, suggesting that differences in demand for RNA polymerase activity could provide a general explanation for the environmental effect on costs for rifampicin resistance mechanisms. Arguably,

in vivo conditions represent a harsher environment, compared to *in vitro*, thus reduced need to grow fast in a live infection could moderate fitness costs.

While the effect of RNA polymerase activity on fitness was not tested here, a fitness benefit due to reduced need to grow fast *in vivo* (harsher environment, compared to broth) could at least partially explain the observed variation in costs here. Although, the elevated costs during gut infection should be treated with caution, as data are largely bimodal (see Figure 3).

Importantly, overall *in vitro* costs were not a reliable predictor for costs in naturalistic infections, and especially those in transmission.

Fitness costs in naturalistic infections. The main challenge in estimating competitive fitness in naturalistic infections was the strong bottleneck effect, or the reduced population size, particularly for oral infections with short-term bacterial exposure (gut infection assay). Where infection was present, hosts were often infected with a single genotype. A test of association revealed that neither genotype, including the wildtype, was marginally more or less successful in mono-colonising larval gut. Outside a laboratory setting, colonising microbes often experience bottlenecks during an infection (Moxon and Murphy, 1978, Caugant *et al.*, 1981, Balmer and Tanner, 2011, van Leeuwen *et al.*, 2015, Mahrt *et al.*, 2021). When a single-pathogen genotype is present within a host, direct within-host competitive ability will be less important. Instead, the relative fitness of a single-strain infection would be determined by the efficiency of between-host transmission (Lipsitch and Samore, 2002).

To determine between-host fitness costs for each genotype, here I measured the population growth for every genotype within mono-colonised hosts; the rate

at which mono-colonised hosts shed infectious propagules through frass and the competitive ability between resistant and wildtype genotypes during direct transmission to microbe-free hosts. I found no significant differences in shedding rate during single-strain infections *in vivo* when controlling for larval size. This trend was also observed for the densities of genotypes shed through frass by mono-colonised hosts. Thus, it can be assumed that there was an equal opportunity for each competing genotype to be transmitted to secondary (microbe-free) hosts. Despite this, fitness costs were present for every resistant genotype during direct transmission, when single-strain (resistant genotype) host carriers were mixed at equal numbers with host carriers infected with the wildtype strain and introduced to a secondary population of bacteria-free hosts. This finding suggests that the fitness costs of the three mutant genotypes in this study are increased during infection and colonization, rather than through the rate of production of infectious material.

While growth rate and competitive ability in mixed infections are typical measures of fitness *in vitro*, there are other components within a live infection, such as total population size and shedding rate of infectious material, which will further impact the ability of resistant pathogens to persist and disseminate (Lipsitch and Samore, 2002). Furthermore, while reduced competitive ability in resistant mutants is an important parameter in mixed-strain infections, it may not necessarily reflect their transmission potential, especially where hosts are colonised with a single genotype. Under this scenario, selection dynamics between hosts will be more important, as within-host selection will depend on pathogen diversification post-infection (Lipsitch and Samore, 2002, Manktelow *et al.*, 2020). Encouragingly, here I show that fitness costs were present within hosts as well as between hosts for every mechanism of resistance tested

relative to the wildtype despite finding no differences in the rates of population growth and shedding from single-strain infected hosts. As long as there are costs present, it can be reasonable to expect that restrictions on antibiotic use and/or cycling strategies may at least slow the rate of dissemination of resistant genotypes (Levin, 2001).

Chapter 2: Social interactions and the collective detoxification of β-lactam antibiotics

Introduction

The most fundamental aim of evolutionary biologists in the study of antibiotic resistance is to be able to understand when the frequency of resistance is going to rise and fall. This is easy to state but challenging to achieve, given the diversity of drugs, resistance mechanisms, and environmental contexts. For example, the evolutionary trajectories of resistance genotypes show variations in the absence of selection - some decline rapidly, while others remain stable, or increase in frequency (Lipsitch, 2001, Enne *et al.*, 2005, Whittles *et al.*, 2017). On the other hand, susceptible genotypes may remain present even under intense selection for resistance (Colijn *et al.*, 2010). Understanding what drives this variation in the evolutionary responses of bacterial lineages in the presence and absence of antibiotics has important implications for antimicrobial stewardship (Andersson and Hughes, 2011, Raymond, 2019).

Some theoretical models predict that, according to the intensity of selection pressure, competitive exclusion should occur (Colijn *et al.*, 2010), resulting in either resistant or susceptible lineages becoming extinct. Yet, empirical data suggests that co-existence of both resistant and susceptible genotypes is common (Goossens *et al.*, 2005, Simonsen, 2018, Núñez-Núñez *et al.*, 2018). For example, the prevalence of some resistant pathogens, such as *Staphylococcus aureus* resistant to penicillin, has been maintained at nearly 100%, while others, such as vancomycin-resistant *Enteroccocus* species or *E. coli* resistant to sulphonamides, have remained at intermediate frequencies for

long periods (Enne *et al.*, 2001, McDonald, 2006). For some groups, for example *S. pneumoniae,* a weak response to selection may have arisen through a fundamental trade-offs between resistance and the duration of infection (Lehtinen *et al.*, 2017).

Near extinction or near fixation can be explained by high fitness costs or advantages, respectively, subject to mutation-selection balance (Colijn *et al.*, 2010). This means that despite high-cost resistance mutations being selected against in the absence of selection, these may still occur at random due to spontaneous mutations or gene acquisition (Cohen and Murray, 2004). Under the same principle, due to random events (genetic drift) favourable mutations may also occasionally become lost (Vilhelmsson *et al.*, 2000). In addition, migration-selection balance may also influence antibiotic resistance evolution (Cohen and Murray, 2004). For instance, the prevalence of resistance frequencies in a hospital setting may be ‘diluted’ by a continuous influx of sensitive strains through patient turnover (Lipsitch *et al.*, 2000). This effect may also occur within hosts (Colijn *et al.*, 2009). For example, the prevalence of resistance-carrying commensals post-antibiotic treatment may be offset through the colonisation of susceptible variants through the intake of food, water, via social contacts, or other sources (Levin *et al.*, 1997, Wade, 2013). Moreover, Davies *et al.* (2019) propose that within-host social dynamics shape the evolution of resistance and enable the co-existence of both resistant and susceptible strains of non-obligate pathogens via negative-frequency-dependent selection.

Experimentally it has been repeatedly shown that the evolution of resistance can be influenced by group-beneficial characteristics, driven by frequency-

dependent selection (Dugatkin *et al.*, 2005). In contrast to simple competitive exclusion (Hardin, 1960), which might occur when mechanisms of resistance are private, when resistance mechanisms are cooperative social interactions between microbes will determine levels of co-existence (Vega and Gore, 2014). For example, the secretion of antibiotic de-activating enzymes, such as *β*-lactamases, reduces the effective drug concentration for the entire community, not solely for conspecifics (Brook, 2009, Murray *et al.*, 2018, Klümper *et al.*, 2019). This group-level protective effect allows non-resistant cells to act as 'cheats' and attain frequency-dependent gains (Dugatkin *et al.*, 2005, Ellis *et al.*, 2007, Amanatidou *et al.*, 2019, Dimitriu *et al.*, 2019), ultimately preventing the fixation of resistance through increased competition under high initial antibiotic doses.

Evidence for sociality has previously been observed in engineered strains of *E. coli* in well-mixed liquid cultures *in vitro,* where cooperative *β*-lactam detoxification is efficient and allows the survival of susceptible genotypes, albeit over a narrow range of antibiotic concentrations (Dugatkin *et al.*, 2005, Perlin *et al.*, 2009, Amanatidou *et al.*, 2019). However, in simple structured environments (agar plates), the action of *β*-lactams is rapid and bactericidal and only a tiny proportion of susceptible bacteria (the viable persisters) can benefit from detoxification by resistant microbes (Medaney *et al.*, 2016). The bactericidal action of *β*-lactams is dependent on active growth (Tuomanen *et al.*, 1986), and subsequent work showed that social protection of susceptible bacteria was more prevalent in biofilms than on solid media where a high proportion of cells are not actively growing, even if they are not strictly persisters (Amanatidou *et al.*, 2019).

Overall, spatial structure, physiology, and environmental conditions can all affect whether or not social detoxification is a significant process in controlled experiments (Medaney *et al.*, 2016, Amanatidou *et al.*, 2019). This begs the question of whether or not social detoxification of *β*-lactam antibiotics actually occurs in live infections, and in turn, whether this phenomenon can explain the continued co-existence of resistant and susceptible cells under intense selection pressure from diverse *β*-lactam antibiotics.

