The social evolutionary dynamics of antibiotic resistance in *Pseudomonas aeruginosa* infections



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A thesis submitted for the degree of

Doctor of Philosophy

Trinity Term 2019

Declaration

I declare that this thesis was composed by myself and that the work contained herein is my own except where explicitly stated in the text. This work has not been submitted for any degree or professional qualification except as specified.

David Crosse, Trinity Term 2019

Acknowledgements

First and foremost I must thank my supervisor, Ashleigh Griffin, both for giving me the chance to do this DPhil and for huge support during the process. This particularly became apparent during the last few years when I have had many external demands on my time. Ashleigh has been very understanding when at times progress must have seemed painfully slow. I must also thank Melanie Ghoul who, although not formally my supervisor, was in effect my second supervisor. Melanie taught me everything in the laboratory, from how to hold a pipette onwards, and she has been continuously on hand for advice – particularly with experimental design and analysis.

I also thank my advisory committee, initially Stu West and Alvaro San Millan, and later Craig MacLean, who all gave helpful advice to guide this project.

I thank Søren Molin and Helle Krogh Johansen from the Danish Technical University for providing the clinical *P. aeruginosa* isolates used in this thesis. It has been a privilege to use such an excellent collection. Thank you also for the hospitality in Copenhagen and useful discussions. From Copenhagen I also thank Linda Jensen for providing me with the antibiotic treatment profiles for each patient, and Sandra Andersen and Lea Sommer for answering many further queries about the data.

I thank Antonio Oliver from Hospital Universitario Son Espases, Spain, for providing the knockout strains used in Chapters 4 and 5.

I must give huge thanks to the microbiology team in the Nuffield Department of Medicine at the John Radcliffe Hospital for welcoming me into their laboratory and giving me some desk space (well, half a desk!) after the untimely closure of the Tinbergen Building. In particular, I thank Ali Vaughan, who arranged all the practicalities.

I would like to thank members of the Theoretical and Experimental Evolutionary Biology (TEEB) groups for many useful discussions over the years. I would like to thank all members of the Griffin and West groups for making it such great environment to work in. The coordinated effort by everyone to make sure I handed in by the deadline on submission day was particularly appreciated…

I would like to thank Heather Green from the Graduate Office for her kind presence and for always knowing which form to fill in.

I also thank Lyn Williamson, my clinical educational supervisor at the Great Western Hospital NHS Trust, for arranging my study leave from the hospital and for ensuring my clinical training never became too much of a burden.

I thank Mary, Josephine and Martha for their patience with me during these years. I thank all those who have helped with childcare, particularly my parents.

I thank the European Research Council for funding. It is worrying that the future of such funding in the UK is, at the time of writing, so uncertain.

Contributions

Chapters 2, 3 and 4.

The collection and sequencing of clinical *Pseudomonas aeruginosa* isolates and associated clinical data was done by collaborators from the Danish Technical University and Copenhagen CF Centre.

Chapter 3.

The laboratory work for approximately half of the resistance measurements for benzylpenicillin, piperacillin and meropenem for the set of 23 isolates was done by Melanie Ghoul.

MIC measurements for the full isolate collection were provided by collaborators at the Danish Technical University.

Chapters 2, 4 and 5.

Knockout strains with tagging were provided by the laboratory of Antonio Oliver, Hospital Universitario Son Espases, Spain.

All chapters are otherwise my own work, supervised by Prof Ashleigh Griffin and with further input from Dr Melanie Ghoul, who both contributed to experimental design, interpretation of results and the manuscript.

Abstract

The maintenance of cooperative behaviours, and understanding why cooperation sometimes breaks down, is one of the most important questions in evolutionary biology. Antibiotic resistance in bacteria can be a cooperative behaviour, so social evolutionary theory may provide an important perspective on the problem of antimicrobial resistance, which is one of the greatest challenges to modern medicine. Bacteria engage in many social behaviours, which often involve production of a public good. One such potential public good is the resistance enzyme beta-lactamase, which degrades beta-lactam antibiotics. Public good producers are vulnerable to exploitation by non-producing cheats, which in this case would be antibiotic-sensitive cells. It is not known whether such cooperator-cheat dynamics are seen in clinical infections, but understanding if sensitive cheats arise, and the circumstances which allow this, could help control antibiotic resistance. In this thesis I examine the social dynamics of antibiotic resistance in chronic infections, concentrating on beta-lactamase production. I use a longitudinally sampled collection of the opportunistic pathogen *Pseudomonas aeruginosa* from cystic fibrosis patients to track how beta-lactamase production and beta-lactam resistance change during infection. I find that there is negligible increase in beta-lactamase in chronic infection. This is contrary to expectation if selection for resistance is the dominant pressure, but consistent with social dynamics. However, I do also find an increase in resistance to beta-lactams, which may reflect the contribution of other resistance mechanisms in addition to beta-lactamase production. I find a divergence in both beta-lactamase production and in beta-lactam resistance, which is correlated with treatment with beta-lactam antibiotics. This suggests beta-lactams are selecting for both high and low beta-lactamase producers, consistent with social dynamics. I compare these results to change in resistance in aminoglycoside and quinolone antibiotics, whose resistance mechanisms have less potential for sociality. In contrast to beta-lactams, I find limited divergence in aminoglycoside resistance and no divergence in quinolone resistance, as predicted by social evolutionary theory. I then take high beta-lactamase producing clinical isolates and show that beta-lactamase is a public good which can protect non-producers from beta-lactam antibiotics, allowing non-producers to invade in some instances. I show that high producers partially compensate for the presence of non-producers by making more beta-lactamase. I also show that the benefits to the non-producer primarily accrue after the cost of synthesis to the producer. These latter two findings may help stabilize cooperation against cheats. Overall, my thesis supports the suggestion that social evolutionary dynamics are important in antibiotic resistance in chronic infections.

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Chapter 1. Introduction

Explaining the maintenance of cooperative behaviours in the face of selection for self-interest, and understanding why cooperation sometimes breaks down, is one of the most important questions in evolutionary biology. Equally, antibiotic resistance has emerged as one of the great challenges to the health of our generation. Since antibiotic resistance in bacteria can be a cooperative behaviour, social evolutionary theory can be used to make some surprising predictions about the evolution of antibiotic resistance, with potential clinical implications. In this thesis I apply social evolutionary theory to the study of antibiotic resistance; it is both a test of fundamental theory and groundwork for future clinical application.

In this introduction I briefly discuss the broad problem of antibiotic resistance before giving a general overview of social evolutionary theory and how this relates to bacterial traits. I then discuss how antibiotic resistance can be considered to be social and the implications this may have. I then introduce *Pseudomonas aeruginosa* in cystic fibrosis as my model system, discussing its clinical context, reviewing existing evidence for sociality and giving a summary of resistance mechanisms. I then give an overview of the proceeding chapters.

The problem of antibiotic resistance

When Alexander Fleming accepted the Nobel Prize for the discovery of penicillin in 1945, he warned that it was '*not difficult to make microbes resistant to penicillin*'

(Fleming, 1945). Indeed, a resistance enzyme had already been discovered prior to the therapeutic use of penicillin (Abraham & Chain, 1940). Antibiotic resistance has been caused by the selection pressure of antibiotic use (Levy & Marshall, 2004). Globally, the association between antibiotic use and resistance has been shown repeatedly (Monroe & Polk, 2000; Goossens *et al.*, 2005). Antibiotic consumption, however, continues to increase (Klein *et al.*, 2018) and resistance has developed to each new class of antibiotic (Molton *et al.*, 2013). New classes of antibiotic themselves have become less frequent (Coates *et al.*, 2011; Fair & Tor, 2014). The rise of resistance and the slow-down in antibiotic discovery have led to the current antibiotic resistance crisis, with warnings of a post-antibiotic era, where infectious diseases become more common and other therapies which rely on antibiotics or immunosuppression – such as surgery, cancer chemotherapy or organ transplantation – become impossible (Davies, 2011). A British government report suggests that by 2050 there may be ten million deaths per year worldwide from antibiotic resistance (O'Neill, 2016). Understanding how bacteria evolve resistance will be vital to tackling this problem.

Social evolutionary theory

Bacteria engage in social behaviours which have evolutionary consequences. A social behaviour is an interaction between two individuals which results in a change in direct fitness of both the actor and the recipient of the behaviour (West *et al.*, 2007b). Direct fitness here broadly equates to reproductive success or the ability to pass on genes to the next generation (Dawkins, 1982). Social behaviours have more generally been studied in animals, but sociality is widespread across different levels of biological organisation, including between single cells (Bourke, 2011) or even genes (Foster, 2011). In recent years, microorganisms have become a new focus for the study of

sociality. This is because they lend themselves to experimental work through of ease of manipulation and short generation times, because the underlying mechanisms are often understood, and because of the important implications for bacterial disease (West *et al.*, 2006).

All social behaviours fall into one of four categories, classified by Hamilton (1964) according to the change to the lifetime direct fitness of the actor and recipient involved in the behavioural interaction (Table 1). If both the actor and recipient gain a direct fitness benefit (+/+), the behaviour is 'mutually beneficial'; if the actor benefits at a cost to the recipient (+/-), the behaviour is 'selfish'; if the behaviour is detrimental to the actor but beneficial to the recipient (-/+), it is 'altruistic'; and if the behaviour has detrimental effects on both actor and recipient (-/-), it is termed 'spiteful'. The fact that it is the 'lifetime' effect of the social interaction means that it is not just about the immediate effects of the behaviour: a behaviour which may be immediately costly to the actor could accrue a direct fitness benefit over the actor's lifetime. If a behaviour has been selected for its beneficial effect on the recipient, it is termed 'cooperative'. Cooperation includes some mutually beneficial behaviours and all altruistic behaviours (West *et al.*, 2007b).

	lifetime direct fitness effect on recipient	
lifetime direct fitness effect on actor	**+**	**-**
+	mutually beneficial	selfish
-	altruism	spite

Table 1. Hamilton's classification of social behaviours. Adapted from West *et al.* (2007b).

The core question of the field of social evolution is the problem of cooperation: why should an individual perform an apparently costly behaviour which benefits other individuals (West *et al.*, 2007b)? All cooperation, whether altruistic or mutually beneficial, is vulnerable to exploitation by 'cheats' – individuals which benefit from the cooperative behaviour of another at a cost to the cooperator's inclusive fitness, due to the cooperative behaviour being directed towards the cheat rather than the intended recipient (Ghoul *et al.*, 2014a). A large body of theory and empirical work studying the problem of cooperation has accumulated (Bourke, 2011), identifying factors which promote or impede cooperation.

Explanations for cooperation revolve around direct and indirect fitness benefits. Hamilton (1964) pointed out that genes can be passed on to the next generation not only in copies in offspring (direct fitness), but in any relatives, such as siblings or cousins. The fitness gained by helping relatives he termed 'indirect fitness' which, together with direct fitness, makes up 'inclusive fitness'. Inclusive fitness is the property that individuals behave as if to maximize (Grafen, 2009; West & Gardner, 2013). This gave rise to his famous rule describing the conditions for altruism to evolve:

$$rB - C > 0$$

where r is the coefficient of relatedness of the actor to the recipient, B is the lifetime direct fitness benefit to the recipient and C is the lifetime direct fitness cost to the actor. This means that altruism can evolve if the benefit to the recipient, weighted by the relatedness, outweighs the cost to the actor. This can only be the case if the actor

and recipient are related (i.e. $r > 0$). It can be generalized for any social behaviour (not just altruism) as below (Bourke, 2011), where the fitness effect is negative for a cost or positive for a benefit:

$$r \times [\text{direct fitness effect on recipient}] + [\text{direct fitness effect on actor}] > 0$$

This equation displays clearly the three factors – relatedness, fitness benefits and costs – that need to be studied to understand social behaviours.

Bacterial social evolution

Bacteria engage in many social behaviours. Cooperative behaviours often take the form of secretion of a public good (West *et al.*, 2007a). In the microbial context, these are secreted molecules that benefit individuals in the vicinity. Examples include pyoverdin for iron acquisition (Griffin *et al.*, 2004), quorum sensing molecules for communication (Keller & Surette, 2006; Diggle *et al.*, 2007), enzymes for nutrient breakdown and acquisition (Greig & Travisano, 2004) and biosurfactants for motility (Daniels *et al.*, 2004). Public good production is a whole group trait – it has the potential to benefit both other individuals and the producer. It therefore has both direct and indirect fitness benefits for the producer, and can be altruistic or mutually beneficial depending on the exact costs and benefits. For example, if the lifetime direct fitness benefits do not outweigh the immediate cost of production, the behaviour is likely to have been selected due to indirect fitness benefits and so would be altruistic.

The problem of cooperation in bacteria is typically a public goods problem. A cheat in this context would be an individual which does not produce the public good while benefiting from the public good made by producer cells. As the cheat gains the benefit without paying the cost of production, it can have a growth advantage and invade a population, potentially leading to a 'tragedy of the commons' scenario (Hardin, 1968), where cheats go to fixation despite being less fit in pure culture.

Public goods problems in bacteria are often investigated with competition assays (West *et al.*, 2006; Ghoul *et al.*, 2014b, 2016). These are simple experiments where strains which perform a cooperative behaviour are competed with selfish mutants in mixed culture. As a control, cooperators and cheaters must be grown in monoculture in an environment where the public good is likely to be beneficial. This is to confirm the assumption that public good production provides a benefit and that cooperators, therefore, have a fitness advantage. In contrast, cheats are predicted to be more fit in mixture when they have the opportunity to gain the benefit of the public good without paying the cost.

Competition experiments have shown that pyoverdin and quorum sensing cheats can invade populations of producer cells (Diggle *et al.*, 2007; Ghoul *et al.*, 2014b), although a number of factors which can limit the ability of cheats to invade have also been found, including effects of low density (Ross-Gillespie *et al.*, 2009), viscosity (Kümmerli *et al.*, 2009a) and frequency dependence (Ross-Gillespie *et al.*, 2007). Crucially, Griffin *et al.* (2004) showed that cooperation can be maintained with high relatedness, as predicted from Hamilton's rule. However, relatedness at the locus of the social behaviour is reduced by *de novo* mutants which do not produce the public

good, or by immigration of non-producers into the system. The durability of the public good molecule (Brown & Taddei, 2007; Kümmerli & Brown, 2010a; Allen *et al.*, 2013; Dobay *et al.*, 2014) and how producers respond to the presence of potential cheats (Harrison, 2013) can all affect the ability of non-producers to invade. It is therefore hard to predict whether any particular public good is going to be vulnerable to cheating without directly performing competition experiments.

Social evolution and antibiotic resistance

Antibiotic resistance can be a social behaviour. Resistance mechanisms differ in the extent to which they protect the focal cell (private mechanisms) or other cells in the vicinity (cooperative mechanisms). This is likely to have an impact on the evolutionary dynamics of different resistance mechanisms. At the social end of the spectrum are extracellular enzymes, such as beta-lactamase, which degrade antibiotics and have the potential to act as public goods (Dugatkin *et al.*, 2005; Clark *et al.*, 2009; Yurtsev *et al.*, 2013; Medaney *et al.*, 2015; Bottery *et al.*, 2016; Frost *et al.*, 2018). Intracellular degradation enzymes, such as aminoglycoside modifying enzymes or chloramphenicol acetyltransferase, have the potential to have some social effect as the concentration of antibiotic in the environment surrounding the focal cell will be reduced. This has been shown in the case of chloramphenicol resistance (Sorg *et al.*, 2016), but not for aminoglycosides (Frost *et al.*, 2018). At the private end of the spectrum, efflux pumps extrude antibiotic unchanged and so protect only the focal cell (Bottery *et al.*, 2016), as do mutations at antibiotic binding sites within cells.

The implications of the sociality of antibiotic resistance are significant. While antibiotic treatment is expected to favour private mechanisms of resistance,

cooperative mechanisms could be vulnerable to invasion by sensitive cheats which do not produce the degrading enzyme. This could lead to a seemingly paradoxical decrease in antibiotic resistance in certain contexts, even in the presence of antibiotics. Bottery *et al.* (2016) and Frost *et al.* (2018) have both shown stronger selection for selfish resistance mechanisms than for cooperative mechanisms at low antibiotic doses. Whether this occurs in clinical infections is unknown. Understanding if sensitive cheats can survive, and the conditions under which this may occur, is vital knowledge in tackling the problem of antibiotic resistance.

Some work has examined beta-lactamase as a social trait in engineered laboratory strains. Broadly, these studies have found that non-producers of beta-lactamase have been protected by producers, but have either remained a very low proportion of the population (Dugatkin *et al.*, 2005; Clark *et al.*, 2009), or have only been able to invade at sub-inhibitory concentrations (Medaney *et al.*, 2015; Bottery *et al.*, 2016; Frost *et al.*, 2018). An exception is Yurtsev *et al.* (2013) who showed invasion by cheats. These studies suggest beta-lactamase can act as a public good, though the conditions for this are unclear. Perlin *et al.* (2009) showed a cross-species protective effect of beta-lactamase. In fact, while not taking a social evolutionary perspective, a number of *in vitro*, *in vivo* and clinical studies going back as far as the 1950s have shown that co-infecting or commensal strains which produce beta-lactamase can protect non-beta-lactamase producers such as *Streptococcus pneumonia* (Tacking, 1954; Simon & Sakai, 1963; Brook & Yokum, 1983; Brook *et al.*, 1983; Brook, 1985). However, no studies have looked systematically over evolutionary time at the potential for beta-lactamase cheats in natural populations from clinical infections.

Model system

To investigate the social evolutionary dynamics of antibiotic resistance, I use *Pseudomonas aeruginosa* in chronic cystic fibrosis infections as my model system. *P. aeruginosa* is an opportunistic pathogen which particularly infects immunocompromised patients (Al-Hasan *et al.*, 2008; Migiyama *et al.*, 2016), burns patients (Mayhall, 2003; Williams *et al.*, 2009) and those in intensive care (Jarvis & Martone, 1992; Spencer, 1996; Richards *et al.*, 1999). It is the primary cause of morbidity and mortality in patients with cystic fibrosis (CF), which is one of the most common genetically inherited conditions, affecting approximately 1 in 2500 live births (Dodge *et al.*, 2007; Folkesson *et al.*, 2012). Patients have a median lifespan of approximately 40 years (Dodge *et al.*, 2007). A defect in epithelial chloride channels results in thick mucus in the airways, impeding mucociliary clearance of bacteria (Gibson *et al.*, 2003). This predisposes to recurrent infections. The inflammatory response causes further damage, impairing lung function still further (Cantin *et al.*, 2015). Once infected, often at a young age, these individuals remain infected for the rest of their lives (Folkesson *et al.*, 2012). *P. aeruginosa* infections in CF therefore represent an ecosystem where the bacteria can evolve over several years, during which they are subjected to regular courses of antibiotics. It is therefore an ideal environment in which to investigate the social evolution of antibiotic resistance.

The isolate collection I used comprised 215 *P. aeruginosa* isolates collected from 17 CF patients between 2002 and 2012 at the Copenhagen CF Centre at Rigshospitalet as part of a long-term study into *P. aeruginosa* in CF by Søren Molin and Helle Krogh Johansen at the Danish Technical University. Patients were aged between 1 and 33 years at the time of sampling. Lung samples were mostly for routine monitoring and

were collected from expectorate, bronchoalveolar lavage and endolaryngeal suction. When *P. aeruginosa* was found, between one and four isolates were selected as representative of the flora, based on observable phenotype. All isolates were genotyped. The 33 clone types represented included DK01, a well-studied transmissible strain. Three other clone types appeared in two patients each, implying transmission. 207 isolates had had resistance data measured for a range of antibiotics. Contextual clinical information – in particular the antibiotic treatment the patients had received – was available. I therefore had a set of longitudinally collected isolates from multiple patients with information on the antibiotic selection pressure. Further information on sampling and the isolates is provided in the Methods section of Chapter 2.

Sociality has already been demonstrated in other traits in *P. aeruginosa*. Cooperation and cheating has been demonstrated with other public goods in the laboratory setting (Griffin *et al.*, 2004; Ghoul *et al.*, 2014b), but it has also been demonstrated in the CF lung. Jiricny *et al.* (2014) showed the loss of many potentially cooperative behaviours in chronic CF infections, including extracellular proteases and quorum sensing molecules. The spread of these loss-of-function mutations has been interpreted as adaptation to the lung environment (Marvig *et al.*, 2015; Schick & Kassen, 2018). However, Andersen *et al.* (2015) demonstrated that non-producers of pyoverdin retain the ability to utilize pyoverdin while producers remain in the population, suggesting that this trait is lost over time as a result of cooperator-cheat dynamics. Furthermore, bacterial populations shift during infection from cooperative iron acquisition by public good mechanisms to private mechanisms, suggesting that bacteria are selected to avoid exploitation (Andersen *et al.,* 2018). This only happened

in infections where pyoverdin had been lost. *P. aeruginosa* in CF lungs therefore seems a good environment to look for sociality of resistance.

Several classes of antibiotic from across the spectrum of sociality of resistance are used to treat *P. aeruginosa* infections in CF patients. First line antibiotics used include the beta-lactams ceftazidime, meropenem, piperacillin-tazobactam and ticarcillin-clavulanate, while second line treatment includes the beta-lactams aztreonam and imipenem (UK Cystic Fibrosis Trust, 2009). To varying degrees these are resisted by beta-lactamases as well as other mechanisms such as efflux (Poole, 2011). The aminoglycoside tobramycin, resisted by intracellular aminoglycoside modifying enzymes, and the quinolone ciprofloxacin, resisted by private efflux pumps and binding site modifications, are also first line treatments (UK Cystic Fibrosis Trust, 2009). Colistin (a polymyxin) is also commonly used, but resistance to this is rare (Falagas & Kasiakou, 2005; Manno *et al.*, 2005). Cystic fibrosis patients receive very regular courses of antibiotics of comparatively long duration, sometimes up to months (UK Cystic Fibrosis Trust, 2009). The selection pressure for resistance is therefore expected to be relatively high and consistent.

Given the high level of antibiotic treatment in CF, it would be difficult to suggest that loss of antibiotic resistance is an adaptation to the CF lung environment, making this a good test of evolutionary theory more broadly. Some existing evidence is suggestive of a loss of beta-lactam resistance in contrast to an increase in resistance to other classes of antibiotics. For example, Smith *et al.* (2006) showed a loss of beta-lactam resistance in a CF patient while resistance to other classes had increased; López-Causapé *et al.* (2013) showed the loss of the chromosomally encoded beta-lactamase

in two genotypes of *P. aeruginosa* in a CF infection; Manno *et al.* (2005) showed that across 224 chronically infected CF patients, *P. aeruginosa* resistance to quinolones, aminoglycosides and a beta-lactam largely resisted by efflux pumps increased, while resistance to two other widely used beta-lactams (resisted by beta-lactamases) remained stable. Whether these studies are representative of antibiotic resistance dynamics, and if all these changes are mediated by beta-lactamase is unclear.

Loss of resistance described above has sometimes been interpreted through the phenomenon of collateral sensitivity. This is where treatment with one antibiotic class selects for resistance to that class and sometimes others, but also leads to increased sensitivity to another antibiotic class. For example, resistance to quinolones can cause sensitivity to beta-lactams and aminoglycosides in *P.aeruginosa* (Imamovic *et al.*, 2018). This is thought to be primarily due to changes in efflux pump expression, via upregulation of MexCD-OprJ efflux pump (for quinolones) and downregulation of OprM for beta-lactams and aminoglycosides (Imamovic *et al.*, 2018). Mulet *et al.* (2011) found no changes in beta-lactamase expression involved in collateral sensitivity, although they suggest increased membrane permeability actually leads to increased extracellular beta-lactamase. Imamovic *et al.* (2018) found one mutation in the AmpC beta-lactamase gene in an experimentally evolved strain, though it is unclear if this was related to collateral sensitivity. The relevance of collateral sensitivity to beta-lactamase expression therefore seems limited. If loss of beta-lactamase could be demonstrated, this would be unlikely to be due to collateral sensitivity.

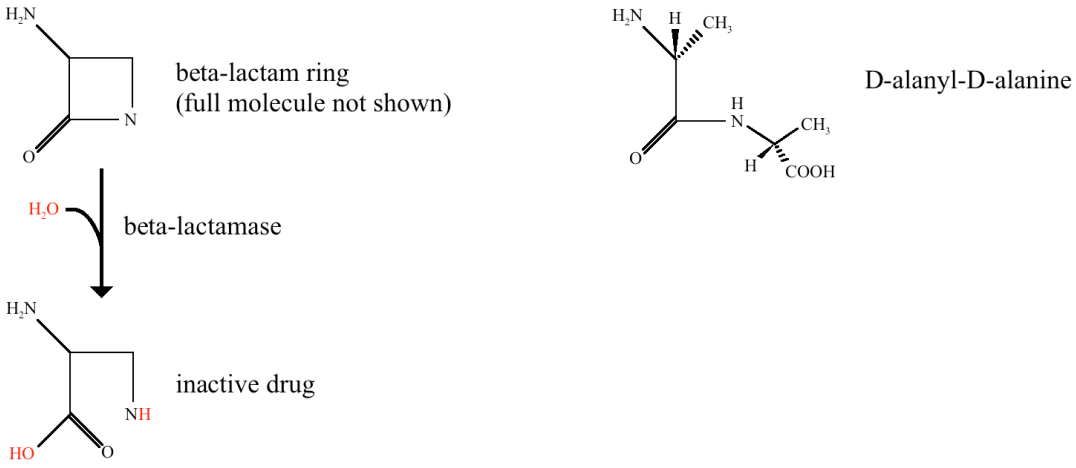
Mechanism of action of beta-lactam antibiotics

Beta-lactam antibiotics act by preventing the cross-linking of the peptidoglycan chains which form the cell wall (Kong *et al.*, 2010). In Gram-negative bacteria such as *P. aeruginosa*, the cell wall is in the periplasmic space. Beta-lactams therefore have to cross the outer membrane to reach their target. They do this by entering the cell by diffusion through porins (transmembrane protein channels). The principal porin by which beta-lactams enter *P. aeruginosa* is OprD, a non-specific porin which allows entry of amino acids and small peptides (Li *et al.*, 2012). Once in the periplasm, beta-lactams irreversibly bind to 'penicillin binding proteins', which are transpeptidases that catalyse the cross-linking of peptidoglycan in the periplasm. The cross-linking occurs between the pentapeptide side chains of the *N*-acetylmuramic acid residues which form part of the backbone of peptidoglycan (alternating with *N*-acetylglucosamine). Beta-lactams bear a structural similarity to D-alanyl-D-alanine (Figure 1), which is the part of the pentapeptide side chain that is the substrate for transpeptidase (Kong *et al.*, 2010). The structural similarity allows beta-lactams to enter and covalently bind to the transpeptidase active site, blocking the enzyme's cross-linking function (Macheboeuf *et al.*, 2006). Prevention of cross-linking disrupts the structural integrity of the peptidoglycan, preventing the continuous recycling of the cell wall seen in Gram-negative bacteria (Goodell, 1985; Park, 1993) and impeding cell division, which requires synthesis of new peptidoglycan. Disruption of peptidoglycan synthesis leads to the death of dividing cells (Cho *et al.*, 2014).

Beta-lactam resistance in Pseudomonas aeruginosa

P. aeruginosa is regarded as having a high level of intrinsic resistance (Strateva & Yordanov, 2009; Murray *et al.*, 2015), and regularly evolves multidrug resistance in clinical settings (Poole, 2011), which has been identified as a particular clinical threat (Livermore, 2002). Against beta-lactams, its principal mechanisms of resistance are degradation by beta-lactamase, efflux, and reduced membrane permeability (Strateva & Yordanov, 2009; Poole, 2011). The relative importance of these mechanisms varies for different classes of beta-lactams and can depend on which beta-lactamases are expressed (Poole, 2011). Beta-lactamases are often regarded as the most important mechanism against penicillins and cephalosporins (Giwercman *et al.*, 1990; Livermore & Brown, 2001; Berrazeg *et al.*, 2015), but less important against the newer carbapenems, where a combination of permeability and beta-lactamase seems important (Livermore, 1992; Pai *et al.*, 2001; Henrichfreise *et al.*, 2007; Lister *et al.*, 2009).

Beta-lactamase deactivates beta-lactam antibiotics by hydrolysing the beta-lactam ring. This ring is both the defining part of beta-lactam molecules and the part that is structurally similar to the D-alanyl-D-alanine of peptidoglycan (Figure 1). On hydrolysis, beta-lactam antibiotics therefore lose their ability to block transpeptidases (penicillin binding proteins).



(a) Hydrolysis of beta-lactams **(b) D-alanyl-D-alanine**

Figure 1 (a) Hydrolysis of the beta-lactam ring by beta-lactamase; (b) beta-lactam antibiotics show a structural similarity to D-alanyl-D-alanine, and so bind irreversibly to the active site of transpeptidases in place of D-alanyl-D-alanine, preventing cross-linking of peptidoglycan. Based on Livermore (1995) and Kong *et al.* (2010).

The principal beta-lactamase in *P. aeruginosa* is the chromosomal AmpC beta-lactamase. This is a Class C beta-lactamase, sometimes termed a cephalosporinase because it has specific activity against cephalosporins, though is also functional against penicillins and, to a lesser extent, against carbapenems (Livermore, 1992). Some antibiotics are inducers of AmpC, including benzylpenicillin and imipenem (Jacoby, 2009). Occasionally mutations can extend the spectrum of AmpC to be more active against newer cephalosporins such as ceftazidime and cefepime (Berrazeg *et al.*, 2015) or imipenem (Rodríguez-Martínez *et al.*, 2009), though these only account for about 1.5% of AmpC (Berrazeg *et al.*, 2015). AmpC beta-lactamase has been shown to be secreted extracellularly in vesicles (Ciofu *et al.*, 2000), which is likely to increase the indirect fitness effects, making it more cooperative.

There are several further beta-lactamases borne by *P. aeruginosa*. OXA-50 (also known as PoxB) is a second chromosomal beta-lactamase present in approximately 57% of isolates (Kong *et al.*, 2005). It is constitutively expressed (Girlich *et al.*, 2004) but seems to have limited clinical relevance (Walther-Rasmussen & Høiby, 2006; Poole, 2011). There are a number of plasmid-borne beta-lactamases, often extended spectrum beta-lactamases derived from TEM and SHV beta-lactamases. These can hydrolyse newer cephalosporins, monobactams and carbapenems (Poole, 2011). However, plasmid-borne beta-lactamases are actually quite rare in *P. aeruginosa*, particularly in Northern Europe (Livermore, 2002). Chen *et al.* (1995) found only 13 of 1991 (0.65%) isolates with 'secondary' beta-lactamases. Therefore, whilst there are a variety of beta-lactamases of different types, the AmpC beta-lactamase remains the most clinically important in *P. aeruginosa*.

Aminoglycoside and quinolone resistance in Pseudomonas aeruginosa

While the focus of this thesis is cooperative beta-lactamase resistance, the contrasting resistance mechanisms of aminoglycosides and quinolones provide a useful comparison. Aminoglycosides are primarily resisted in *P. aeruginosa* by aminoglycoside modifying enzymes and efflux pumps (Poole, 2005). Modifying enzymes are intracellular (Shaw *et al.*, 1993). While intracellular enzymes could have a public good effect (Sorg *et al.*, 2016), this is not necessarily the case (Dugatkin *et al.*, 2005), and Frost *et al.* (2018) showed no public good effect in aminoglycoside resistance. Modifying enzymes are also rare in CF (Henrichfreise *et al.*, 2007). Therefore while there theoretically may be some capacity for cooperative resistance of aminoglycosides, it is likely this will be less than for beta-lactamase. Quinolones, by contrast, are not resisted by any cooperative mechanisms. Their primary

mechanisms of resistance are efflux pumps and binding site mutations (Lister *et al.*, 2009). Henrichfreise *et al.* (2007) shows that efflux is the more important mechanism in CF. Quinolone resistance is therefore evolutionarily exclusively private and will provide a useful comparison.

Summary

In summary, tackling the global problem of antimicrobial resistance requires an understanding of the selective forces on resistance mechanisms. Resistance mechanisms are on a spectrum of sociality which is likely to lead to divergent evolutionary pressures. For example, while evolutionarily private mechanisms of resistance are likely to be selected by repeated exposure to antibiotics, cooperative resistance mechanisms such as beta-lactamase enzymes may be vulnerable to exploitation by sensitive cheats. The extent to which social evolutionary dynamics are important in infections is unknown.

Thesis aims and outline

In this thesis I examine the role of social evolutionary dynamics in chronic infections, using *P. aeruginosa* in cystic fibrosis as a clinical model system. I concentrate on beta-lactamase production, although I also make comparisons with aminoglycoside and quinolone resistance. Across the thesis I build up a picture of how social evolutionary dynamics might influence antibiotic resistance in infections.

In Chapter 2 I characterize patterns of long-term evolution of beta-lactamase in CF lungs. I measure extracellular beta-lactamase production in longitudinally sampled

isolates from patients, enabling me to see changes in extracellular beta-lactamase production across the course of infection. I then ask whether my observations are consistent with predictions based on beta-lactamase acting as a public good. I find negligible increase in beta-lactamase production, even in strains which have experienced up to ten years of regular treatment with beta-lactams. By contrast, I find beta-lactamase production diverges over time, and that this divergence is positively correlated with beta-lactam treatment. This suggests antibiotics are selecting for both higher and lower producers, which is consistent with social dynamics and cells adopting cooperative and cheating strategies in the lung for this trait.

In Chapter 3 I relate beta-lactamase production to beta-lactam resistance. I find beta-lactamase production is correlated with resistance, confirming it is beneficial in natural isolates. I look at how beta-lactam resistance itself changes in patients over time, and compare this to aminoglycoside and quinolone resistance to see if resistance patterns in each antibiotic class are consistent with the sociality of the resistance. Consistent with a social evolutionary view of antibiotic resistance, I find that while cooperative beta-lactam resistance diverges over time, the effect is less in aminoglycosides and non-existent in privately-resisted quinolones.

In Chapter 4 I take clinical isolates which produce high levels of beta-lactamase and examine their ability to protect lower producers, and see if lower producers can exploit the production of high producers. I find that extracellular beta-lactamase from high producers can protect low producers, and that in some instances low producers can invade the population.

In Chapter 5 I look at how producer cells respond to the presence of non-producers. I also measure the durability of beta-lactamase. I find that producers partially compensate for the presence of non-producers by making extra beta-lactamase. I also find that beta-lactamase is a durable molecule but is degraded by cells. I consider the implications these findings may have for social dynamics.

Finally, in Chapter 6 I summarize the main findings from Chapters 2 to 4 to show the importance of social evolutionary dynamics to antibiotic resistance in infections. I then discuss the implications of the results and directions for future research.

Chapter 2. Dynamics of beta-lactamase production in chronic infections

Introduction

Given the urgent need to understand the evolution of antibiotic resistance, and the social nature of antibiotic resistance mechanisms discussed previously, it is imperative that social factors in the evolution of antibiotic resistance are studied in natural populations of bacteria. Here, as a starting point in the study of social factors in real-world antibiotic resistance, I explore how beta-lactamase, a social resistance mechanism, evolves during chronic infection.

Social antibiotic resistance is predicted to be under two selection pressures in the presence of antibiotics: i) selection for increased resistance and ii) selection to avoid the cost of resistance through cheating. Beta-lactamase is an antibiotic degradation enzyme produced by many bacteria, including *P. aeruginosa*. Intense selection pressure from antibiotic treatment is predicted to lead to an increase in resistance (Levy & Marshall, 2004) and a corresponding increase in beta-lactamase activity during chronic infection. But it is also a public good (Dugatkin *et al.*, 2005; Perlin *et al.*, 2009; Yurtsev *et al.*, 2013; Medaney *et al.*, 2015; Bottery *et al.*, 2016; Frost *et al.*, 2018) and so cheats which do not produce beta-lactamase should gain an advantage. The relative importance of each selection pressure in a chronic infection is not known, but existing work has focussed on understanding selection for resistance alone.

Evidence for selection for increased resistance due to exposure to antibiotics comes from epidemiological studies, laboratory experiments and patient trials across a variety of species and infection types. Epidemiologically, beta-lactamase associated resistance has increased since penicillin was introduced in the 1940s, seen particularly in *Staphylococcus aureus* (Lowy, 2003) and Enterobacteriaceae, *Pseudomonas* and *Acinetobacter* (Livermore & Woodford, 2006). A population level association between beta-lactam treatment and beta-lactamase production has been shown in *Moraxella nonliquefaciens* from the upper respiratory tract flora (Mölstad *et al.*, 1988) and *Moraxella catarrhalis* from middle ear infections (Nissinen *et al.*, 1995). Resistance to antibiotics including beta-lactams is easily selected in the laboratory, as shown by Sanders *et al.* (1984) in *Klebsiella pneumonia*, *Enterobacter cloacae*, *Serratia marcescens* and *P. aeruginosa*, and by Cabot *et al.* (2016) and Barbosa *et al.* (2017) in *P. aeruginosa*, and exposure to a course of beta-lactam antibiotics selects for short-term resistance in patients, shown in *H. influenzae* from the throat (Chung *et al.*, 2007), *P. aeruginosa* from intubated patients (Reinhardt *et al.*, 2007) and *E. coli* from gut flora (Raum *et al.*, 2008). Reinhardt *et al.* (2007) showed that increased beta-lactamase expression was responsible for some of the increase in resistance observed, although the isolates used were only from two patients. However, there is a lack of information on beta-lactamase production in chronic infections. Within-patient trends are not necessarily the same as trends at the epidemiological scale (Simpson, 1951; Pearl, 2014) and results may differ from laboratory studies, and short-term patient studies, both because of the increased spatial complexity shown in chronic infections (Kirketerp-Møller *et al.*, 2008; Winstanley *et al.*, 2016), and because of the increased length of infection, over months or years, which often comes with a complex selection pressure of multiple courses of different antibiotics.

In contrast to the increasing beta-lactamase expression that might be predicted from the studies above, social evolution theory (Ghoul *et al.*, 2014a), experimental evolution studies (Griffin *et al.*, 2004; Diggle *et al.*, 2007), and studies of social iron acquisition in the lung (Andersen *et al.*, 2015) show that exploitation by non-producing cheats can drive down population level expression of a social trait. This could manifest itself in two ways: first, there could be a decline in beta-lactamase production if non-producers gain a competitive advantage by not paying the cost of beta-lactamase production while gaining the benefit from producers. This is a "public good dilemma" scenario (West *et al.*, 2006; Frank, 2010), where non-producing cheats might go to fixation (discussed in Chapter 1). Alternatively, social dynamics could result in the coexistence of higher and lower producer lineages if the fitness of the non-producers is frequency dependent (Ross-Gillespie *et al.*, 2007). The evolution of coexisting lineages of higher and lower producers could be detectable as an increase in the variance of beta-lactamase production over time.

The extent to which social dynamics influence beta-lactamase evolution will depend on the location of the beta-lactamase – whether it remains associated with the cell (intracellular or periplasmic), or is released extracellularly (see Chapter 1). Increased public good diffusion decreases the direct fitness benefit to the producer and allows non-producing cheats to survive (Allison, 2005; Kümmerli *et al.*, 2009a). Beta-lactamase was thought to be restricted to the periplasm in Gram-negative bacteria such as *Pseudomonas* (Nikaido & Normark, 1987; Livermore, 1995), but more recent work has shown that it can be secreted outside the outer membrane (Ciofu *et al.*, 2000). Matsumura *et al.* (2000) and Giwercman *et al.* (1992) have also shown extracellular beta-lactamase activity in *P.aeruginosa*. The protective effect of cell-

associated beta-lactamase will primarily benefit the focal cell. By contrast, extracellular beta-lactamase will break down antibiotic over a wider area, protecting neighbours as well as the focal cell. The presence of extracellular beta-lactamase would make social dynamics more important in the evolution of beta-lactamase-mediated resistance. However, there has been no comprehensive study of extracellular beta-lactamase in isolates from chronic infections, and on how this may change over time.

One of the reasons such a study has not been done is the difficulty involved with longitudinal sampling of sufficient numbers of patients, over a sufficient number of years, to detect long-term patterns. The isolate collection also requires good contextual information on treatment duration and frequency. Existing studies do suggest that beta-lactamase production and resistance increase with duration of infection but this result is not reported consistently between studies. Beta-lactamase was shown to be higher after the introduction of regular antibiotic treatment in a repeated cross-sectional study of 51 to 100 patients (Ciofu *et al.*, 1994), and resistance was shown to be higher in older patients (Llanes *et al.*, 2013); beta-lactamase has also been shown to increase over the short-term, in days and weeks, after antibiotic exposure (Giwercman *et al.*, 1990; Juan *et al.*, 2005). Longitudinal bioinformatic studies of data from a larger patient population, however, suggest a more complex picture (Markussen *et al.*, 2014; Marvig *et al.*, 2015). Marvig *et al.* (2015) showed convergent evolution in ten known antibiotic resistance genes across 34 patients with cystic fibrosis. Surprisingly, none were genes known to have a direct effect on beta-lactamase production, but instead were genes involved in efflux of antibiotics, outer membrane permeability, and quinolone binding sites.

Here, I investigate the long-term evolution of beta-lactamase in a human host using isolates collected from chronic *P.aeruginosa* infections in cystic fibrosis patients. The isolate collection includes longitudinal sampling from patients. This enables me to see the change in beta-lactamase activity over time across all clone types within a patient, but also enables me to follow change in individual clone types (see Methods for definition of clone types). I directly measure beta-lactamase production using a colorimetric nitrocefin assay. I ask (i) how extracellular beta-lactamase production, including the variance of production, changes over time at the population level and how it changes within clone types; and (ii) whether these changes can be explained by the beta-lactam antibiotics given to the patients. In addition, some beta-lactam antibiotics induce higher levels of beta-lactamase production (Nordström & Sykes, 1974; Livermore & Yang, 1987; Jacoby, 2009) and this could affect the social consequences. In particular, induction has been shown to increase extracellular beta-lactamase (Matsumura *et al.*, 2000), potentially making more public good available. Therefore, I measure beta-lactamase production both in the presence and absence of an inducing antibiotic to estimate the upper and lower bounds of beta-lactamase production. For each question (i) and (ii) above, I ask how the results are affected by the presence or absence of an inducing antibiotic.

Methods

Isolate collection

My strain collection comprised 215 *Pseudomonas aeruginosa* lung isolates, collected from 17 cystic fibrosis (CF) patients. This was a subset of a larger collection of

isolates from 34 patients. The original isolates had been collected repeatedly from CF patients from the Copenhagen CF Centre at Rigshospitalet over 10 years, mostly for routine monitoring. Lung samples were from expectorate, bronchoalveolar lavage and endolaryngeal suction. All samples were examined by microscopy to evaluate the origin of the bacteria and cultured on selective and diagnostic agar plates. All bacteria considered as pathogenic were cultured and relevant antibiotic testing was performed (Hoiby & Frederiksen, 2000). In case of growth of *P. aeruginosa*, one to four isolates were then selected as representative of the bacterial flora. The *P. aeruginosa* isolates were all sequenced at BGI.

I selected the patients where at least one clone type was present in the lung for more than three years, and where there were at least seven lung samples overall – this was to ensure there were enough data points to examine trends within clone types in patients. A clone type was defined as a lineage of closely related isolates whose genomes differed by approximately 100-1000 single nucleotide polymorphisms (SNPs). Genomes between clone types differed by >10,000 SNPs (Marvig *et al.*, 2015). 33 clone types were represented, although 15 of these only

patient code	clone type	number of isolates
P0504	DK13	7
P0804	DK09	10
P1404	DK12	7
P1905	DK15 DK16	17 1
P2104	DK06 DK37	14 1
P2305	DK31	7
P2505	DK04 DK05	15 1
P2605	DK12 DK25 DK26 DK33	3 6 1 1
P3004	DK34 DK35	1 6
P3602	DK01 DK15 DK53	10 3 10
P4103	DK02 DK19 DK20	1 20 1
P4104	DK03	10
P7204	DK19 DK38 DK47 DK48 DK49	7 1 1 1 1
P7604	DK40 DK41	1 10
P8203	DK32	12
P9604	DK27 DK28 DK29 DK30	8 1 9 1
P9803	DK36	9
17 patients	**33 clone types**	**215 isolates**

Table 1. Strain collection: clone types and isolates by patient. Coloured clone types each appear in two patients.

appeared once. Of the remaining 18 which appeared multiple times, three appeared in two patients (all other clone types were present in one patient only). This means there were 21 sequentially sampled clone types within patients (Table 1).

Laboratory and knockout strains

In addition to the clinical isolates, I used some laboratory and knockout strains for comparison. I used PAO1, a wild type strain commonly used in laboratories. Derived from this I used a strain with the chromosomal AmpC beta-lactamase knocked out to be representative of a very low beta-lactamase producer (PAO1Δ*ampC*, henceforth referred to as Δ*ampC* or the *ampC* knockout) (Moya *et al.*, 2008). I also used an *ampD* knockout as a moderate constitutive producer (PAOΔ*ampD*, referred to as Δ*ampD* or the *ampD* knockout) (Juan *et al.*, 2006).

Measuring beta-lactamase activity

I measured beta-lactamase production using a standard nitrocefin assay (O'Callaghan *et al.*, 1972). I prepared cultures from freezer stock in 6ml of King's B medium (KB) and grew them overnight at 37˚C, 200rpm. 1ml of overnight culture was then diluted into 4ml of fresh KB for uninduced samples, and into 4ml of fresh KB with subinhibitory benzylpenicillin (final concentration 500mg/l) for induced samples, based on the methods of Giwercman *et al.* (1990) and Ciofu *et al.* (2000). These were then incubated for a further three hours at 37˚C, 200rpm. I then standardized cell density so that a 1 in 5 dilution of the culture in M9 had an OD of 0.1 (so the final OD in the test wells is 0.1). I centrifuged the standardized culture to obtain the supernatant in which to measure extracellular beta-lactamase levels (some culture was separated

off prior to this to be used to measure the final OD – see below). Filter-sterilization of the supernatant in a subset of six isolate cultures made no significant difference to the results and so this step was not performed (Figure 1).

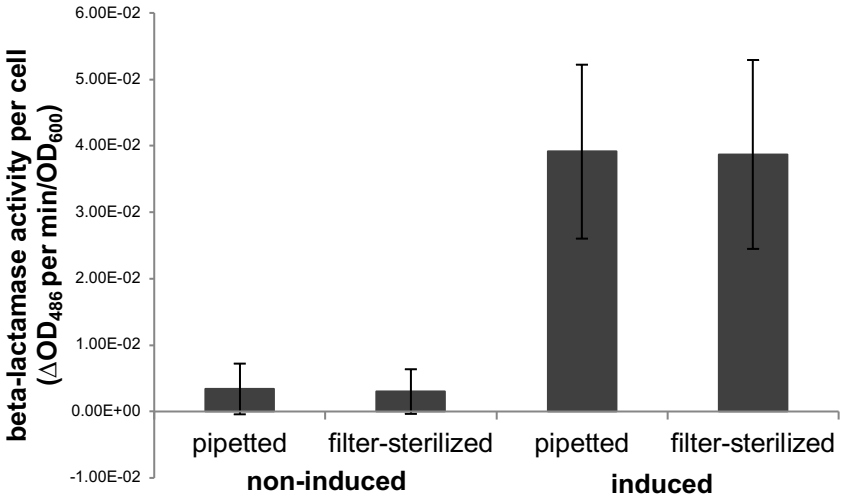


Figure 1. Filter-sterilizing the supernatant (as opposed to removing it with a pipette) makes no difference to the measurement of beta-lactamase activity, in either non-induced or induced samples. Non-induced: paired t-test, $n = 6$, $t = 1.37$, $p = 0.23$. Induced: paired *t*-test, $n = 6$, $t = 0.32$, $p = 0.76$. Error bars are 95% confidence intervals.

After preparing a 96-well plate with 100µl of 150µg/ml nitrocefin, I added 200µl of test sample, consisting of 60µl of the standardized culture or supernatant and 140µl M9, to start the reaction. The final concentration of nitrocefin was 50µg/ml, as per O'Callaghan *et al.* (1972), Giwercman *et al.* (1990) and Piriz *et al.* (2004). I then immediately transferred the test plate to the spectrophotometer and took absorbance readings at 486nm every minute for 10 minutes and then every 10 minutes up to 60 minutes. I calculated the gradient of the linear section of the reaction curve (the rate of change of absorbance) to give a quantitative measurement of beta-lactamase activity. I took the linear section to be the readings where OD_{486} is less than 0.4 (Figure 2). In a few cases a reading just above an OD_{486} of 0.4 had to be used to calculate the gradient and in two cases of very high producers the readings had to be

done separately with a shorter time interval between readings to capture the colour change. To get a 'per cell' measurement for the supernatants (where the cells have been removed and $OD_{600} \approx 0$), the gradients were divided by the mean OD_{600} of the culture divided off prior to centrifuging, which had then been diluted in the same way as the test supernatant (as this would represent the concentration of cells which would have been present, had they not been removed by centrifuging).

Four technical replicates of the nitrocefin assay were done on one day, and four on a separate day from new overnight cultures. This was to reduce error from conditions which could vary from day to day, such as the temperature in the laboratory, or the length of time to set up the 96-well plates (during which more beta-lactamase could be produced by the cells, although culture vials were refrigerated to minimize this effect).

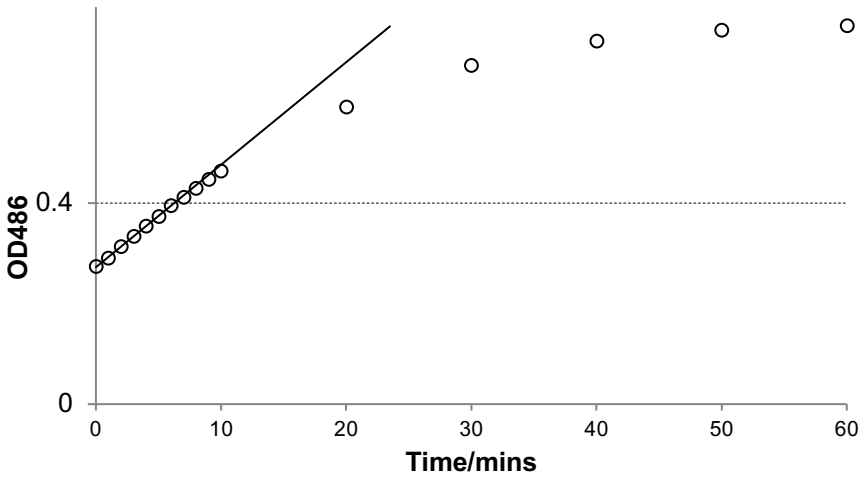


Figure 2. A typical nitrocefin reaction curve. The initial, linear section of the reaction curve gives a quantitative measurement of beta-lactamase activity. I used a cut off of absorbance of 0.4 OD_{486} to give the linear part of the curve.

Statistical methods

Beta-lactamase assay

I used linear mixed effects models to analyse the beta-lactamase data. These models have many advantages over other statistical methods because they can account for the repeated sampling of genotypes and patients. I used the data to answer two separate questions: (i) how beta-lactamase production changes with the length of time a clone type had been in the lung and (ii) how beta-lactamase production changes with the overall length of infection. I therefore analysed the data separately for these two questions as the random factors in the model are different (clone type nested within patient for (i) versus patient alone for (ii)). While this raises the issue of multiple tests on the same dependent variable, the key results still hold with a Bonferroni correction. Ultimately eight models were associated with each dataset, leading to a significance threshold of 0.05/8 = 0.00625. The key positive finding of the chapter is the significant interaction between the intensity of beta-lactam treatment and clone infecting time in explaining change in variance of induced beta-lactamase, for which the *p*-value is 0.0034, which therefore remains significant.

Linear mixed effects models assume a normal distribution of residuals and homogeneity of variance. These conditions were not met with the raw data. However, after log transformation the induced beta-lactamase results sufficiently met the assumptions of a linear model (see diagnostic plots in Supplementary Information, Figures S1-S4, pages 56-58). However, this was not the case for the non-induced beta-lactamase data. For this data, I therefore resorted to both non-parametric tests

and tests involving alternative distributions. Below, I first discuss the linear mixed effects models and then the alternative tests for the non-induced data.

For the linear mixed effects models for the induced data, I compared random intercept with random intercept-and-gradient models and used the latter if it explained the data significantly better (final models chosen can be seen in Supplementary Information, Tables S1-S3, pages 59-60. Divergence of beta-lactamase activity over time was assessed using the variance of activity, for which I took the absolute value of the residuals of a linear mixed effects model on the original (not log transformed) data, allowing random intercepts and gradients for random factors, and then ran a linear model on the log transformed residuals over time.

The non-induced data was highly skewed and did not meet the assumptions of a linear model, even after log transformation. For the change in non-induced beta-lactamase over time, I used a generalized linear mixed effects model with a binomial distribution for constitutive versus non-constitutive production, using the glmer function from the lme4 package in R. Constitutive producers were defined as isolates producing significantly more beta-lactamase (in the absence of an inducer) than the *ampD* knockout strain, which shows moderate constitutive production. While this partitioning into constitutive and non-constitutive producers loses some information, it accurately reflects the distribution of results and allows binomial analysis.

However, I was unable to use this method for the change in variance (for non-induced data) over time because of the lack of a biologically meaningful way of partitioning the variance data (unlike constitutive versus not). This data was difficult to analyse as

it violates the assumptions of many tests. After obtaining the absolute value of the residuals of a linear mixed effects model on the original (not log transformed) data as my metric of variance (as with induced data), I then used a non-parametric Kruskal-Wallis test to compare variance across time blocks. I divided the data into early, middle and late time blocks (2.42 years each for clone infecting time, 10.95 years for time since first colonization). When significant, I then used the Dunn Test with Bonferroni correction for non-parametric *post hoc* comparison between time blocks. For completeness and comparison purposes I also performed these tests on the induced data (in addition to the linear models). The Kruskal-Wallis test, though, cannot be used for interactions and so I was unable to investigate the effect of beta-lactam treatment on variance of non-induced beta-lactamase. While this test accounts for non-normality of the distribution of residuals, it is not robust to heteroscedastic data, and so as a further confirmation of results I also used a linear model with a non-normal (gamma) distribution for the non-induced variance results. This showed the same results. Interestingly, though, heteroscedasticity in the results in effect confirms the presence of an increase in variance. This observation and use of two alternative ways to analyse the data, both of which produce the same overall results, suggests validity in this approach.

Estimating length of infection

Two different estimates of 'time' were calculated. First, the *clone infecting time* of an isolate is the time that a particular *Pseudomonas aeruginosa* clone type has been in the patient's lung – i.e. it is the time from the first recorded isolate of that clone type to the sampling time of the isolate being considered. This reflects the amount of time that clone has had to adapt to its host lung. I also did our analysis using the *time since*

first colonization. This is the total length of known *Pseudomonas aeruginosa* colonization up to that point, i.e. the time from the first identified *P.aeruginosa* isolate (of any clone type) to the collection of the isolate being considered. Although sometimes the same, infecting time and time since first colonization can be different if there are different clone types of *P.aeruginosa* infecting the same patient. Also, one patient had been infected with *P.aeruginosa* for approximately 25 years before sequencing of samples began. Values for clone infecting time are therefore likely to be artificially small, as it is likely the same clone types were present for at least some of the preceding unsequenced years. All the isolates sampled after 25 years since first colonization (visible clearly in Figures 7, 8, 11 and 12) come from this one patient. I used time since first colonization when analysing patterns across all lineages within a patient, rather than patterns just within clone type.

Effect of treatment by beta-lactam antibiotics

Treatment by beta-lactam antibiotics was quantified in two ways for each patient: i) total beta-lactam duration, which was the total number of weeks of beta-lactam treatment a patient received during the time period of study (i.e. from the first collected isolate to the last); and ii) intensity of beta-lactam treatment, which was the total beta-lactam duration divided by the time period of study in years (which varied between patients). Beta-lactam duration and beta-lactam intensity were then added as interaction terms into the linear models of beta-lactamase activity or variance of beta-lactamase activity discussed above.

High and low producers

When identifying co-infecting high and low producers, I defined a high producer as one which produces more than the median induced level of beta-lactamase and a

coexisting low producer as one which was sampled from the same patient on the same day and which produces less than half the beta-lactamase of the high producer.

Borderline significance

In two instances I refer to 'borderline' results. In these cases the *p*-values are between 0.05 and 0.055, very close to the 'conventional' significance of $p < 0.05$. While categorizing results as significant or not can be useful for clarity, recent writing has criticized the arbitrary nature of *p*-values (Nuzzo, 2014; Hector, 2015; Wasserstein & Lazar, 2016). While making it very clear these results were borderline, I chose to report these particular borderline results as they are of interest in the context of theory and other results. The results must be treated with caution (as should a result with a *p*-value just under 0.05 as well), and are simply used as a point of discussion, and do not represent 'key' results. Ultimately, the statistical power of this research was limited by the number of clinical samples available and consideration of borderline results can help shape future research (Dorey, 2011).

Statistical analysis was done in R (www.r-project.org), using the lme4 package for the mixed effects models.

Results

Extracellular beta-lactamase in the presence and absence of an inducing antibiotic

In the presence of an inducing antibiotic, isolates produce nine times more extracellular beta-lactamase per cell on average than in their non-induced state (paired *t*-test, $t = 12.05$, $df = 214$, mean difference 0.237, $p < 2.2\times10^{-16}$, $n = 215$ isolates,

Figure 3). 53% of isolates (n = 215) produce significantly more beta-lactamase than induced PAO1. 10% produce more than ten times the beta-lactamase of PAO1, with the highest producer having a beta-lactamase activity 37 times higher than induced PAO1 (see Figure 4B for distribution). 97% of induced isolates produced more than double the extracellular beta-lactamase of non-induced Δ*ampD* (a moderate constitutive producer).

Most non-induced isolates produce negligible extracellular beta-lactamase (Figure 3): in the absence of an inducing antibiotic, there is no detectable difference in extracellular beta-lactamase production between 70% (n = 215) of clinical isolates and an *ampC* beta-lactamase knockout strain. However, 10% (n = 215) of isolates act as constitutive producers, producing significantly more extracellular beta-lactamase than the *ampD* knockout strain (from 1.45 to 410 times higher). They form the long tail of the highly right-skewed distribution (Figure 4A). Only these constitutive producers have any notable extracellular activity without induction.

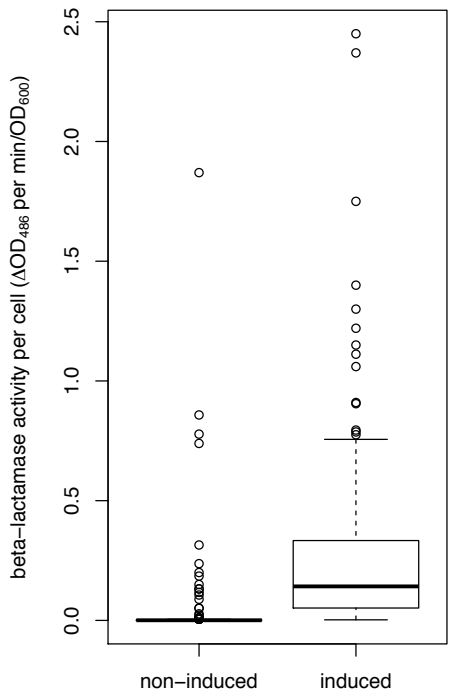


Figure 3. Effect of an inducing antibiotic on extracellular beta-lactamase. Exposure to the inducing antibiotic benzylpenicillin for three hours increases extracellular activity nine-fold (paired t-test, t = 12.05, df = 214, mean difference 0.237, $p < 2.2\times10^{-16}$).

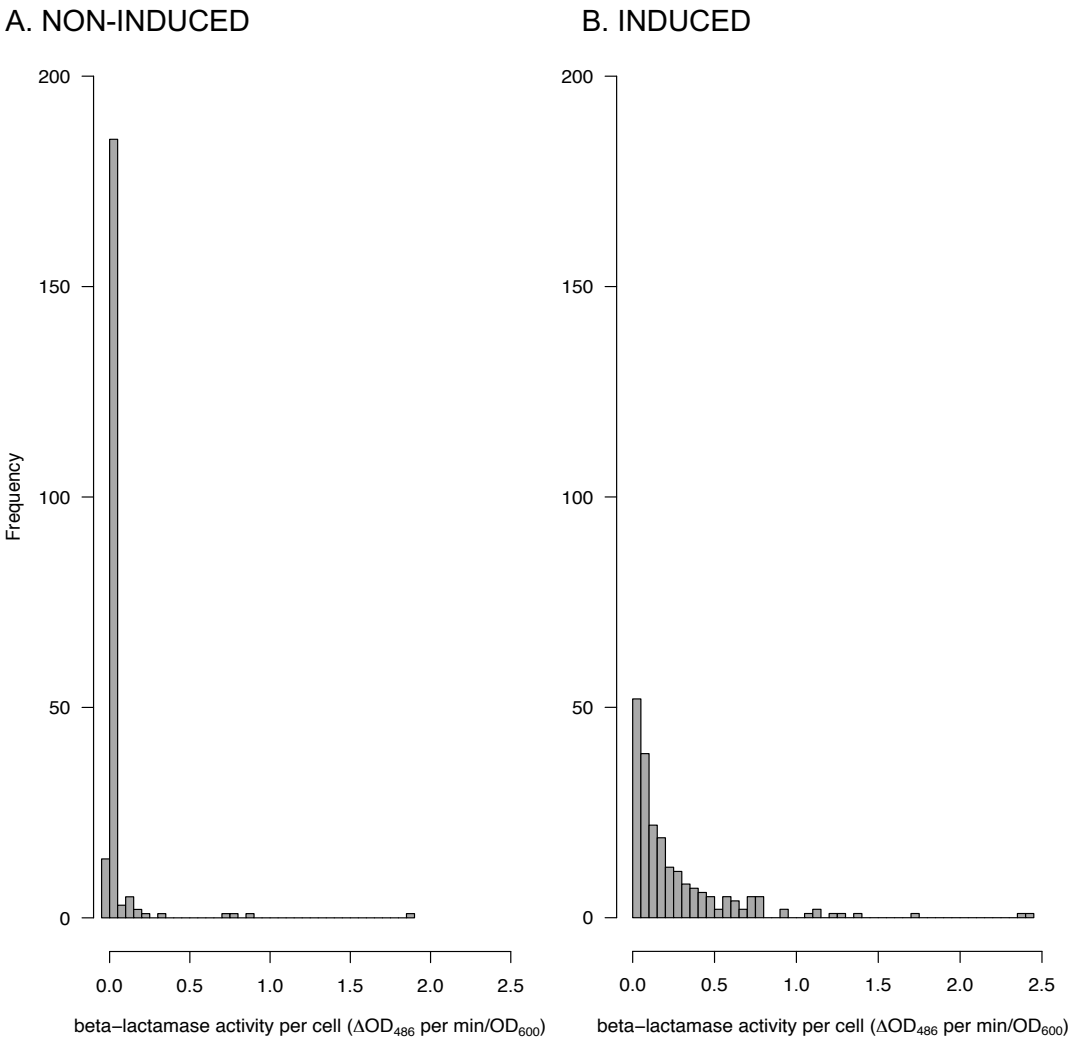


Figure 4. Distribution of extracellular beta-lactamase activity per cell in (A) non-induced and (B) induced states. In the basal, non-induced state, extracellular beta-lactamase production is generally very low with a few constitutive producers, giving a heavily right-skewed distribution. The presence of an inducing antibiotic increases beta-lactamase production in most isolates, reducing but by no means eliminating the skew. The small number of negative beta-lactamase values in the non-induced state are an artefact of the assay, representing very low values close to zero.

Beta-lactamase dynamics within clone-type

Analysis treating clone-type as independent replicates, showed that only non-induced beta-lactamase production increased with clone infecting time, in terms of increased numbers of constitutive producers when clone types had been in the lung for longer (p = 0.025, n = 215, Figures 5 and S5). Even when constitutive producers are removed from the analysis there is an increase in beta-lactamase ($F_{1, 184}$ = 9.68, p = 0.0022, n =

193 isolates), although the average increase amongst these isolates is only two-fold over the seven years (10% per year). In contrast, levels of induced beta-lactamase did not increase over time ($F_{1, 203} = 1.50$, $p = 0.222$, $n = 215$ isolates, Figure 6).

When I included more detailed information on antibiotic treatment duration and intensity, there was still no evidence of selection for beta-lactamase over time in relation to this treatment. There was no significant interaction between clone infecting time and either total duration of beta-lactam courses, or intensity of beta-lactam treatment (average beta-lactam antibiotics per year), in explaining levels of induced beta-lactamase (intensity: $p = 0.142$, $n = 215$ isolates; duration: $p = 0.057$, $n = 215$ isolates). It was not possible to determine an effect on levels of non-induced (constitutive) production as the models failed to converge.

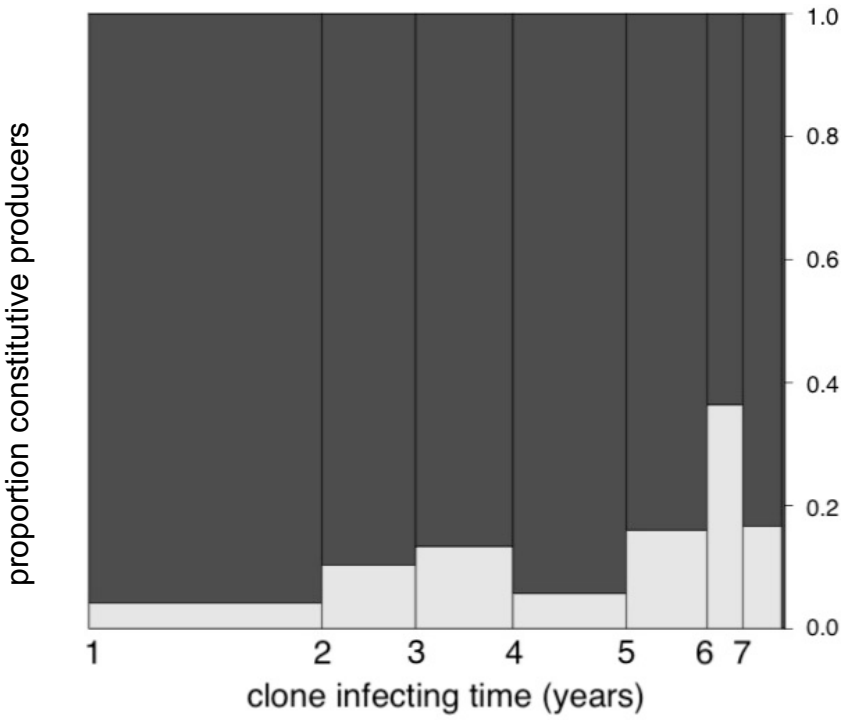


Figure 5. The proportion of constitutive beta-lactamase producers increases with clone infecting time ($p = 0.025$, $n = 215$). Light grey represents constitutive producers as a proportion of the isolates in each time block. Block 1 is the first year (i.e. 0-1 years), block 2 the second year (1-2 years) etc. The total size of each block is proportional to the total number of isolates in the time block. For raw data, please see Supplementary Information, Figure S5.

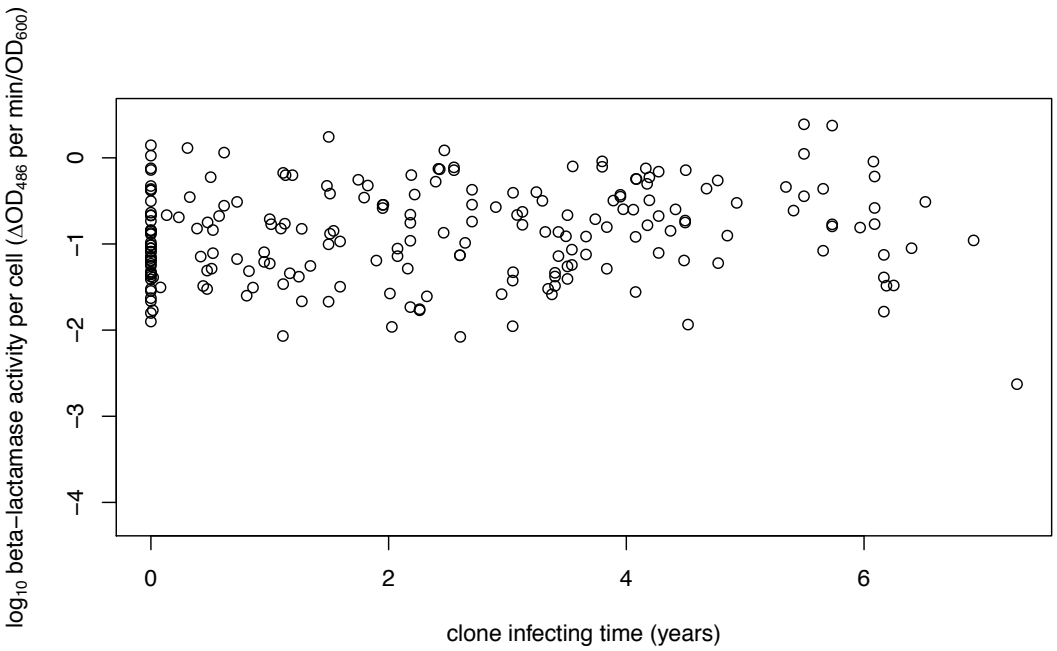


Figure 6. Induced extracellular beta-lactamase activity does not change with clone infecting time ($F_{1, 203} = 1.50$, $p = 0.222$). Each point represents the $\log_{10}$ of the beta-lactamase production of one isolate (n = 215) from 17 different patients who were longitudinally sampled, covering 33 different clone types. For raw data, please see Supplementary Information, Figure S6.

Beta-lactamase dynamics within patient

Analysis treating patient infection as an independent replicate revealed patterns more clearly consistent with selection favouring increased beta-lactamase over time in the lung environment. Induced extracellular beta-lactamase activity within patients (across clone-types) increased on average with time since first colonization ($F_{1, 47.6} = 4.91$, $p = 0.0315$, n = 215 isolates, Figures 7 and S7). I found an annual increase of 5% in induced beta-lactamase production, such that isolates produced 5.5 times more beta-lactamase after 33 years of infection, compared to when they were first sampled. Patient P3602 had been infected for approximately 25 years longer than the other 16 patients; however, the increase in beta-lactamase was still significant after removing this patient from the analysis ($F_{1, 189.8} = 5.75$, $df = 189$, $p = 0.0174$, n = 192 isolates). Non-induced beta-lactamase also increased in terms of numbers of constitutive

producers, (p = 0.018, n = 215 isolates, Figure 8 and S8). This holds even when patient P3602 is removed from the analysis (p = 0.0064, n = 192 isolates). When constitutive producers are removed from the analysis there is a small increase in beta-lactamase of 5% per year ($F_{1, 39}$ = 7.86, p = 0.0078, n = 193 isolates). Constitutive producers were not observed until four years after colonization. Again, neither the total duration of beta-lactam courses, nor the intensity of beta-lactam selection (beta-lactam duration per year) added any explanatory power to the model for induced or non-induced beta-lactamase production with time since first colonization (induced beta-lactamase: effect of beta-lactam duration, p = 0.33; effect beta-lactam intensity, p = 0.66; non-induced beta-lactamase: effect of beta-lactam duration, model failed to converge; effect beta-lactam intensity, p = 0.39).

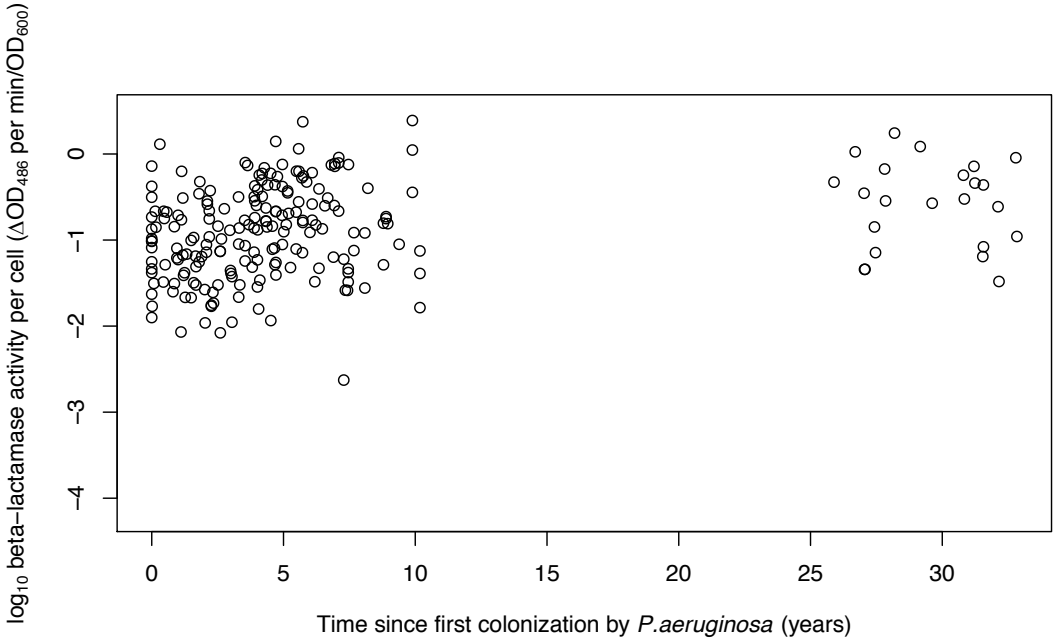


Figure 7. Induced extracellular beta-lactamase activity increases with time since first colonization by any clone type ($F_{1, 47.6}$ = 4.91, p = 0.0315). Each point represents the $\log_{10}$ of the beta-lactamase production of one isolate (n = 215) from 17 different patients who were longitudinally sampled, covering 33 different clone types. The isolates collected around 30 years all come from one patient (see Methods). For raw data, please see Supplementary Information, Figure S7.

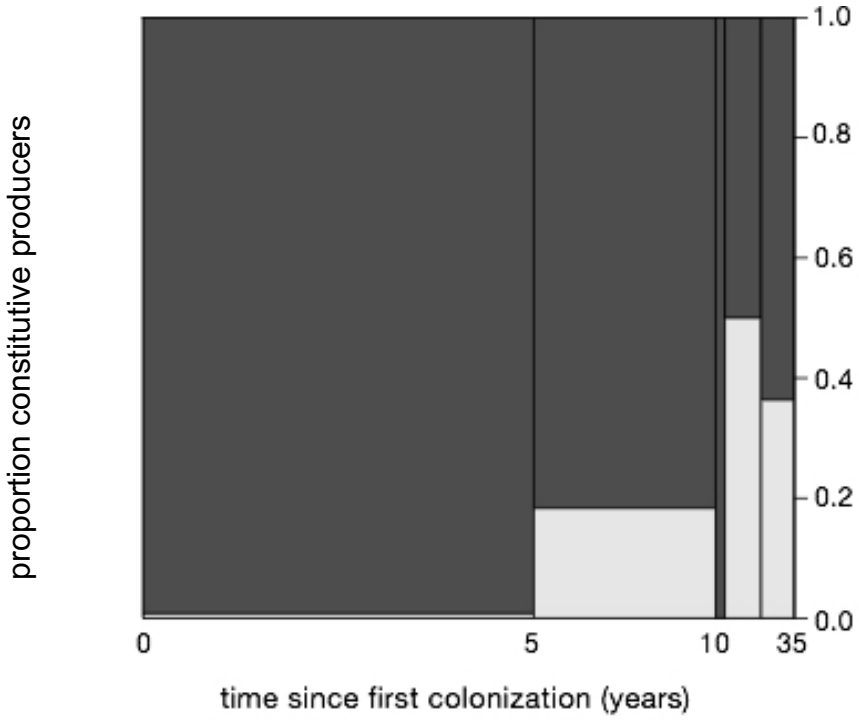


Figure 8. The proportion of constitutive beta-lactamase producers increases with time since first colonization (p = 0.018, n = 215). Light grey represents constitutive producers as a proportion of the isolates in each time block. The total size of each block is proportional to the total number of isolates in the time block. For raw data, please see Supplementary Information, Figure S8.

Variance of beta-lactamase activity over time[1]

Variance in beta-lactamase production increased over time. This was found in all circumstances (induced and non-induced, with clone infecting time and time since first colonization) when comparing blocks of early and late isolates (see Table 2 for statistics; Figures 9-11). This was confirmed in linear models on the variance for induced beta-lactamase, albeit with only borderline significance for induced beta-lactamase with clone infecting time ($F_{1,\ 213}$ = 3.76, p = 0.0537, n = 215 isolates, Figure 12; with time since first colonization: $F_{1,\ 213}$ = 15.5, p = 0.00011, n = 215 isolates, Figure 13). Linear models were not done on non-induced variance of beta-lactamase because the data remained non-normal after log transformation (although analysis with a non-normal (gamma) distribution confirmed significant increases in

[1] For updated analysis, please see Ghoul & Crosse *et al.* (manuscript in preparation). Social mechanisms of antibiotic resistance and long-term evolution of resistance in *Pseudomonas aeruginosa* infections.

variance of non-induced beta-lactamase: clone infecting time $t = 2.2$, $p = 0.026$; time since first colonization $t = 5.6$, $p = 7.5\times10^{-8}$, $n = 215$. See also Supplementary Information Figures S9-S10 for figures of raw data).

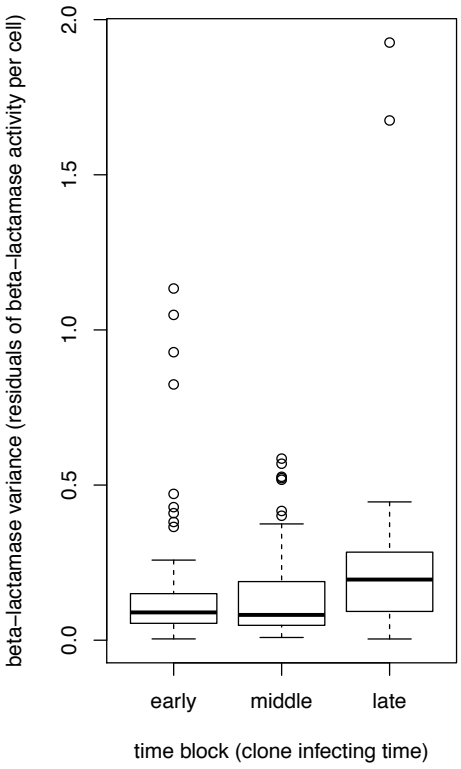
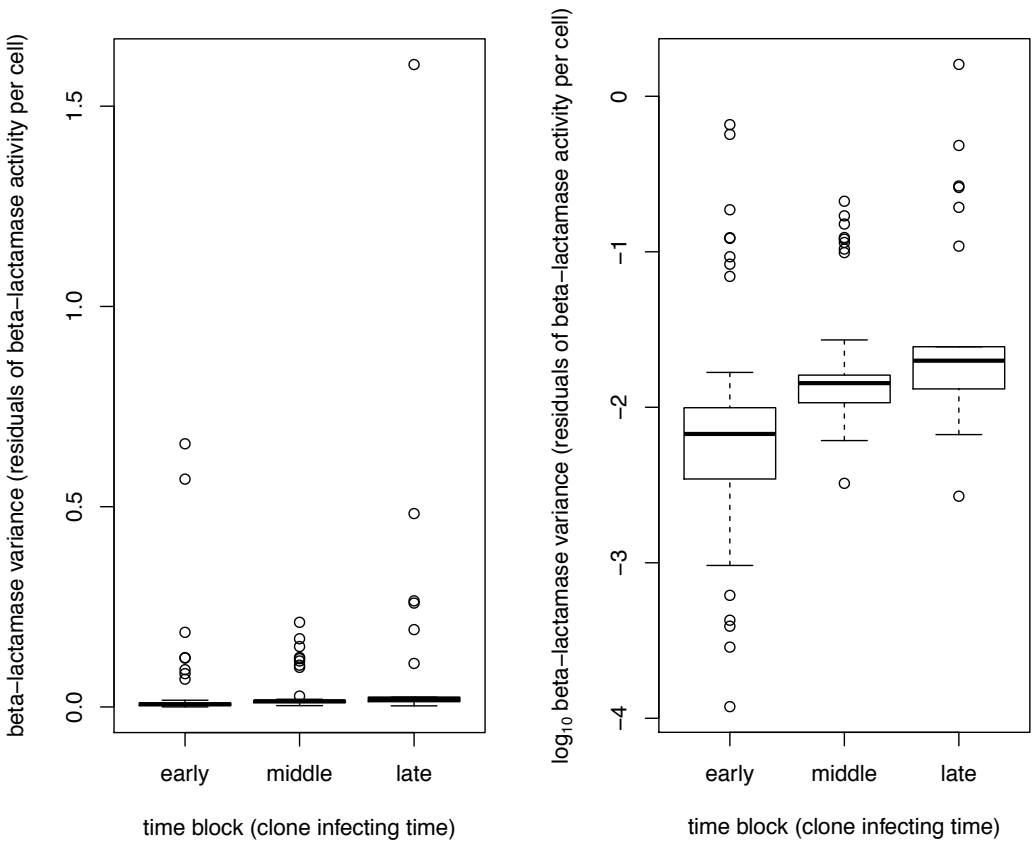


Figure 9. Variance of induced beta-lactamase increases with clone infecting time. Variance is higher in the late time block than the early ($p = 0.0067$) and middle ($p = 0.021$) time blocks, $n = 215$ isolates from 17 patients. See Table 2 for full statistics. Each time block is 2.42 years.