In this study, I investigated the demographic and environmental conditions under which constitutively expressed AmpC *β*-lactamases produced a shared social benefit of detoxification in mixed genotype cultures. This mode of resistance is typical of clinical *E. cloacae* and widespread in the *Enterobacteriacae* (Kuga *et al.*, 2000, Kaneko *et al.*, 2005, Jacoby, 2009) and imposes small fitness costs *in vivo* (see Chapter 1). AmpC *β*-lactamases along with extended-spectrum *β*-lactamases (ESBLs), are the two main types of resistance mechanisms to cephalosporins, including third-generation cephalosporins (Meini *et al.*, 2018). Yet, despite the importance of these enzymes, AmpC *β*-lactamases receive disproportionally little attention.

Here, I examined the capacity of cooperative clearance by spontaneous *β*-lactam-resistant *E. cloacae* (CEF) in the presence of cefotaxime - a highly active third-generation cephalosporin, recognised as a critically important drug, categorised as ‘highest priority’ by the World Health Organisation (WHO, 2019). Social protection was assessed in broth, in biofilms *in vitro*, and in a natural system using the larvae of the diamondback moth, *P. xylostella*. I measured the competitive fitness of the susceptible strain in the presence of the resistant genotype (CEF) over a range of concentrations and with varying genotype frequencies of the initial population. I tested the hypothesis that a natural mixed

gut infection should facilitate social protection of the wildtype over a wider concentration range of antibiotics, similar to previous experiments with biofilms *in vitro* (Amanatidou *et al.*, 2019).

Methods

Bacterial strains

The susceptible *E. cloacae* ancestor strain (WT) and the cefotaxime-resistant mutant (CEF), previously characterised (see Chapter 1), were used for experiments in this chapter.

Staring cultures were prepared in 5 ml LB broth, containing selective antibiotic (4 µg/ml cefotaxime) for the resistant mutant and no selective agents for the wildtype.

Starter cultures

Initial inocula (starter cultures) were prepared by mixing diluted (1000-fold) cultures, as appropriate to give proportions of 1:9, 1:1, and 9:1 (susceptible to resistant strains), the susceptible strain was also included as a single strain to serve as a control. Initial and final mixtures were plated on both selective (4 µg/ml cefotaxime) and non-selective media and incubated overnight at 30°C to obtain cell densities.

Antibiotic-dose clearance *in vivo*

P. xylostella eggs were surface-sterilised as previously described (Chapter 1) and incubated for 48 hours at 25°C. Post incubation, the eggs were transferred to wheatgerm diet inoculated with 150 µl of the wildtype (susceptible ancestor) strain at a 1000-fold dilution from an overnight culture in 5 ml LB. In addition,

150 µl of 0.85% saline was used as a control. Diet was allowed to dry in a Class 2 safety cabinet for 45 minutes before adding *P. xylostella* eggs (around 100 eggs per plate). Plates containing inoculated diet and eggs were left at 25°C. After 24 hours, when a sufficient number of nymphs had emerged, the remaining eggs were removed. Larvae were returned at 25°C for another 48 hours. At hour 72 from initial infection, single larvae were transferred to individual chambers containing freshly prepared wheatgerm diet supplemented with either no antibiotic, 0.25 µg/ml, 1 µg/ml, 4 µg/ml, 16 µg/ml, 64 µg/ml, 256 µg/ml, or 1024 µg/ml cefotaxime. Uninfected larvae were transferred on 0 µg/ml cefotaxime to ensure larval sterility. Infected, but untreated larvae were also included as a positive control. Larvae were left to feed individually at each dose for a total of 4 days when single larvae were homogenised in 500 µl 0.85% saline and plated on both selective and non-selective media to obtain final counts and check for spontaneous resistance emergence. Each treatment (dose) included 16 independent replicates.

To visualise gut infections in *P. xylostella*, 4th instar larvae carrying WT infection were dissected. Larval guts were teased out using fine entomology pins, and fixed in methanol for 10 minutes. Giemsa's staining solution (20%) (Fisher Scientific, 1992) was applied for 30 minutes and rinsed in phosphate-buffered saline. Microscopy image was taken using 60x magnification, under bright field illumination with an Olympus BX61. Uninfected larvae were also stained as a negative control.

Sociality in broth (*in vitro*)

Starter cultures (mixes and WT only) were inoculated (10 µl) to 1 ml LB broth containing cefotaxime at the following concentrations: 0 µg/ml (control), 0.0625

µg/ml, 0.25 µg/ml, 1 µg/ml, or 4 µg/ml. This experiment was replicated six times per starter culture and antibiotic dose. Plates were sealed and left to incubate for 24 hours at 30°C, 180 rpm. Post incubation, final cell densities were obtained, as described above.

Sociality in biofilms

Experiments used a membrane-grown biofilm system described in previous studies of social detoxification (Amanatidou *et al.*, 2019). Polycarbonate filter membranes (Whatman Nucleopore Track-Etch Membranes) with pore size 0.2 µm and 25 mm diameter were placed on non-selective LB agar plates and inoculated with 10 µl of each starter culture. Biofilms were left to grow at 30°C for 24h. Post incubation, biofilms were transferred to LB agar plates containing cefotaxime at the following concentrations: 0 µg/ml (control), 1 µg/ml, 1000 µg/ml, 3000 µg/ml and incubated at 30°C for another 24 hours. Biofilms were transferred daily to fresh plates for three days. At this stage, biofilms were disrupted in 10 ml 0.85% saline and vortexed until no traces were visible on the membrane. Final cell densities were obtained, as described above.

Sociality in *P. xylostella* hosts

Eggs were sterilised as previously described (Chapter 1) and incubated at 25°C for 48 hours. Post incubation, eggs were transferred to sterile non-selective wheatgerm diet prepared by covering the surface of each diet block with 150 µl of a starter culture: either the susceptible ancestor (WT), a culture mixture at the appropriate proportions, or 0.85% saline (control). The diet was allowed to dry for 45 minutes in a Class 2 safety cabinet before transferring eggs. Petri dishes were sealed with parafilm, and larvae left to feed on initial cultures at 25°C for a

total of 72 hours. Parafilm lining, along with any remaining unhatched eggs were removed from the Petri dishes after 24 hours.

Post completing 72 hours of incubation, infected larvae were transferred to bio-assay trays, containing sterile wheatgerm diet, containing cefotaxime at the following concentrations: 0 µg/ml (control), 1 µg/ml, 1000 µg/ml. Larvae (48 replicates per culture type and antibiotic dose) were placed in individual wells, where they were left to feed for four days when each larva was surface sterilised in 70% ethanol and homogenised in a bead beater (Qiagen TissueLyser II) at 30Hz per second for 4 minutes in 500 µl (0.85%) saline and a lysing ball. Final cell densities were obtained, as described above.

Statistical analysis

Initial and final cell densities were calculated from colony counts on LB agar (total counts) and resistant colony counts on agar plates, supplemented with 4 µg/ml cefotaxime. When the count of resistant colonies exceeded the total colony count, wildtype cells were assumed to be extinct. Fitness here was calculated in terms of selection rate constant (*r*), or the difference of the Malthusian parameters, as opposed to the ratio (W). This change was necessary due to declines in abundance of both competitors as a result of the antibiotic challenge (Lenski, 1991, Travisano and Lenski, 1996). Initial densities for biofilms were calculated from the starter biofilm culture before allowing the biofilm to grow on non-selective media. As the initial infection bottleneck in insects is assumed to be around 50 cells (van Leeuwen *et al.*, 2015, Zhou *et al.*, 2020), the proportions of susceptible and resistant strains were adjusted based on the proportions of the starter cultures.

The number of generations of susceptible bacteria were calculated as [$\log_{10}$(number of final cell densities) - $\log_{10}$(number of initial cell densities)]/$\log_2$. Log_{10} transformed doses were fitted as covariates in statistical models, unless dose responses were clearly non-linear, in which case dose was fitted as factor. Initial proportions of susceptible cells were fitted as covariates.

Statistical tests used generalised linear models in R v1.4.1106 (R Core Team, 2021). Sequential removal of non-significant terms was used to produce minimal adequate model and graphical analysis of residuals was used to test assumptions of normality and homoscedasticity (Crawley, 2012).

Results

Antibiotic clearance *in vivo*

This experiment aimed to establish the appropriate oral dose of antibiotics that would either clear or suppress *E. cloacae* infections *in vivo,* in order to establish meaningful experimental doses. After treating individually housed infected *P. xylostella* larvae through diet supplemented with a wide range of cefotaxime concentrations (0 – 1024 µg/ml), a small number of gut infections remained viable at each antibiotic concentration. Despite this, suppression of gut commensal infections significantly increased with dose ($F_{7,248}$ = 35.06, P > 0.001, Figure 5).