A. Raw data

B. Logged data
(presented for visualization only)

Figure 10. Variance of non-induced beta-lactamase increases with clone infecting time. Variance is higher in the late and middle time blocks than the early time block (early:late $p = 2.3\times10^{-11}$; early:middle $p = 8.9\times10^{-14}$) , $n = 215$ isolates from 17 patients. See Table 2 for full statistics. Each time block is 2.42 years. See Figure S10 for raw data.

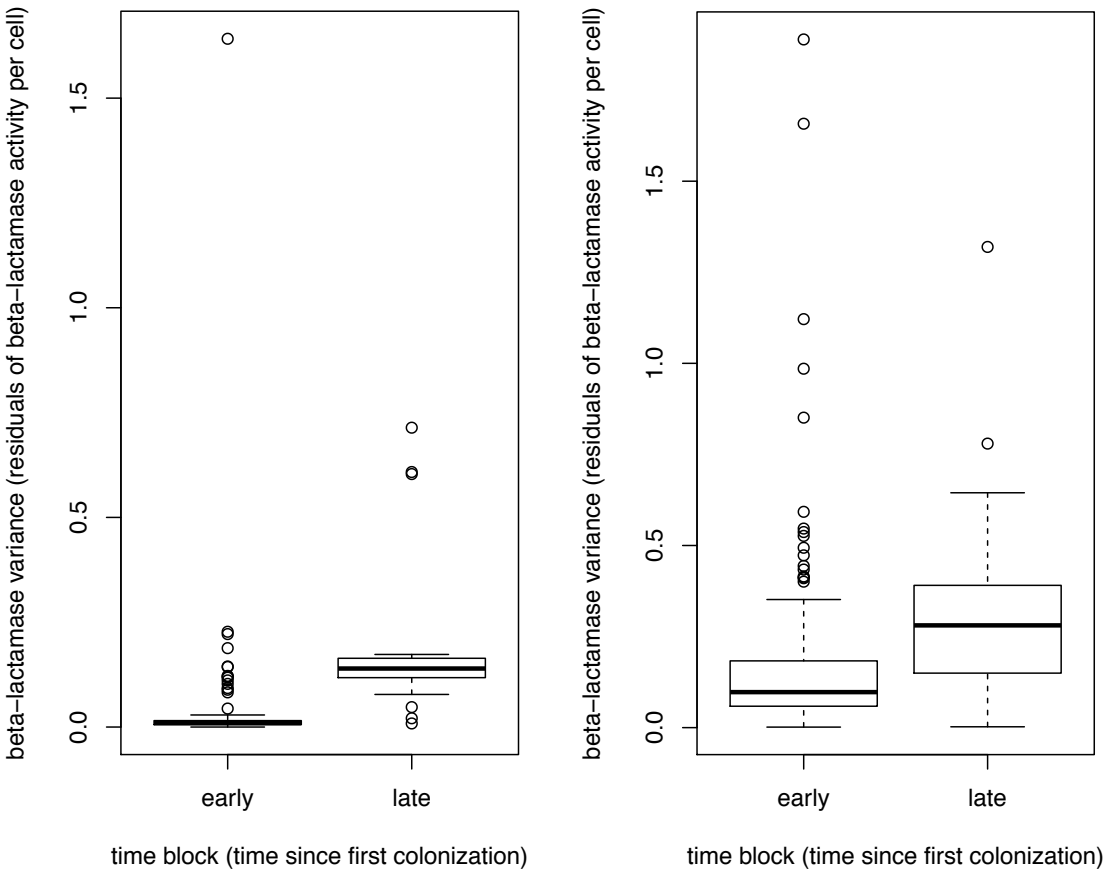


Figure 11. Variance of non-induced and induced beta-lactamase increases with time since first colonization. Non-induced ($p = 4.3\times10^{-12}$), induced ($p = 0.00015$), $n = 215$ isolates from 17 patients. See Table 2 for full statistics. Each time block is 11 years; the middle block contained no isolates. This was due to patient P3602 having been infected 25 years longer than any other patient (see Figure 15). All late isolates are therefore from this patient. However, the effect remains significant if patient P3602 is removed from the analysis before calculating the variance and dividing into time blocks (results not shown). See Figure S11 and S12 for raw data.

		χ	*df*	*p*
variance of induced beta-lactamase activity	**clone infecting time**	9.6	2	0.0083

		z	*p*	*p* adjusted (Bonferroni)
post hoc pairwise comparisons between time blocks	early-late	-3.1	0.0022	0.0067
	early-mid	-0.33	0.74	1
	late-mid	2.7	0.0070	0.021

		χ	*df*	*p*
variance of induced beta-lactamase activity	**time since first colonization**	14.4	1	0.00015

		z	*p*	*p* adjusted (Bonferroni)
post hoc pairwise comparisons between time blocks	early-late	-3.8	0.00015	0.00015

		χ	*df*	*p*
variance of non-induced beta-lactamase activity	**clone infecting time**	84.0	2	$<2.2\times10^{-16}$

		z	*p*	*p* adjusted (Bonferroni)
post hoc pairwise comparisons between time blocks	early-late	-6.8	7.5×10^{-12}	2.3×10^{-11}
	early-mid	-7.6	3.0×10^{-14}	8.9×10^{-14}
	late-mid	1.6	0.11	0.32

		χ	*df*	*p*
variance of non-induced beta-lactamase activity	**time since first colonization**	48.0	1	4.3×10^{-12}

		z	*p*	*p* adjusted (Bonferroni)
post hoc pairwise comparisons between time blocks	early-late	-6.9	4.3×10^{-12}	4.3×10^{-12}

Table 2. Effect of time on variance of beta-lactamase production. Kruskal-Wallis on differences between time blocks, with *post hoc* Dunn Test on pairwise comparisons between blocks. When considering the time since first colonization, no isolates fall in the middle time block. This is due to one patient having been infected 25 years longer than all other patients (see Figure 15). While this therefore does not make a good comparison (as all the 'late' isolates are from one patient), the results remain significant for time since first colonization if patient P3602 is removed from the analysis before calculating the variance.

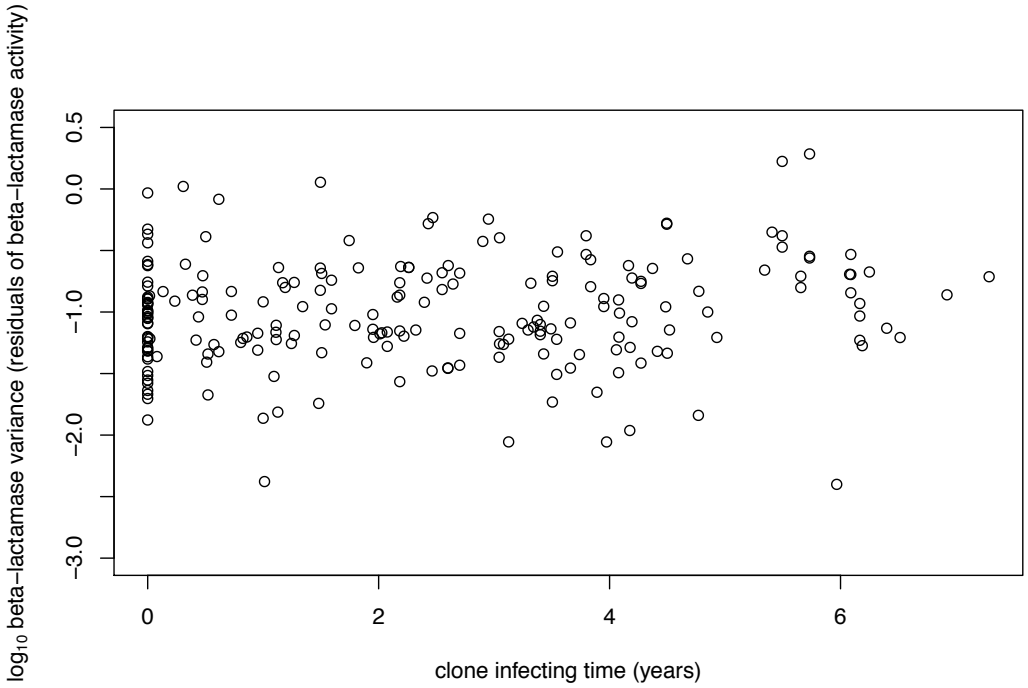


Figure 12. The increase in variance of induced extracellular beta-lactamase activity with clone infecting time approaches significance ($F_{1,\ 213}$ = 3.76, p = 0.0537). Each point represents the $\log_{10}$ of an isolate's residual beta-lactamase activity not explained by a linear model. The similarity with Figure 6 is because the original model does not explain the data well, resulting in large residuals. n = 215, from 17 different patients who were longitudinally sampled, covering 33 different clone types. See Figure S9 for raw (unlogged) data.

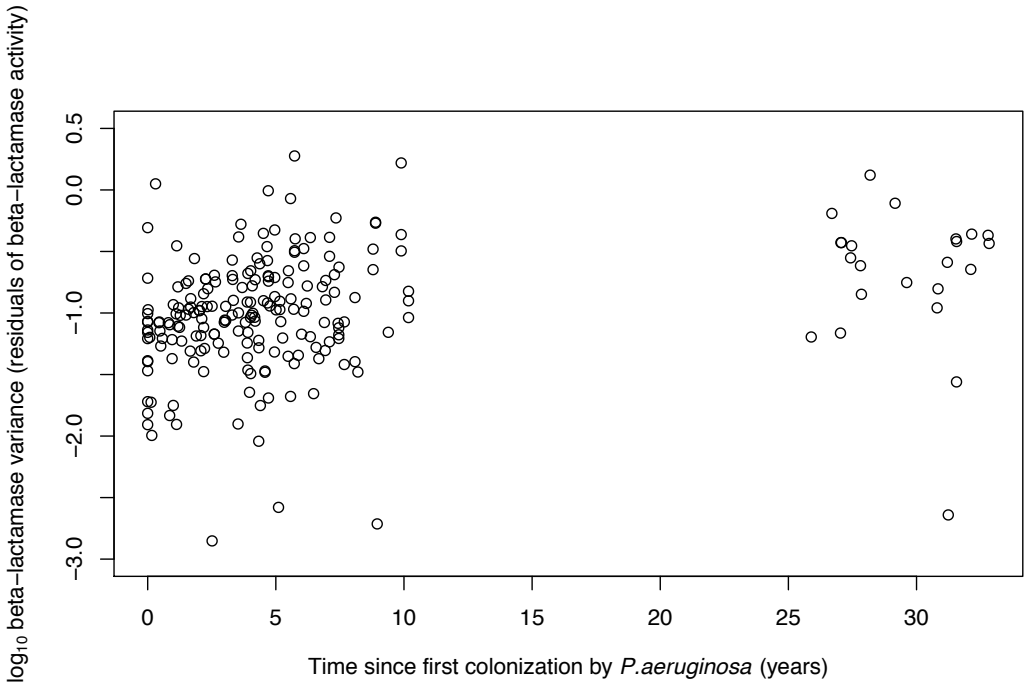


Figure 13. The variance of induced extracellular beta-lactamase activity increases with time since first colonization by any clone type ($F_{1,\ 213}$ = 15.5, p = 0.00011). Each point represents the $\log_{10}$ of an isolate's residual beta-lactamase activity not explained by a linear model. The similarity with Figure 7 is because the original model does not explain the data well, resulting in large residuals. n = 215, from 17 different patients who were longitudinally sampled, covering 33 different clone types. The isolates collected around 30 years all come from one patient (see Methods). See Figure S12 for raw (unlogged) data.

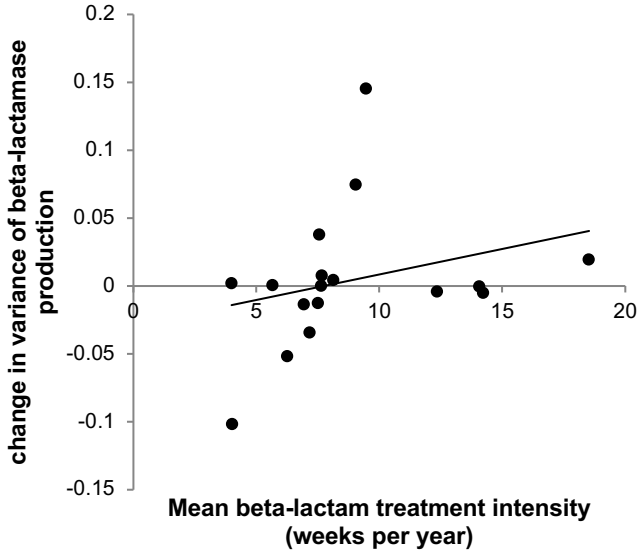


Figure 14. Interaction between beta-lactam treatment intensity and the change in variance of induced beta-lactamase production. Each point represents one patient. The x-axis is the mean beta-lactam treatment intensity that the patient received; the y-axis is the gradient of the change in beta-lactamase variance for that patient. The higher the intensity of beta-lactam treatment, the higher the increase in beta-lactamase variance in that patient. Statistics were done on the full dataset (interaction: t = 3.0, p = 0.0034, n = 215) rather than on the condensed data for the figure.

Within clone-types, treatment intensity was found to significantly increase variance of induced beta-lactamase activity with clone infecting time (t = 3.0, p = 0.0034, n = 215 isolates, Figure 14). After removing this effect of antibiotics, the variance of induced beta-lactamase significantly decreased over time (t = -2.1, p = 0.036, n = 215 isolates). Within patients, there was no interaction between treatment intensity and time since first colonization in explaining the variance of induced beta-lactamase activity, although the result was of borderline significance (t = 2.0, p = 0.0507). The total duration of beta-lactam treatment (as opposed to intensity of treatment) did not interact with either clone infecting time (t =1.8, p = 0.07, n = 215) or time since first colonization (t =0.8, p = 0.42, n = 215) to explain induced beta-lactamase variance in any instance. Interaction with antibiotics was not was not investigated with variance

of non-induced beta-lactamase as the data violated the assumptions of linear regression.

Coexistence of high and low producing isolates

In the presence of an inducer, in nine out of 17 patients at least one example of coexistence between high and low producers was observed (for examples, see patients P4103, P4104 and P8203, Figure 15). Under non-induced conditions only two of 17 patients had coexisting high and low producers. These were constitutive high producers in patients P3602 and P8203 (Figure 16).

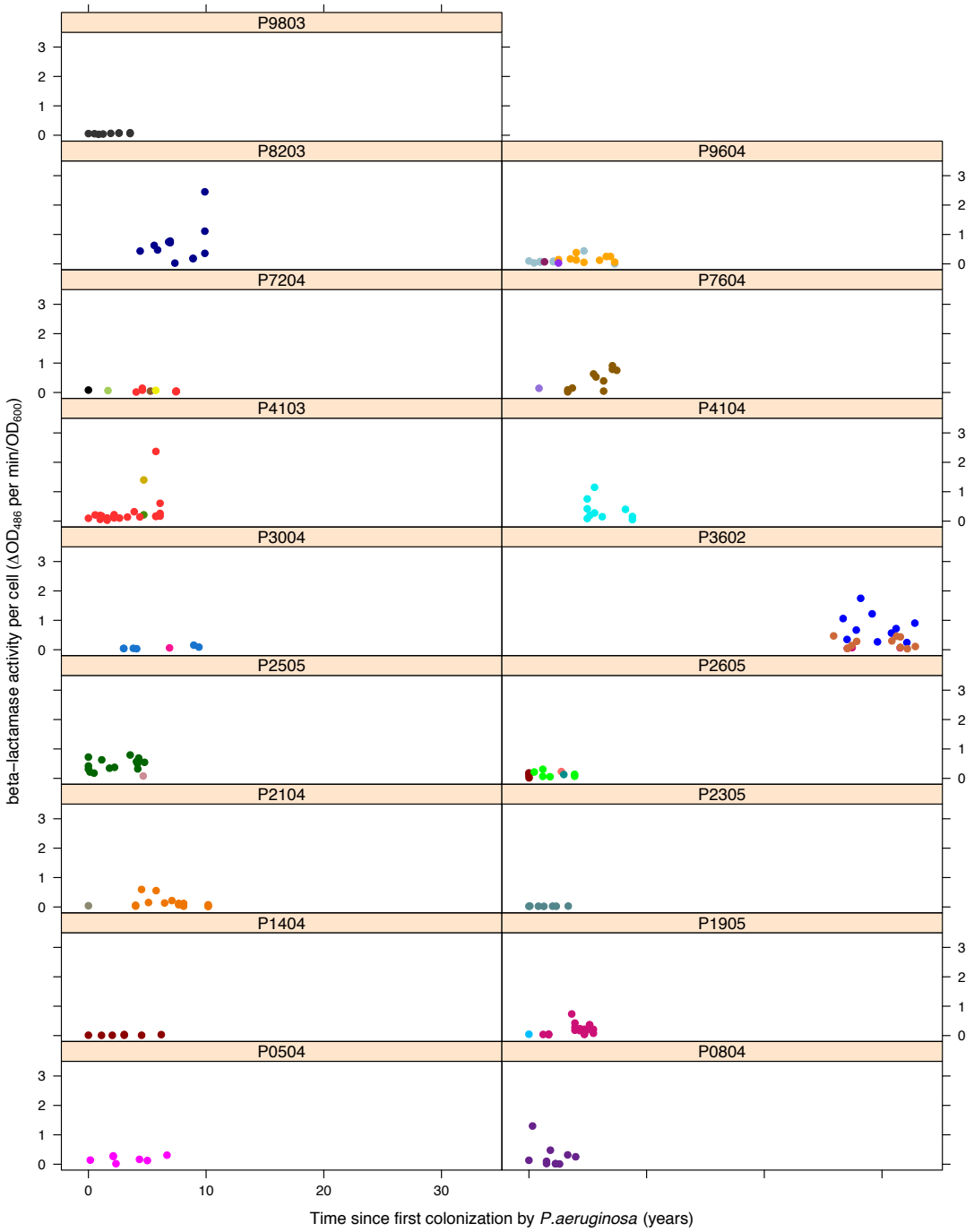


Figure 15. Induced extracellular beta-lactamase production over time since first colonization by *Pseudomonas aeruginosa* (of any clone type) for individual patients (P numbers are the patient reference numbers). Colours represent different clone types. When no isolate appears at time zero, this is because routine sampling and genotyping had not commenced in that patient, though *P.aeruginosa* infection had been identified at time zero. Nine patients (P1905, P2505, P2605, P3602, P4103, P4104, P7604, P8203 and P9604) out of 17 had at least one example of coexisting high and low producers.

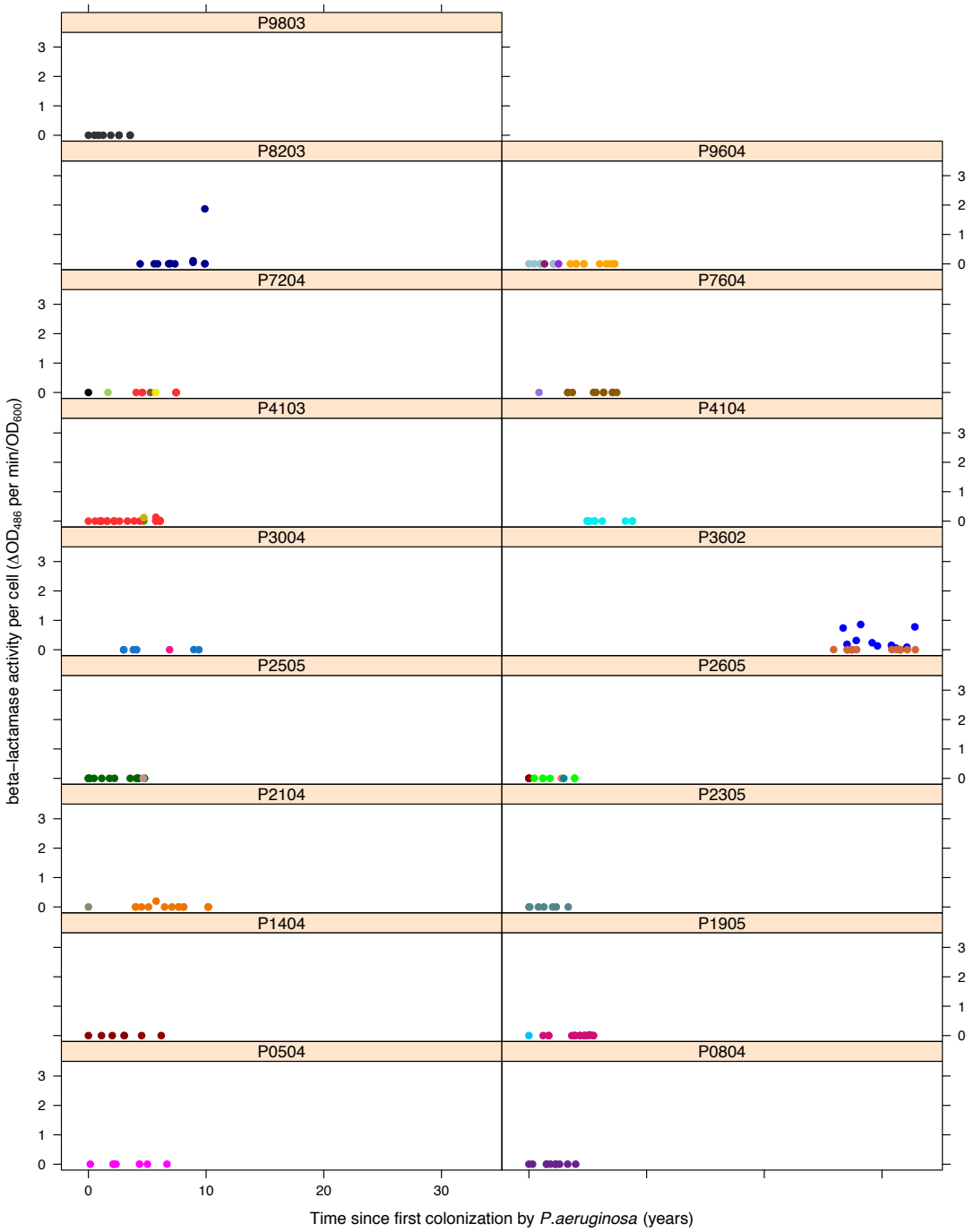


Figure 16. Non-induced extracellular beta-lactamase production over time since first colonization by *Pseudomonas aeruginosa* (of any clone type) for individual patients (P numbers are the patient reference numbers). Colours represent different clone types. When no isolate appears at time zero, this is because routine sampling and genotyping had not commenced in that patient, though *P.aeruginosa* infection had been identified at time zero. There are coexisting high and low producers in patients P3602 and P8203 only.

A. Overall trends

	Clone infecting time		Time since first colonization	
	mean	variance	mean	variance
Induced		↑	↑	↑
Non-induced	↑	↑	↑	↑

B. Trends with beta-lactam duration

	Clone infecting time		Time since first colonization	
	mean	**variance**	**mean**	**variance**
Induced *Effect of beta-lactam duration:*				
Non-induced *Effect of beta-lactam duration:*	(interaction not tested due to model limitations)			

C. Trends with beta-lactam intensity

	Clone infecting time		Time since first colonization	
	mean	**variance**	**mean**	**variance**
Induced *Effect of beta-lactam intensity:*		↓ ↑		↑
Non-induced *Effect of beta-lactam intensity:*	(interaction not tested due to model limitations)			

Table 3. Results summary. Arrows indicate direction of trend. Grey arrows indicate results of borderline significance ($p > 0.05, < 0.06$). Blank boxes indicate the model was not significant.

Discussion

Despite being exposed to prolonged and intensive antibiotic treatment regimes, bacterial cells isolated from the lungs of cystic fibrosis patients showed little or no increase in beta-lactamase production with the time spent in the lung (Figures 6 and S5). An exception is that there were more constitutive producers later in infection, although overall these only make up 10% of isolates in the study (Figure 5). Instead,

there is a clearer pattern of divergence in production levels, indicated by an increase in variance over time (Figures 9-13). This result might be thought to be the result of weak selection on beta-lactamase production despite intensive treatment; however, the pattern in induced isolates correlated with antibiotic treatment (Figure 14), and further, when the effect of antibiotics was removed from the analysis, the opposite pattern of variance reduction over time was observed. This suggests that antibiotic treatment may be selecting for both higher and lower producers of beta-lactamase, and I found high and low producers sampled from the same lungs at the same time (Figures 15 and 16). Taken together, these results suggest that beta-lactamase production is not simply responding to selection from antibiotics to increase and conditions exist which potentially would allow social interactions between higher and lower producers (all results summarized in Table 3).

Beta-lactamase patterns within clone type

Within clone types, there was a lack of evidence for positive selection on beta-lactamase production, but evidence for a divergence in beta-lactamase production over time. There was no increase in induced beta-lactamase levels over time. Though there was an increase in non-induced beta-lactamase, particularly with the emergence of constitutive producers, in general production remains low with 70% of isolates producing negligible extracellular beta-lactamase in the absence of an inducing antibiotic. There was, however, an increase in the variance of induced beta-lactamase activity with time in the lung, showing that beta-lactamase production diverges. Furthermore, there was a positive interaction with the intensity of beta-lactam antibiotic treatment, suggesting antibiotic treatment is selecting for this divergence, and indeed the model suggests variance would decrease if antibiotics were not given.

One possible explanation for all these results is the emergence of cooperator-cheat dynamics. Although antibiotics may be selecting for higher public good production, the increased cost of this cooperation drives selection to cheat, since non-production (or low production) gains a fitness advantage over producers (Jiricny *et al.*, 2010; Andersen *et al.*, 2018). On average there is, therefore, no increase in beta-lactamase production, while there is a divergence. Overall these results appear inconsistent with selection simply for increased resistance, and more consistent with cooperator-cheat dynamics.

Beta-lactamase patterns within patient

Beta-lactamase patterns within patients over time since first colonization (considering all genotypes together) are broadly similar to those with clone infecting time (see Table 3), in part because normally one clone type dominates in a lung. Importantly there is still a divergence in beta-lactamase production with time, though the association of higher treatment intensity with a higher divergence in induced beta-lactamase levels was not quite significant. Another difference is that induced beta-lactamase across all isolates increases, when there was no significant increase within clone types. Whilst this could imply that later infecting isolates have higher induced beta-lactamase activities when they first infect, there was no evidence for this as there is no significant difference between the initial beta-lactamase activities of clone types present at first colonization, and those which infect a year or more later (unpaired *t*-test, $p = 0.50$). It could reflect the larger number of data points available when all clone types within a patient are considered together.

Evidence for social interactions

In addition to the patterns of beta-lactamase production over time, discussed above, which are consistent with social dynamics, I show that there is both extracellular beta-lactamase activity and also coexistence of higher and lower beta-lactamase producers – both features which are consistent with social evolutionary dynamics. Despite evidence to the contrary (Ciofu *et al.*, 2000; Matsumura *et al.*, 2000), it is still the prevailing opinion in books (e.g. Bax & Griffin, 2012) and reviews (e.g. Iredell *et al.*, 2016) that beta-lactamase in Gram-negative bacteria is periplasmic. This study shows considerable extracellular beta-lactamase activity in clinical *P. aeruginosa* isolates – particularly in the presence of an inducing antibiotic, but also in a subset of isolates even in the absence of an inducing antibiotic. This gives the potential for beta-lactamase to act as a public good involved in a social mechanism of antibiotic resistance. Furthermore, coexistence of high and low producers was found in nine out of 17 patients under induced conditions, although it was found in only two patients under non-induced conditions. Given my strict definition of 'coexistence' referring to the limited number of isolates sampled from the same patient on the same day, it is likely that this is an underestimate of coexistence. The demonstration of coexistence shows that conditions potentially exist for social interactions between higher and lower producers.

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While the above results are consistent with the involvement of social dynamics in the evolution of beta-lactamase-mediated resistance, there are a number of alternative explanations. Regional isolation is an alternative cause of the divergence in beta-lactamase production over time. Jorth *et al.* (2015) showed that strains from one initial clone type end up inhabiting different regions of the cystic fibrosis lung and

evolve in isolation. Not only does this mean that seemingly 'coexisting' isolates may not actually be socially interacting due to spatial separation, it also provides an alternative cause of the divergence in beta-lactamase production over time. Since cystic fibrosis lungs are known to have perfusion defects (Amaxopoulou *et al.*, 2017), different isolates could have been exposed to different levels of antibiotic. Equally, slow growing bacteria – such as those growing anaerobically deep in the biofilm – are under less antibiotic selection pressure since beta-lactams only kill dividing cells (Bagge *et al.*, 2004b). Differing antibiotic selection pressure could result in spatially separated high and low producers in lungs and could explain the increase in variance of beta-lactamase production over time. However, if this were the case, an increase in variance of resistance should be seen across all antibiotic classes, regardless of the sociality of the resistance mechanism. This provides a useful test for sociality and is examined further in Chapter 3.

More fine-tuned analysis of the antibiotic treatment regimes would potentially help to clarify the evolutionary processes described in my results. First, in my analysis I did not distinguish between different types of beta-lactam antibiotic, such as inducing versus non-inducing antibiotics, or those prescribed with and without beta-lactamase inhibitors. Second, patients treated with more beta-lactam antibiotics may also be prescribed more antibiotics of other classes, which could select for broader spectrum resistance mechanisms (Vestergaard *et al.*, 2016) in preference to beta-lactamase, hence making a relationship between beta-lactam treatment and beta-lactamase less clear. However, explanations based on possible switching to alternative resistance mechanisms would not explain the effect of beta-lactam treatment on the variance of

beta-lactamase activity, supporting social dynamics as the explanation for this key finding.

Concluding remarks

The results present a complex picture suggestive of a role for social dynamics in beta-lactamase production. It is unclear how this affects antibiotic resistance because beta-lactamase is not the only resistance mechanism: we do not know how other resistance mechanisms may change over time, perhaps compensating for changes in beta-lactamase. It is also unclear if cheating can actually occur when beta-lactamase producers and non-producers are in competition. The next three chapters first relate beta-lactamase production to resistance, and then examine the costs and benefits of beta-lactamase production to both producers and non-producers exposed to beta-lactam antibiotics, to show whether social cheating can or cannot occur.

Supplementary information

Diagnostic plots for linear models

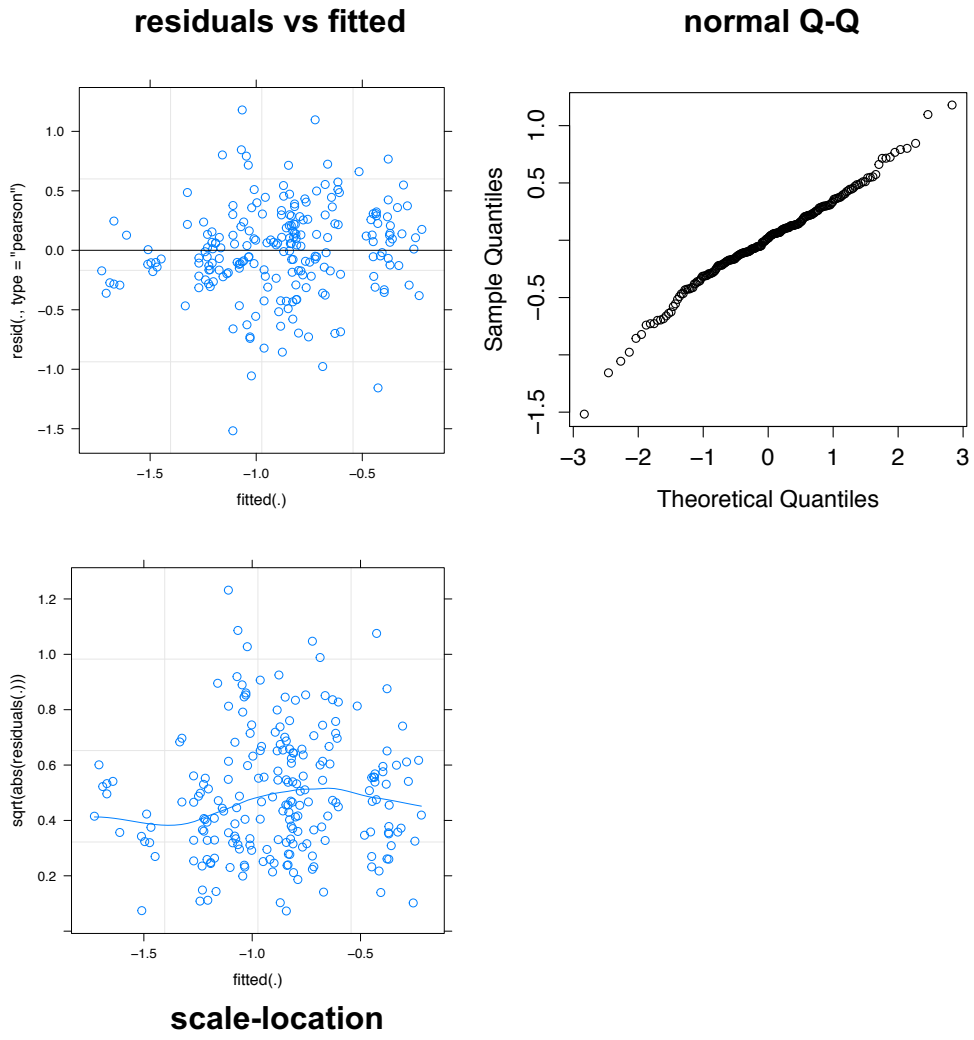


Figure S1. Diagnostic plots for linear mixed effects model of induced beta-lactamase activity and clone infecting time (see Figure 6).

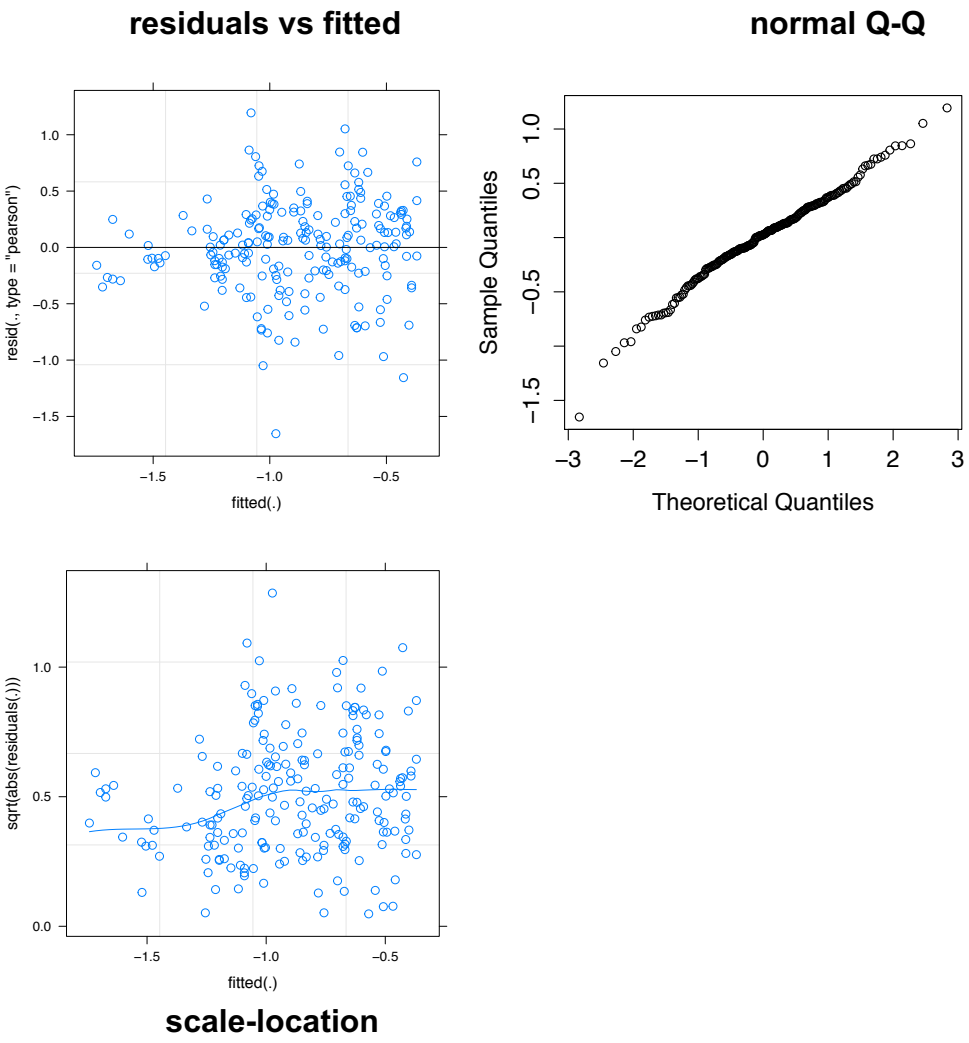


Figure S2. Diagnostic plots for linear mixed effects model of induced beta-lactamase activity and time since first colonization (see Figure 7).

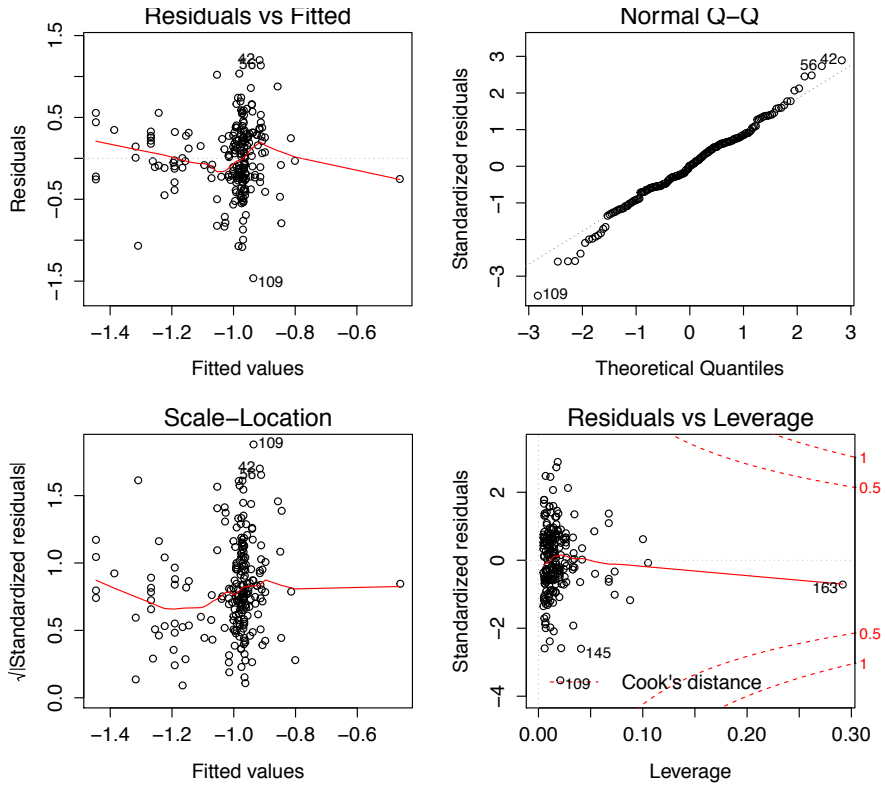


Figure S3. Diagnostic plots for linear mixed effects model of variance of induced beta-lactamase activity and clone infecting time interacting with intensity of beta-lactam treatment (see Figures 12 and 14).

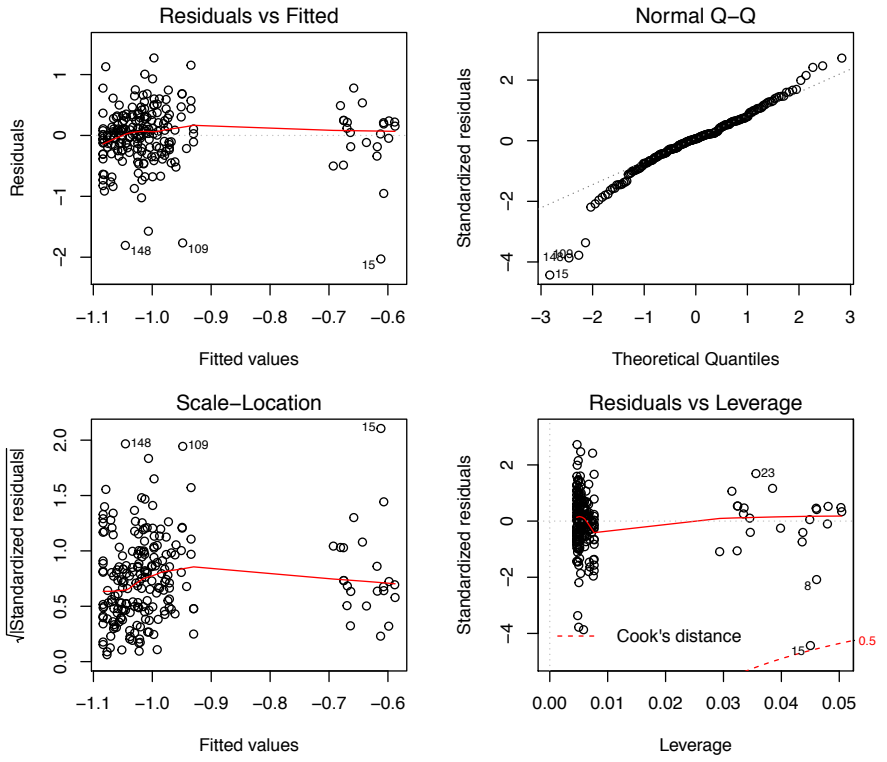


Figure S4. Diagnostic plots for linear mixed effects model of variance of induced beta-lactamase activity and time since first colonization (see Figure 13).

Statistical models

	Dependent variable	Fixed effects	Random effects	Model type
1.	$\log_{10}$(induced beta-lactamase activity)	clone infecting time	clone type nested within patient	random intercepts, fixed gradients model
2.	constitutive beta-lactamase production (i.e. non-induced)	clone infecting time	clone type nested within patient	binomial distribution; random intercepts, fixed gradients model
3.	$\log_{10}$(induced beta-lactamase activity)	time since first colonization	patient	random intercepts, fixed gradients model
4.	constitutive beta-lactamase production (i.e. non-induced)	time since first colonization	patient	binomial distribution; random intercepts and fixed gradients model

Table S1. Linear mixed effects models of change in beta-lactamase over time. Random intercepts and random gradient models were used if significantly better than random intercepts, fixed gradients models. Final models were then compared with models with beta-lactam treatment duration or beta-lactam treatment intensity as an interaction term.

	Dependent variable	Fixed effects	Random effects	Model type
Model A	induced beta-lactamase	clone infecting time	clone type nested within patient	random intercepts and random gradients
Model B	non-induced beta-lactamase	clone infecting time	clone type nested within patient	random intercepts and random gradients
Model C	induced beta-lactamase	time since first colonization	patient	random intercepts and random gradients
Model D	non-induced beta-lactamase	time since first colonization	patient	random intercepts and random gradients

Table S2. Linear mixed effects models used to calculate the variance of beta-lactamase activity

	Dependent variable	Fixed effects/ Factor	Random effects	Model type
1.	$\log_{10}$(residuals of **Model A**)	clone infecting time	none	linear model
2a.	residuals of **Model B**	time block derived from clone infecting time	N/A	Kruskal-Wallis
2b.	alternative model used: residuals of **Model B**	clone infecting time	none	linear model with gamma distribution and log link
3.	$\log_{10}$(residuals of **Model C**)	time since first colonization	none	linear model
4a.	residuals of **Model D**	time block derived from time since first colonization	N/A	Kruskal-Wallis
4b.	alternative model used: residuals of **Model D**	time since first colonization	none	linear model with gamma distribution and log link

Table S3. Models of change in variance with time, using residuals of models from Table S2.

Raw data plots

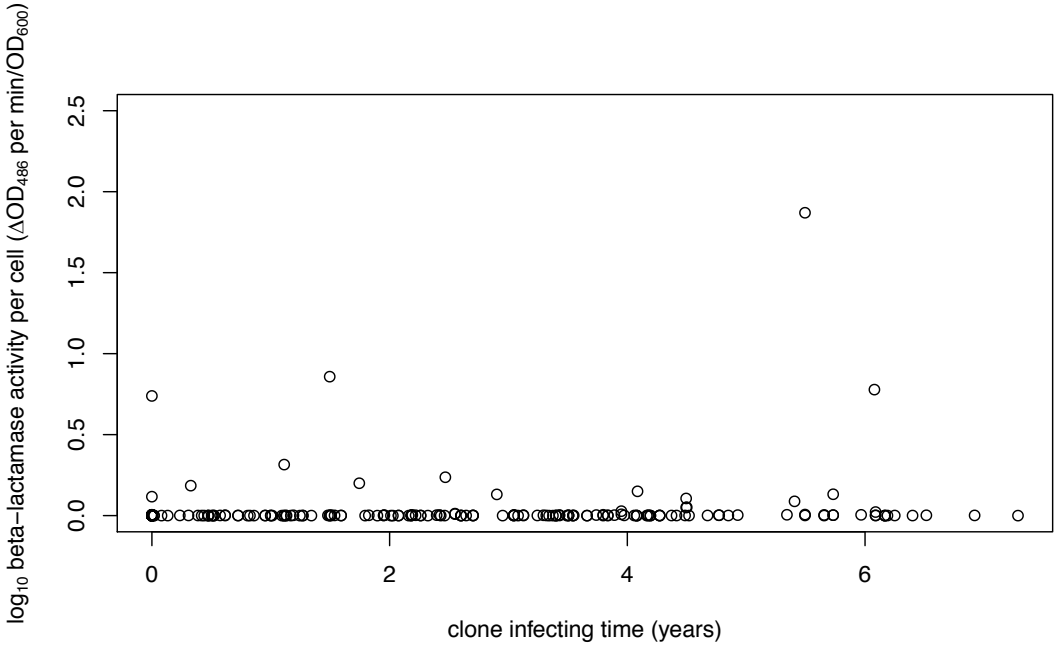


Figure S5. Non-induced extracellular beta-lactamase activity and clone infecting time. Apart from a small number of constitutive beta-lactamase producers, numbers of which increase with time (see Figure 5), beta-lactamase production is generally low. Each point represents the beta-lactamase production of one isolate (n = 215) from 17 different patients who were longitudinally sampled, covering 33 different clone types.

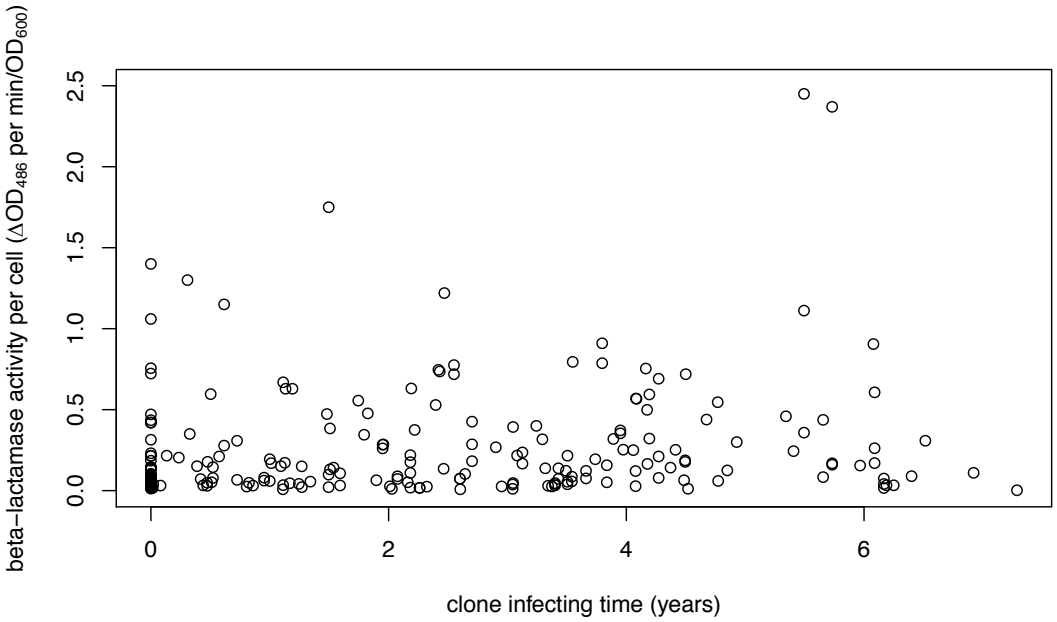


Figure S6. Induced extracellular beta-lactamase activity does not change with clone infecting time ($F_{1, 203} = 1.50$, $p = 0.222$). Each point represents the beta-lactamase production of one isolate ($n = 215$) from 17 different patients who were longitudinally sampled, covering 33 different clone types. See also Figure 6 for logged data as analysed.

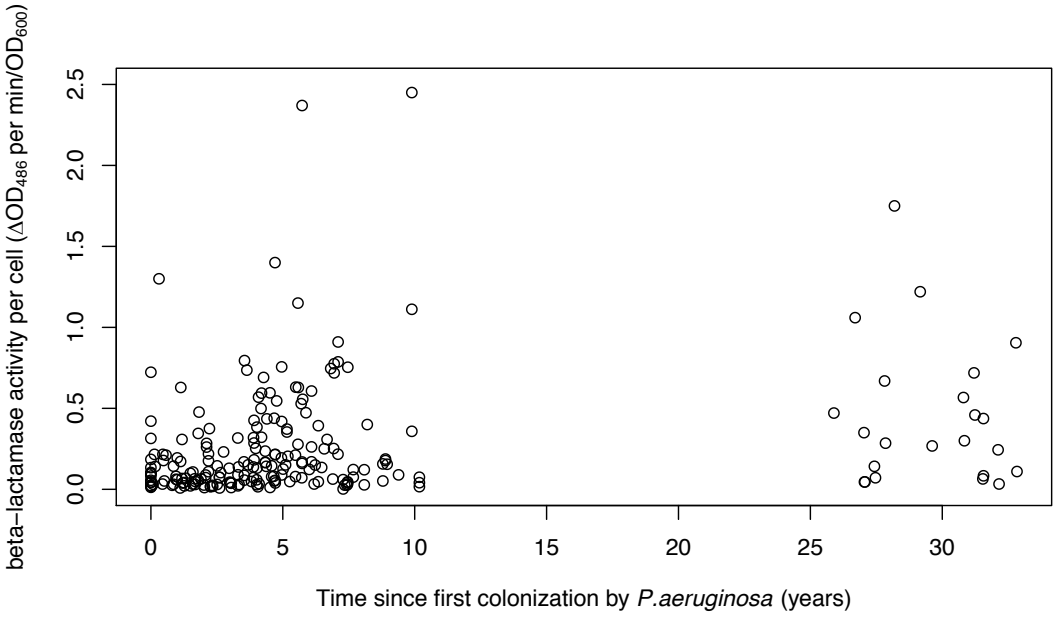


Figure S7. Induced extracellular beta-lactamase activity increases with time since first colonization by any clone type ($F_{1, 47.6} = 4.91$, $p = 0.0315$). Each point represents the beta-lactamase production of one isolate ($n = 215$) from 17 different patients who were longitudinally sampled, covering 33 different clone types. The isolates collected around 30 years all come from one patient (see Methods). See also Figure 7 for logged data as analysed.

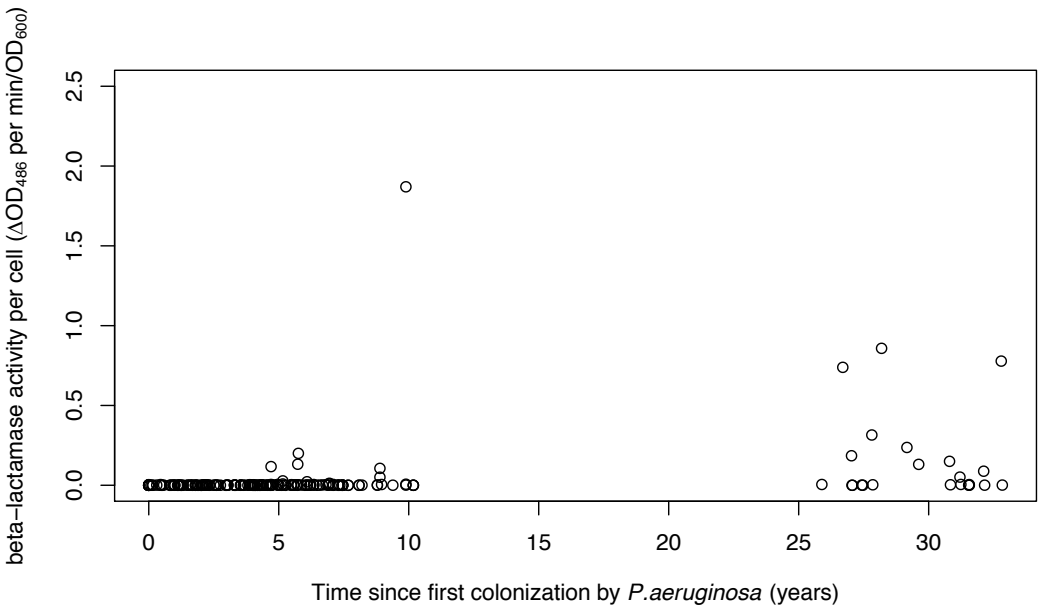


Figure S8. Non-induced extracellular beta-lactamase activity with time since first colonization by any clone type. Each point represents the beta-lactamase production of one isolate (n = 215) from 17 different patients who were longitudinally sampled, covering 33 different clone types. The isolates collected around 30 years all come from one patient (see Methods). There are no constitutive producers until after four years into the infection. See also Figure 8 for constitutive versus non-constitutive isolates as analysed.

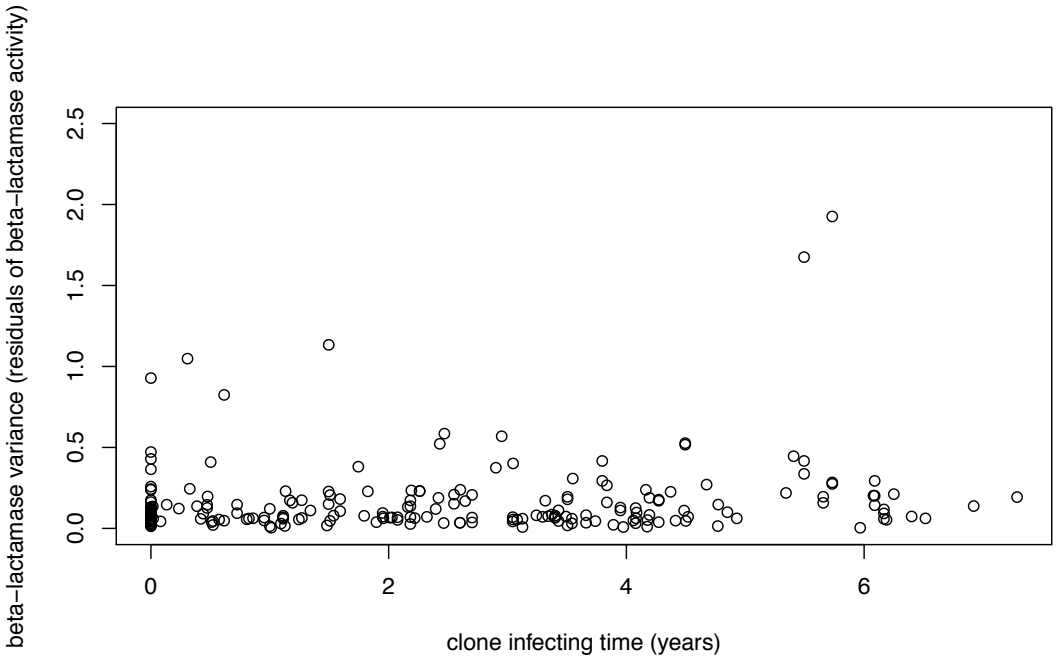


Figure S9. The increase in variance of induced extracellular beta-lactamase activity with clone infecting time approaches significance (linear model on logged data, see Figure 12; $F_{1, 213} = 3.76$, $p = 0.0537$). Each point represents an isolate's residual beta-lactamase activity not explained by a linear model. The similarity with Figure S6 is because the original model does not explain the data well, resulting in large residuals. n = 215, from 17 different patients who were longitudinally sampled, covering 33 different clone types. See Figures 9 and 12.

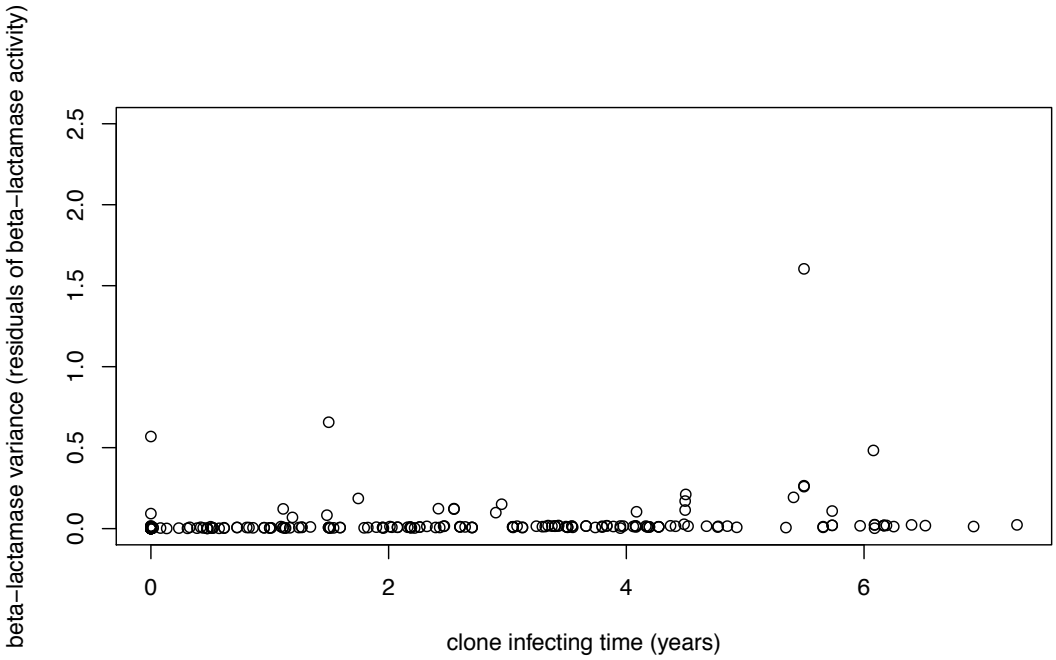


Figure S10. The variance of non-induced extracellular beta-lactamase activity increases with clone infecting time (linear model with gamma distribution and log link, $t = 2.2$, $p = 0.026$). Each point represents an isolate's residual beta-lactamase activity not explained by a linear model. The similarity with Figure S5 is because the original model does not explain the data well, resulting in large residuals. $n = 215$, from 17 different patients who were longitudinally sampled, covering 33 different clone types. See also Figure 10 for blocked data as analysed.

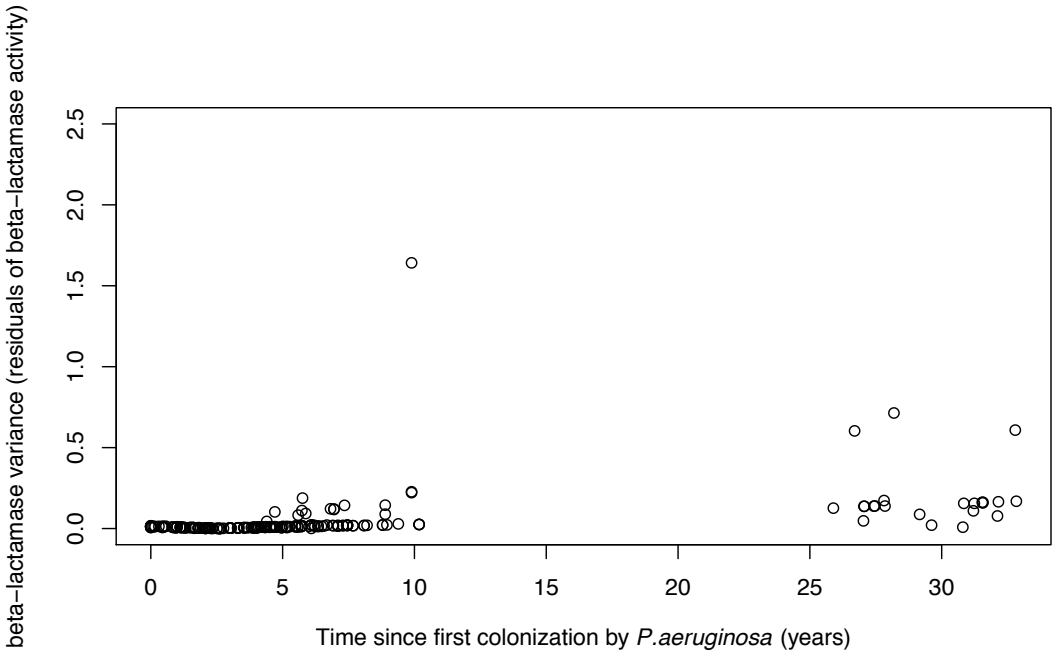


Figure S11. The variance of non-induced extracellular beta-lactamase activity increases with time since first colonization by any clone type ($F_{1, 213} = 158.5$, $p < 2.2\times10^{-16}$). Each point represents an isolate's residual beta-lactamase activity not explained by a linear model. The similarity with Figure S8 is because the original model does not explain the data well, resulting in large residuals. $n = 215$, from 17 different patients who were longitudinally sampled, covering 33 different clone types. The isolates collected around 30 years all come from one patient (see Methods). See also Figure 11 for blocked data as analysed.

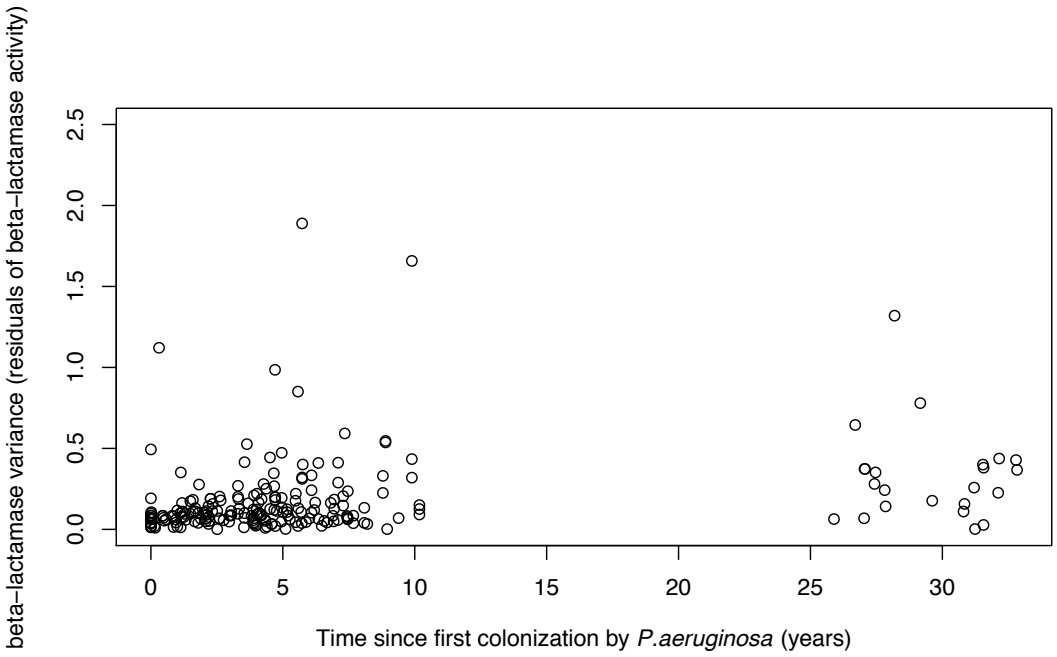


Figure S12. The variance of induced extracellular beta-lactamase activity increases with time since first colonization by any clone type (linear model on logged data, see Figure 13; $F_{1,213} = 15.5$, $p = 0.00011$). Each point represents an isolate's residual beta-lactamase activity not explained a linear model. The similarity with Figure S7 is because the original model does not explain the data well, resulting in large residuals. $n = 215$, from 17 different patients who were longitudinally sampled, covering 33 different clone types. The isolates collected around 30 years all come from one patient (see Methods). See also Figures 11 and 13.

Chapter 3. Antibiotic resistance in chronic infections

Introduction

In Chapter 2, I showed that beta-lactamase production generally does not increase with time during a chronic infection, but that the variance of beta-lactamase activity does. This result is surprising given that the cystic fibrosis lung is an environment exposed to high levels of antibiotic and is, therefore, likely to be an environment where resistance is under positive selection (Folkesson *et al.*, 2012; for fuller discussion, please see Chapter 2).

One potential explanation is that resistance does not correlate with beta-lactamase production. This may arise if low beta-lactamase production can be compensated for by selection for alternative resistance mechanisms. It is well known that beta-lactam antibiotics are resisted by multiple resistance mechanisms, such as efflux pumps (Lister *et al.*, 2009), but specific evidence that lower beta-lactamase is compensated by an increase in other resistance mechanisms is lacking. Some evidence comes from Barbosa *et al.* (2017), whose experimental evolution work indirectly suggests that resistance to antibiotics – particularly beta-lactams and aminoglycosides – may be achieved through different molecular mechanisms even under identical antibiotic selection pressures. The data I collected as part of my study therefore offers a valuable opportunity to test the underlying assumption that beta-lactamase production correlates with resistance in infection.

The second pattern I observed in the previous chapter is an increase in variance of beta-lactamase production. Under strong selection for resistance, we might instead have expected directional selection towards higher production (Giwercman *et al.*, 1990). A potential explanation for this observation is spatial separation within the lung, such that strains evolve in isolation and under different selection pressures depending on local antibiotic concentrations. Though several recent papers demonstrate phenotypic divergence in *P. aeruginosa* in chronic cystic fibrosis infections (Workentine *et al.*, 2013; Clark *et al.*, 2015; Darch *et al.*, 2015; Williams *et al.*, 2015; see also reviews by Folkesson *et al.* (2012) and Winstanley *et al.* (2016)), evidence that spatial separation leads to divergence comes primarily from Jorth *et al.* (2015), who showed that an initial infecting clonal lineage evolves independently in the different regions of the lung it inhabits, leading to divergent phenotypes – including those for antibiotic resistance. While divergence may be due to drift (Jorth *et al.*, 2015; Winstanley *et al.*, 2016), selection pressures are likely to vary between different areas of the CF lung. With regard to antibiotic selection pressure, the perfusion defects of the CF lung (Amaxopoulou *et al.*, 2017) are likely to lead to different levels of antibiotic exposure in different regions. On a finer scale, bacteria in different locations within the biofilm may be exposed to different levels of antibiotic (Bagge *et al.*, 2004a), and slower growth rates deep in anaerobic layers of the biofilm could also reduce antibiotic selection pressure, since many antibiotics only kill dividing cells (Walters *et al.*, 2003; Borriello *et al.*, 2004).

The two patterns in mean and variance of beta-lactamase are also consistent with predictions if this trait were being shaped by cooperator-cheat competition. If cheats were able to survive, this could have the effect of suppressing beta-lactamase

production on average at the population level. The coexistence of cells adopting cooperator and cheating strategies would increase the variance in beta-lactamase production, even if this trait were under strong directional selection. This could be consistent with the phenomenon of heteroresistance, where resistant subpopulations exist amongst more susceptible cells, which has been described in beta-lactam antibiotics (Nicoloff *et al.*, 2019). The evolutionary pressures leading to heteroresistance are unclear, but social evolutionary pressures could provide an answer. In order to distinguish social effects from the alternative explanations described above, it is first necessary to establish the relationship between beta-lactamase and resistance. If the failure to observe an increase in beta-lactamase production over time were due to a switch to alternative resistance mechanisms, we might expect a weakened relationship between beta-lactamase production and resistance. However, if there is a relationship between beta-lactamase production and resistance, we would then expect a divergence in beta-lactam resistance over time (to mirror the finding in Chapter 2 of a divergence in beta-lactamase over time). If spatial separation were the main cause of a divergence in antibiotic resistance over time, then such a divergence should be seen for resistance to all antibiotic classes used, regardless of the potential for social effects (see Chapter 1) – unless there are mechanistic constraints in certain resistance mechanisms, and not others, that have not yet been described.

Here I investigate the long-term evolution of antibiotic resistance in a human host using longitudinally sampled isolates from chronic *P.aeruginosa* infections in cystic fibrosis patients. I use available resistance data to four beta-lactam antibiotics – all non-inducers of beta-lactamase – for each isolate to ask whether beta-lactam

resistance is correlated with beta-lactamase production. To enable further types of beta-lactam antibiotics – including inducers – to be tested, and to confirm that resistance is in part mediated by beta-lactamase, I take a subset of isolates and measure their resistance both with and without beta-lactamase inhibitors. I then use resistance data for each isolate to the four beta-lactams mentioned, but also to two aminoglycosides and a quinolone, to see how resistance and variance of resistance change over time within and across lineages in patients, and to compare the findings in the different antibiotic classes. I also ask if any changes in resistance, or variance of resistance, over time correlate with the antibiotic treatment given.

Methods

Isolate collection

The strain collection comprised 215 *Pseudomonas aeruginosa* lung isolates, collected from 17 cystic fibrosis (CF) patients at the Copenhagen CF Centre at Rigshospitalet. Patients were sampled longitudinally over 10 years with one to four *P. aeruginosa* isolates selected per sample. 33 clone types were represented, although 15 of these only appeared once. Please see Chapter 2 for further details.

Measuring beta-lactamase activity

I measured beta-lactamase in the 215 isolates using a standard nitrocefin assay (O'Callaghan *et al.*, 1972). Please see Chapter 2 for further details.

Measuring resistance

I had two resistance datasets available which I used to correlate resistance and beta-lactamase production. One was a large dataset with resistance data for 207 out of the 215 isolates for which I had beta-lactamase data. This had been collected previously by clinicians at the Copenhagen CF Centre at Rigshospitalet. This provided resistance data for four beta-lactams, two aminoglycosides and one quinolone. The beta-lactam data was for: aztreonam (a monobactam), ceftazidime (a cephalosporin), meropenem (a penem), and piperacillin with tazobactam (a penicillin with a beta-lactamase inhibitor, although it does not inhibit any of the common *P.aeruginosa* beta-lactamases (Jacoby, 2009; Drawz & Bonomo, 2010)). The aminoglycosides were gentamicin and tobramycin, and the quinolone was ciprofloxacin. Minimum inhibitory concentrations (MICs) had been determined using E-test strips (Marley *et al.*, 1995). A 0.5 McFarland standard suspension was inoculated onto Mueller-Hinton agar plates. Up to four E-test strips were placed on each plate in accordance with the manufacturer's instructions (Liofilchem, Italy). The concentrations measured were: aztreonam: 0.016-256 µg/ml, ceftazidime: 0.016-256 µg/ml, meropenem: 0.002-32µg/ml, piperacillin: 0.016-256µg/ml (+tazobactam 4µg/ml), gentamicin: 0.016-256µg/ml, tobramycin: 0.016-256µg/ml, and ciprofloxacin: 0.002-32 µg/ml.

This dataset had the advantage of a large sample size and resistance data for three classes of antibiotics. However, it was limited by only having data for non-inducing beta-lactam antibiotics and for only having resistance in terms of minimum inhibitory concentrations, which is not the most sensitive measurement of resistance (Soothill *et al.*, 1992). I therefore separately measured resistance in two inducing antibiotics

(imipenem and benzylpenicillin), as well as repeating the measurements for piperacillin and meropenem, in a subset of 23 out of the full collection of 215 isolates. This enabled me to gather data suitable for calculating IC_{50} values, which is the antibiotic concentration at which there is half the full inhibitory effect, as well is MICs. IC_{50} is a more sensitive measure than MIC, suitable for detecting subtle differences in antibiotic resistance (Soothill *et al.*, 1992). A third benefit of measuring resistance in this subset was to allow me to measure resistance with and without beta-lactamase inhibitors in order to demonstrate whether beta-lactamase contributes to the resistance.

I selected 23 out of the full collection of 215 isolates for this experiment, with relatively low, medium and high levels of beta-lactamase production. This categorization was based on the mean of the total basal and total induced beta-lactamase activity per cell (Chapter 2, Figures 3 and 4). The overall lowest and overall highest beta-lactamase producer were also included. Two very slow growers were later removed from the analysis as there was only measurable growth at 48 hours, by which time the antibiotics is likely to have significantly degraded such that the results may not be comparable to standard measurements at 18 hours. This left 21 isolates in the experiment.

The beta-lactamase inhibitors used were (i) cloxacillin, which inhibits chromosomal class C beta-lactamases (including AmpC) (Jacoby, 2009; Tan *et al.*, 2009), and tazobactam, which inhibits class A beta-lactamases (generally plasmid-borne TEM and SHV beta-lactamases (Bush *et al.*, 1993; Payne *et al.*, 1994)) and is only a weak inhibitor of AmpC (Akova *et al.*, 1990). The inducing antibiotics I used were

imipenem, a strong inducer of AmpC beta-lactamase, which is hydrolysed relatively slowly by AmpC, and benzylpenicillin, a strong inducer of AmpC beta-lactamase which is rapidly hydrolysed by it (Jacoby, 2009).

I measured MICs using the broth microdilution method (Andrews, 2001). I added either beta-lactamase inhibitor (cloxacillin, final concentration 200mg/l, or tazobactam, final concentration 4mg/l) or deionised water (the no inhibitor treatment) to 12 serial dilutions of antibiotic in fresh KB in 96-well plates. I then added culture that had been standardised to 0.1OD_{600} and then diluted 100 times into fresh KB. The plates were then incubated at 37°C for 18 hours, when growth was measured (OD_{600}). MIC is usually defined as the lowest antibiotic concentration at which no visible growth occurs, though I based it on the lowest antibiotic concentration where OD_{600} at 18 hours was below 0.1 (no visible growth in the microtitre well). The experiment comprised three technical replicates.

Statistical methods

Correlation between resistance and beta-lactamase

Using the full dataset of 207 isolates, I used linear mixed effects models to assess the effect of beta-lactamase on resistance. Since resistance was measured using MICs, which are measured on a scale of halving antibiotic concentrations, the $\log_2$ of the MIC was used for analysis. As this study was prompted by the finding that induced beta-lactamase in clone types does not increase over time, I am interested in how

resistance within a clone type may vary with beta-lactamase. Therefore I include clone type nested within patient as a random factor. Analysis was performed as one mixed effects model for the four antibiotics, with antibiotic included as a fixed effect interacting with beta-lactamase production. I then simplified the model, removing any non-significant terms.

For the subset of 21 isolates, I tested the effect of beta-lactamase production on resistance using a simple linear model, using $\log_2$ of the IC_{50} or the MIC as the dependent variable. The model included a three-way interaction between beta-lactamase production, antibiotic, and presence or absence of the beta-lactamase inhibitors cloxacilin or tazobactam. I used separate models for induced beta-lactamase and non-induced beta-lactamase. I also used a paired *t*-test with Bonferroni correction on the $\log_2$ of the IC_{50} and the MIC, with and without the inhibitors, to look at the effect of beta-lactamase inhibition.

Change in resistance and variance of resistance over time

I used linear mixed effects models to assess the effect of time on resistance. When change in resistance within clone type was being considered, the random factor of clone type nested within patient was used, and I used 'clone infecting time' as the measure of time (see Chapter 2, page 32 for definitions). When change in resistance across all clone types was being considered, patient was the random factor and 'time since first colonization' was the measure of time (see Chapter 2). I compared random intercept models with random intercept-and-gradient models and used the latter if it explained the data significantly). I included all seven antibiotics in one model, with an

interaction between time and antibiotic, before simplifying the model and removing non-significant terms.

Divergence of resistance over time was assessed using the variance of MIC, for which I took the absolute value of the residuals of a linear mixed effects model on the original data, allowing random intercepts and random gradients, and then ran a simple linear model on the residuals over time. After inspection of the diagnostic plots it was apparent that using a $\log_2$ transformation of variance was too powerful a transformation for the amount of skew. I therefore used a square root transformation of the MIC residuals.

To investigate the relationship between resistance, time and beta-lactamase production I used a linear mixed effects model with a three-way interaction between $\log_2$[MIC], beta-lactamase production (non-induced only – as the antibiotics were non-inducers), and time (clone infecting time and time since first colonization in separate models).

Effect of antibiotic treatment

The amount of antibiotic treatment was quantified for each of the three classes of antibiotics in this study. As in Chapter 2, antibiotic treatment was quantified in two ways: (i) total antibiotic duration (of a particular class), which was the total number of weeks of treatment by that antibiotic class which a patient received during the time period of study (i.e. from the first collected isolate to the last); and (ii) intensity of antibiotic treatment, which was the total duration of treatment by a particular

antibiotic class, divided by the time period of study in years (which varied between patients). Antibiotic treatment duration and antibiotic treatment intensity of the appropriate class were then added as interaction terms into the linear models of resistance, or variance of resistance, discussed above (i.e. beta-lactam treatment duration and intensity were added to the model for beta-lactam antibiotics, aminoglycoside treatment was added to models for aminoglycoside antibiotics etc.). Biologically meaningless interactions were deleted at the outset (such as any interaction between treatment duration and treatment intensity), followed by model simplification, leaving only significant terms in.

Comment on statistical methods

Linear mixed effects models are a powerful method for analysing data with repeated sampling (Harrison *et al.*, 2018), such as from patients over time. I inspected diagnostic plots and transformed data as necessary, either with a log or square root transformation, to reduce skew in order to satisfy the assumptions of linear regression.