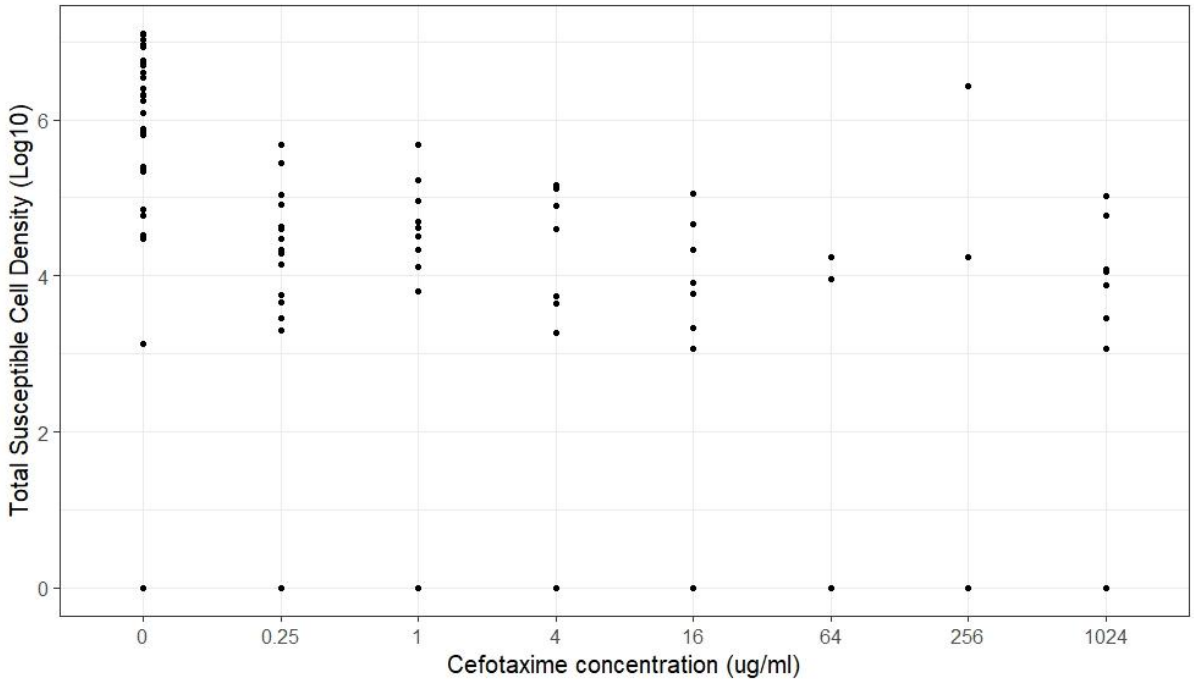


Figure 5. Total susceptible cell density recovered from larvae fed on artificial diet containing a range of antibiotic concentrations. Each dot represents an independent replicate.

Planktonic cells as well as bacterial aggregates were visible in dissected *P. xylostella* larvae guts, infected with WT, as shown in the image below.

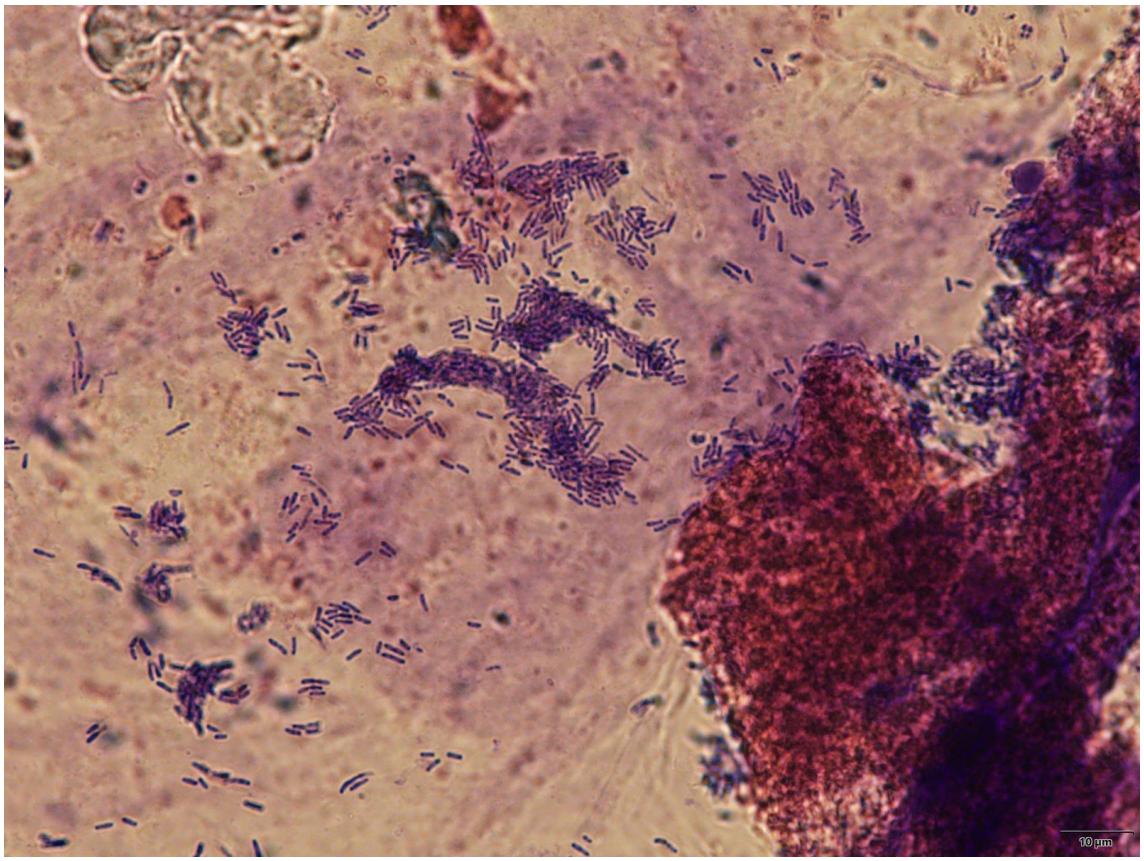


Figure 6. Microscopy image of dissected *P. xylostella* larvae gut, infected with wildtype *Enterobacter cloacae*. Bacterial rods are stained in purple. Microscopy slide was prepared and photographed by Prof. Ben Raymond.

Sociality experiments

Bacterial cultures, either as a single strain (WT) or mixed cultures (WT & CEF), were challenged with a range of cefotaxime concentrations in a planktonic state (*in vitro*), as biofilms, or within a live infection (*P. xylostella*). Control treatments with no antibiotic challenge were included at every stage. Obtained were the initial and final cell densities, either directly (broth), or indirectly (biofilms & live infection), allowing the estimation of numbers of generations of the susceptible strain WT, as well as the selection rate constant (r).

Sociality in broth

There are two ways of testing for the presence of social interactions in these experiments. First, can we see evidence of cross-protection of susceptible cells by resistant genotypes, as evidenced by survival or growth of susceptible cells in mixed cultures at doses that would normally preclude survival? There was clear evidence of cross-protection in that the susceptible *E. cloacae* strain was able to survive and grow at the MIC dose (cefotaxime 0.25 µg/ml) and above when the resistant mutant CEF was present. In addition, the susceptible strain grew better overall when the initial proportion of resistant cells was most abundant (frequency treatment, $F_{1,79} = 9.90$, $P = 0.002$). There was a clear effect of cefotaxime concentration on susceptible cell replication ($F_{1, 80} = 21.71$, $P > 0.001$) but no significant dose x frequency interaction ($F_{1,78} = 0.13$, $P = 0.72$).