This chapter uses multiple statistical models which could increase the chance of Type I error. Because of this I have minimized multiple testing by constructing models with all antibiotics together and antibiotic included as an interaction term. However, as in Chapter 2, I have made separate statistical models for clone infecting time and time since first colonization since the data is being used to answer different questions (change in resistance within a clone type and change within a patient respectively), which therefore have different random effects (clone type nested within patient versus just patient). I also constructed separate models for each antibiotic class when

antibiotic treatment was included in the model, and separate models for induced and non-induced beta-lactamase. Again, this is because separate questions are being addressed (for example, the effect of beta-lactam treatment or aminoglycoside treatment or quinolone treatment). When I made a complex model with all antibiotic types and all treatments in one, the step() function in R failed to simplify the model.

The many questions addressed in this chapter do generate multiple tests (25 linear or mixed effects models) and increase the risk of a Type I error. However, approaches to multiple testing vary (Feise, 2002). Also these separate models have been produced through model simplification and so represent the most parsimonious model in each case. Equally, most of the key results are highly significant and would remain significant if the conservative Bonferroni correction had been applied. Apart from the test of the effect of beta-lactamase inhibitors (to which a Bonferroni correction was applied), this chapter is an exploratory analysis rather than a controlled experimental test, where adjustment for multiple testing is considered 'less critical' (Althouse, 2016). The only important result which might have changed is the significant interaction between quinolone treatment and the increase in quinolone resistance with clone infecting time ($p = 0.011$), but this alone does not change the overall inferences gained from this exploratory analysis.

Statistical analysis was done in R (www.r-project.org), using the lme4 package for the mixed effects models.

Results

Resistance and beta-lactamase production

Beta-lactam resistance correlates with beta-lactamase activity. Resistance to the four non-inducing beta-lactams (aztreonam, piperacillin with tazobactam, ceftazidime and meropenem) correlated with non-induced beta-lactamase levels within genotypes within patients (t = 6.3, $p = 6.6\times10^{-10}$, Figure 1, Table 1). The correlation disappeared when constitutive producers were removed from the analysis (t = -0.96, p = 0.34, Table 2). There is no significant correlation between induced beta-lactamase and resistance in ceftazidime (t = 0.51, p = 0.61), meropenem (t = 0.91, p = 0.36) and piperacillin (t = -2.0, p = 0.0504), and a negative correlation between aztreonam resistance and induced beta-lactamase (t = -4.0, $p = 6.2\times10^{-5}$) (Table 3).

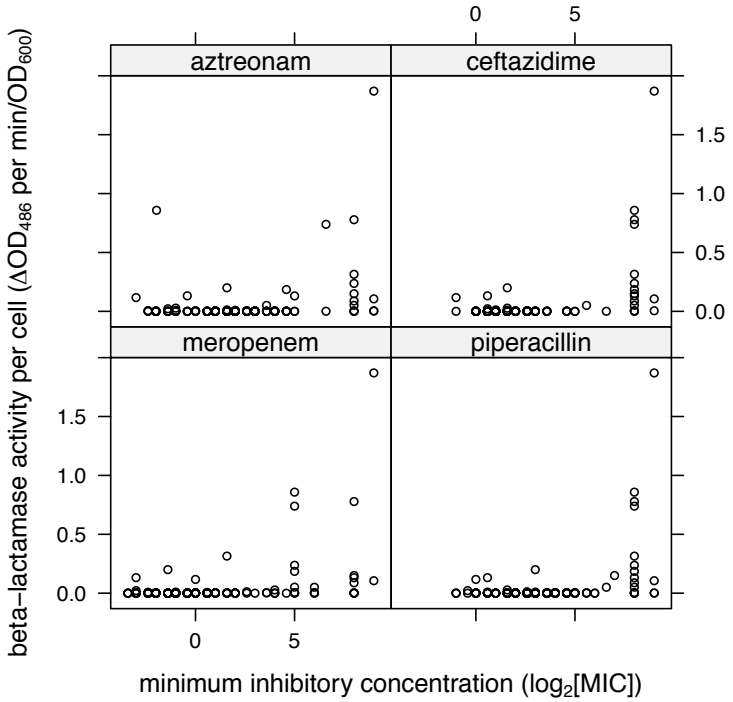


Figure 1. Non-induced beta-lactamase and resistance. Non-induced beta-lactamase activity correlates with resistance (t = 6.3, $p = 6.6\times10^{-10}$). Each point represents the beta-lactamase production and resistance of one isolate (n = 207) from 17 different patients, covering 33 different clone types.

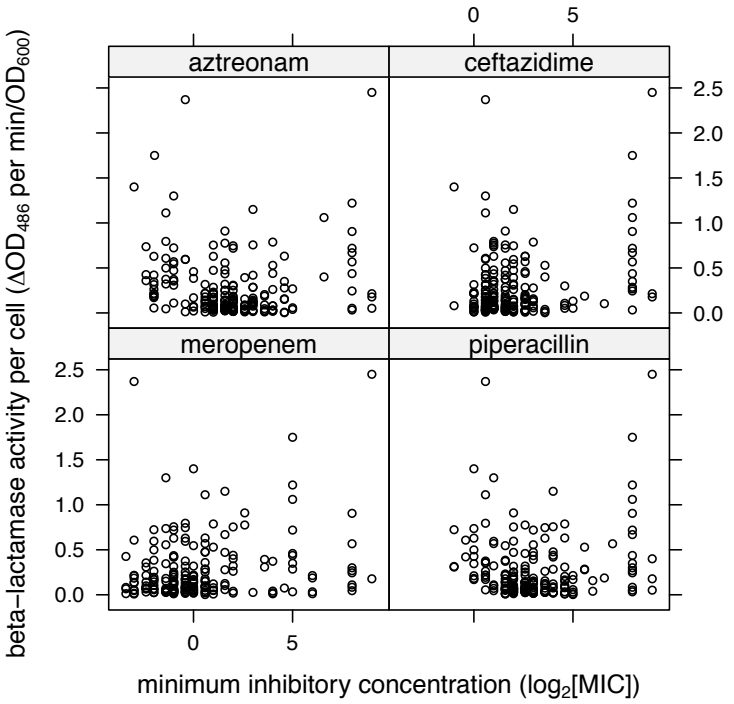


Figure 2. Induced beta-lactamase and resistance. Induced beta-lactamase activity does not correlate with resistance in ceftazidime (t = 0.51, p = 0.61), meropenem (t = 0.91, p = 0.36) or piperacillin (t = -2.0, p = 0.0504), and there is a negative correlation between aztreonam resistance and induced beta-lactamase (t = -4.0, $p = 6.2\times10^{-5}$). Each point represents the beta-lactamase production and resistance of one isolate (n = 207) from 17 different patients, covering 33 different clone types.

	Gradient	*df*	*T*	*p*
log2(resistance)				
non-induced beta-lactamase	2.67	805.1	6.3	6.6×10^{-10}

Table 1. Relationship between non-induced beta-lactamase activity and resistance. Beta-lactamase production and resistance to four beta-lactam antibiotics (aztreonam, ceftazidime, meropenem, piperacillin-tazobactam) was measured in 207 isolates, making 828 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients. Results of a linear mixed effects model with genotype nested within patient as a random factor. There is one gradient for all four beta-lactams tested as there was no interaction between antibiotic type and beta-lactamase production in explaining resistance. Each antibiotic had a difference intercept (not shown).

	Gradient	*df*	*T*	*p*
log2(resistance)				
non-induced beta-lactamase	-52.7	734.9	-0.96	0.34

Table 2. Relationship between non-induced beta-lactamase activity and resistance without constitutive producers. Beta-lactamase production and resistance to four beta-lactam antibiotics (aztreonam, ceftazidime, meropenem, piperacillin-tazobactam) was measured in 207 isolates, making 828 isolate-antibiotic pairs. 22 isolates which showed constitutive beta-lactamase production were then excluded from the analysis, leaving 185 isolates and 740 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients. Results of a linear mixed effects model with genotype nested within patient as a random factor. There is one gradient for all four beta-lactams tested as there was no interaction between antibiotic type and beta-lactamase production in explaining resistance. Each antibiotic had a difference intercept (not shown).

	Gradient	*df*	*T*	*p*
$\log_2$(resistance)				
induced beta-lactamase				
aztreonam	-1.45	800.9	-4.0	6.2×10^{-5}
ceftazidime	0.183	800.9	0.51	0.61
meropenem	0.329	800.9	0.91	0.36
piperacillin	-0.71	800.9	-1.96	0.0504

Table 3. Relationship between induced beta-lactamase activity and resistance. Beta-lactamase production and resistance to four beta-lactam antibiotics (aztreonam, ceftazidime, meropenem, piperacillin-tazobactam) was measured in 207 isolates, making 828 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients. Results of a linear mixed effects model with genotype nested within patient as a random factor. Each antibiotic had a difference intercept (not shown).

When examining the subset of isolates (n = 21, chosen from all genotypes), resistance of the inducing antibiotic imipenem increased with both induced and non-induced beta-lactamase in terms of both IC_{50} (induced: t = 3.6, p = 0.00044; non-induced: t = 6.0, $p = 9.4\times10^{-9}$, Figures 3 and 4, Tables 4 and 5) and MIC (induced: t = 3.2, p = 0.0021; non-induced: t = 5.3, $p = 2.6\times10^{-7}$, Tables 6 and 7). The relationship though, is driven by one very high beta-lactamase producer which is highly resistant; if this isolate is removed from the analysis, there is no significant correlation (t = -0.35, p = 0.73). There was no correlation between either induced or non-induced beta-lactamase and resistance in terms of either MIC or IC_{50} for the second inducing antibiotic, benzylpenicillin (Figures 3 and 4, Tables 4-7). Of the non-inducing antibiotics, meropenem showed a correlation with non-induced beta-lactamase levels (IC_{50} t = 4.0, $p = 9.4\times10^{-5}$, MIC t = 2.4, p = 0.017, Tables 4 and 6), but again this relationship disappears when one very high producer is removed from the analysis. Piperacillin here showed no correlation with either IC_{50} or MIC (Figure 4, Tables 4-7), even though there had been a correlation in the larger dataset. All statistics are in Tables 4-7.

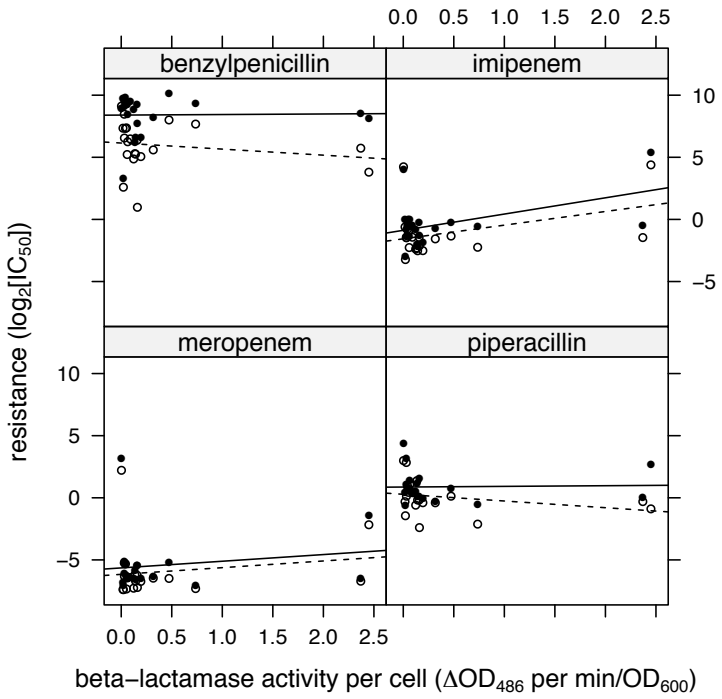


Figure 3. Induced beta-lactamase activity and resistance (IC_{50}). Filled circles and solid line show the relationship in the absence of a beta-lactamase inhibitor. Open circles and the dotted line show the relationship in the presence of cloxacillin, an AmpC beta-lactamase inhibitor. Imipenem resistance increases with induced beta-lactamase (t = 3.6, p = 0.00044), but there is no correlation between induced beta-lactamase and resistance in benzylpenicillin (t = -0.83, p = 0.41), meropenem (t = 1.6, p = 0.11) or piperacillin (t = -1.1, p = 0.29).

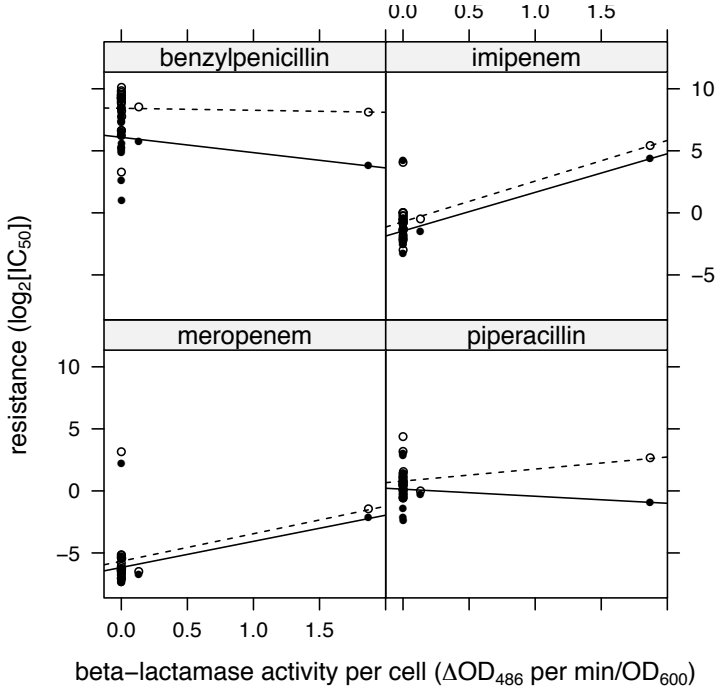


Figure 4. Non-induced beta-lactamase activity and resistance (IC_{50}). Filled circles and solid line show the relationship in the absence of a beta-lactamase inhibitor. Open circles and the dotted line show the relationship in the presence of cloxacillin, an AmpC beta-lactamase inhibitor. Imipenem resistance increases with non-induced beta-lactamase activity (t = 6.0, 9.4×10^{-9}), as does meropenem (t = 4.0, $p = 9.4\times10^{-5}$). These correlations disappear when the outlier is removed. There is no correlation between non-induced beta-lactamase and resistance in benzylpenicillin (t = -1.4, p = 0.16) or piperacillin (t = -0.06, p = 0.95).

	Estimate	num *df*	den *df*	*T*	*F*	*p*
log_2(resistance [IC_{50}])		9	239		212.7	2.2×10^{-16}
non-induced beta-lactamase (gradient)						
imipenem	3.23			6.0		9.4×10^{-9}
benzylpenicillin	-0.757			-1.4		0.16
meropenem	2.16			4.0		9.4×10^{-5}
piperacillin	-0.0336			-0.06		0.95
inhibitor (intercept)						
cloxacillin	-1.14			-4.3		2.7×10^{-5}
tazobactam	-0.383			-1.4		0.15

Table 4. Non-induced beta-lactamase activity and resistance (IC_{50}). Beta-lactamase production and resistance to four beta-lactam antibiotics (imipenem, benzylpenicillin, meropenem, piperacillin) was measured in 21 isolates. Resistance was also measured with cloxacillin (an AmpC beta-lactamase inhibitor) or tazobactam (a TEM and SHV beta-lactamase inhibitor), giving 252 isolate-antibiotic-inhibitor combinations. Results of a linear model are shown above. The first line shows the significance of the model. The estimate for each antibiotic is the gradient of the relationship between $log_2[IC_{50}]$ for each antibiotic and non-induced beta-lactamase production. The estimate for the inhibitors is the difference in intercept from the no inhibitor treatment. Each antibiotic also had a difference intercept (not shown).

	Estimate	num *df*	den *df*	*T*	*F*	*p*
log_2(resistance [IC_{50}])		9	239		183.2	2.2×10^{-16}
induced beta-lactamase (gradient)						
imipenem	1.20			3.6		0.00044
benzylpenicillin	-0.279			-0.83		0.41
meropenem	0.538			1.6		0.11
piperacillin	-0.360			-1.1		0.29
inhibitor (intercept)						
cloxacillin	-1.14			-4.0		8.2×10^{-5}
tazobactam	-0.383			-1.3		0.18

Table 5. Induced beta-lactamase activity and resistance (IC_{50}). Beta-lactamase production and resistance to four beta-lactam antibiotics (imipenem, benzylpenicillin, meropenem, piperacillin) was measured in 21 isolates. Resistance was also measured with cloxacillin (an AmpC beta-lactamase inhibitor) or tazobactam (a TEM and SHV beta-lactamase inhibitor), giving 252 isolate-antibiotic-inhibitor combinations. Results of a linear model are shown above. The first line shows the significance of the model. The estimate for each antibiotic is the gradient of the relationship between $log_2[IC_{50}]$ for each antibiotic and induced beta-lactamase production. The estimate for the inhibitors is the difference in intercept from the no inhibitor treatment. Each antibiotic also had a difference intercept (not shown).

	Estimate	num *df*	den *df*	*T*	*F*	*p*
log_2(resistance [MIC])		9	242		186.7	2.2×10^{-16}
non-induced beta-lactamase (gradient)						
imipenem	2.99			5.3		2.6×10^{-7}
benzylpenicillin	-0.743			-1.3		0.189
meropenem	1.36			2.4		0.0168
piperacillin	0.0630			0.11		0.911
inhibitor (intercept)						
cloxacillin	-1.05			-3.8		0.00018
tazobactam	-0.298			-1.1		0.28

Table 6. Non-induced beta-lactamase activity and resistance (MIC). Beta-lactamase production and resistance to four beta-lactam antibiotics (imipenem, benzylpenicillin, meropenem, piperacillin) was measured in 21 isolates. Resistance was also measured with cloxacillin (an AmpC beta-lactamase inhibitor) or tazobactam (a TEM and SHV beta-lactamase inhibitor), giving 252 isolate-antibiotic-inhibitor combinations. Results of a linear model are shown above. The first line shows the significance of the model. The estimate for each antibiotic is the gradient of the relationship between log_2[MIC] for each antibiotic and non-induced beta-lactamase production. The estimate for the inhibitors is the difference in intercept from the no inhibitor treatment. Each antibiotic also had a difference intercept (not shown).

	Estimate	num *df*	den *df*	*T*	*F*	*p*
log_2(resistance [MIC])		9	242		167.3	2.2×10^{-16}
induced beta-lactamase (gradient)						
imipenem	1.07			3.2		0.0021
benzylpenicillin	-0.183			-0.53		0.596
meropenem	0.183			0.53		0.596
piperacillin	-0.160			-0.47		0.642
inhibitor (intercept)						
cloxacillin	-1.05			-3.6		0.00034
tazobactam	-0.298			-1.0		0.303

Table 7. Induced beta-lactamase activity and resistance (MIC). Beta-lactamase production and resistance to four beta-lactam antibiotics (imipenem, benzylpenicillin, meropenem, piperacillin) was measured in 21 isolates. Resistance was also measured with cloxacillin (an AmpC beta-lactamase inhibitor) or tazobactam (a TEM and SHV beta-lactamase inhibitor), giving 252 isolate-antibiotic-inhibitor combinations. Results of a linear model are shown above. The first line shows the significance of the model. The estimate for each antibiotic is the gradient of the relationship between log_2[MIC] for each antibiotic and induced beta-lactamase production. The estimate for the inhibitors is the difference in intercept from the no inhibitor treatment. Each antibiotic also had a difference intercept (not shown).

Contribution of beta-lactamase to resistance

Experimentally comparing growth in cultures with and without beta-lactamase inhibitors, in the presence of antibiotic, revealed that AmpC beta-lactamase contributes to resistance. The AmpC beta-lactamase inhibitor cloxacillin was a significant factor causing a reduction in resistance in terms of both MIC and IC_{50} in the models. Tazobactam, which inhibits TEM and SHV beta-lactamases, however had no significant effect (see Tables 4-7).

Looking at the effect of cloxacillin on each antibiotic individually with *post hoc t*-tests, all four beta-lactam antibiotics tested (benzylpenicillin, imipenem, piperacillin, and meropenem) had reduced IC_{50}s, with the greatest reduction in the inducing antibiotics benzylpenicillin and imipenem (72% and 38% reduction respectively, versus 27% and 31% in the non-inducers meropenem and piperacillin; Table 8). In terms of MIC, cloxacillin only significantly reduced the resistance of the inducing antibiotics (benzylpenicillin and imipenem, Table 9). Tazobactam is not included in Figures 3 and 4 for clarity as there was no significant effect. The results suggest AmpC beta-lactamase is the most important beta-lactamase in clinical *P.aeruginosa* resistance. It should be noted that the inhibitors did not affect the gradient of the relationships between beta-lactamase and resistance in any instance (interaction not significant and removed during model simplification).

To confirm that the effect of the inhibitors is through their inhibition of beta-lactamase and not through direct inhibition of the cells, I grew the isolates in cloxacillin and tazobactam in the absence of antibiotic. Cloxacillin only had a

significant direct inhibitory effect (in the absence of antibiotic) on one isolate only, and even in this instance growth with the cloxacillin was 88% of the growth in its absence. Tazobactam had a direct inhibitory effect on two isolates, which grew to 87% and 94% of growth without tazobactam. This confirms that the inhibitors had a very limited direct inhibitory effect on the isolates, and their main effect was to reduce resistance in the presence of beta-lactam antibiotics.

antibiotic	**effect of cloxacillin**				
	t	***df***	**% change**	***p***	***p* adjust**
benzylpenicillin	7.34	20	72	4.2×10^{-7}	3.4×10^{-6}
imipenem	7.49	20	38	3.1×10^{-7}	2.5×10^{-6}
meropenem	4.79	20	27	0.00013	0.0010
piperacillin	3.34	20	31	0.0032	0.026

Table 8. Effect of beta-lactamase inhibitors on resistance (IC_{50}). Paired *t*-tests on the $\log_2$ of the IC_{50} of 21 isolates with and without the inhibitor cloxacillin. Adjustment for multiple tests is by Bonferroni correction (for eight comparisons).

antibiotic	**effect of cloxacillin**			
	t	***df***	***p***	***p adjust***
benzylpenicillin	6.83	20	1.2×10^{-6}	9.6×10^{-6}
imipenem	7.17	20	6.1×10^{-7}	4.9×10^{-6}
meropenem	2.91	20	0.0086	0.069
piperacillin	2.28	20	0.034	0.27

Table 9. Effect of beta-lactamase inhibitors on resistance (MIC). Paired *t*-tests on the $\log_2$ of the MIC of 21 isolates with and without the inhibitor cloxacillin. Percentage reduction in resistance, which was included in the IC_{50} results (Table 8), is not included as a percentage reduction in resistance does not make sense in the context of the discrete scale of MICs. Adjustment for multiple tests is by Bonferroni correction (for eight comparisons).

Change in antibiotic resistance over time

Antibiotic resistance increased in patients with both clone infecting and time since first colonization for all antibiotics tested (four beta-lactams, two aminoglycosides and one quinolone) (Figures 5 and 6; see Tables 10 and 11 for statistics). The increase

in beta-lactam resistance was not correlated with the beta-lactam treatment the patients received: there was no significant interaction between time and beta-lactam treatment duration or treatment intensity for any of the beta-lactam antibiotics (see Tables 12 and 13 for statistics and Figure 7 for effect of treatment intensity). This contrasts with the general patterns found in aminoglycosides and quinolone antibiotics. Intensity of aminoglycoside treatment correlated with the increase in aminoglycoside resistance over time in both aminoglycosides tested (significant interaction between clone infecting time and aminoglycoside treatment intensity: gentamicin, $t = 4.8$, $p = 7.9\times10^{-5}$ and tobramycin $t = 3.0$, $p = 0.0065$, Table 14; time since first colonization: gentamicin and tobramycin, $t = 5.8$, $p = 4.8\times10^{-8}$, Table 15). Similarly, the intensity of quinolone treatment correlated with the increase in the resistance of ciprofloxacin (a quinolone) over both clone infecting time and time since first colonization (significant interaction between intensity of quinolone treatment and clone infecting time ($t = 2.6$, $p = 0.011$, Table 16) and also a significant interaction between intensity of quinolone treatment and time since first colonization ($t = 2.3$, $p = 0.023$, Table 17). With clone infecting time, the interaction with treatment intensity drives the entire increase in resistance, since the main effect of clone infecting time is no longer significant once treatment intensity is in the model ($t = 0.9$, $p = 0.37$, Table 16). With aminoglycosides only, there is a small negative interaction between aminoglycoside treatment duration and time (clone infecting time: gentamicin, $t = -4.3$, $p = 0.00028$ and tobramycin $t = -2.5$, $p = 0.021$, Table 14; time since first colonization: $t = -5.7$, $p = 2.8\times10^{-7}$, Table 15). In all other cases, antibiotic treatment duration has no significant effect. (See Figure 7 for effect of antibiotic treatment intensity on change in resistance within a clone type over time).

I also asked if there was any interaction between time and non-induced beta-lactamase production in explaining resistance, given the correlation between non-induced beta-lactamase and resistance in these non-inducing antibiotics. There was a positive interaction between non-induced beta-lactamase production and clone infecting time in explaining resistance (p = 0.008), but this was in explaining aztreonam resistance only (t = 3.9, p = 0.00013). No such interaction was found in the remaining three antibiotics (ceftazidime, meropenem, piperacillin) (see Table 18). Therefore the effect of non-induced beta-lactamase increases with clone infecting time in aztreonam only. There was no such interaction between time since first colonization and non-induced beta-lactamase (p = 0.448).

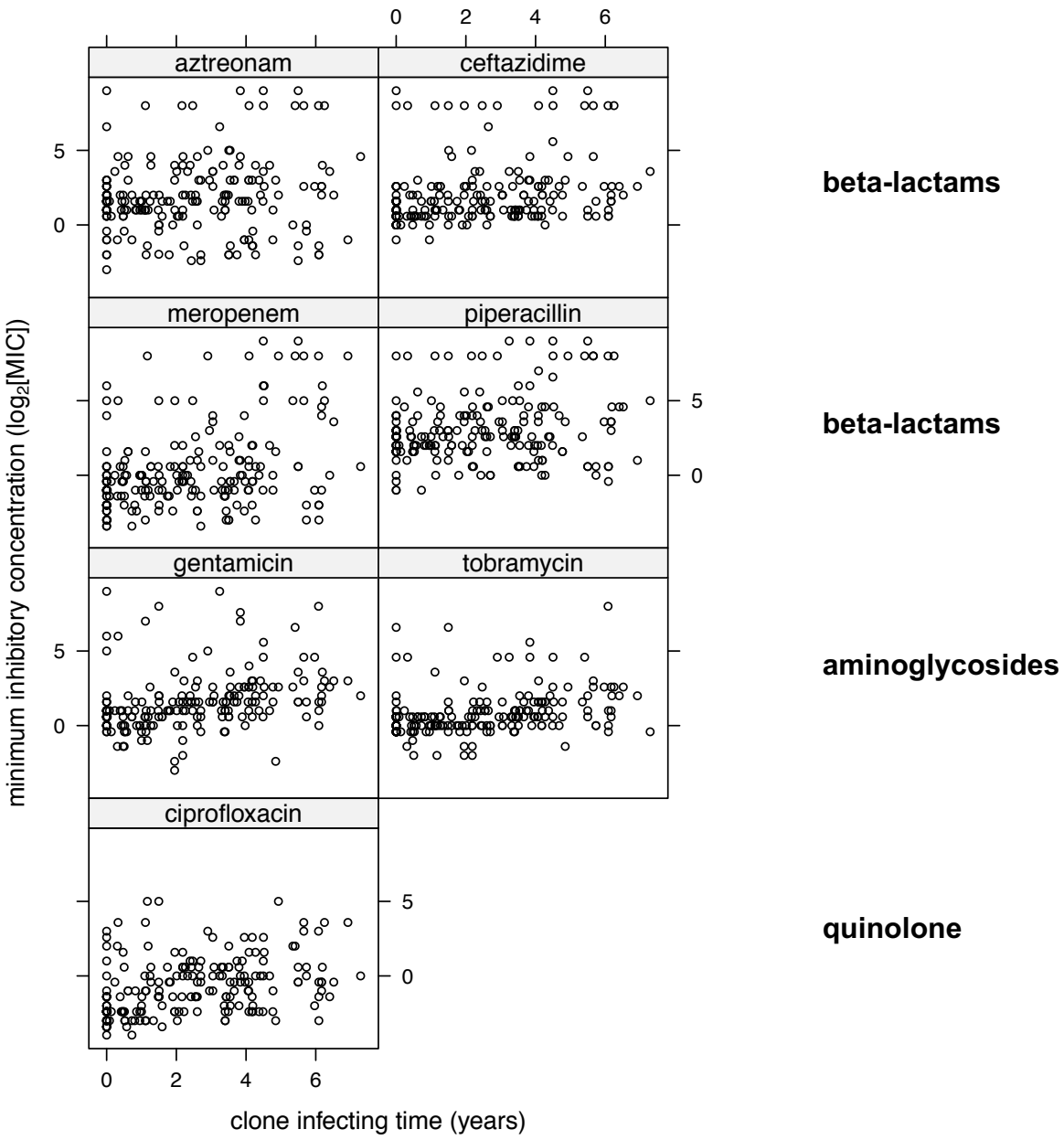


Figure 5. Change in resistance within clone types over time. Resistance within clone types in patients increased to all seven antibiotics tested (four beta-lactams, two aminoglycosides and one quinolone; see Table 10 for statistics). Each point represents the minimum inhibitory concentration of an isolate (n = 207) from 17 different patients, covering 33 different clone types.

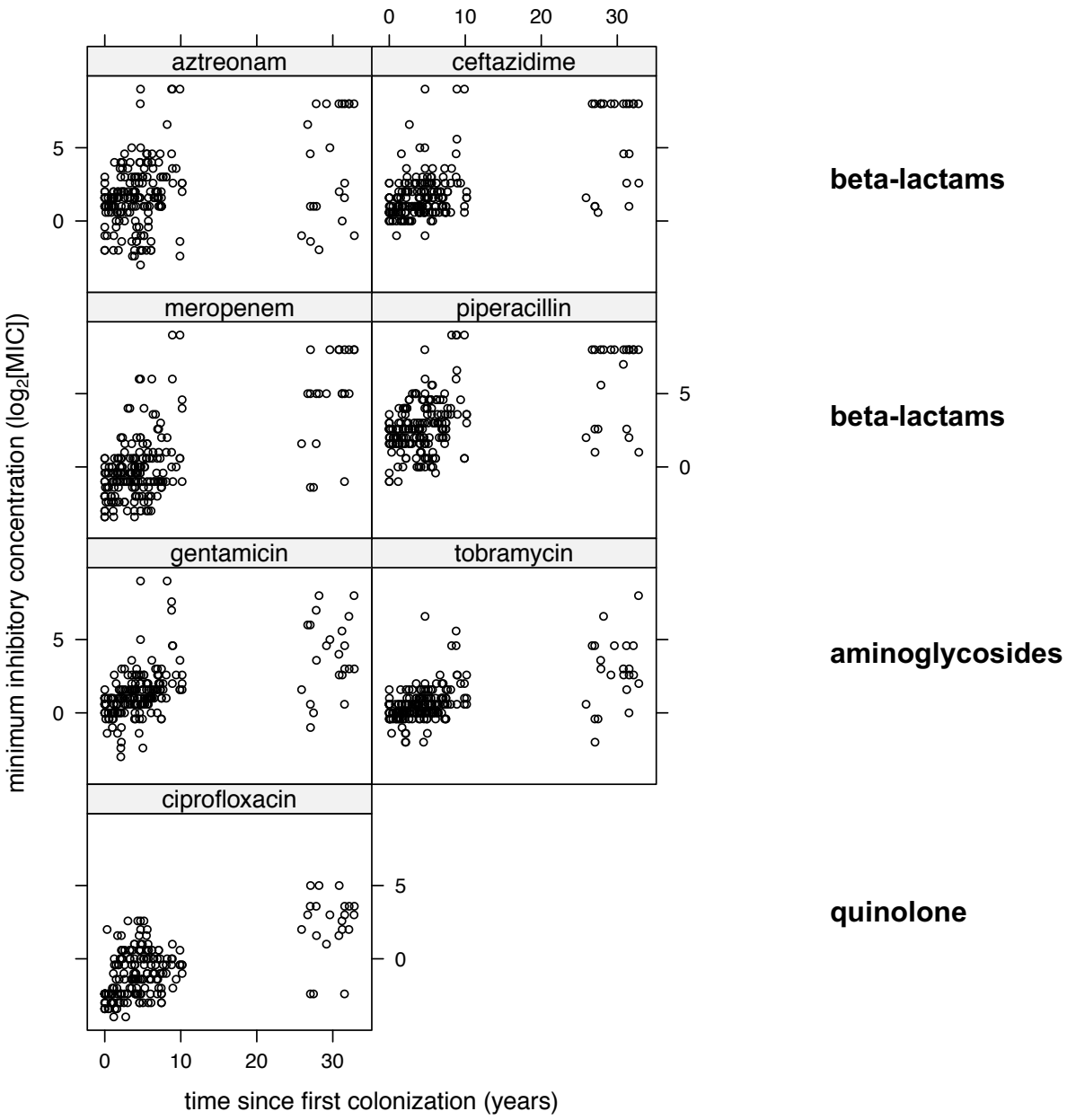


Figure 6. Change in resistance within patients over time. Resistance within patients (considering all clone types together) increased to all seven antibiotics tested (four beta-lactams, two aminoglycosides and one quinolone; see Table 11 for statistics). Each point represents the minimum inhibitory concentration of an isolate (n = 207) from 17 different patients, covering 33 different clone types. The isolates collected around 30 years all come from one patient (see description in Chapter 2, Methods).

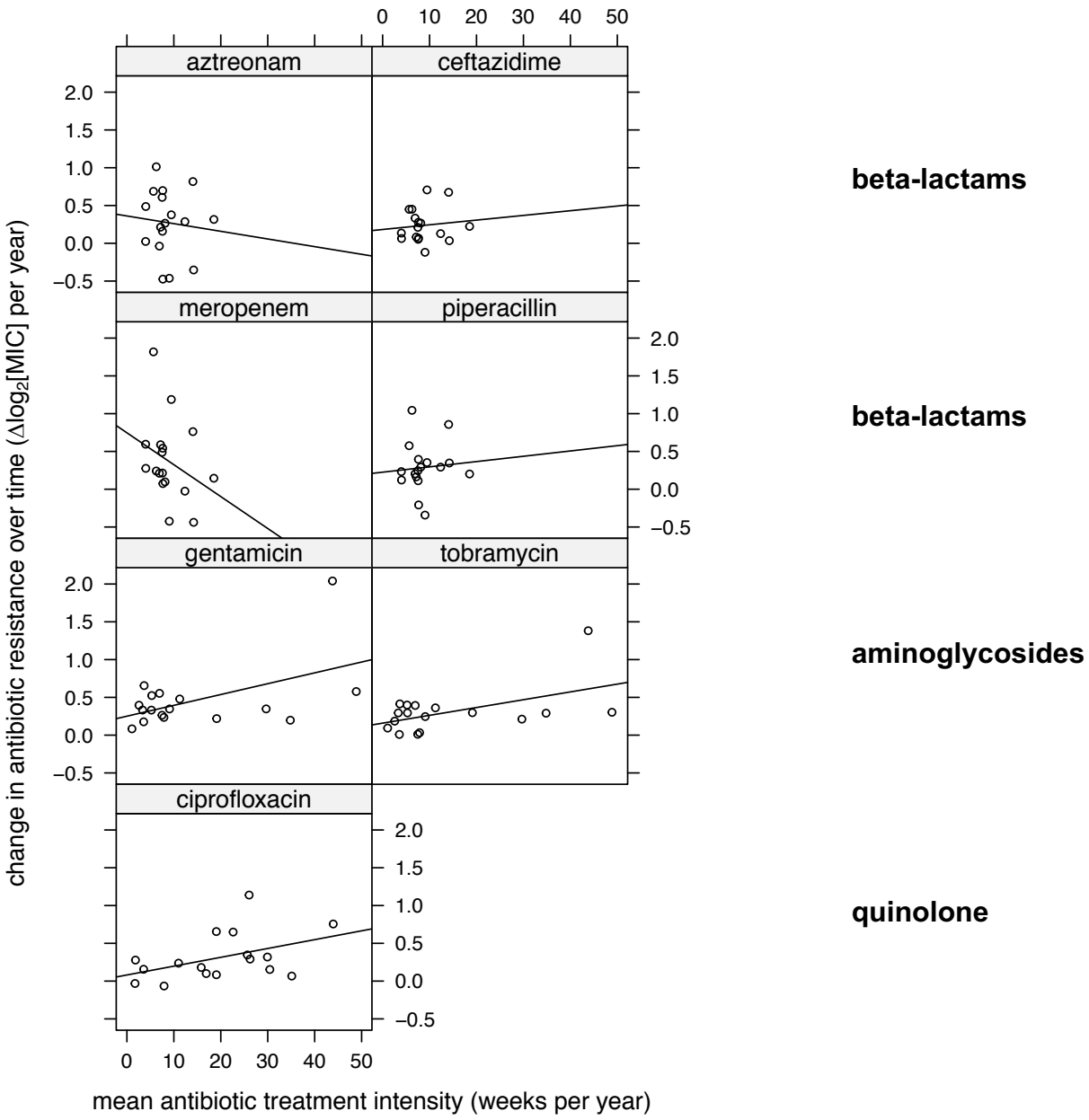


Figure 7. Interaction between antibiotic treatment intensity and the change in antibiotic resistance within clone types over time. Each point represents one patient (n = 17). The y-axis is the gradient of the change in antibiotic resistance over time for each patient, and the x-axis is the mean treatment intensity (weeks of antibiotic treatment per year) for the relevant antibiotic class that that patient received. In the beta-lactams there is no significant relationship between treatment intensity and change in resistance (interaction term dropped from the model). In ciprofloxacin (a quinolone) and the aminoglycosides (gentamicin and tobramycin) there is a greater increase in resistance in patients who have received a higher intensity of treatment by quinolone and aminoglycoside antibiotics respectively (ciprofloxacin: p = 0.011; gentamicin: 7.9×10^{-5}; tobramycin: p = 0.0065). For full statistics, please see the interaction terms in Tables 12, 14 and 16.

		Gradient	*df*	*T*	*p*
log_2(resistance)					
clone infecting time					
beta-lactams:	aztreonam	0.160	52.5	2.1	0.040
	ceftazidime	0.270	52.5	3.5	0.00083
	meropenem	0.498	52.5	6.6	2.5×10^{-8}
	piperacillin	0.214	52.5	2.8	0.0068
aminoglycosides:	gentamicin	0.309	52.5	4.1	0.00016
	tobramycin	0.219	52.5	2.9	0.0058
quinolone:	ciprofloxacin	0.360	52.5	4.7	1.7×10^{-5}

Table 10. Resistance and clone infecting time. Results of a linear mixed effects model with clone infecting time and antibiotic as fixed effects, and genotype nested within patient as a random factor. The gradient for each antibiotic is given. Resistance to seven antibiotics in 207 isolates was measured, giving 1449 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients.