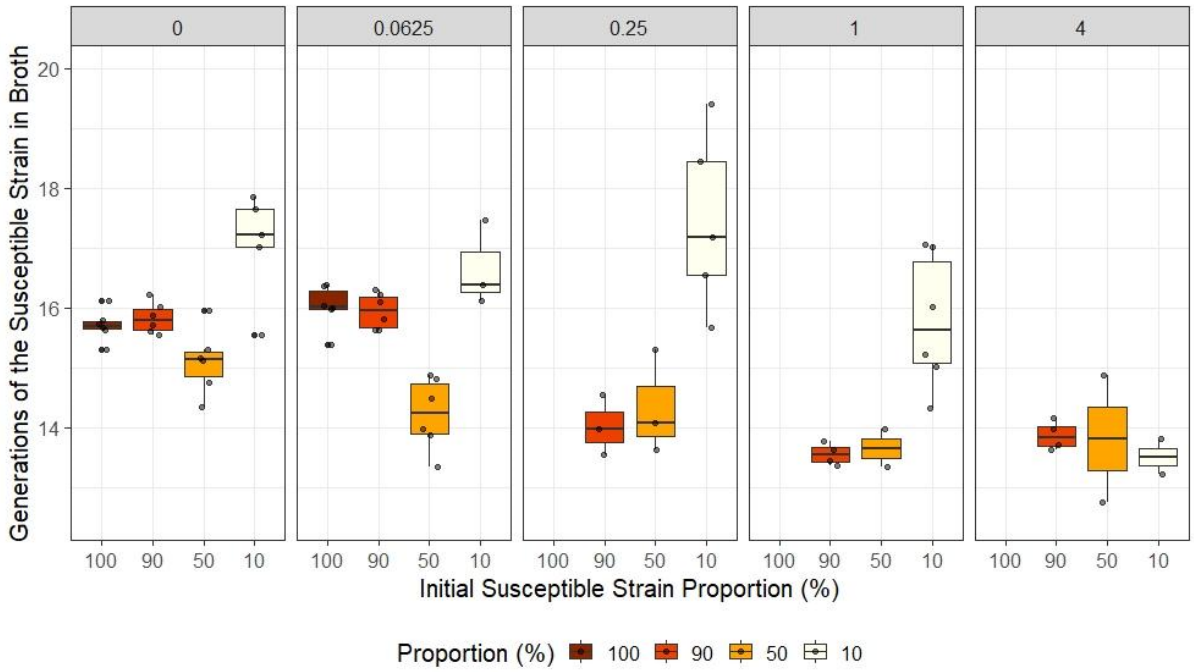


Figure 7. Mean susceptible *E. cloacae* (WT) generations, cultured in broth supplemented with either 0 µg/ml (control), 0.0625 µg/ml, 0.25 µg/ml, 1 µg/ml, or 4 µg/ml cefotaxime, with varying initial proportions of the susceptible strain:

either 100%, or co-cultures with the cefotaxime-resistant mutant strain CEF at initial proportions of the susceptible at 90%, 50%, and 10%.

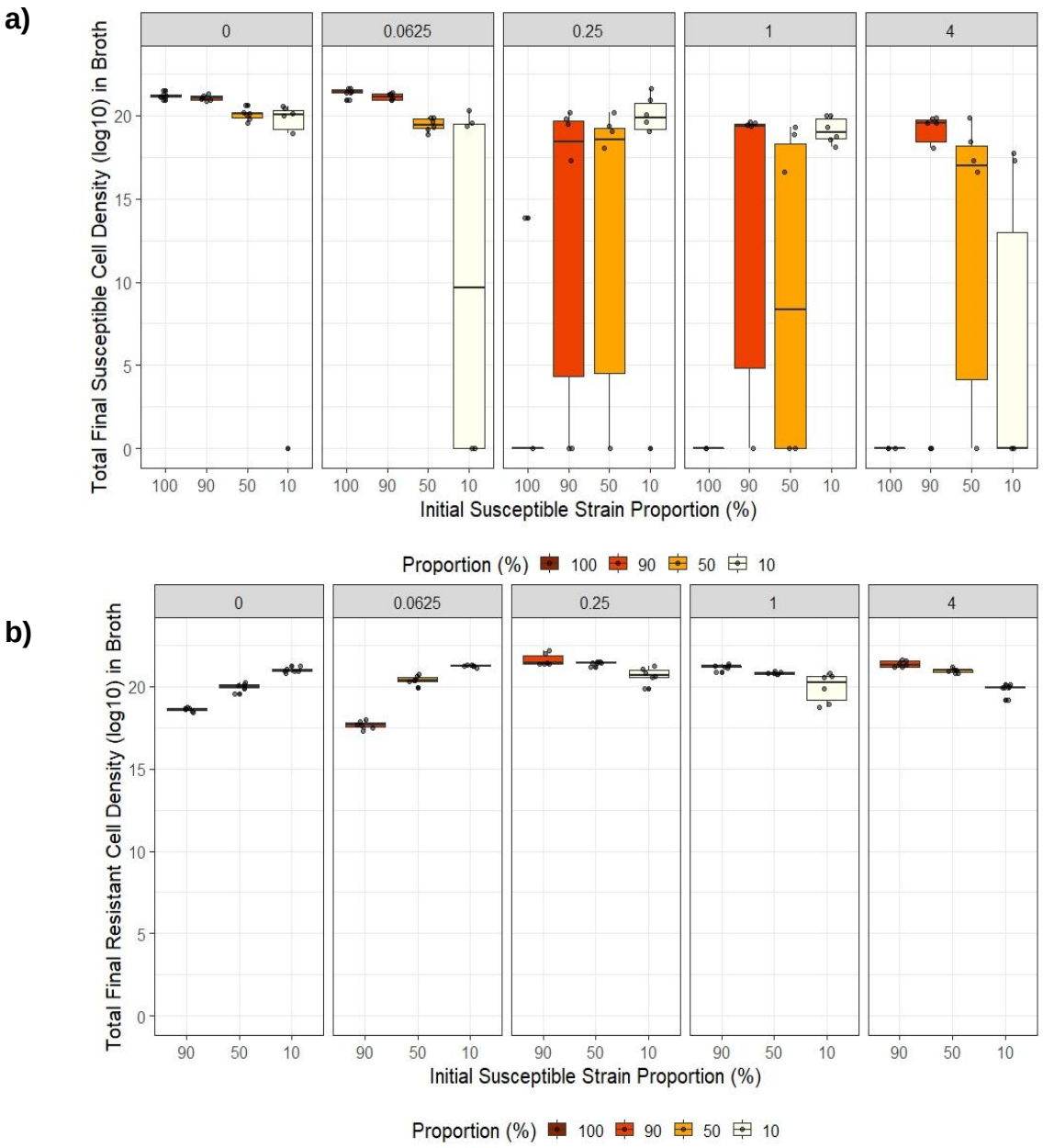


Figure 8. Total final cell density of the susceptible genotype (**a)**, and the resistant genotype (**b)** across antibiotic concentrations (0-4 µg/ml), and at varying initial population proportions.

The second test of sociality is whether or not there is evidence of frequency-dependent selection on the susceptible cells. If resistant cells are providing a public good in terms of detoxifying growth medium then the fitness of susceptible cells should increase as the proportion of cells providing this public

good increases. These experiments found frequency-dependent fitness (expressed as selection rate constant, r), as predicted but also that frequency-dependent fitness changed with dose (dose x frequency interaction $F_{1, 65} = 5.94$, $P = 0.018$). This is important as the difference in fitness between frequency treatments typically increased at doses above MIC.

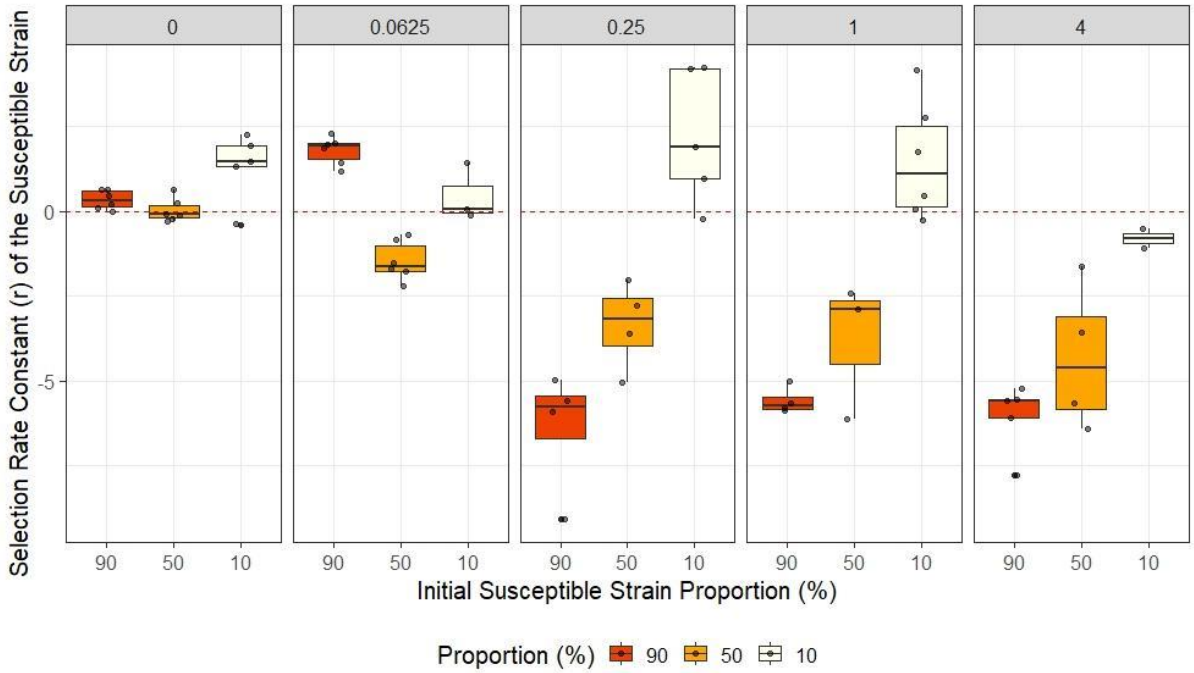


Figure 9. Selection rate constant (r) of the susceptible strain *E. cloacae* (WT) in broth, competed in mixed cultures with the cefotaxime-resistant mutant strain CEF at initial proportions of the susceptible strain of 90%, 50%, 10% in broth supplemented with either 0 µg/ml (control), 0.0625 µg/ml, 0.25 µg/ml, 1 µg/ml, or 4 µg/ml cefotaxime. Dashed line at zero indicates when resistant and susceptible cells have the same fitness.