		Gradient	*df*	*T*	*p*
log_2(resistance)					
time since first colonization					
beta-lactams:	aztreonam	0.222	15.7	4.2	0.00064
	ceftazidime	0.281	15.7	5.4	6.5×10^{-5}
	meropenem	0.336	15.7	6.4	9.2×10^{-6}
	piperacillin	0.259	15.7	5.0	0.00015
aminoglycosides:	gentamicin	0.241	15.7	4.6	0.00031
	tobramycin	0.219	15.7	4.2	0.00071
quinolone:	ciprofloxacin	0.264	15.7	5.0	0.00013

Table 11. Resistance and time since first colonization. Results of a linear mixed effects model with time since first colonization and antibiotic as fixed effects, and patient as a random factor. The gradient for each antibiotic is given. Resistance to seven antibiotics in 207 isolates was measured, giving 1449 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients.

factors included in maximal model					
dependent variable:	resistance (log_2[MIC])				
independent variables:	clone infecting time treatment intensity treatment duration antibiotic (categorical)	**Estimate (log_2)**	*df*	*T*	*p*
outputs from minimal adequate model					
clone infecting time					
aztreonam		0.157	40.3	1.8	0.085
ceftazidime		0.249	40.3	2.8	0.0079
meropenem		0.469	40.3	5.3	4.9×10^{-6}
piperacillin-tazobactam		0.199	40.3	2.2	0.031
clone infecting time × beta-lactam intensity					*not sig*
clone infecting time × beta-lactam duration					*not sig*

Table 12. Resistance to beta-lactam antibiotics over clone infecting time. Resistance to four beta-lactam antibiotics (aztreonam, ceftazidime, meropenem, piperacillin-tazobactam) was tested in 207 isolates, making 828 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients. Results are from a linear mixed effects model with genotype nested within patient as a random effect. 'Not sig' means the terms were dropped during model simplification as they did not significantly improve the model. Some outputs not of direct relevance to this study, such as different intercepts for different antibiotics, are not included in the table.

factors included in maximal model					
dependent variable:	resistance (log_2[MIC])				
independent variables:	time since first colonization treatment intensity treatment duration antibiotic (categorical)	**Estimate (log_2)**	***df***	***T***	***p***
outputs from minimal adequate model					
time since first colonization					
aztreonam		0.183	15.1	2.7	0.017
ceftazidime		0.242	15.1	3.5	0.0029
meropenem		0.297	15.1	4.3	0.00057
piperacillin-tazobactam		0.220	15.1	3.2	0.0056
time since first colonization × beta-lactam intensity					*not sig*
time since first colonization × beta-lactam duration					*not sig*

Table 13. Resistance to beta-lactam antibiotics over time since first colonization. Resistance to four beta-lactam antibiotics (aztreonam, ceftazidime, meropenem, piperacillin-tazobactam) was tested in 207 isolates, making 828 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients. Results are from a linear mixed effects model with patient as a random effect. 'Not sig' means the terms were dropped during model simplification as they did not significantly improve the model. Some outputs not of direct relevance to this study, such as different intercepts for different antibiotics, are not included in the table.

factors included in maximal model					
dependent variable:	resistance (log_2[MIC])				
independent variables:	clone infecting time treatment intensity treatment duration antibiotic (categorical)	**Estimate (log_2)**	***df***	***T***	***p***
outputs from minimal adequate model					
clone infecting time					
gentamicin		0.302	23.3	2.2	0.034
tobramycin		0.200	23.3	3.4	0.0025
gentamicin					
clone infecting time × aminoglycoside intensity		0.0598	21.8	4.8	7.9×10^{-5}
clone infecting time × aminoglycoside duration		-0.00953	22.0	-4.3	0.00028
tobramycin					
clone infecting time × aminoglycoside intensity		0.0371	21.8	3.0	0.0065
clone infecting time × aminoglycoside duration		-0.0055	22.0	-2.5	0.021

Table 14. Resistance to aminoglycoside antibiotics over clone infecting time. Resistance to the aminoglycosides (gentamicin and tobramycin) was tested in 207 isolates, making 414 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients. Results are from a linear mixed effects model with genotype nested within patient as a random effect. Some outputs not of direct relevance to this study, such as different intercepts for different antibiotics, are not included in the table.

factors included in maximal model					
dependent variable:	resistance (log_2[MIC])				
independent variables:	time since first colonization treatment intensity treatment duration antibiotic (categorical)	**Estimate (log_2)**	***df***	***T***	***p***
outputs from minimal adequate model					
time since first colonization		0.222	358.9	5.3	1.6×10^{-7}
time since first colonization × aminoglycoside intensity		0.0341	133.4	5.8	4.8×10^{-8}
time since first colonization × aminoglycoside duration		-0.0053	71.6	-5.7	2.8×10^{-7}

Table 15. Resistance to aminoglycoside antibiotics over time since first colonization. There was no interaction between antibiotic and time, so there is one gradient for both antibiotics (gentamicin and tobramycin). Resistance to the aminoglycosides (gentamicin and tobramycin) was tested in 207 isolates, making 414 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients. Results are from a linear mixed effects model with patient as a random effect. Some outputs not of direct relevance to this study, such as different intercepts for different antibiotics, are not included in the table.

factors included in maximal model					
dependent variable:	resistance (log_2[MIC])				
independent variables:	clone infecting time treatment intensity treatment duration	**Estimate (log_2)**	***df***	***T***	***p***
outputs from minimal adequate model					
clone infecting time		0.0707	184.4	0.90	0.370
clone infecting time × quinolone intensity		0.0103	182.0	2.6	0.011
clone infecting time × quinolone duration					*not sig*

Table 16. Resistance to quinolone antibiotics over clone infecting time. Resistance to the quinolone ciprofloxacin was tested in 207 isolates. Isolates were from 33 clone types in 17 patients. Results are from a linear mixed effects model with genotype nested within patient as a random effect. 'Not sig' means the terms were dropped during model simplification as they did not significantly improve the model. Some outputs not of direct relevance to this study, such as the fixed effect of antibiotic treatment (as opposed to the interaction effect) are not included in the table.

factors included in maximal model					
dependent variable:	resistance (log_2[MIC])				
independent variables:	time since first colonization treatment intensity treatment duration	**Estimate (log_2)**	***df***	***T***	***p***
outputs from minimal adequate model					
time since first colonization		0.145	16.8	4.2	0.00056
time since first colonization × quinolone intensity		0.00617	92.1	2.3	0.0233
time since first colonization × quinolone duration					*not sig*

Table 17. Resistance to quinolone antibiotics over time since first colonization. Resistance to the quinolone ciprofloxacin was tested in 207 isolates. Isolates were from 33 clone types in 17 patients. Results are from a linear mixed effects model with patient as a random effect. 'Not sig' means the terms were dropped during model simplification as they did not significantly improve the model. Some outputs not of direct relevance to this study, such as the fixed effect of antibiotic treatment (as opposed to the interaction effect) are not included in the table.

factors included in maximal model				
dependent variable:	resistance (log_2[MIC])			
independent variables:	non-induced beta-lactamase clone infecting time antibiotic (categorical)	**Estimate (log_2)**	***df***	***T***	***p***
outputs from minimal adequate model				
aztreonam				
non-induced beta-lactamase		-5.06	582.2	-2.8	0.0059
clone infecting time		0.0985	42.8	1.1	0.263
non-induced beta-lactamase × clone infecting time		1.43	612.2	3.9	0.00013
ceftazidime					
non-induced beta-lactamase		2.868	582.2	1.6	0.118
clone infecting time		0.240	42.8	2.8	0.0084
non-induced beta-lactamase × clone infecting time		-0.0033	612.2	-0.009	0.993
meropenem					
non-induced beta-lactamase		0.629	582.2	0.34	0.732
clone infecting time		0.453	42.8	5.2	5.2×10^{-6}
non-induced beta-lactamase × clone infecting time		0.499	612.2	1.3	0.179
piperacillin					
non-induced beta-lactamase		0.908	582.2	0.50	0.620
clone infecting time		0.186	42.8	2.1	0.038
non-induced beta-lactamase × clone infecting time		0.220	612.2	0.59	0.554

Table 18. Effect of non-induced beta-lactamase and clone infecting time on beta-lactam resistance. Resistance to four beta-lactam antibiotics (aztreonam, ceftazidime, meropenem, piperacillin-tazobactam) was tested in 207 isolates, making 828 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients. Results are from a linear mixed effects model with non-induced beta-lactamase and clone infecting time as continuous predictor variables, antibiotic as a categorical variable and genotype nested within patient as a random effect. Some outputs not of direct relevance to this study, such as different intercepts for different antibiotics, are not included in the table.

Change in variance of antibiotic resistance over time[1]

The variance of antibiotic resistance increased with clone infecting time for all beta-lactam antibiotics and both aminoglycosides, but not for the quinolone ciprofloxacin, for which there was no significant change over time (Figure 8, Table 19). Variance of resistance increased with time since first colonization for all antibiotics of all types tested (Figure 9, Table 20), although for ciprofloxacin and gentamicin this effect was abolished when patient P3602 was removed from the analysis (Table 21). This patient had been infected for approximately 25 years longer than any other in the dataset.

[1] For updated analysis, please see Ghoul & Crosse *et al.* (manuscript in preparation). Social mechanisms of antibiotic resistance and long-term evolution of resistance in *Pseudomonas aeruginosa* infections.

When antibiotic treatment was included in the initial model, the increase in variance in beta-lactams was found to be correlated with the intensity of beta-lactam treatment (positive interaction between clone infecting time and treatment intensity, $t = 3.3$, $p = 0.0008$, Table 22), accounting for the overall increase in variance over time reported above (the effect of clone infecting time itself was no longer significant in this model ($t = 1.1$, $p = 0.28$). There was also a small negative interaction with total duration of beta-lactam treatment ($t = -3.0$, $p = 0.003$). There was no correlation between antibiotic treatment (either duration or intensity) and the change in variance of resistance over clone infecting time for aminoglycosides or quinolones (Tables 23 and 22).

The results with time since first colonization were similar for beta-lactams and quinolones. There was a positive interaction between intensity of beta-lactam treatment and time since first colonization accounting for the increase in variance of beta-lactam resistance ($t = 6.0$, $p = 2.8\times10^{-9}$), with a smaller negative interaction with beta-lactam treatment duration ($t = -4.6$, $p = 5.8\times10^{-6}$) (Table 25). There was no effect of treatment (either duration or intensity) on variance of quinolone resistance over time since first colonization (Table 26). In contrast to clone infecting time there was, however, a positive interaction between aminoglycoside treatment intensity and time since first colonization in their effect on variance of resistance ($t = 3.0$, $p = 0.0034$), and a smaller negative interaction with treatment duration ($t = -2.6$, $p = 0.0094$) (Table 27).

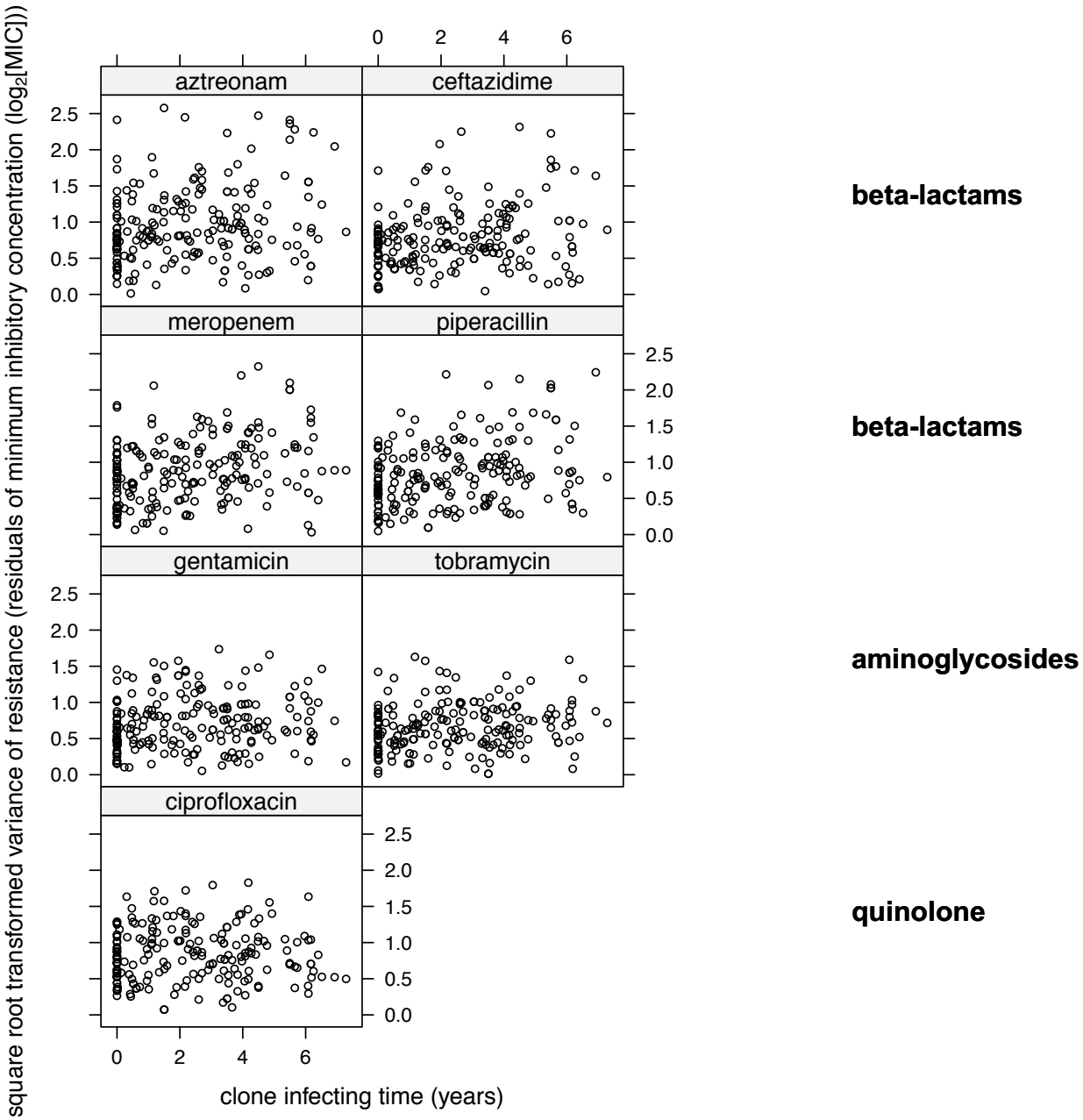


Figure 8. Change in variance of resistance within clone types over time. Variance of resistance within clone types in patients increased in all four beta-lactams tested (aztreonam: $p = 7.9\times10^{-5}$; ceftazidime: $p = 0.00074$; meropenem: $p = 2.2\times10^{-6}$; piperacillin with tazobactam: $p = 1.7\times10^{-6}$) and both aminoglycosides (gentamicin: $p = 0.045$, and tobramycin: $p = 0.025$). There was no increase in variance in the quinolone, ciprofloxacin ($p = 0.84$). See Table 19 for full statistics. Each point represents an isolate's residual minimum inhibitory concentration not explained by the linear mixed model (a higher value therefore represents greater variance from the mean). $n = 207$ isolates from 17 different patients, covering 33 different clone types. The y-axis has been square root transformed, reflecting the analysis.

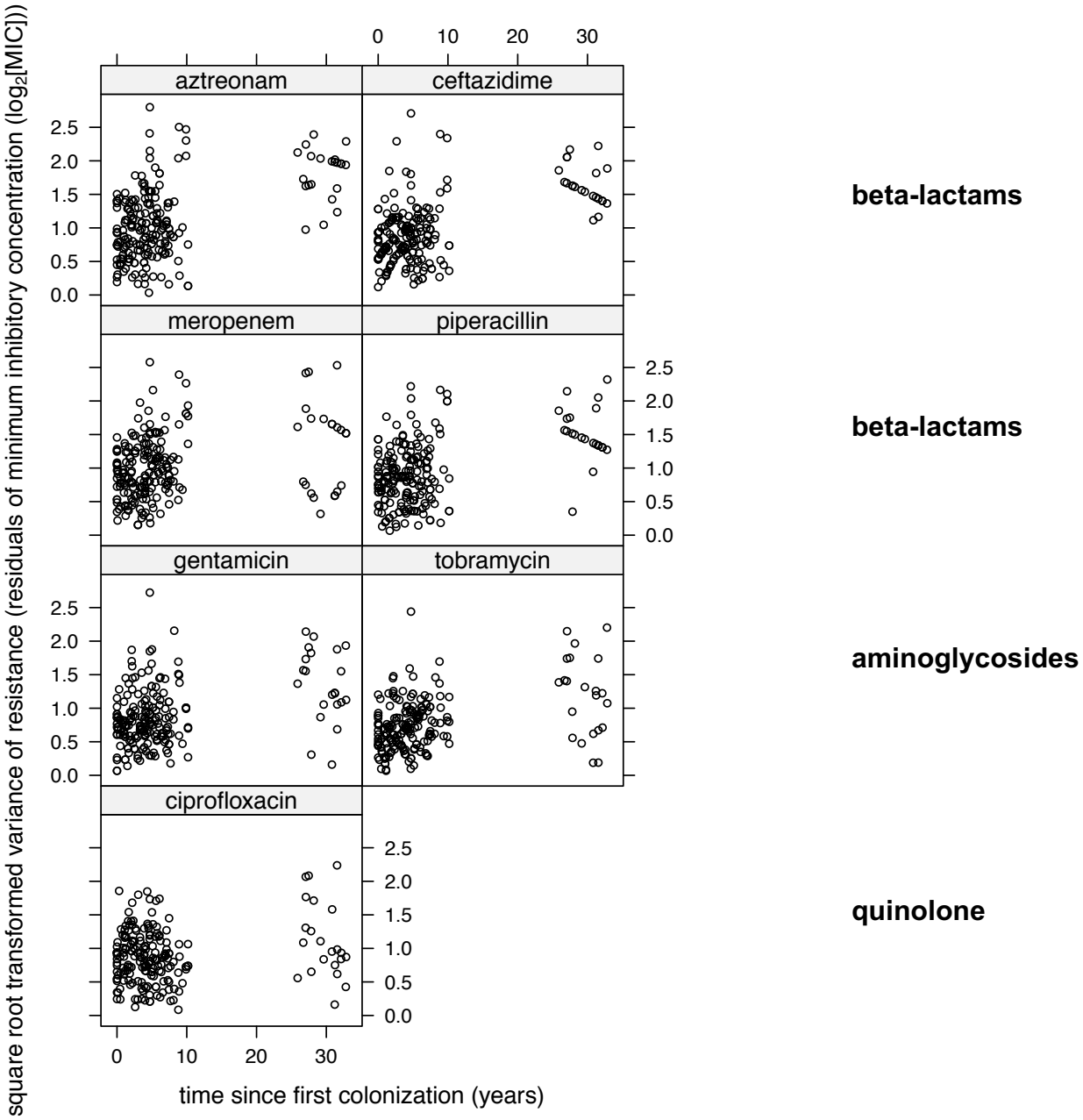


Figure 9. Change in variance of resistance within patients over time. Variance of resistance within patients increased in all antibiotics of all classes tested: the four beta-lactams (aztreonam: $p = <2\times10^{-16}$; ceftazidime: $p = 4.1\times10^{-16}$; meropenem: $p = 1.8\times10^{-6}$; piperacillin with tazobactam: $p = 1.9\times10^{-12}$); the two aminoglycosides (gentamicin: $p = 5.1\times10^{-8}$ and tobramycin: $p = 1.2\times10^{-7}$) and the quinolone, ciprofloxacin: ($p = 0.035$) (see Table 20). Removal of isolates from patient P3602 (all the points clustered around 30 years) abolished the effect in gentamicin and ciprofloxacin (Table 21). Each point represents an isolate's residual minimum inhibitory concentration not explained by the linear model (a higher value therefore represents greater variance from the mean). $n = 207$ isolates from 17 different patients, covering 33 different clone types. The y-axis has been square root transformed, reflecting the analysis.

		Estimate √	T	$F_{13, 1435}$	p
variance of resistance				12.7	$<2\times10^{-16}$
beta-lactams:	aztreonam	0.0565	4.0		7.9×10^{-5}
	ceftazidime	0.0483	3.4		0.00074
	meropenem	0.0680	4.8		2.2×10^{-6}
	piperacillin	0.0688	4.8		1.7×10^{-6}
aminoglycosides:	gentamicin	0.0287	2.0		0.045
	tobramycin	0.0322	2.3		0.025
quinolone:	ciprofloxacin	0.0028	0.2		0.84

Table 19. Change in variance of resistance (MIC) with clone infecting time for each antibiotic (linear model of effect of clone infecting time on the square root transformed variance of resistance, with antibiotic type included as a categorical variable). The top line is the significance of the overall model; the lines below represent the results for each antibiotic, with the *p*-value the significance in the difference of the gradient from zero.

		Estimate √	T	$F_{13, 1435}$	p
variance of resistance				27.3	$<2\times10^{-16}$
beta-lactams:	aztreonam	0.0312	8.9		$<2\times10^{-16}$
	ceftazidime	0.0292	8.2		4.1×10^{-16}
	meropenem	0.0170	4.8		1.8×10^{-6}
	piperacillin	0.0252	7.1		1.9×10^{-12}
aminoglycosides:	gentamicin	0.0195	5.5		5.1×10^{-8}
	tobramycin	0.0189	5.3		1.2×10^{-7}
quinolone:	ciprofloxacin	0.00751	2.1		0.035

Table 20. Change in variance of resistance (MIC) with time since first colonization for each antibiotic (linear model of effect of time since first colonization on the square root transformed variance of resistance, with antibiotic type included as a categorical variable). The top line is the significance of the overall model; the lines below represent the results for each antibiotic, with the *p*-value the significance in the difference of the gradient from zero.

		Estimate √	T	$F_{13, 1281}$	p
variance of resistance					$<2\times10^{-16}$
beta-lactams:	aztreonam	0.038	3.4		0.00066
	ceftazidime	0.022	2.0		0.043
	meropenem	0.055	4.9		9.6×10^{-7}
	piperacillin	0.031	2.8		0.0055
aminoglycosides:	gentamicin	0.010	0.93		0.36
	tobramycin	0.027	2.4		0.016
quinolone:	ciprofloxacin	-0.011	-0.99		0.32

Table 21. Change in variance of resistance (MIC) with time since first colonization for each antibiotic, with Patient 3602 removed from the analysis (linear model of effect of time since first colonization on the square root transformed variance of resistance, with antibiotic type included as a categorical variable). The top line is the significance of the overall model; the lines below represent the results for each antibiotic, with the *p*-value the significance in the difference of the gradient from zero.

factors included in maximal model					
dependent variable:	variance of resistance				
independent variables:	clone infecting time treatment intensity treatment duration antibiotic (categorical)	**Estimate** √	***T***	$F_{8, 819}$	***p***
outputs from minimal adequate model				11.7	6.7×10^{-16}
clone infecting time		0.0227	1.1		0.28
clone infecting time × beta-lactam intensity		0.0150	3.3		0.00085
clone infecting time × beta-lactam duration		-0.00166	-3.0		0.0030

Table 22. Variance of resistance to beta-lactam antibiotics over clone infecting time. There was no interaction between antibiotic and time, so there is one gradient for all four antibiotics (aztreonam, ceftazidime, meropenem, piperacillin-tazobactam). Resistance to four antibiotics was tested in 207 isolates, making 828 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients. Variance was quantified from the residuals of a linear mixed effects model of change in resistance (MIC) over time, with genotype nested within patient as a random effect. Variance was then square root transformed for the final linear model. Estimates given are for the square root model. Some outputs not of direct relevance to this study, such as different intercepts for different antibiotics, are not included in the table.

factors included in maximal model					
dependent variable:	variance of resistance				
independent variables:	clone infecting time treatment intensity treatment duration antibiotic (categorical)	**Estimate** √	***T***	$F_{2, 411}$	***p***
outputs from minimal adequate model				11.1	2.1×10^{-5}
clone infecting time		0.0274	3.3		0.0012
clone infecting time × aminoglycoside intensity					not sig
clone infecting time × aminoglycoside duration					not sig

Table 23. Variance of resistance to aminoglycoside antibiotics over clone infecting time. There was no interaction between antibiotic and time, so there is one gradient for both antibiotics (gentamicin and tobramycin). Resistance to both antibiotics was tested in 207 isolates, making 414 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients. Variance was quantified from the residuals of a linear mixed effects model of change in resistance (MIC) over time, with genotype nested within patient as a random effect. Variance was then square root transformed for the final linear model. Estimates given are for the square root model. 'Not sig' means the terms were dropped during model simplification as they did not significantly improve the model. Some outputs not of direct relevance to this study, such as different intercepts for different antibiotics, are not included in the table.

factors included in maximal model					
dependent variable:	variance of resistance				
independent variables:	clone infecting time treatment intensity treatment duration	**Estimate** √	***T***	$F_{1, 205}$	***p***
outputs from minimal adequate (null) model				3.3	0.072
clone infecting time		0.0041	1.8		0.072
clone infecting time × quinolone intensity					not sig
clone infecting time × quinolone duration					not sig

Table 24. Variance of resistance to quinolone antibiotics over clone infecting time. Resistance to the antibiotic ciprofloxacin was tested in 207 isolates. Isolates were from 33 clone types in 17 patients. Variance was quantified from the residuals of a linear mixed effects model of change in resistance (MIC) over time, with genotype nested within patient as a random effect. Variance was then square root transformed for the final linear model. Estimates given are for the square root model. 'Not sig' means the terms were dropped during model simplification as they did not significantly improve the model. No significant terms remained within this model after simplification (values for the effect of clone infecting time are presented in the table, while not significant).

factors included in maximal model					
dependent variable:	variance of resistance				
independent variables:	time since first colonization treatment intensity treatment duration antibiotic (categorical)	**Estimate** √	***T***	$F_{14, 813}$	***p***
outputs from minimal adequate model				20.6	$<2.2\times10^{-16}$
time since first colonization					
aztreonam		-0.00605	-0.50		0.62
ceftazidime		-0.00635	-0.52		0.60
meropenem		-0.0205	-1.7		0.093
piperacillin-tazobactam		-0.0118	-0.97		0.33
time since first colonization × beta-lactam intensity		0.0154	6.0		2.7×10^{-9}
time since first colonization × beta-lactam duration		-0.00152	-4.6		5.8×10^{-6}

Table 25. Variance of resistance to beta-lactam antibiotics over time since first colonization. Resistance to four antibiotics (aztreonam, ceftazidime, meropenem, piperacillin-tazobactam) was tested in 207 isolates, making 828 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients. Variance was quantified from the residuals of a linear mixed effects model of change in resistance (MIC) over time, with patient as a random effect. Variance was then square root transformed for the final linear model. Estimates given are for the square root model. Some outputs not of direct relevance to this study, such as different intercepts for different antibiotics, are not included in the table.

factors included in maximal model					
dependent variable:	variance of resistance				
independent variables:	length of infection treatment intensity treatment duration	**Estimate** √	***T***	$F_{1, 205}$	***p***
outputs from minimal adequate model				5.1	0.025
length of infection		0.0075	2.3		0.025
length of infection × quinolone intensity					not sig
length of infection × quinolone duration					not sig

Table 26. Variance of resistance to quinolone antibiotics over time since first colonization. Resistance to the antibiotic ciprofloxacin was tested in 207 isolates. Isolates were from 33 clone types in 17 patients. Variance was quantified from the residuals of a linear mixed effects model of change in resistance (MIC) over time, with patient as a random effect. Variance was then square root transformed for the final linear model. Estimates given are for the square root model. 'Not sig' means the terms were dropped during model simplification as they did not significantly improve the model.

factors included in maximal model					
dependent variable:	variance of resistance				
independent variables:	time since first colonization treatment intensity treatment duration antibiotic (categorical)	**Estimate** √	***T***	$F_{6, 407}$	***p***
outputs from minimal adequate model				18.8	$<2.2\times10^{-16}$
time since first colonization		0.0157	1.45		0.147
time since first colonization × aminoglycoside intensity		0.00369	3.0		0.00034
time since first colonization × aminoglycoside duration		-0.000479	-2.6		0.00094

Table 27. Variance of resistance to aminoglycoside antibiotics over time since first colonization. There was no interaction between antibiotic and time, so there is one gradient for both antibiotics (gentamicin and tobramycin). Resistance to both antibiotics was tested in 207 isolates, making 414 isolate-antibiotic pairs. Isolates were from 33 clone types in 17 patients. Variance was quantified from the residuals of a linear mixed effects model of change in resistance (MIC) over time, with patient as a random effect. Variance was then square root transformed for the final linear model. Estimates given are for the square root model. Some outputs not of direct relevance to this study, such as different intercepts for different antibiotics, are not included in the table.

Discussion

In this chapter, I describe the relationship between beta-lactamase production and beta-lactam resistance, and then long-term dynamics in antibiotic resistance. I show that beta-lactamase production is correlated with resistance (Figures 1 and 3), and that this resistance is in part mediated by AmpC beta-lactamase (Tables 8 and 9). This confirms that beta-lactamase is beneficial in an environment where selection pressure from beta-lactam antibiotics is likely to be a significant. I then show that beta-lactam resistance increases over time (Figure 5 and 6), contrasting with the finding in Chapter 2 where beta-lactamase production does not increase. This suggests that resistance to beta-lactam antibiotics is not simply a result of beta-lactamase production, but is likely to involve other mechanisms. The effect of (non-induced) beta-lactamase production on resistance increased with time for one beta-lactam only (positive interaction for aztreonam, Table 18) but not for the other three beta-lactams, suggesting that the relative importance of beta-lactamase in the majority of cases does not change with time. No effect of beta-lactam treatment on the increase in beta-lactam resistance is found (Figure 7). This is in contrast to the relationship between treatment and resistance found in other antibiotic classes (also Figure 7), suggesting that beta-lactam antibiotics may not simply select for higher resistance, while aminoglycosides and quinolones do. Finally, and crucially, I show that while there is an increase in variance in beta-lactam resistance over time and that this correlates with intensity of treatment with beta-lactams, there is less of a divergence with aminoglycosides, and no divergence in quinolone resistance (Figure 8) – and no interaction with treatment in the latter two classes. This is consistent with predictions

based on the differences in the potential for social dynamics between the three classes of antibiotics (see Chapter 1).

Resistance and beta-lactamase

The results reported in this chapter suggest that beta-lactamase is an important mechanism of resistance, even when multiple other resistance mechanisms are potentially operating. Non-induced beta-lactamase production correlates with resistance in non-inducing beta-lactams (Figure 1), and induced beta-lactamase correlates with resistance in the inducing antibiotic imipenem (Figure 3). These correlations show that if beta-lactamase production is low, cells do not fully compensate for the reduced resistance with other mechanisms. In the case of the non-inducing antibiotics, constitutive producers are important in the relationship: when these are removed, the correlation disappears. For the inducer imipenem, one very high beta-lactamase producer is crucial for the relationship. The fact that the correlations exist but are reliant on a comparatively small number of isolates underlines the contribution of other resistance mechanisms. This is consistent with the literature where beta-lactamase has been shown to be an important resistance mechanism in *P. aeruginosa* in both laboratory strains (Juan *et al.*, 2006) and clinical isolates (Cavallo *et al.*, 2000; Juan *et al.*, 2005) (see also reviews by Jacoby (2009) and Lister *et al.* (2009)), but that other mechanisms of resistance are important (Köhler *et al.*, 1999; Cavallo *et al.*, 2000; Lister *et al.*, 2009; Strateva & Yordanov, 2009; Tomás *et al.*, 2010). Indeed, relative contributions of each resistance mechanism can vary for each individual antibiotic (Masuda *et al.*, 1999; Srikumar *et*

al., 1999) and are not even necessarily consistent within subclasses of beta-lactams (Köhler *et al.*, 1999).

The effect of non-induced beta-lactamase production on resistance increased with clone infecting time for one beta-lactam only (positive interaction for aztreonam, Table 18) but not for the other three beta-lactams. This suggests that the relative importance of beta-lactamase in the majority of cases does not change with time. From a social evolutionary perspective it might have been expected that non-producers with other resistance mechanisms should arise over time, potentially weakening the relationship between beta-lactamase and resistance. The results do not support this, and even goes against this with aztreonam. The results do not preclude a social effect, but more direct testing of the contribution of different resistance mechanisms at different stages of infection would be valuable in understanding the evolutionary dynamics.

While a correlation between resistance of the inducer imipenem and induced beta-lactamase was shown, no such correlation was found in benzylpenicillin, the second inducing antibiotic used in this study. This is likely to reflect the fact that benzylpenicillin is highly labile to AmpC beta-lactamase (Jacoby, 2009) such that even low beta-lactamase producers are resistant to concentrations thousands of times higher than typical clinical breakpoints for benzylpenicilin (Li *et al.*, 1994; EUCAST, 2019).

These results also confirm the importance specifically of AmpC beta-lactamase. Addition of cloxacillin, an AmpC beta-lactamase inhibitor, reduced resistance in all

cases, even when no correlation between beta-lactamase and resistance had been shown. Tazobactam, which primarily inhibits plasmid-borne TEM and SHV beta-lactamases had no significant effect, supporting the idea that AmpC is the most important beta-lactamase in these clinical isolates. This is consistent with previous studies which have shown that AmpC is ubiquitous in *P. aeruginosa* but secondary beta-lactamases, such as TEM and SHV, are comparatively rare (Livermore, 1995).

Long-term dynamics and antibiotic treatment

While resistance to all antibiotics tested - beta-lactams, aminoglycosides and a quinolone – increased over time, in beta-lactams the level of increase was not correlated with beta-lactam treatment. Increase in aminoglycoside and quinolone resistance was positively correlated with aminoglycoside and quinolone treatment intensity respectively. This suggests that while beta-lactams do not simply select for higher resistance, aminoglycosides and quinolones do. A meta-analysis by Bell *et al.* (2014) found a weaker relationship between beta-lactam consumption and resistance than in other antibiotic classes, and the strongest correlation for a particular class was in quinolone resistant *E. coli*. Both my findings and those of Bell *et al.* are consistent with social dynamics where beta-lactams select for higher beta-lactamase production, but then lower-producing cheats survive as they are protected by the beta-lactamase of higher producers. However, the lack of a clear relationship in beta-lactams could also be because beta-lactams are resisted by other mechanisms – such as efflux pumps – which are selected by antibiotics of other classes, making the relationship less clear. If this were the case, it remains unclear why aminoglycosides would not be affected in the same manner as beta-lactams, except that they are substrates for a narrower range

of efflux pumps (Lister *et al.*, 2009). In the current study it is also the case that the mean beta-lactam treatment duration is less than the aminoglycoside or quinolone treatment duration, and the variance of beta-lactam treatment duration is smaller, perhaps making a correlation less likely to find rather than there being a biological difference, although this is very unlikely to be the case in all the studies included in Bell *et al.* Beta-lactamase could simply be lost between courses of beta-lactam antibiotics if it were particularly costly. Beta-lactam resistance has been shown to be lost once treatment has ceased (Chung *et al.*, 2007; Reinhardt *et al.*, 2007; Raum *et al.*, 2008), although in other cases it has persisted (Karami *et al.*, 2008). Why beta-lactamase should be lost, while aminoglycoside and quinolone resistance mechanisms are not, remains unclear. This is particularly the case when costs could potentially be offset by compensatory adaptations, although there is only limited evidence for this clinically (Karami *et al.*, 2008; MacLean & Vogwill, 2015). While the results are suggestive of social dynamics in antibiotic resistance, there are alternative explanations.

Variance over time

That beta-lactam resistance diverged over time, while aminoglycosides showed some divergence and quinolones did not, supports the role of social dynamics in antibiotic resistance evolution. Furthermore, the divergence in beta-lactams correlated with treatment intensity, while there was no effect in quinolones, and only an effect in aminoglycosides when looking at divergence within patients rather than clone type, suggesting treatment in beta-lactams selects for divergence but not in quinolones. Divergence is unlikely to be simply due to regional isolation, as then divergence

should be seen in all classes of antibiotic. While it could be argued that as fewer beta-lactams were given to patients on average, genetic drift in isolated regions would play a larger role, leading to greater divergence, this seems unlikely as mutations in antibiotic resistance mechanisms are not likely to be neutral, even in the absence of antibiotics. In fact, giving fewer antibiotics in the context of regional variation in antibiotic availability could lead to less divergence (the fewer antibiotics that are given, the less the regional variation in antibiotic availability and so the less the divergence). My finding, therefore, that beta-lactam resistance diverges over time in contrast to quinolones, despite fewer beta-lactams being given than quinolones, points strongly to a mechanism other than regional isolation.

Heteroresistance is a phenomenon where cells from a single population – and, by some definitions, from a single clonal isolate – exhibit different resistances, usually with a small, resistant subpopulation amongst a more sensitive wider population (El-Halfawy & Valvano, 2015; Andersson *et al.*, 2019). It therefore describes a process whereby bacteria of differing antibiotic resistance coexist. It is, however, not an evolutionary explanation, nor is it a unified process as it includes a number of distinct phenomena (Andersson *et al.*, 2019). How my findings relate to heteroresistance is intriguing. Whether social dynamics may play a role in heteroresistance is unclear. Notably, heteroresistance has been observed in beta-lactams and aminoglycosides, with particularly high rates in cephalosporins and carbapenems (both beta-lactams) (Nicoloff *et al.*, 2019), but not in quinolones (Andersson *et al.*, 2019). However, social evolution tends to suppose that high resistance evolves and is then lost in some lineages, whereas heteroresistance is described as a process where highly resistant lineages emerge out of sensitive ones. While beta-lactamase has been demonstrated to

be involved in heteroresistance (Søgaard, 1985; Pournaras *et al.*, 2010; Lee *et al.*, 2011), efflux and outer membrane permeability changes have also been shown to be involved (Ikonomidis *et al.*, 2008; He *et al.*, 2018), implying heteroresistance, at least in some instances, is distinct from social resistance processes. Further work is needed to clarify the relationship between social dynamics and heteroresistance.

Concluding remarks

Together, the results in this chapter support the hypothesis that social evolution can explain aspects of antibiotic resistance in chronic infections. The power of this approach appears not just confined to those with cooperative resistance mechanisms such as beta-lactams, but it can be used as a framework to compare and contrast resistance evolution across the social spectrum of resistance, from cooperative to private mechanisms. Further comparison of resistance evolution across more examples of each antibiotic class would help clarify the evolutionary pressures. Research into the respective costs of contrasting resistance mechanisms would help clarify the evolutionary pressures.

The social evolutionary approach is not only useful for studying different mechanisms of resistance against different classes of antibiotic, but for different mechanisms of resistance against the same class. While the findings show beta-lactamase is clearly important for beta-lactam resistance, they also suggest an important role for other resistance mechanisms. Future work should consider how the contributions of different mechanisms may change across the course of infection (see Future Directions, Chapter 6).

The results here help relate the beta-lactamase findings from Chapter 2 to clinical resistance. The results are consistent with predictions based on the potential effects of cooperator-cheat dynamics in resistance to different classes of antibiotics. However, the results do not show if low beta-lactamase producers can be protected by higher producers, or gain an advantage when in competition with higher producers. The next two chapters look at the potential ability of lower producers to gain an advantage through cheating.

Chapter 4. Protection and competition mediated by beta-lactamase production

Introduction

In Chapters 2 and 3 I showed that beta-lactamase evolution in chronic cystic fibrosis infections is consistent with social dynamics. I found relatively high levels of extracellular beta-lactamase production by isolates in my collection, particularly in the presence of antibiotics which induce beta-lactamase production. I also showed that high and low producers of beta-lactamase coexist within patients' lungs and that higher producers are on average more resistant, which would potentially require low producers to exploit protection conferred by coexisting with high producers. Here I ask if high beta-lactamase producers can be exploited by low producers acting as evolutionary cheats.

There are two aspects of the interactions between producing and non-producing strains that need to be considered in order to assess the potential for cooperator-cheat dynamics to emerge. First, whether there is a public benefit to the trait and, second, whether the cost of cooperation creates the potential for cheats to invade. A number of studies have investigated the potential for beta-lactamase to act as a public good, but results have been inconclusive. While Yurtsev *et al.* (2013) showed non-producers could invade a population of producers at antibiotic concentrations above the MIC, other studies have shown non-producers remaining only a very small proportion of the population (Dugatkin *et al.*, 2005; Clark *et al.*, 2009), or else invading only at sub-

inhibitory antibiotic concentrations (Medaney *et al.*, 2015; Frost *et al.*, 2018). Others have suggested a protective effect, but an equivalent monoculture control for comparison is required to confirm this (Bottery *et al.*, 2016), while Nicoloff & Andersson (2016) looked at changes in MIC rather than quantifying fitness. Furthermore, these studies used artificially constructed high or low producers (albeit sometimes using natural resistance plasmids), rather than natural clinical isolates. This is important, because many factors might determine fitness differences between strains in nature, or strains may have evolved mechanisms for reducing cross-protection or competition (Zhang *et al.*, 2009; Kümmerli *et al.*, 2015). The potential for public good dynamics to take place between beta-lactamase producers and non-producers in natural clinical populations therefore remains untested. One important next step is to show that beta-lactamase from clinical isolates can protect lower producers which coexist in the same patient, and also to conduct competition assays with clinical isolates, in antibiotic concentrations where survival of non-producers is severely reduced.