Sociality in biofilms

The overall doublings of susceptible cells in biofilms was found to be dependent on the antibiotic dose ($F_{1,102} = 29.32$, $P > 0.001$), and initial proportion of susceptible bacteria ($F_{1, 101}=5.02$, P = 0.027), with no dose x frequency interaction ($F_{1,100} = 2.11$, $P = 0.149$). Note that all the numbers of generations

in these figures are positive as we measured growth from initial plate inoculation to biofilm maturation and through to subsequent antibiotic challenge. Population sizes in antibiotic-treated biofilms were 2-3 orders of magnitude lower when compared to controls indicating slow death of bacteria in antibiotic-treated biofilms. However, there was also considerable variation in mortality rates of all cells between experimental replicates, and this produced much greater variation than seen in broth data. Total death of both susceptible cells and high mortality of resistant cells in some replicates at 1000 µg/ml, or 3000 µg/ml cefotaxime indicates the resistance mechanism in the CEF mutant was not fully effective at these concentrations.

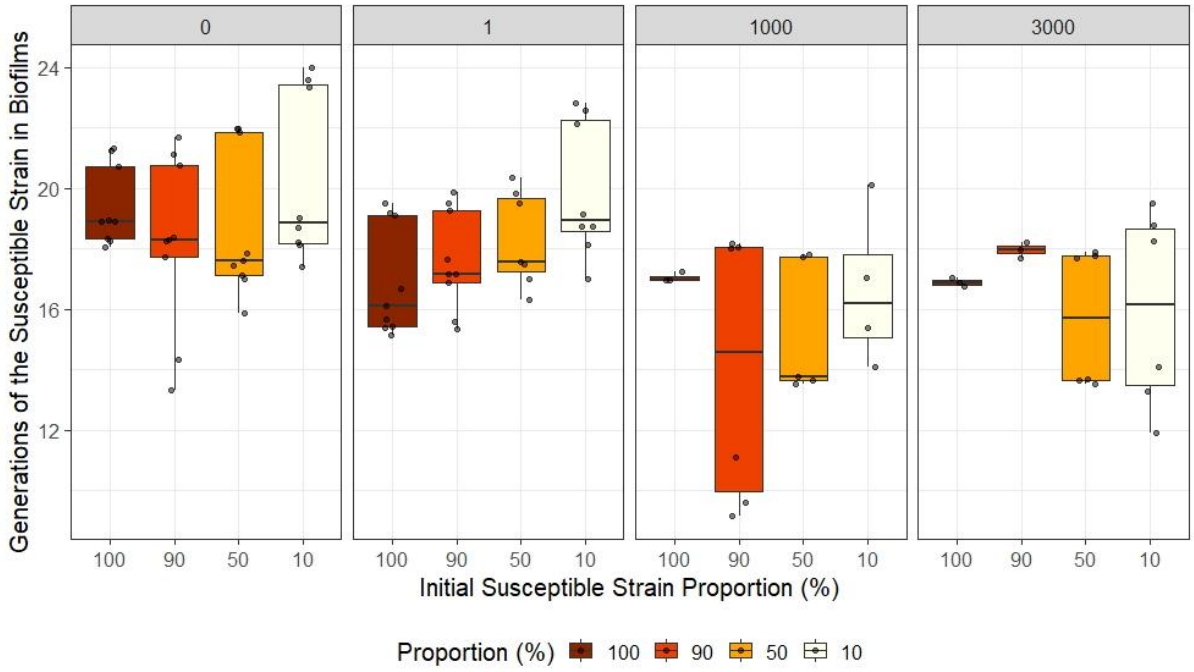


Figure 10. Mean susceptible E. cloacae generations (WT), cultured in biofilms and challenged with either 0 µg/ml (control), 1 µg/ml, 1000 µg/ml, or 3000 µg/ml. Biofilms were established with either 100% susceptible cells, or in mixed cultures with the cefotaxime-resistant mutant strain (CEF) at initial wild type proportions of 90%, 50%, 10%, supplemented with fresh media every 24 hours for a total of 4 days.

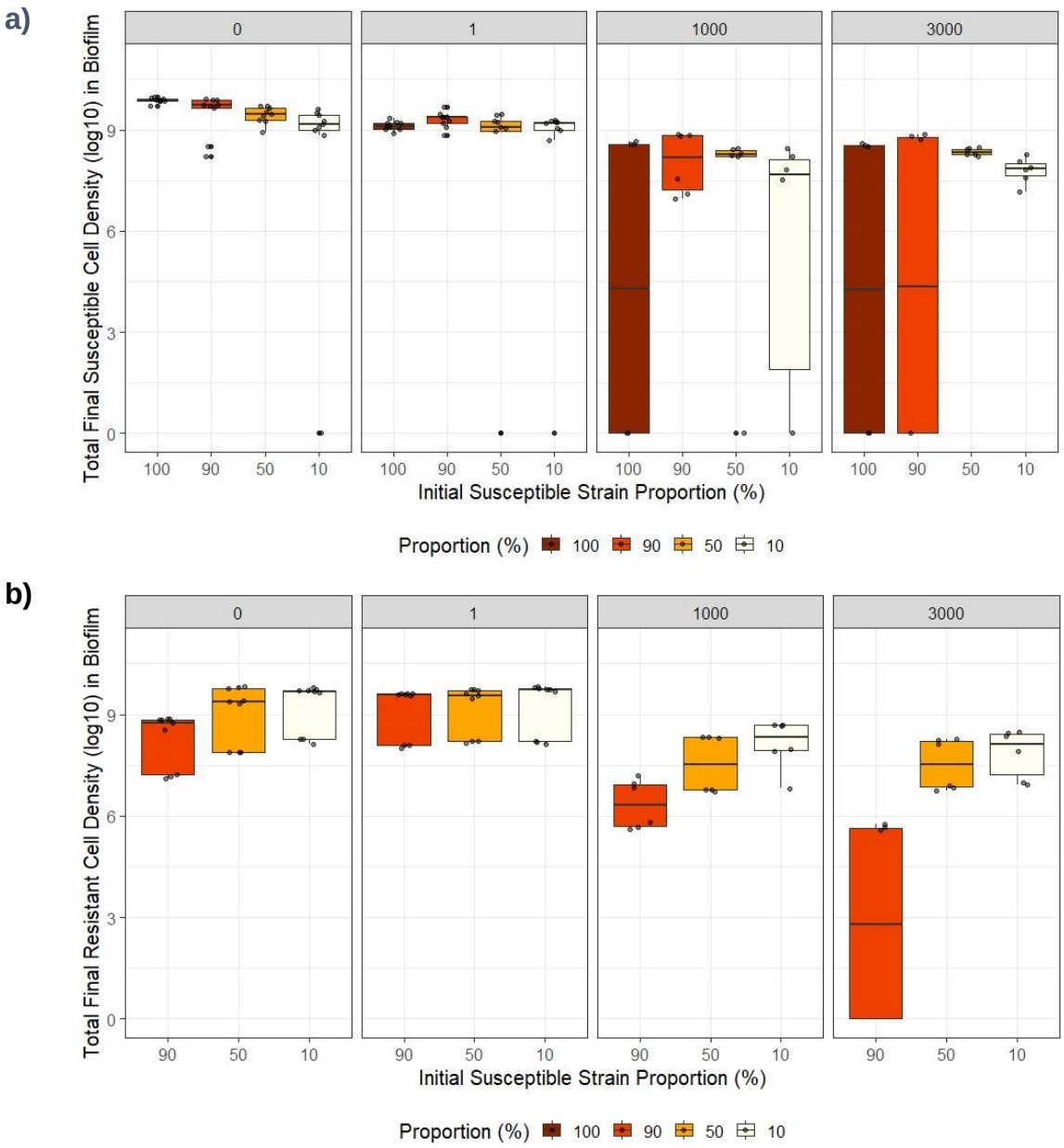


Figure 11. Total final cell density of the susceptible genotype (**a**), and the resistant genotype (**b**) cultured as biofilms across antibiotic concentrations (0-3000 µg/ml), and at varying initial population proportions.

In biofilms, the pattern of increasing susceptible fitness at low frequencies was seen only when cultures were challenged with 1 µg/ml cefotaxime (see Figure 12). However, susceptible cells tended to have higher fitness than resistant cells at high doses across all frequencies, suggesting more prevalent conditions for social exploitation (possible cheating) at these doses. Selection rate (r) in biofilms was also affected by the dose x initial proportion interaction ($F_{1,76}$ =

6.62, P = 0.012, Figure 12). After removing the interaction, *r* increased with dose ($F_{1, 78}$ = 6.35, P = 0.014) but was not dependent on initial proportion ($F_{1,77}$ = 3.27, P = 0.074).

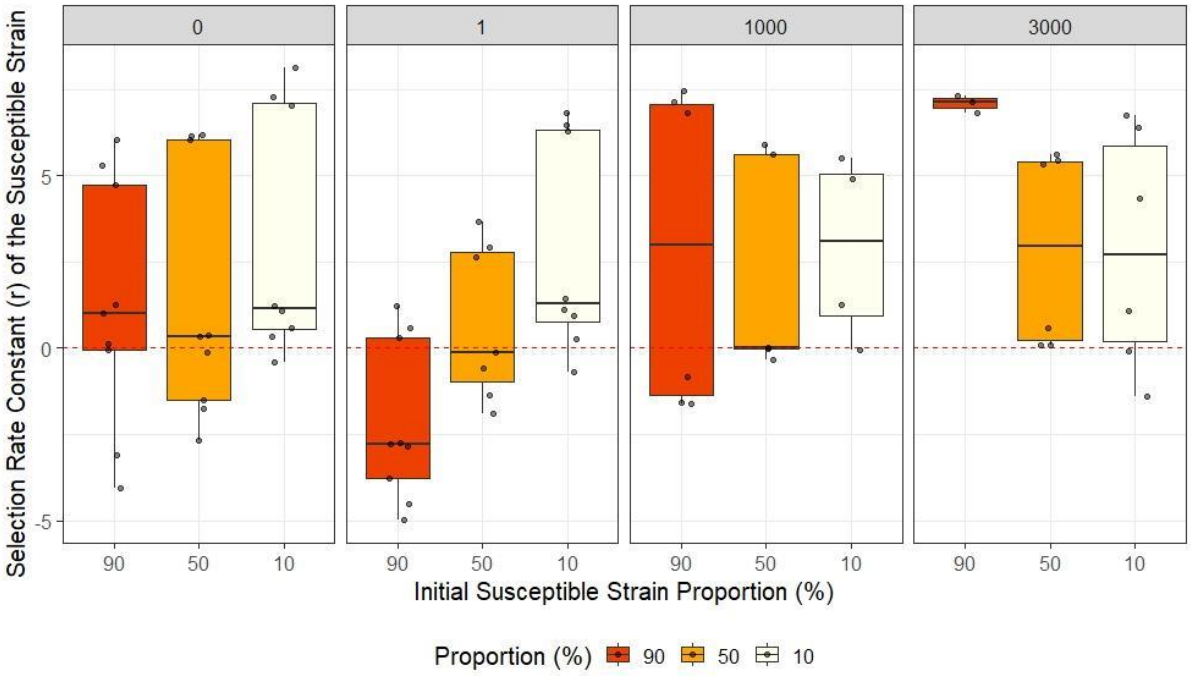


Figure 12. Selection rate constant (r) of the susceptible cells in mixed biofilms, established from varying initial culture proportions (90%, 50% and 10%) of the susceptible (WT) strain. Biofilms were established on non-selective media, then transferred onto fresh media, supplemented with either 0 µg/ml (control), 1 µg/ml, 1000 µg/ml, or 3000 µg/ml cefotaxime for three days.

Sociality *in vivo*

Susceptible cells cultured *in vivo* (*P. xylostella* larvae) grew better in mixed cultures with a high frequency of resistant bacteria, and showed stronger frequency effects as dose increased ($F_{1, 172}$ = 8.52, *P* = 0.004, Figure 15). Few susceptible cells and no resistant cells were recovered from larvae fed on diet supplemented with 1000 µg/ml cefotaxime, where susceptibles were most abundant. However, a small number of susceptible infections persisted at this high dose despite being established from mono-cultures.

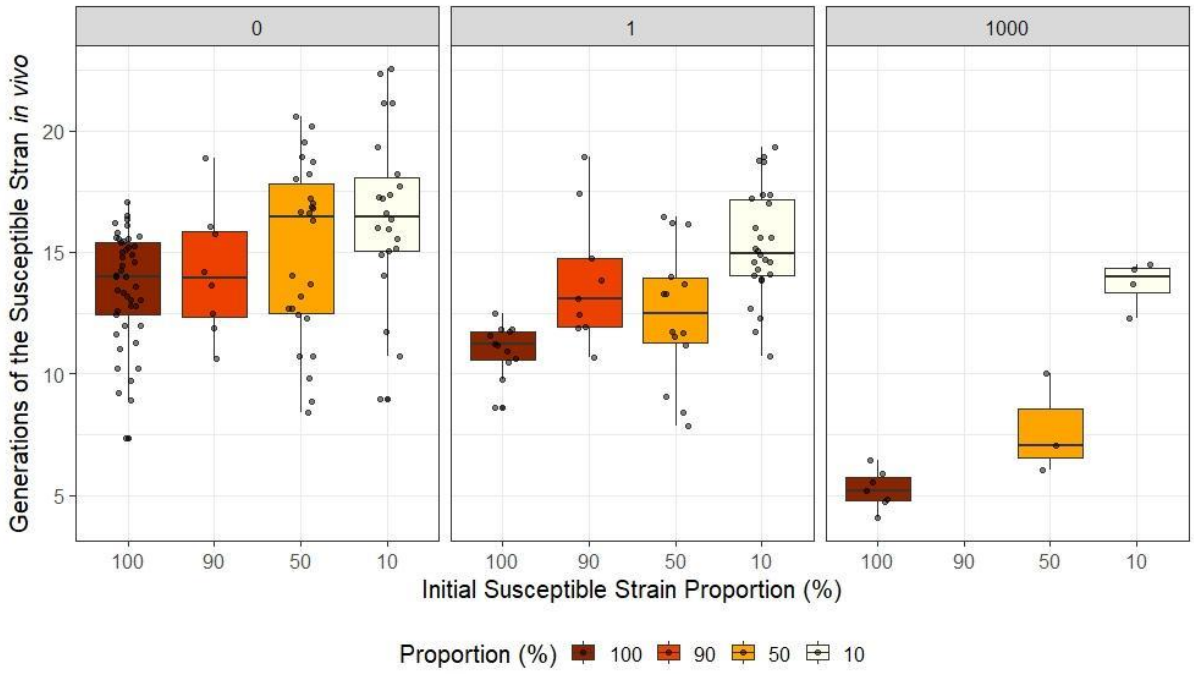
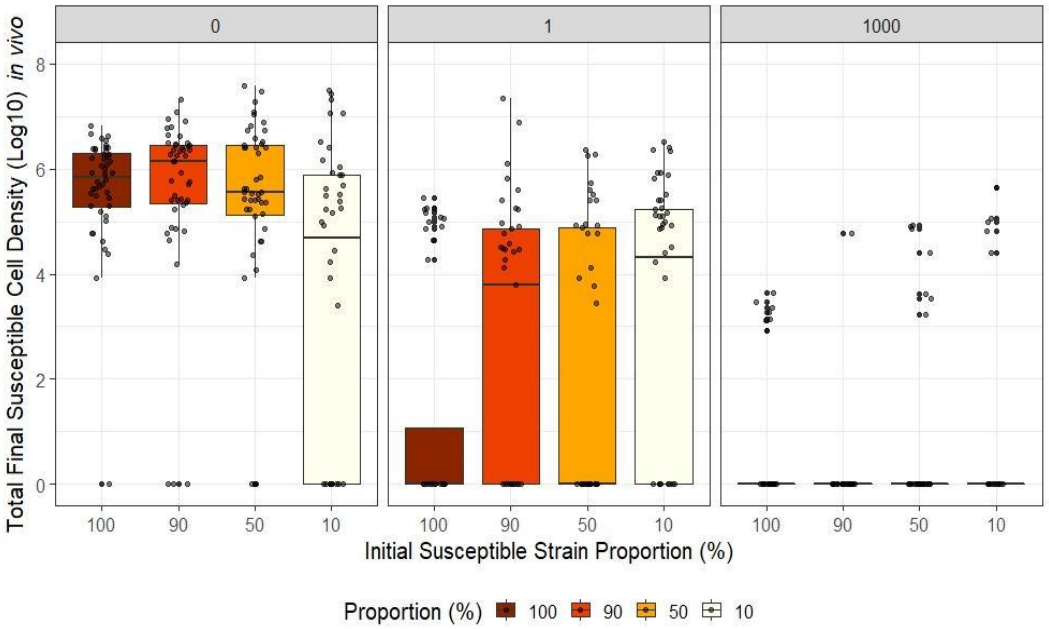