In this chapter, I examine whether high producing beta-lactamase isolates from chronic infections can protect lower producing isolates, and whether these lower producers can act as cheats. First, I ask if the supernatant of a high beta-lactamase producer can protect an *ampC* beta-lactamase knockout strain (an artificial low producer), and naturally coexisting low beta-lactamase producing clinical isolates, in the presence of antibiotic at concentrations lethal to the non-producer. I then perform a series of competition assays (West *et al.*, 2006; Ghoul *et al.*, 2014b) between clinical high producers and the low producing *ampC* knockout, to further demonstrate any benefit to low producers from being grown in mixed culture with high producers,

while also measuring the cost to the high producers. Comparison is made with growth in monocultures of low producers and high producers. I perform all experiments in the presence and absence of a beta-lactam antibiotic and in the presence and absence of cloxacillin, an inhibitor of AmpC beta-lactamase. The former is necessary to show if the beta-lactamase is protective against antibiotics or if there could be another unidentified benefit; the latter will show if any benefit seen is mediated by AmpC beta-lactamase.

Methods

Bacterial strains

The full clinical strain collection comprised 215 *Pseudomonas aeruginosa* lung isolates, collected from 17 cystic fibrosis (CF) patients at the Copenhagen CF Centre at Rigshospitalet, Denmark. Patients were sampled longitudinally over 10 years with one to four *P. aeruginosa* isolates selected per sample. 33 clone types were represented, although 15 of these only appeared once (see Chapter 2 for further details). In addition, I used PAO1 (a wild-type laboratory strain) as a beta-lactamase producer, and an otherwise isogenic strain with AmpC beta-lactamase knocked out (PAO1Δ*ampC*) as a low producer in the cross-protection experiment (Moya *et al.*, 2008). In competition assays, I also used the *ampC* knockout tagged with gentamicin resistance (PAO1Δ*ampC*::Gm) in order to distinguish the strains using gentamicin agar plates. There is no fitness cost to the tagging (Moya *et al.*, 2008) (and Figure 1).

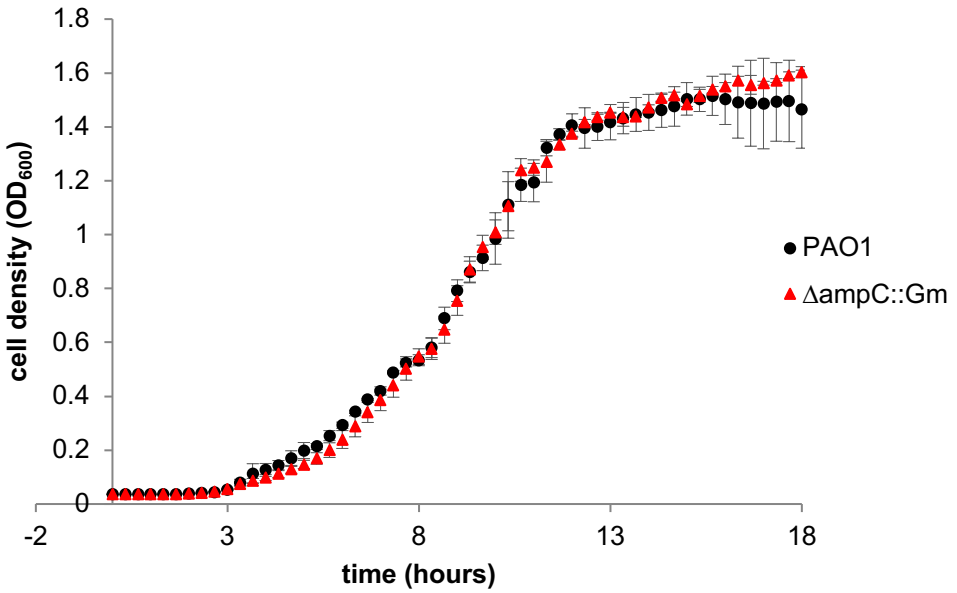


Figure 1. Growth curve for PAO1 and Δ*ampC*::Gm. In the absence of antibiotic, there is no difference in growth between PAO1 and the beta-lactamase knockout tagged with gentamicin resistance (Δ*ampC*::Gm), indicating there is no cost in carrying the gentamicin resistance cassette, and no difference in growth under these conditions with *ampC* knocked out. Growth curves done in KB media in 96-well plates at 37ºC. Each point is three replicates.

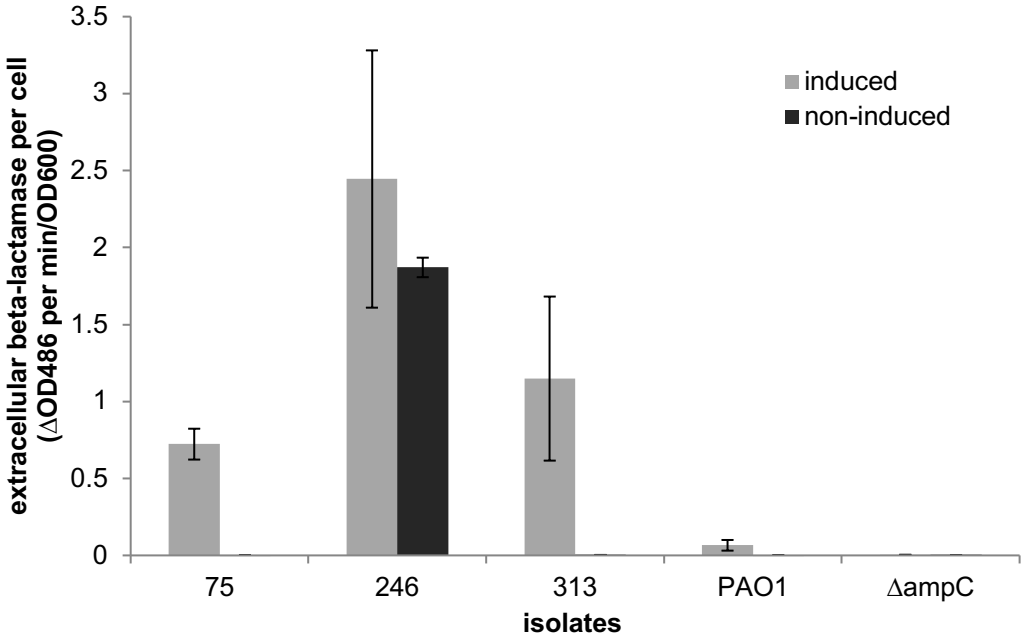


Figure 2. Induced and non-induced beta-lactamase production of isolates used in the competition assays. Although PAO1 produces much less beta-lactamase than the clinical isolates, it still produces 20 times more than Δ*ampC*. Only isolate 246 is a constitutive producer of extracellular beta-lactamase.

For the cross-protection experiment, I selected nine pairs of high and low producing isolates from the main clinical collection. Each pair came from the same clone type within a patient. Each of the nine pairs came from a different patient. I selected 'high producing' isolates from the top quartile of extracellular beta-lactamase production and, from the same patients, I then selected lower producers which were isolated within six months of the high producer. I gave preference to pairs with a shorter time interval between the isolates to increase the chance that the isolates were genuinely coexisting within the patients; for five of the pairs, the high and low producers were sampled on the same day. When there were multiple potential pairs to choose from within a patient, I used the pair with the highest-producing high-producer. In all cases, the higher producer always made more than double the amount of extracellular beta-lactamase than the lower producer. I also used the laboratory strain PAO1 (as the higher beta-lactamase producer) and a beta-lactamase knockout strain (PAO1Δa*mpC*) as the low producer.

For use in the competition assays, I selected three high-producing isolates from the nine used in the cross-protection assay. Of the original nine, one was discounted due to slow growth – there was insufficient growth after 18 hours to accurately measure the MIC. Two further high producers were discounted as their growth was clearly inhibited by the beta-lactamase knockout strain in a spot inhibition assay. Trial mixed cultures were done of the remaining six, and in three further cases the beta-lactamase knockout strain partially inhibited growth of the high producer. This left three suitable clinical high producers and PAO1 which could be grown in mixed culture with the beta-lactamase knockout. Figure 2 shows their level of extracellular beta-lactamase production.

Measuring beta-lactamase activity

I estimated beta-lactamase production per cell for each isolate using a standard nitrocefin assay (O'Callaghan *et al.*, 1972); see Chapter 2 for further details.

Cross-protection assays

First, I prepared sterile supernatant of high producing isolates. To do this, I cultured high producing isolates from freezer stock in 6ml of KB, grown overnight at 37˚C, 200rpm. Beta-lactamase production is highest during growth, so I took 1ml of overnight culture and diluted it into 4ml of fresh KB containing sub-inhibitory benzylpenicillin (final concentration 500mg/l). I incubated these for a further three hours to induce beta-lactamase production. After centrifuging at 7385rpm for five minutes, I filter-sterilized the supernatant.

I then prepared cultures of low producing isolates, which I also had cultured from freezer stock in 6ml of KB, grown overnight at 37˚C, 200rpm. I standardized the undiluted overnight culture to 0.1 OD_{600} to create the inoculum, which I then added to the appropriate supernatant at a 10^{-2} dilution. I added the low producer inoculum to both high producer supernatant, and back into its own supernatant as a control. I added 75µl of this inoculum/supernatant mix into a 96-well plate with 50µl of 12 serial dilutions (in fresh KB) of the beta-lactam antibiotic piperacillin (final concentrations from 512mg/l to 0.25mg/l). This enabled me to find the MICs of the low-producing isolates in their own supernatant. For the purposes of this experiment, I defined MIC as less than 0.1OD_{600} growth (no visible growth in wells). I could then compare this with growth at the same antibiotic concentration when the low producer

was inoculated into supernatant from the high producer. I added a further 25µl of either dH_2O or AmpC beta-lactamase inhibitor (cloxacillin, final concentration 200mg/l) to find out if the protective effect was mediated by AmpC beta-lactamase. I quantified growth by measuring OD_{600} after 18 hours incubation at 37ºC. One pair of slow growing isolates then had to be reincubated up to a total of 66 hours for growth to be measurable. Three technical replicates were completed.

Competition assays

I cultured isolates from freezer stock in 6ml of KB, grown overnight at 37˚C, 200rpm, before standardizing to 0.2 OD_{600}. I used these cultures to inoculate fresh media with either single or mixed strain inoculums. For mixed cultures, I added 2µl of the high producer culture and 2µl of a 10^{-1} dilution of the low producer to 148µl KB liquid media in a 96-well plate, making a 10:1 starting ratio; for monocultures, I added 2µl of 0.2 OD_{600} culture and 2µl of M9. These were then incubated at 37˚C for three hours to move the cells out of lag phase, before adding 25µl imipenem and 25µl of dH_2O for the standard competition assay, 25µl imipenem and 25µl of the cloxacillin for the treatment with beta-lactamase inhibitor, or 50µl dH_2O for the no antibiotic or inhibitor treatment. In treatments with imipenem, the final concentration was 0.3125mg/l, which is 25% above the MIC of imipenem for the Δ*ampC*::Gm beta-lactamase knockout strain (Figure 3). The final concentration of cloxacillin was 50mg/l, a concentration which has no direct effect on the growth of Δ*ampC*::Gm measured after 18 hours at 37ºC (Figure 4). I thoroughly mixed the wells and removed a sample from each for the initial cell counts (see below). The 96-well plates were then reincubated at 37˚C for 24 hours, when I took samples to plate out for the final cell counts. While preliminary work had shown that beta-lactamase production

generally peaked within the first eight hours of competition (Figure 5), end counts were done at 24 hours after a full period of exponential growth.

This preliminary work (Figure 5) had been done by measuring beta-lactamase activity from samples removed every two hours from cultures of each high producer grown in glass vials at 37°C, 200rpm. Cultures consisted of 4440µl KB, 60µl 0.2 OD_{600} culture, 750µl imipenem (final concentration 0.3125mg/l) and 810µl dH_2O, which was the same proportions of contents as the competition assay monocultures, but scaled up into glass vials to allow repeated sampling. I used a standard nitrocefin test to measure activity in these samples (see Chapter 2 Methods for further details; unlike in Chapter 2, samples were not standardized as these measurements were for total beta-lactamase activity rather than activity per cell). I measured the first 12 hours in a separate culture from the second 12 hours to avoid overnight sampling.

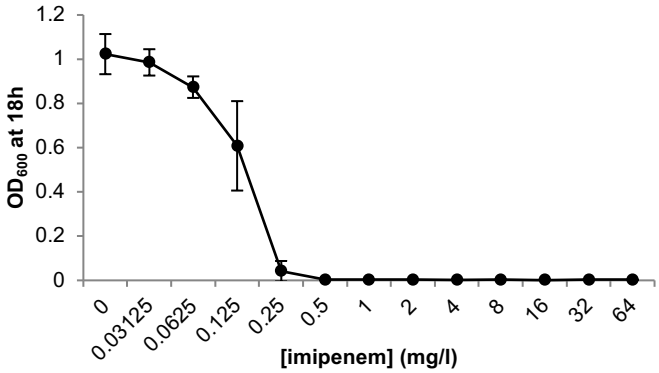


Figure 3. Minimum inhibitory concentration curve for beta-lactamase knockout strain tagged with gentamicin resistance (PAO1Δ*ampC*::Gm). MIC is 0.25mg/l. Each data point is six replicates. Error bars are 95% confidence intervals. MICs were determined by broth microdilution as described in Chapter 3.

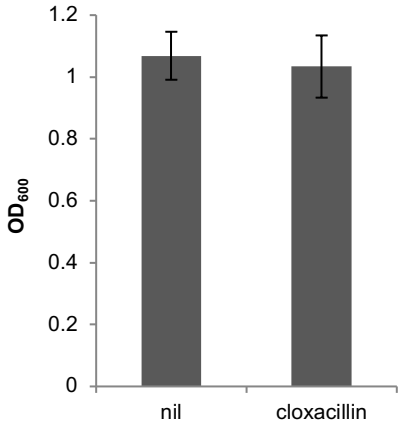


Figure 4. Beta-lactamase inhibitor cloxacillin has no direct inhibitory effect on growth of the beta-lactamase knockout strain (PAO1Δ*ampC*::Gm) (*t*-test, $t = 0.53$, $df = 9$, $p = 0.61$). OD_{600} was measured after 18 hours of growth at 37ºC in KB media with and without 50mg/l cloxacillin. Methods as per MIC determination by broth microdilution (see Chapter 3 Methods). Treatment without cloxacillin is seven replicates, with cloxacillin is four replicates. Error bars are 95% confidence intervals.

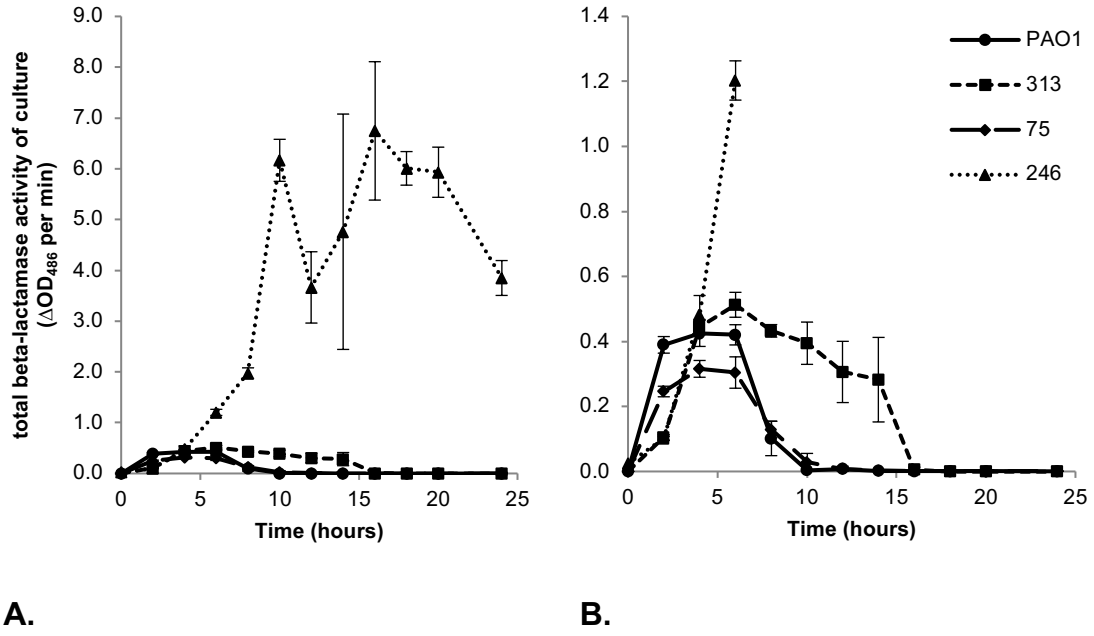


Figure 5. Total beta-lactamase activity of growing cultures over 24 hours. Beta-lactamase activity in the growing cultures of PAO1 and three clinical isolates (isolates 75, 246 and 313) exposed to 0.3125mg/l imipenem. **(A)** Isolate 246 is a constitutive producer and very high levels of beta-lactamase accumulate in the culture over 24 hours obscuring the results for the other isolates. **(B)** only shows the first six hours for isolate 246 so that the curves for the other isolates (PAO1, 75 and 313) are clearer. For theses three isolates, activity peaks around four to six hours and then declines. I measured the first 12 hours in a separate culture from the second 12 hours, which may account for the obvious disjunction at 12 hours for isolate 246. Each data point is three replicates (from separate cultures). Error bars are 95% confidence intervals.

I made serial dilutions of each sample taken after 0 and 24 hours of growth, and plated four 8µl spots of a range of dilutions onto KB agar plates. Due to the different growth rates of the clinical isolates they could not all be incubated at the same temperature for the same length of time before they could be counted; they were therefore either incubated at between 30 and 37˚C for approximately 12-18 hours, or left at room temperature overnight for approximately 18 hours until colonies were easily countable. I plated out the mixed cultures of PAO1 and Δ*ampC*::Gm on normal KB agar and on KB plates with 30mg/l gentamicin. The gentamicin inhibits growth of PAO1, leaving the gentamicin-resistance tagged beta-lactamase knockout to be counted. I then subtracted this count from the total count on the normal KB plate to give the PAO1 count. When mixed cultures of the clinical strains with Δ*ampC*::Gm were plated onto normal KB, the relatively fast growth of Δ*ampC*::Gm obscured the growth of the comparatively slow growing clinical isolates, and so an accurate combined count was not possible. Therefore, in addition to plating on gentamicin plates to obtain the Δ*ampC*::Gm counts, the clinical mixed cultures were plated onto KB plates with 256mg/l benzylpenicillin, which inhibits growth of Δ*ampC*::Gm, enabling the clinical high producers to be counted. Despite Δ*ampC*::Gm being resistant to gentamicin, it was found that the gentamicin plates on average reduced Δ*ampC*::Gm counts to 80.75% of their true value (n = 16), and so a correction factor of 1/0.8075 was applied to Δ*ampC*::Gm counts from gentamicin plates. No such partial inhibitory effect of benzylpenicillin plates on the high producers was found.

I did six replicates of each competition assay for the four pairs. In a few individual instances, more than six replicates were done – in one case to check an outlier and in another case to compensate for partial loss of a replicate due to a pipetting error.

Statistical methods

For the cross-protection assays, I used paired *t*-tests to compare growth between the different conditions.

For the competition assays, I calculated the relative fitness (ω) (change in frequency over time) of the low beta-lactamase producer relative to the high producer in competition. This is given by $\omega = x_2(1-x_1)/x_1(1-x_2)$ where x_1 is the mean initial proportion of low producers at time zero and x_2 is the final proportion measured at 24 hours (Ross-Gillespie *et al.*, 2007). If $\omega > 1$, the low producer has a higher fitness than the beta-lactamase producer and so is a cheat, and a value $\omega < 1$ indicates the low producers have a lower fitness than cooperators, and so it cannot invade the population.

I also calculated the growth rate constant k of the high and low producers, taken from the exponential growth equation $N = N_0e^{kt}$, where N_0 is the number of cells at time zero and N is the number of cells at time point t. From the cell counts, k is calculated from *ln2/doubling time*. I also used k to calculate the selection rate of the low producer (r), which is the $k_{(\text{low producer})} - k_{(\text{high producer})}$ (Lenski *et al.*, 1991) to ensure results are consistent across different fitness metrics. I did not use Lenski *et al.*'s (1991) method for calculating relative fitness with the ratio of growth rate constants (rather than the difference, as for r) since this can produce erroneous results when one or both populations decline – as is expected in experimental work with antibiotics.

I treated each competition assay with a particular high producer as its own separate experiment, and compared growth rate between treatments with paired *t*-tests. I also analysed the data across all competitions as one experiment, using *post hoc* Tukey-HSD analysis on a three-way ANOVA on selection rate. I also used a two-way ANOVA on the change in selection rate to compare the change in antibiotic to the change with antibiotic and beta-lactamase inhibitor.

I did not use relative fitness in a three-way ANOVA as relative fitness is problematic with this form of analysis. As relative fitness is a ratio it tends to magnify differences due to sampling error, in contrast to selection rate which is purely the difference in values and so less sensitive to error (Travisano & Lenski, 1996). Large error becomes particularly problematic when comparing across multiple different experiments under different conditions with very different fitness values. For example, fitness is one to two orders of magnitude higher in the absence of antibiotic, rendering smaller changes in relative fitness in antibiotic as not significant. I also did not use growth rate for this analysis since selection rate is simply the difference in the growth rate constants – growth rate is therefore already reflected directly in this analysis.

Results

Beta-lactamase is cross-protective

Supernatant of high beta-lactamase producers protects low-producers and allows them to grow at antibiotic concentrations that would otherwise inhibit their growth. Clinical high producers protected low producers sampled from the same patient within six

months of each other (Figure 6A; paired *t*-test, $n = 9$, $t = 3.1$, $df = 8$, mean difference in $OD_{600} = 0.26$, $p = 0.016$), and protected the *ampC* beta-lactamase knockout (Figure 6B; paired *t*-test, $n = 9$, $t = 13.7$, $df = 8$, mean difference in $OD_{600} = 0.55$, $p = 7.7\times10^{-7}$). In both cases the protective effect is abolished by the presence of cloxacillin, an AmpC beta-lactamase inhibitor (paired *t*-tests: clinical pairs, $n = 9$, $t = 0.6$, $df = 8$, mean difference in $OD_{600} = 0.003$, $p = 0.54$; knockout strain: $n = 9$, $t = 1.6$, $df = 8$, mean difference in $OD_{600} = 0.07$, $p = 0.13$), indicating that the effect is mediated by beta-lactamase.

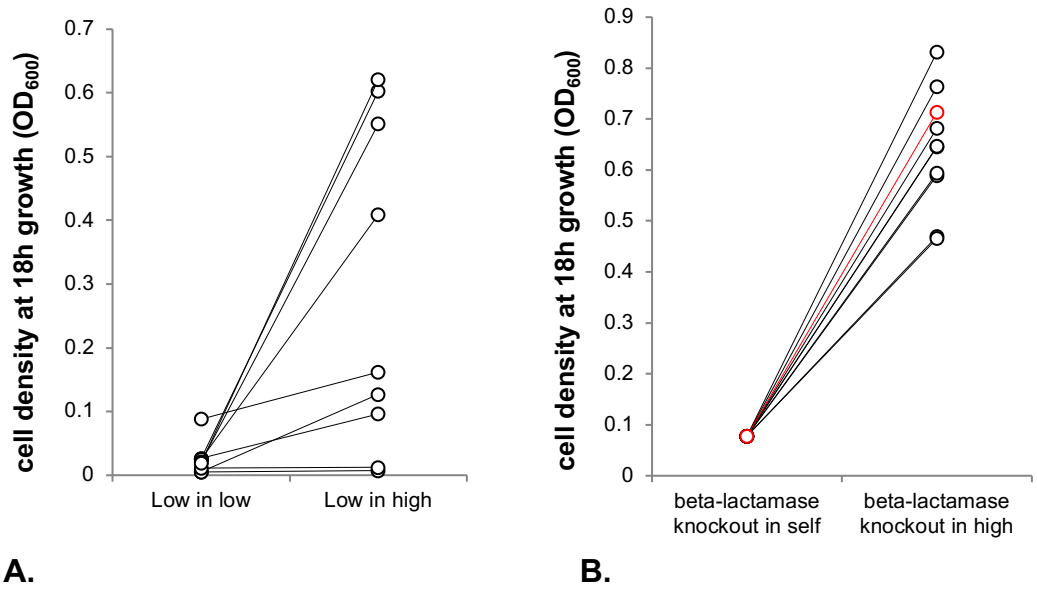


Figure 6. Low beta-lactamase producers grow better in the supernatant of high producers. (A) Clinical low producers grow better in the supernatant of a coexisting high beta-lactamase producer rather than in its own supernatant, at the low producer's minimum inhibitory concentration of piperacillin (paired *t*-test, $n = 9$, $t = 3.1$, $df = 8$, mean difference in $OD_{600} = 0.26$, $p = 0.016$). **(B)** The *ampC* beta-lactamase knockout grows better in the supernatant of a high beta-lactamase producer than in its own supernatant, at its minimum inhibitory concentration of piperacillin (paired *t*-test, $n = 9$, $t = 13.7$, $df = 8$, mean difference $OD_{600} = 0.55$, $p = 7.7\times10^{-7}$; statistics for clinical high producers only). The black data points represent the effect of the clinical isolates, the red point is for PAO1.

Low producers have higher fitness in mixed culture

The beta-lactamase knockout persisted over 24 hours in mixed cultures with each of the three clinical high producers when it would otherwise die in monoculture at the same imipenem concentration (Figures 7A, 8A and 9A including statistics). With two of the three high producing clinical isolates, the knockout was able to invade, with a relative fitness above one at 24 hours, albeit of borderline significance for isolate 75 (one sample, one-sided *t*-tests; isolate 75: $t = 1.9$, $df = 5$, $p = 0.057$, Figures 10B; isolate 313: $t = 4.8$, $df = 5$, $p = 0.0025$, Figure 11B). In competition with PAO1 as the high beta-lactamase producer, the knockout was able to survive and grow despite being unable to survive in monoculture ($t = 11.2$, $df = 5$, $p = 9.8\times10^{-5}$, Figure 13A). It did not grow well enough to outcompete PAO1 (Figure 14).

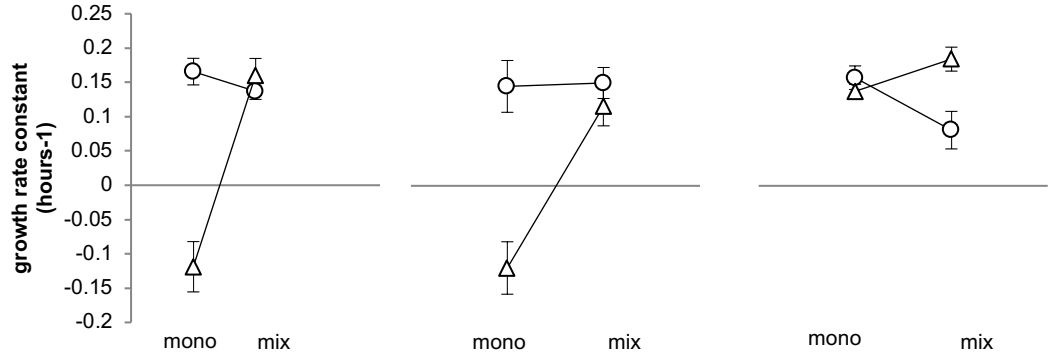
Post hoc Tukey-HSD analysis on a three-way ANOVA on selection rate confirmed higher selection for low producers in antibiotic in mixed culture, compared with monoculture ($p < 0.0000001$ for each pair of isolates).

The increase in relative fitness of the beta-lactamase knockout in mixed culture is mediated at least in part by AmpC beta-lactamase. In three of the four competitions, the knockout's competitive ability in mixed culture was reduced by the presence of the beta-lactamase inhibitor cloxacillin (at 24 hours, with PAO1: $t = 6.1$, $df = 5$, $p = 0.0017$; with 75: $t = 3.6$, $df = 5$, $p = 0.016$; with 313: $t = 5.6$, $df = 5$, $p = 0.0024$; not significant with 246: $t = 2.3$, $df = 5$, $p = 0.073$, Figures 10-12 and 14), even though cloxacillin did not reduce the growth rate of any of the high producers (one-tailed paired *t*-tests: PAO1: $t = 0.86$, $df = 5$, $p = 0.22$; 75: $t = 1.2$, $df = 5$, $p = 0.14$; 246: $t =$ -

1.1, df = 5, p = 0.83; 313: t = -2.6, df = 5, p = 0.98; compare high producer monoculture growth rates in sections A and B of Figures 7-9 and 13).

Post hoc Tukey-HSD analysis on a two-way ANOVA on selection rate supports this by showing that the selection rate of the beta-lactamase knockout is lower in mixed culture with cloxacillin than mixed culture with just antibiotic (p <0.05). However, this effect is due to isolates 313 (p = 0.0000096) and 246 (p = 0.0000005) only, not 75 (p = 0.346) or PAO1 (p = 0.996).

The data show a possible benefit to the knockout of growing in mixed culture with high-producing clinical isolates (but not with PAO1) even in the absence of antibiotic. Statistically treated as separate experiments, there is an increased growth rate in mixed culture with all three clinical high producers and an increase in relative fitness in two (see 'no antibiotic or inhibitor' columns of statistics tables in Figures 7-9, and 'Nil' treatment at 24 hours in statistics tables in Figures 10 and 11). In one of these, the increase in relative fitness is at least partially caused by the high producer appearing to grow less well in mixed culture (isolate 75, t = 6.8, df = 5, p = 0.001, Figures 7C). The magnitude of the increase in growth rate of the knockout strain is two to six times less than in the treatments with antibiotic (unpaired t-tests: knockout with isolate 75, t = 15.8, df = 10, $p = 2.2\times10^{-8}$; knockout with isolate 246, t = 5.7, df = 11, p = 0.00014; knockout with isolate 313, t = 9.7, df = 10, $p = 2.1\times10^{-6}$). *Post hoc* Tukey-HSD analysis on a three-way ANOVA of selection rate suggests there is only increased selection of the low producer in the absence of antibiotic when grown with two isolates, 75 (p = 0.014) and 246 (p <0.0000001) (see Discussion).

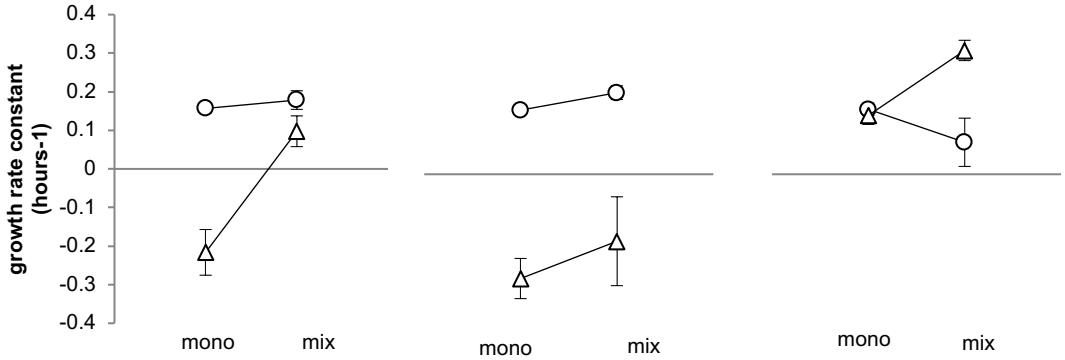


A. imipenem **B. imipenem + cloxacillin** **C. no antibiotic or inhibitor**

○ Isolate 75
△ Δ*ampC*::Gm

		imipenem				imipenem + cloxacillin				no antibiotic or inhibitor			
		t	***df***	**mean diff**	***p***	***t***	***df***	**mean diff**	***p***	***t***	***df***	**mean diff**	***p***
24 hours	**75**	**2.60**	**5**	**0.0286**	**0.048**	0.283	5	0.0048	0.789	**6.84**	**5**	**0.074**	**0.001**
	Δ*ampC*::Gm	**31.5**	**5**	**0.279**	**6.1×10^{-7}**	**21.3**	**5**	**0.228**	**4.2×10^{-6}**	**3.83**	**5**	**0.046**	**0.012**

Figure 7. Growth rate of producer (isolate 75) and non-producer (Δ*ampC*::Gm) in mono- and mixed culture over 24 hours. The table shows the results of paired *t*-tests comparing growth in monoculture and mixed culture (the comparisons are connected by lines in the graphs).

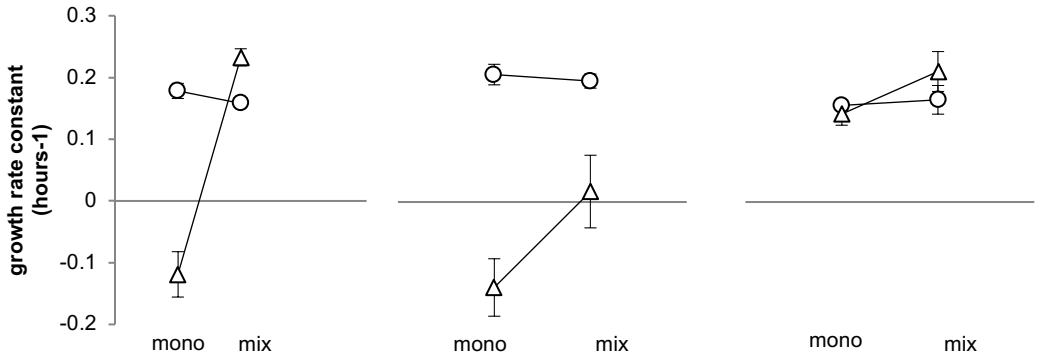


A. imipenem **B. imipenem + cloxacillin** **C. no antibiotic or inhibitor**

○ Isolate 246
△ Δ*ampC*::Gm

		imipenem				imipenem + cloxacillin				no antibiotic or inhibitor			
		t	***df***	**mean diff**	***p***	***t***	***df***	**mean diff**	***p***	***t***	***df***	**mean diff**	***p***
24 hours	**246**	1.96	5	0.0217	0.107	**4.10**	**5**	**0.0437**	**0.0094**	**2.66**	**6**	**0.083**	**0.037**
	Δ*ampC*::Gm	**12.6**	**5**	**0.314**	**5.5×10^{-5}**	2.32	5	0.0934	0.0683	**13.1**	**6**	**0.163**	**1.2×10^{-5}**

Figure 8. Growth rate of producer (isolate 246) and non-producer (Δ*ampC*::Gm) in mono- and mixed culture over 24 hours. The table shows the results of paired *t*-tests comparing growth in monoculture and mixed culture (the comparisons are connected by lines in the graphs).

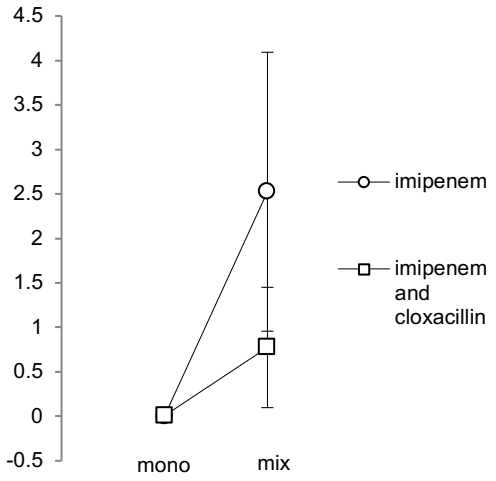


A. imipenem **B. imipenem + cloxacillin** **C. no antibiotic or inhibitor**

○ Isolate 313
△ Δ*ampC*::Gm

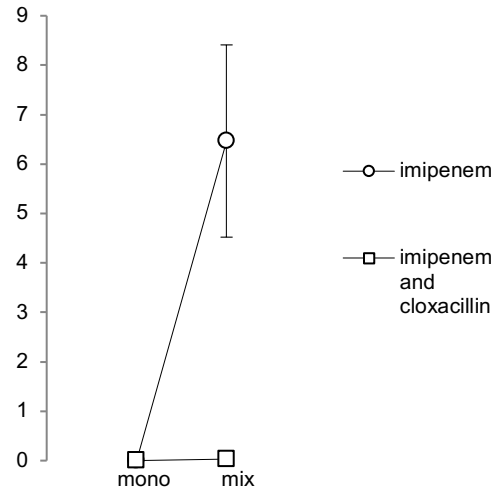
		imipenem				imipenem + cloxacillin				no antibiotic or inhibitor			
		t	***df***	**mean diff**	***p***	***t***	***df***	**mean diff**	***p***	***t***	***df***	**mean diff**	***p***
24 hours	**313**	**3.37**	**5**	**0.0201**	**0.020**	0.861	5	0.0101	0.429	0.63	5	0.00877	0.554
	Δ*ampC*::Gm	**14.0**	**5**	**0.351**	**3.4×10^{-5}**	**5.07**	**5**	**0.150**	**0.0039**	**4.43**	**5**	**0.0666**	**0.0068**

Figure 9. Growth rate of producer (isolate 313) and non-producer (Δ*ampC*::Gm) in mono- and mixed culture over 24 hours. The table shows the results of paired *t*-tests comparing growth in monoculture and mixed culture (the comparisons are connected by lines in the graphs).



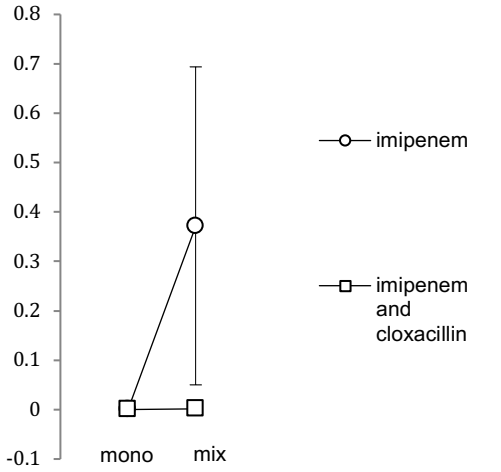
	t	***df***	**mean diff**	***P***
Imipenem	3.16	5	2.52	0.025
Imip + clox	2.23	5	0.767	0.075
Nil	8.35	5	10.8	0.00040

Figure 10. Relative fitness of isolate 75 v Δ*ampC*:Gm. Relative fitness of non-producers to producers at 24 hours. The table shows the results of paired *t*-tests comparing relative fitness in monoculture and mixed culture (the comparisons are connected by lines in the graphs).



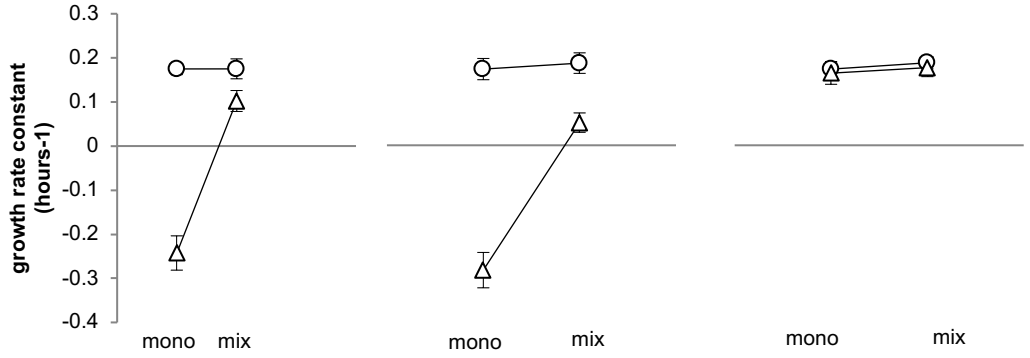
	t	*df*	mean diff	*p*
Imipenem	5.64	5	6.46	0.0024
Imip + clox	2.91	5	0.0297	0.033
Nil	2.94	5	3.80	0.032

Figure 11. Relative fitness of isolate 313 v Δ*ampC*:Gm. Relative fitness of non-producers to producers at 24 hours. The table shows the results of paired *t*-tests comparing relative fitness in monoculture and mixed culture (the comparisons are connected by lines in the graphs).