Figure 13. Mean susceptible *E. cloacae* (WT) generations, cultured within a live infection (*P. xylostella*), where the initial gut infection was established by either the susceptible strain (WT) (100%), or mixed cultures with the cefotaxime-resistant mutant strain CEF introduced at initial proportions of 90%; 50%; 10% of the susceptible strain. Bacteria were then challenged through the larvae diet, supplemented with 0 µg/ml (control), 1 µg/ml, or 1000 µg/ml cefotaxime for a total of 4 days.

a)



b)

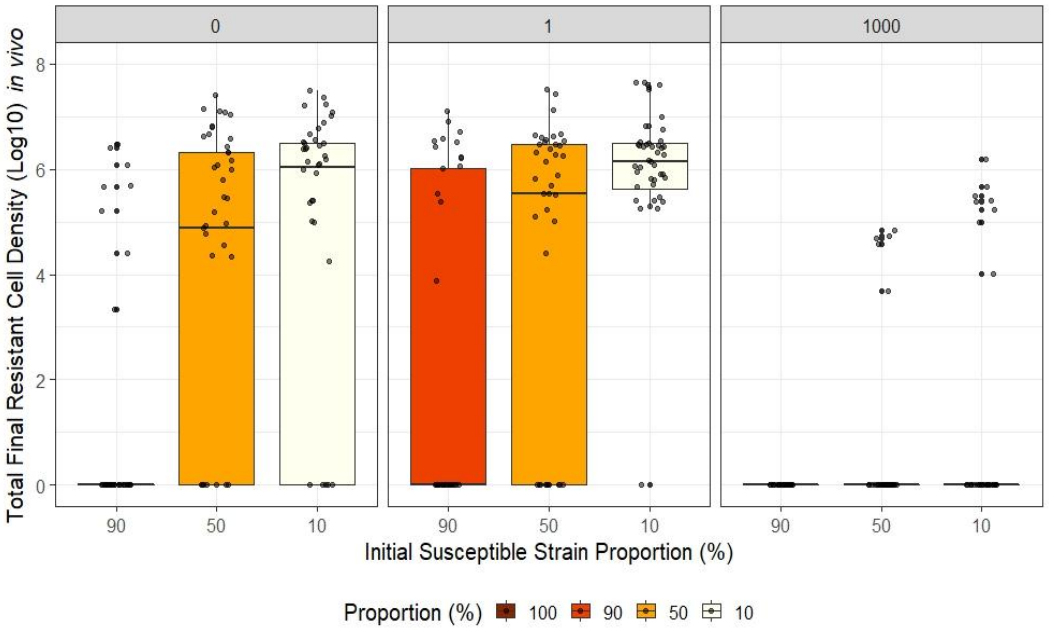


Figure 14. Total final cell density of the susceptible genotype (**a**), and the resistant renotype (**b**) recovered from *in vivo* infections (*P. xylostella*) treated with cefotacime at 0 µg/ml, 1 µg/ml and 1000 µg/ml, and at varying initial population proportions.

In contrast, the rate of selection of the susceptible strain *in vivo* was strongly dependent by the initial population proportion ($F_{1,108} = 24.04$, $P > 0.001$), but no effect was found of dose ($F_{1,108} = 1.32$, $P > 0.253$), or the interaction between

the two variables ($F_{1,107} = 2.34$, $P > 0.129$). In controls without antibiotics, and at high frequencies of resistant cells the median fitness of susceptible cells was very close to 0, i.e. equal fitness to resistant bacteria (Figure 15).

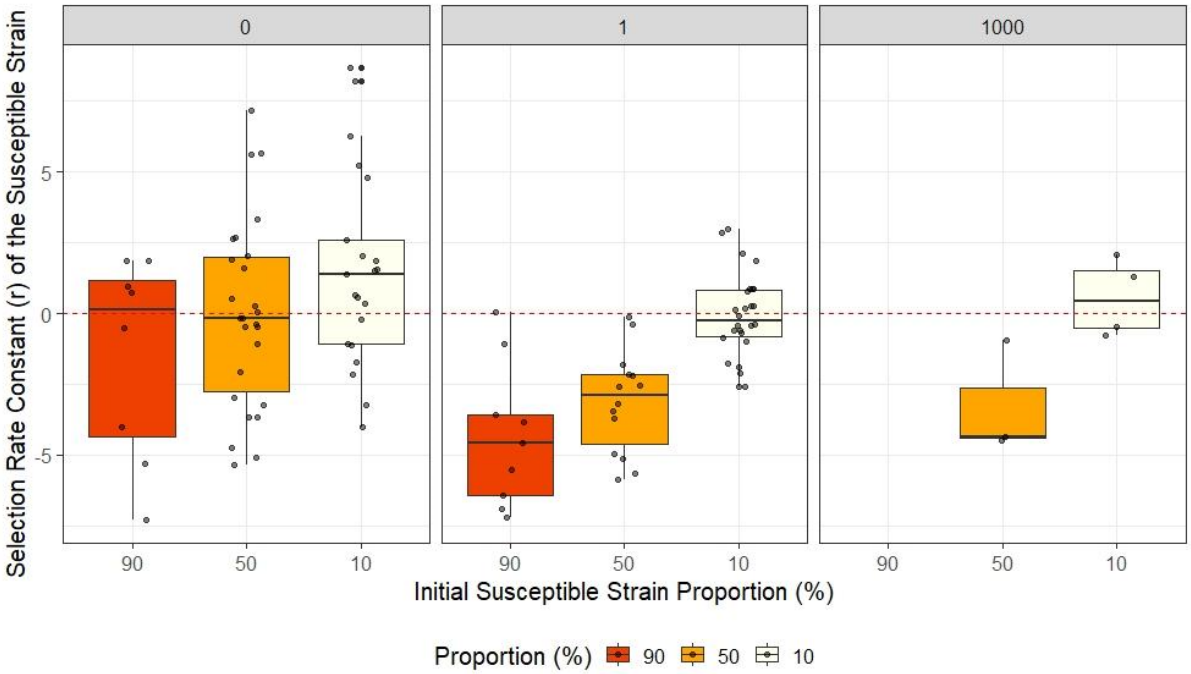


Figure 15. Selection rate constant (r) of the susceptible strain *in vivo* (*P. xylostella*), established from varying initial culture proportions with to the resistant mutant CEF. Gut infections were challenged by introducing diet supplemented with either 0 µg/ml (control), 1 µg/ml, or 1000 µg/ml cefotaxime for a total of 4 days.

Discussion

Understanding how the frequency of resistant and susceptible lineages responds to the presence and absence of selection across diverse ecological contexts has important implications for antimicrobial stewardship. Previous work on social dynamics of antibiotic resistance focused on engineered plasmid-carrying *E. coli* strains (Dugatkin *et al.*, 2005, Yurtsev *et al.*, 2013, Medaney *et al.*, 2016, Amanatidou *et al.*, 2019). Here, I explored the sociality of a spontaneous *E. cloacae* mutant, constitutively expressing AmpC-*β*-lactamases at low cost. The findings presented here suggest strong frequency-dependent selection in the presence of the resistant strain *in vitro*, which decreased at higher antibiotic doses, indicative of social cefotaxime detoxification. There is some evidence of frequency-dependent selection in biofilms as well as *in vivo*, however the effects of frequency and dose were less clear, particularly at the higher doses.

Physiology had an important role in the way bacteria responded to cefotaxime, despite the bactericidal effect of this cephalosporin (Olsen, 2015, Yan and Bassler, 2019). As shown above, susceptible cells in a planktonic state were not able to grow in monocultures at or beyond their MIC (0.25 µg/ml). In contrast, established biofilms formed by susceptible cells from the same starter culture were able to survive exposure to antibiotic concentrations as high as 3000 µg/ml challenged over the course of 4 days, albeit not consistently between experimental replicates. This finding reiterates the resilience but also variability in biofilms responding to environmental stressors, including to the toxicity of antibiotics (Davey and O'toole, 2000, Ciofu *et al.*, 2022). This is important, as outside an experimental setting, as much as 80% of bacterial growth is

estimated to occur within biofilms (Flemming and Wuertz, 2019), including over 60% of nosocomial infections (Davey and O'toole, 2000, Fux *et al.*, 2003, Silva *et al.*, 2022).

The biofilm experimental conditions presented a number of challenges in this experimental work. Resistant cells also suffered substantial mortality in the biofilm set-up at high doses, which could preclude effective detoxification. Experimental repeats gave quite distinctive results in terms of whether or not all the cells in biofilms died, suggesting that subtle differences in bacterial physiology at the start of experiments have implications for susceptibility later on. A wide range of options for biofilms exists (Amanatidou, 2017). In general, clearer results from a biofilm context might require a substantial investment of time in order to optimise experimental conditions. Despite these limitations, the trends in the biofilm experiment is at least in broad agreement with the other two experiments suggesting this could be a fruitful area of future work.

Similar to biofilms *in vitro*, a proportion of susceptible cells *in vivo* remained viable within gut infections after larvae were fed on a diet supplemented with 1000 µg/ml cefotaxime for a total of 4 days. While the mechanisms of pathogenicity in *E. cloacae* are still not well-understood, biofilm formation is considered a virulence feature in this non-obligate pathogen (Zhou *et al.*, 2014, Zurob *et al.*, 2019). Evidence of bacterial aggregates lining the larval gut can be seen in Figure 6. Forming biofilm infections *in vivo* could at least partially explain how some susceptible monocultures were able to survive high cefotaxime doses without acquired resistance. Other contributing factors will be differences in pharmacokinetics (Andes and Lepak, 2017) and increased environmental stressors (i.e. poor nutrient availability, host interactions) *in vivo*

(MacLean *et al.*, 2010, Sousa *et al.*, 2012, Durão *et al.*, 2015, Hall *et al.*, 2015, Cardoso *et al.*, 2019).

Despite this, susceptible cells under selection grew overall better in mixed cultures when the prevalence of AmpC *β*-lactamase producers was high, compared to growth in monocultures. The evidence for social detoxification observed in broth, biofilms, and *in vivo* experiments is notable, given the number of differences in biological conditions between these set-ups.

Previously, co-existence and social 'cheating' in planktonic cultures were considered limited to non-lethal initial antibiotic doses and high frequencies of resistant cells detoxifying the antibiotics, while some susceptible cells remained dormant until the level of antibiotic was sufficiently reduced (Lenski and Hattingh, 1986, Dugatkin *et al.*, 2005, Medaney *et al.*, 2015). In this study, I demonstrated that sensitive cells were able to grow in co-cultures with the *β*-lactamase producing strain, at antibiotic doses which prevented growth of susceptible monocultures in broth and *in vivo*. This can justify cefotaxime degradation as a cooperative trait, as it permits the persistence and reinvasion of susceptible cells, once the effective concentration of the antibiotic is sufficiently reduced. Cooperative detoxification was less clear with *in vitro* biofilms, as some susceptible monoculture replicates were able to withstand even the highest concentration of 3000 µg/ml, while no susceptible growth could be recovered from others.

Previous studies on cooperative antibiotic deactivation used either ampicillin (Medaney *et al.*, 2015, Amanatidou *et al.*, 2019, Dimitriu *et al.*, 2019, Gjonbalaj *et al.*, 2020) or a combination of ampicillin with a *β*-lactam inhibitor (Yurtsev *et al.*, 2013), as social detoxification for cefotaxime was considered unlikely due to

the high toxicity of the antibiotic, as demonstrated by Medaney *et al.* (2015) who showed that resistant *E. coli* can readily exclude susceptible cells on agar plates *in vitro* at concentrations far below MIC due to the high sensitivity of this species. In natural environments (Wellington *et al.*, 2013, Amos *et al.*, 2014) and in hospital settings (Yang and Zhang, 2008, Moore *et al.*, 2014), however, *β*-lactamase secreting bacteria are widespread. Moreover, cooperative detoxification may be operated by members of the *Enterobacteriacae* family, which are found in the mammalian gut, suggesting that gut infections may be shielded during treatment by cooperative detoxification, as indicated by Gjonbalaj *et al.* (2020) and O'Brien *et al.* (2021).

In support, Murray *et al.* (2018) showed that social protection of sensitive *E. coli* under cefotaxime selection can occur in the presence of a complex sewage-derived microbial community. Whilst here, I show that in a simpler system of a mixed genotype co-culture, the presence of resistant cells is enough to cross-protect sensitive genotypes from the toxicity of this third generation cephalosporin across different environmental contexts.

Cefotaxime is critically important and recognised as antibiotic of highest priority in antimicrobial stewardship (WHO, 2019), thus understanding the implications of sociality in resistance dynamics during cefotaxime treatment has implications for resistance management. In addition, *E. cloacae* is highlighted as an 'ESKAPE' pathogen, an acronym for resistant *Enterococcus faecium, Staphylococcys aureus, K. pneumoniae, Acinetobacter baumanii, P. aeruginosa* and *Enterobacter* spp., or clinically important pathogens able to 'escape' the toxicity of highly bactericidal antibiotics (Pendleton *et al.*, 2014). The *E. cloacae* complex, in particular, are frequent causative agents of clinical bloodstream

infections (Polsfuss *et al.*, 2011, Mezzatesta *et al.*, 2012), presenting additional challenges for treatment management.

The importance of the ecological context for antibiotic resistance dynamics, including the intensity of selection and environmental heterogeneity, has been previously recognised from work *in vitro* on single-species populations (Drlica and Zhao, 2004, Gullberg *et al.*, 2011). However, it is unclear to what extent the study of resistance evolution within monocultures can be extrapolated to natural environments, as this approach overlooks the role of microbial interactions on resistance dynamics. Few experimental studies suggest that inter-and intra–species interactions, such as competition and cooperation, along with the physiological alterations of cells within microbial communities can affect how bacteria respond to antibiotics by altering the strength of selection through social protection over susceptible strains, ultimately increasing competition (Yurtsev *et al.*, 2013, Sorg *et al.*, 2016, Medaney *et al.*, 2016, Murray *et al.*, 2018, Klümper *et al.*, 2019, Amanatidou *et al.*, 2019, Gjonbalaj *et al.*, 2020).

Concluding Remarks

Fitness costs are undoubtedly important in shaping resistance dynamics by generating negative selection in antibiotic-free environments, such as within hosts which have ceased antibiotic use post-treatment, or during colonisation of new untreated hosts (Vogwill and MacLean, 2015). However, fitness costs show high variability, determined by the interplay of the genetic background of resistance and growth conditions (Hall *et al.*, 2015, Durão *et al.*, 2018, Manktelow *et al.*, 2020). As a result, some strains may show high costs *in vitro*, but have small costs *in vivo*, as was the case with rifampicin resistance, assessed in Chapter 1. The opposite may also occur, when fitness costs are elevated *in vivo* (Lindgren *et al.*, 2005), similar to what was observed here for *β*-lactam resistance, as the CEF strain was fitter in broth but generated some cost during gut infection and transmission in *P. xylostella* hosts.

Moreover, the same mutation in different genotypes of the same bacterial species may have completely contrasting outcomes. For example, Luo *et al.* (2005) demonstrated that one genotype of *Campylobacter jejuni* with a *gyrA* mutation was fitter than the susceptible genotype in the absence of selection *in vivo*, while a second genotype carrying the same mutation exhibited deleterious effects in an identical set-up. The underlying mechanisms behind these differences are not well understood. Regardless, what is evident is that confining fitness cost assessments to a single condition will result in a limited or inaccurate depiction of the true cost of resistance. In addition, estimating different components of fitness, such as colonisation, competitiveness, or transmission, may reveal potentially exploitable physiological weaknesses (Andersson, 2006, Paulander *et al.*, 2009).

The important role of the ecological context was further reiterated in the study of social detoxification by *β*-lactamase secretion, as a potential mechanism for co-existence of mixed genotype infections during antibiotic treatment. Evidence for cooperative protection was found in every experimental system, despite using a highly toxic third-generation cephalosporin, suggesting that co-infection interactions play a major part in the long-term population dynamics of mixed-genotype infections.

In monocultures, the survival of susceptible genotypes in the presence of antibiotics was highly dependent on bacterial physiology and population structure. Bacteria in live infections and in biofilms established by susceptible monocultures were able to survive cefotaxime concentrations as high as 4000-fold above their MIC, although this was not consistently repeatable. Previous work has shown an association between reduced metabolism and antibiotic tolerance in cells within mature biofilms *in vitro* and in live infections (Hassett *et al.*, 2002, Amanatidou, 2017, Wan *et al.*, 2018), which could at least partially explain the antibiotic tolerance observed here. Whether cells in still-forming biofilms will be able to endure the toxicity of cefotaxime, is unclear.

Finally, with this thesis, I presented a tractable *in vivo* experimental system, utilising the larvae of the diamondback moth, *P. xylostella,* and the non-obligate gut pathogen *E. cloacae*. This host represented a simplified bacteria-free *in vivo* environment, which proved especially valuable in assessing within and between host fitness components as well as the effect of social dynamics on antibiotic resistance evolution by applying uncomplicated amendments to the experimental setup. The low cost and maintenance, quick generation turnover, and versatility make this invertebrate host attractive for tackling questions on

the ecology and evolution of antibiotic resistance. Of course, this system may not represent a fully accurate picture with regard to conditions within mammalian infection, but it nonetheless provides grounds for obtaining valuable base-level scientific data, whilst still advancing the 3Rs policy.

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