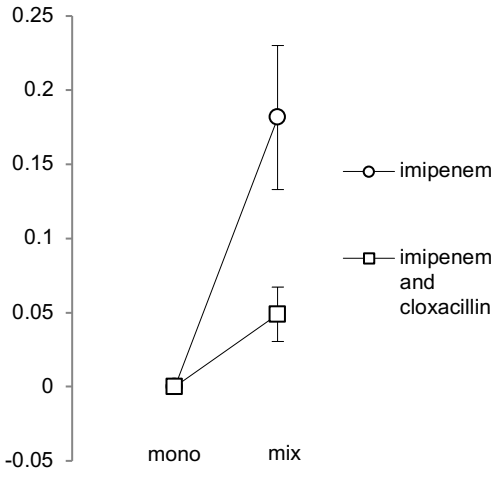
	t	*df*	mean diff	*p*
Imipenem	2.26	5	0.371	0.073
Imip + clox	2.04	5	0.0014	0.097
Nil	1.95	6	595.5	0.099

Figure 12. Relative fitness of isolate 246 v Δ*ampC*:Gm. Relative fitness of non-producers to producers at 24 hours. The table shows the results of paired *t*-tests comparing relative fitness in monoculture and mixed culture (the comparisons are connected by lines in the graphs).



		imipenem				imipenem + cloxacillin				no antibiotic or inhibitor			
		t	*df*	mean diff	*p*	*t*	*df*	mean diff	*p*	*t*	*df*	mean diff	*p*
24 hours	**PAO1**	0.017	5	0.00012	0.987	2.2	5	0.0131	0.0787	**2.66**	**8**	**0.0139**	**0.029**
	Δ*ampC*::Gm	**11.2**	**5**	**0.344**	**9.8×10^{-5}**	**11.0**	**5**	**0.323**	**0.00011**	0.91	8	0.0121	0.386

Figure 13. Growth rate of producer (PAO1) and non-producer (Δ*ampC*::Gm) in mono- and mixed culture over 24 hours. The table shows the results of paired *t*-tests comparing growth in monoculture and mixed culture (the comparisons are connected by lines in the graphs).



	24 hours			
	t	*df*	mean diff	*p*
Imipenem	7.32	5	0.181	0.0007
Imip + clox	5.21	5	0.0488	0.0034
Nil	0.362	8	0.125	0.73

Figure 14. Relative fitness of isolate PAO1 v Δ*ampC*:Gm. Relative fitness of non-producers to producers at 24 hours. The table shows the results of paired *t*-tests comparing relative fitness in monoculture and mixed culture (the comparisons are connected by lines in the graphs).

Discussion

The results of the experiments described in this chapter suggest that beta-lactamase from clinical isolates has the potential to act as a public good in infection. The cross-protection experiment shows a clear benefit to both clinical low producers and to a beta-lactamase knockout from the extracellular products of high beta-lactamase producers. This benefit is again clearly seen in the competition assays, where the knockout strain shows improved growth in mixed culture. In two out of three competitions with high producing clinical isolates, the knockout was observed to invade. In competition with an isogenic PAO1, this growth advantage in mixture was not observed, despite its increased growth rate and increased relative fitness in mixed culture. This could be related to specific features of PAO1, such as lower levels of beta-lactamase production than clinical isolates (Figure 2), or be related to the costs and benefits when competing isogenic strains. In both the cross-protection and competition assays, addition of the beta-lactamase inhibitor cloxacillin reduces the benefit derived by the low producers, confirming that AmpC beta-lactamase is responsible for the competitive dynamics observed.

The results here differ in some respects from previous studies, which may reflect the clinical isolates I used as well as aspects of experimental design. I showed improved growth rates in the non-producer above its MIC, with invasion in some specific instances, which contrasts with previous studies which only showed invasion at sub-inhibitory concentrations (Medaney *et al.*, 2015; Bottery *et al.*, 2016, Frost *et al.*, 2018), or showed a stable but very low population of non-producers above their MIC (Dugatkin *et al.*, 2005; Clark *et al.*, 2009). My results imply that susceptible non-producers can survive antibiotic treatment, and in some circumstances can even be

selected in the presence of antibiotics. Although the other studies did not quantify beta-lactamase production or did not do so in a comparable way, it is possible my results differ because induced clinical isolates produce high levels of beta-lactamase, allowing greater protection of non-producers. Also, unlike Medaney *et al.* (2015) and Frost *et al.* (2018), I used liquid cultures. This could have two opposing effects: liquid cultures may allow higher mixing of producers and non-producers and greater diffusion of beta-lactamase, potentially favouring non-producers (Kümmerli *et al.*, 2009a), or could reducing the density of cells and of the public good (since they are distributed evenly throughout the media) making it harder for non-producers to gain an advantage (Ross-Gillespie *et al.*, 2009, Yurtsev *et al.*, 2013). The overall effect of using liquid cultures is unclear. A further difference is that, in contrast to Dugatkin *et al.* (2005) who kept antibiotic concentration constant and used a separate inducing agent, I gave a one-off dose of inducing antibiotic. This one-off dose captures the dynamics of the more realistic scenario of antibiotic concentration falling between doses. Overall, the exact reasons why my results differ from previous studies are unclear, but is likely to revolve around the nature of the clinical isolates used and the effects of the experimental set-up as described.

There is a trade-off with working on competitive dynamics with clinical isolates in that they help us to understand what is happening in natural populations, but the dynamics are also likely to be more complex than between near-isogenic laboratory strains. In laboratory strains differing only in the gene for the social trait, it is clear that any advantage gained by the cheat is from gaining the benefit while not paying the cost of the cooperative trait. Clinical isolates, however, differ from the defined beta-lactamase knockout across multiple traits beyond just beta-lactamase production.

Clinical isolates from chronic CF infections are characterized by slow growth (Yang *et al.*, 2008; La Rosa *et al.*, 2018; Lozano *et al.*, 2018) which could be caused by many metabolic differences (Yang *et al.*, 2008) rather than just antibiotic resistance (La Rosa *et al.*, 2018). While the results unequivocally show that beta-lactamase is a public good from which non-producers can benefit in mixed culture (in the presence of antibiotic), it is not necessarily the case that the growth advantage it attains in mixed culture is derived from the cost saved by not producing beta-lactamase. This is less relevant in other experimental systems where a constant environment is maintained which requires the cooperative trait (such as an iron-limited media in pyoverdin studies). It is more relevant in my system as the cooperative trait changes the environment by removing antibiotic such that non-producing cheats will no longer be inhibited. If at this point the non-producers have an 'inherent' growth rate (in the absence of antibiotics) that exceeds that of the producer for any variety of metabolic reasons (not just antibiotic resistance phenotypes), it will be able to invade at this late stage in the competition. This could explain the two instances where the knockout can invade the high producing clinical isolates (Isolate 75, Figure 10B and Isolate 313, Figure 11B) in contrast to its inability to invade the isogenic PAO1, despite gaining some benefit (Figure 14B). I begin to investigate the timing of costs and benefits in isogenic strains in Chapter 5, but future work should clarify this further in non-isogenic clinical isolates.

The potential differences in 'inherent' growth rate could explain the finding of a small but significant increase in non-producer relative fitness in mixed culture even in the absence of antibiotic in the same two strains (75 and 313) as discussed in the previous paragraph (see statistics for the 'nil' antibiotic treatment at 24 hours in Figures 10 and

11). A social effect due to beta-lactamase is not possible as the isolates do not produce beta-lactamase in the absence of antibiotic (Figure 2). However, an apparent advantage in mixed culture could derive from any 'inherent' growth rate differences which results in scramble competition when directly competed (Hibbing *et al.*, 2010), which does not occur in the monocultures. This is likely to explain the fitness benefit observed. Analysis by ANOVA on selection rate gives similar results, except that one of the isolates where the advantage is seen is different (246 rather than 313). The difference is likely to reflect the borderline nature of some of the results.

While the results strongly support a role for social interaction driving evolution of resistance in infections in principle, it is difficult to establish exactly how representative my competition assays were of dynamics in infection. For example, although the antibiotic concentration I used was above the MIC of the low producing strain, it was only 25% above this value at 0.3125mg/L. This could be significantly lower than cells are likely to experience in the lung: serum imipenem concentrations can range between troughs of 1mg/L (Drusano *et al.*, 1984; MacGregor *et al.*, 1986) to peaks of approximately 50mg/L (Drusano *et al.*, 1984; MacGregor *et al.*, 1986; Jaruratanasirikul *et al.*, 2005), although lung concentrations are lower (Benoni *et al.*, 1987) and exact biofilm concentrations in CF are unknown. Higher antibiotic concentrations could reduce the ability of non-producers to survive in the presence of high producers, as in (Bottery *et al.*, 2016). Also, only a one-off dose was given, in contrast to a typical beta-lactam course of at least two weeks (UK Cystic Fibrosis Trust, 2009). The effect of repeated doses is less clear than that of concentration, but it does not prevent non-producers surviving: both Clark *et al.* (2009) and Yurtsev *et*

al. (2013) showed non-producer survival in mixed culture with high producers after five days and eight days of antibiotic respectively.

Another difference between my competition experiments and infection is that the liquid cultures I used do not allow the structured biofilm mode of growth seen in the lung, although the impact of this is uncertain. As mentioned above, well-mixed liquid cultures could make cheating more likely (viscosity effect (Kümmerli *et al.*, 2009a)) or less likely (density effect (Ross-Gillespie *et al.*, 2009; Yurtsev *et al.*, 2013)). Frost *et al.* (2018) saw no benefit to non-producers in liquid cultures, unlike their competitions on agar plates, suggesting importance of the effect of density. Cell density is likely to be high by the time an infection becomes clinically apparent. It therefore seems likely that my experimental set-up prejudices against non-producing cheats, making it more likely that non-producers could benefit in real biofilm infections.

There are important clinical implications from this work. Whether or not low producers invade, it shows that their improved survival (increased absolute fitness) in mixed culture is possible. Recent papers have emphasized the importance of absolute fitness in contrast relative fitness (Day *et al.*, 2015; Bell & MacLean, 2018). In this instance, improved absolute fitness may mean that susceptible strains survive to the end of the antibiotic course. Susceptible non-producers then have a chance to repopulate the chronically infected lung, particularly since they might have a growth advantage in the absence of antibiotic. This could explain the observation that beta-lactam resistance often decreases after antibiotic selection pressure is removed (Reinhardt *et al.*, 2007), unlike certain other classes which maintain resistance

(Malhotra-Kumar *et al.*, 2007). Considering optimization of conditions to allow survival of sensitive strains during courses of antibiotic treatment would reduce selection for resistance.

The results raise a number of further questions about the nature and timing of the benefits and costs. For example, in the competition between PAO1 and the knockout, which are isogenic apart from the gene for the social trait and the tagging, it is important to consider why only a limited benefit is seen to the knockout, in contrast to pyoverdin studies where the low producer is able to invade (Griffin *et al.*, 2004; Ross-Gillespie *et al.*, 2007; Ghoul *et al.*, 2014b). The knockout will only be able to grow once the antibiotic concentration drops below its MIC, so it seems likely there will be a delay before the knockout can accrue any benefit. Furthermore, in this experiment with an inducing antibiotic, the production of beta-lactamase by the high producer falls once the antibiotic concentration drops. Therefore, at the point at which the non-producer could benefit, the high producer stops paying the cost of production. A further consideration that could change the costs and benefits would be if high producers compensated for the presence of non-producers by upregulating their own beta-lactamase production. Additional data (not presented) showed a reduced growth rate at eight hours (while still in exponential phase) in PAO1 and one clinical isolate which could be for this reason. While pyoverdin producers partially compensate for the presence of pyoverdin cheats (Harrison, 2013), the response of beta-lactamase producers to beta-lactamase cheats is unknown. Partial compensation stabilizes cooperation (Houston & Davies, 1985; Harrison *et al.*, 2009) and so its presence could help explain why high beta-lactamase producers are present late in chronic

infections (see Chapter 2). The timing of the costs and benefits, and compensation, are examined in the next chapter.

Chapter 5. Temporal dynamics and the response to exploitation

Introduction

The preceding chapters have identified the potential role of social dynamics in the evolution of antibiotic resistance by beta-lactamase in infection. Chapter 4 showed that beta-lactamase-producing clinical isolates can be exploited by non-producers, leading to a reduction in fitness. This chapter examines in more detail how producers respond to the presence of non-producers and how this affects the costs and benefits of producing beta-lactamase. The outcome of competition, and so the clinical relevance of social dynamics, is dependent on the factors considered here.

It is known there is a degree of plasticity in beta-lactamase production: higher antibiotic concentrations induce higher levels of beta-lactamase (Nordström & Sykes, 1974; Livermore & Yang, 1987; Giwercman *et al.*, 1991). It seems possible, therefore, that beta-lactamase production could be adjusted in response to other environmental factors, such as the presence of non-producers in the social environment. Such phenotypic plasticity has been shown in pyoverdin, where producer cells compensate for the presence of cheats by increasing their public good production (Kümmerli *et al.*, 2009b). Full compensation would increase the cost to the producer cells, potentially making it easier for cheats to invade. Alternatively, partial compensation can stabilize cooperation (Houston & Davies, 1985). Unlike full compensation, it reduces the amount of public good available to both producers and non-producers, limiting population growth. Ross-Gillespie *et al.* (2007) identified this

as a cause of frequency-dependence, limiting the advantage to non-producers by reducing the period of growth over which they could gain an advantage. The stabilizing effect of partial compensation has been shown in models of biparental care in birds (Houston & Davies, 1985; McNamara *et al.*, 2003), and it has been demonstrated both in birds (Harrison *et al.*, 2009) and with pyoverdin production in bacteria (Harrison, 2013). If partial compensation were shown with beta-lactamase, it could help explain the maintenance of cooperative antibiotic resistance mechanisms in chronic infections. It is not known how the presence of non-producers affects beta-lactamase production in producer cells.

The response of producer cells may also affect the timing of the costs and benefits associated with beta-lactamase production. For example, the absence of compensation would keep the cost of production low, but decrease the benefit, taking longer to clear antibiotic from the environment. If an antibiotic-sensitive non-producer can only grow once the antibiotic concentration falls below its minimum inhibitory concentration, then the length of time taken to reduce the antibiotic concentration is crucial to its ability to gain a benefit. In the case of an inducing antibiotic, beta-lactamase production by the high producer will also fall as antibiotic concentration drops. These dynamics, mediated by the production of beta-lactamase as a public good, may lead to a scenario where the high producer stops paying the cost of production at the point at which the non-producer benefits. This disjunction between benefits to the non-producer and costs to the producer may make it difficult for the non-producer to invade, unless there were an inherent cost to the producer of carrying the beta-lactamase gene, or other sources of fitness differences, as with the clinical isolates in Chapter 4. This disjunction is not expected to occur where the benefit from a public

good is concurrent with the cost paid for its production, for example in pyoverdin (Ross-Gillespie *et al.*, 2007; Ghoul *et al.*, 2014b) and quorum sensing (Diggle *et al.*, 2007) where cheats have been shown to invade. Understanding when non-producers benefit, and relating this to the production of beta-lactamase in mixed culture, will help to more accurately predict the invasion ability of beta-lactamase cheats.

The timing of costs and benefits can only be understood in the context of the molecular properties of the public good. In particular, public good durability has been shown to have an impact on the costs and benefits (Brown & Taddei, 2007; Kümmerli & Brown, 2010a; Allen *et al.*, 2013; Dobay *et al.*, 2014). In addition to the inherent durability of the molecule, the overall durability will also be affected by extrinsic rates of removal (Brown & Taddei, 2007), such as breakdown by proteases. Durability has been shown to both promote (Kümmerli & Brown, 2010a; Allen *et al.*, 2013; Dobay *et al.*, 2014) and impede (Brown & Taddei, 2007; Dobay *et al.*, 2014) cooperation in different circumstances. While these conflicting findings would make the effect of durability difficult to predict, basic information about the durability of beta-lactamase and the potential breakdown and recycling of beta-lactamase by cells is lacking.

Here I ask (i) how the presence of non-producers affects beta-lactamase production in producer cells, (ii) when in a growth cycle the non-producers benefit the most, and (iii) how durable the beta-lactamase molecule is. First I compared the amount of beta-lactamase per producer cell in a mixed culture of producers and non-producers, with the beta-lactamase per cell in a producer monoculture. I varied the starting ratio of producers to non-producers from 10:1 to 1:1 and 1:10 to see if increasing the burden

of non-producers increased the amount of beta-lactamase each producer cell makes. Then I measured relative fitness of the non-producers in mixed culture at two hour intervals at the three starting ratios above to determine when non-producers gain the highest benefit during a growth cycle, in order to relate this to the beta-lactamase production at different points in the growth cycle. And finally, to study how durable beta-lactamase is, I took beta-lactamase and recorded how long its activity lasted in pure supernatant and in supernatant with cells.

Methods

I set up competition assays as described in Chapter 4 (see pages 116-119), but taking samples every two hours to measure beta-lactamase activity and cell counts. I used cell counts to estimate beta-lactamase activity per producer cell and calculate relative fitness or growth rates for every two-hour period. Another difference is that I used three different starting ratios of producers to non-producers: 10:1 (as in Chapter 4's competition assays), 1:1, and 1:10, while keeping the overall initial cell density constant across treatments (so in fact 10:1, 5.5:5.5 and 1:10). This was to show the effect of increasing the burden of non-producers on the producer cells. Competitions were run for 12 hours, as this covers the period of beta-lactamase production (see Figure 5B in Chapter 4).

Competition assay

I used PAO1, a wild-type *P. aeruginosa* laboratory strain, as the beta-lactamase producer and competed this with an *ampC* beta-lactamase knockout of PAO1 as the

non-producer. The non-producer had been tagged with gentamicin resistance (PAO1Δ*ampC*::Gm – abbreviated below to Δ*ampC*::Gm).

I prepared cultures from freezer stock in 6ml of KB and grew them overnight at 37˚C, 200rpm, before standardizing to 0.2 OD_{600}. For the 10:1 producer:non-producer mixed cultures, I added 60µl of the high producer culture and 60µl of a 10^{-1} dilution of the low producer to 5190µl KB liquid media in a glass vial; I reversed the dilutions for the 1:10 ratio. For the 1:1 ratio, I took 550µl of each of the 0.2 OD_{600}-standardized cultures and made them up to 1000µl with dH_2O, and I added 60µl of each to 5190µl KB liquid media. I inoculated monocultures to have the same density of producers or non-producers as in each of the respective mixed cultures ('10', '5.5' and '1'), so that there were equal numbers of producers in monoculture as in its comparison mixed culture, and equal numbers of non-producers in monoculture as in its comparison mixed culture. I also added 60µl of dH_2O in place of the second culture to make the monoculture volume equal to the mixed culture. I added 750µl imipenem to all of the vials to give a final imipenem concentration of 0.3125mg/l, which is 25% above the MIC of imipenem for the Δ*ampC*::Gm beta-lactamase knockout strain (Chapter 4, Figure 3). I then incubated these vials at 37˚C, 200rpm, removing 225µl of culture every two hours to test beta-lactamase activity with a nitrocefin assay as in Chapter 1, and a further sample for serial dilutions to plate out for cell counts. The two-hourly cell counts enable me to calculate both beta-lactamase activity per producing cell every two hours, and the relative fitness of the low-producers in every two-hour period. This allowed me to detect the time points at which the low-producers gained an advantage, in relation to levels of beta-lactamase production per cell.

Six replicates of each competition assay were set-up at the three different starting ratios. Each of the six replicates was done on a different day.

Nitrocefin test

I measured beta-lactamase activity using a standard colorimetric nitrocefin assay (O'Callaghan *et al.*, 1972) – see Chapter 2 for further details. I diluted Nitrocefin in M9 to a final concentration of 50µg/ml, and added this solution to 60µl of culture in three wells of a 96-well plate (final volume in well 300µl). Absorbance readings were taken in a spectrophotometer at 486nm every 20 seconds for 20 minutes. I calculated the gradient of the linear part of the reaction curve (the rate of change of absorbance) to give a quantitative measurement of beta-lactamase activity. As in Chapter 2, I took the linear part to be the readings where OD_{486} is less than 0.4 (Chapter 2, Figure 2); however, this only works at low cell densities since cells also absorb at OD_{486} (to a lesser extent than at the usual OD_{600}). As I was using a growing culture, samples from later time points often had an OD_{486} greater than 0.4, even at the start of the reaction. I therefore instead took the part of the curve from time zero which gave the highest gradient (which gives the linear part before the nitrocefin concentration becomes a limiting factor). This method does not work well at low densities with low levels of beta-lactamase where noise can result in spuriously high readings (since selecting the highest gradient can result in a smaller number of points being used than if the 0.4 OD cut off was used). Throughout I therefore used both methods to calculate the gradient and took the values from the method which had the most data points for calculating the gradient. Generally this means that for low cell densities I took the result from the 0.4 OD cut off method, and at high cell densities I used the maximum rate method. Three technical replicates were done for each nitrocefin test.

Cell counts

Every two hours I extracted samples from each competition assay and serially diluted the samples. I then plated out four 8µl spots of a range of dilutions onto KB agar plates. I plated out a range of dilutions each time to ensure there would be a dilution suitable for counting. These were then incubated for approximately 14 hours overnight until colony growth was sufficient for accurate counting of colonies in each spot. The mixed cultures of PAO1 and Δ*ampC*::Gm were plated out on normal KB agar and on KB plates with 30mg/l gentamicin. The gentamicin inhibits growth of PAO1, leaving the gentamicin-resistance tagged beta-lactamase knockout to be counted. This count was then subtracted from the total count on the normal KB plate to give the PAO1 count. Despite Δ*ampC*::Gm being resistant to gentamicin, I had previously found that the gentamicin plates reduced Δ*ampC*::Gm counts to 80.75% of their true value (Chapter 4, Methods), and so I applied the same correction factor of 1/0.8075 to Δ*ampC*::Gm counts from gentamicin plates.

Durability of beta-lactamase

Using a nitrocefin assay, I measured beta-lactamase activity from samples of supernatant over 24 hours to determine its half-life under two different conditions at 37ºC: i) supernatant only, to see how long the molecule lasts when it is not being used or broken down and ii) supernatant with cells added back in, as cells may secrete proteases (Nicas & Iglewski, 1986) which break down and recycle amino acids. I used three different types of cells: a standard *Pseudomonas aeruginosa* laboratory strain (PAO1), a beta-lactamase knockout strain to see if non-producers degrade beta-

lactamase any differently (Δ*ampC*), and a protease deficient knockout strain to see if proteases were responsible for breakdown of beta-lactamase (Δ*lasR*).

I grew cultures of the constitutive AmpC beta-lactamase producer (PAO1Δ*ampD*Δ*dacB*, Pérez-Gallego *et al.*, 2016) overnight in KB media at 37°C. This results in cultures with high concentrations of beta-lactamase. I then centrifuged and filter-sterilized cultures to extract the cell-free supernatant. I put 4ml of supernatant into glass vials, to which I added: 1ml of cell culture standardized to 1.0 OD_{600} for the treatments with cells, or 1ml M9 in supernatant only treatments. I incubated the vials at 37°C, 200rpm. Every two hours up to 12 hours I removed 3 × 75µl samples from each vial which I used for three technical replicates for a nitrocefin test, following the same protocol as above, to measure beta-lactamase activity. I took a further sample at 24 hours. I did four biological replicates for the no cell, PAO1 and Δ*ampC* treatments, and three replicates for Δ*lasR*.

Statistical methods

Beta-lactamase per producer cell was calculated every two hours by dividing the beta-lactamase activity from the nitrocefin assay by the producer cell counts. The area under this curve represents the beta-lactamase available to breakdown antibiotic over the 12 hour time period of measurement, per producer cell. I use this as an estimate of production, though strictly speaking it is a function of production and degradation (see Discussion). I calculated the difference in beta-lactamase available per producer cell in the mixed cultures compared with the equivalent producer monocultures by taking the area between the mixed and monoculture curves of beta-lactamase activity per cell (see Figure 1A). I calculated the area using the trapezium rule separately for

the six replicates. To assess the effect of increasing the burden of non-producers, I used a one-way ANOVA blocked by replicate, with starting ratio as a factor. I also used a paired *t*-test to assess if the difference in beta-lactamase per cell between mixed and monocultures was significant.

To compare total beta-lactamase levels between mixed and monoculture, I did a paired *t*-test comparing the areas under the mixed and monoculture curves. As above, these areas were calculated using the trapezium rule separately for the six replicates.

I calculated the growth rate constant k of the high and low producers, taken from the exponential growth equation $N = N_0 e^{kt}$ where N_0 is the number of cells at time zero and N is the number of cells at time point t. From the cell counts, k is calculated from $ln(N/ N_0)/t$. I used paired *t*-tests to make comparisons at particular time points. In particular, to calculate the cost (in terms of growth rate over 12 hours) to the producer cells from being in mixed culture, I subtracted k for producers in monoculture from k for producers in mixed culture, and then did a one-way ANOVA of the effect of starting ratio on the results.

To assess how non-producers benefit during the growth cycle, I calculated relative fitness and selection rate of the non-producer for each two-hour time block (see Chapter 4 Methods). I analysed the data with a linear mixed effects model, using the lme4 package in R. The model had a three-way interaction between time block, starting ratio, and sociality (for mixed versus monoculture). Replicate nested within starting ratio was included as a random factor. I log transformed relative fitness due to non-normality of residuals; this, however, was not necessary for selection rate. I then

performed *post hoc* pairwise comparison on the effect of sociality at each time point for each starting ratio. For this I used the emmeans package in R, which uses estimated marginal means. I then applied a Bonferroni correction by multiplying *p*-values by 15 to account for the 15 pairwise comparisons made (comparing mixed and monoculture at five different time points for three different starting ratios; this multiplication accounts for the unusual '< 0.0015' outputs, which originally appeared as < 0.0001). A few additional comparisons not covered by the mixed effects model were performed as *t*-tests (for example, to test if relative fitness exceeded 1). I only included data up to 10 hours, as in the 10-12 hour time block there was some growth in monoculture (not visible), despite the antibiotic concentration being above the MIC.

Owing to the use of gentamicin agar plates to count Δ*ampC*::Gm cells and plain agar plates to count total cell counts (PAO1 and Δ*ampC*::Gm), it is possible, due to experimental error, to get negative PAO1 counts. This happened twice, and prevents calculation of a meaningful relative fitness or selection rate. These two replicates, both in the 0-2 hour time block, were therefore excluded from the analysis.

For visualization (rather than statistical) purposes, I also calculated and plotted the improvement in relative fitness gained by being in mixed culture by subtracting the relative fitness of the low producer in monoculture from the relative fitness of the low producer in mixed culture.

For the durability of beta-lactamase, I first standardized initial beta-lactamase activity to 1 for each replicate, to calculate and portray half-life more easily as the point when

activity equals 0.5. I calculated half-life on the basis of a straight line between points (as plotted), which will give a small overestimate at lower values. I used a one-way ANOVA to assess the effect of the presence of cells on the half-life. I also used a linear mixed effects model to look at the effect of cells at each time point. Replicate nested within each treatment was included as a random factor.

I calculated all statistics in R (www.r-project.org).

Results

Burden of non-producers

Producer cells increased beta-lactamase production in the presence of high levels of non-producers: at the 1:1 and 1:10 producer:non-producer ratios, producer cells make significantly more beta-lactamase (1:1, $t = 5.75$, $df = 5$, $p = 0.0022$; 1:10, $t = 11.03$, $df = 5$, $p = 0.00011$, Figure 1). The extra beta-lactamase made per producer cell (compared with monoculture) increased with the starting proportion of non-producers (ANOVA, $F_{2,14} = 22.9$, $p = 3.8\times10^{-5}$; Figure 1B). This compensation is only partial over the time period of measurement, as total beta-lactamase levels in these mixed cultures are less than in the equivalent high-producer monoculture (1:1 ratio: $t = -8.05$. $df = 5$, $p = 0.00048$; 1:10 ratio: $t = -7.48$, $df = 5$, $p = 0.00068$, Figure 2). In the treatment with the lowest relative frequency of non-producers (10:1 ratio), producers do not increase production of beta-lactamase (10:1 ratio, $t = 0.69$, $df = 5$, $p = 0.52$, Figure 1), and total beta-lactamase levels are only borderline different from an equivalent monoculture of the high producer (10:1, $t = -2.57$, $df = 5$, $p = 0.0501$, Figure 2).

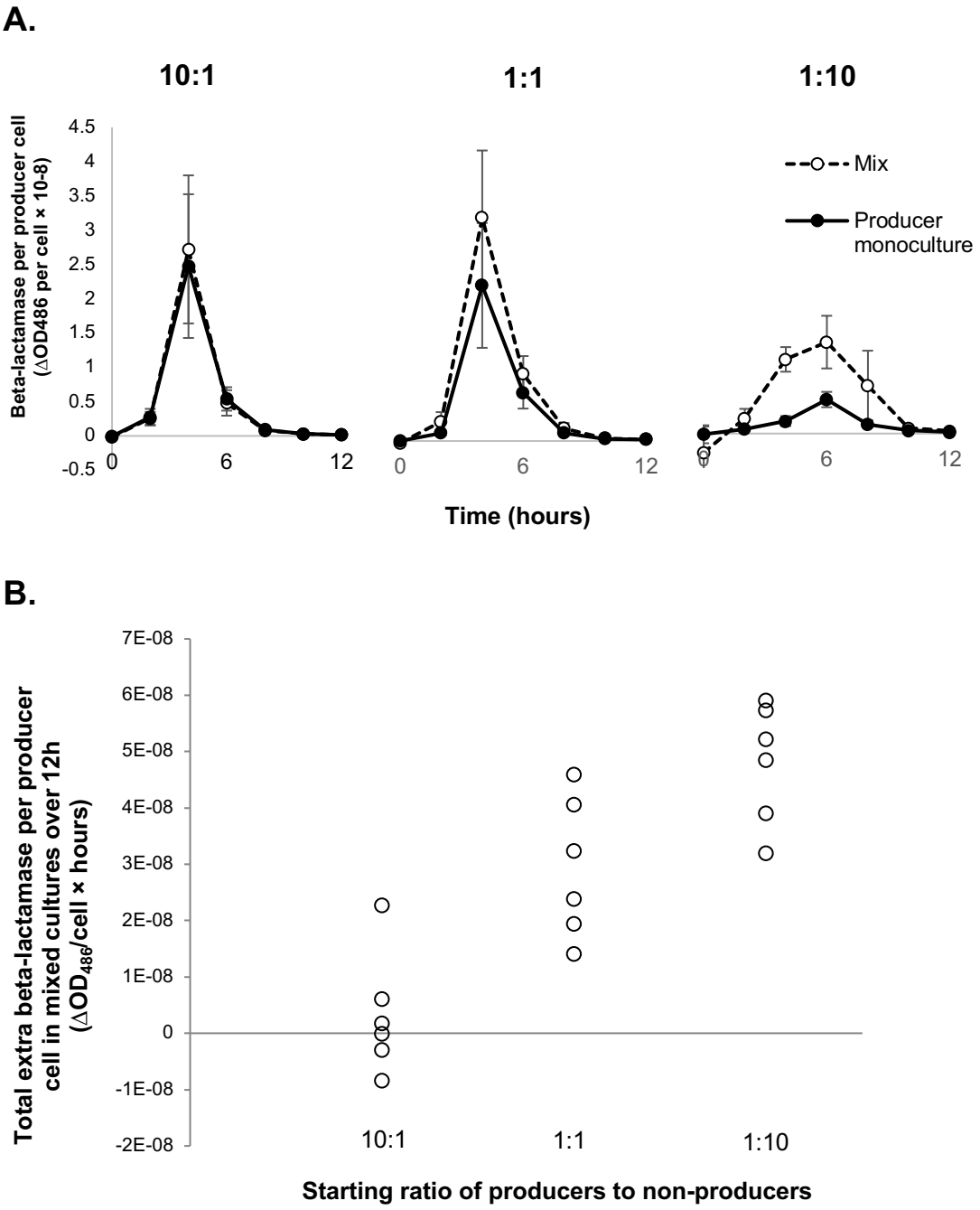


Figure 1. (A) Beta-lactamase activity per producer cell over 12 hours at three different producer:non-producer starting ratios. Producer cells have to work harder to compensate when they are rare. Each point is the mean of six replicates. Error bars are 95% confidence intervals. At the 10:1 ratio, no extra beta-lactamase is produced (t = 0.69, df = 5, p = 0.52), while at 1:1 and 1:10, producer cells make more beta-lactamase (t = 5.75, df = 5, p = 0.0022 and t = 11.03, df = 5, p = 0.00011 respectively); t-tests compare the difference in area under the curves between mixed and monoculture and are equivalent to a one-sample t-test comparing difference from zero in (B). (**B). Total extra beta-lactamase per producer cell in mixed culture compared with monoculture at three different producer:non-producer starting ratios.** Data points are from the area between the lines in (A), calculated separately for each of the six replicates. Increasing the proportion of non-producers increases the cost to the producer cells, which have to produce more beta-lactamase per producer cell. One-way ANOVA blocked design, $F_{2,14}$ = 22.9, p = 3.8×10^{-5}. All six replicates for each of the three starting ratios are displayed.

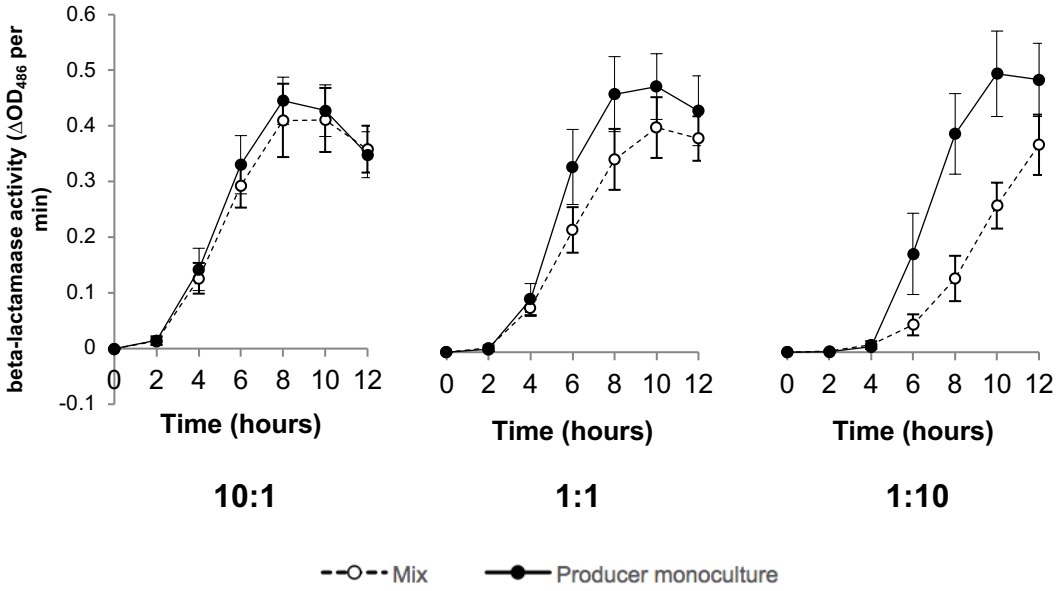


Figure 2. Compensation is only partial. Total beta-lactamase activity over 12 hours of growth in mixed culture and monocultures. Producers did not fully compensate for the presence of non-producers, particularly when non-producers were more frequent. To determine the difference between the mixed and monoculture curves for each ratio, a paired *t*-test was performed on the area under the curve. At the 10:1 ratio, total beta-lactamase is borderline significantly less in mixed culture (t = -2.57, df = 5, p = 0.0501). At 1:1 and 1:10, the mixed culture has lower total beta-lactamase levels (t = -8.05. df = 5, p = 0.00048 and t = -7.48, df = 5, p = 0.00068).

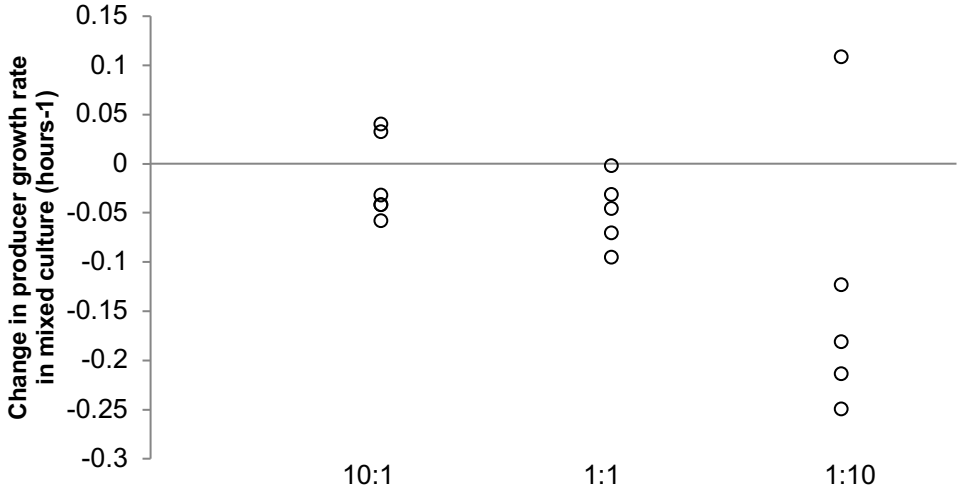


Figure 3. Growth rate of producer cells in the presence of non-producers. There is no significant change in producer growth rate when the proportion of non-producers is increased (one-way ANOVA, $F_{2,13}$ = 1.2, p = 0.33), although if the 1:10 ratio outlier is removed, there is a highly significant decrease in growth ($F_{2,13}$ = 16.3, p = 0.0004). Points represent the growth rate constant in mixed culture minus the growth rate constant in monoculture (negative values represent a slower growth rate in mixed culture). n = 6 for each treatment. One data point was excluded from the 1:1 and 1:10 ratios because of a negative initial cell count calculated from the gentamicin plates (see Methods).

This increased beta-lactamase production is reflected in a fitness cost during the period of high production: between two and four hours, producer growth rate in mixed culture is lower than in monoculture at the 10:1 ratio and 1:10 ratio (paired *t*-test: 10:1 ratio, $t = -6.4$, $df = 5$, $p = 0.0014$; 1:10 ratio, $t = -8.4$, $df = 5$, $p = 0.0004$, Figure 4A). Curiously, no cost is shown at the 1:1 ratio ($t = -1.1$, $df = 5$, $p = 0.32$). However, there is no significant effect of the burden of non-producers on average growth rate over the 12-hour period (one-way ANOVA on the effect of starting ratio on the change in growth rate between mixed and monoculture $F_{2,13} = 1.2$, $p = 0.33$, although removal of an outlier makes the result highly significant, $F_{2,13} = 16.3$, $p = 0.0004$, Figure 3).

Timing of benefit

Increasing the proportion of non-producers in mixed culture delays the improvement in the growth rate of non-producers (Figure 4A). There is an interaction between time block, starting ratio and sociality (mixed versus monoculture) ($F = 6.0$, $p = 1.0\times10^{-6}$), and *post hoc* analysis reveals that non-producers grow faster in mixed culture than monoculture from 2-4 hours at the 10:1 ratio ($p < 0.0015$) and at the 1:1 ratio ($p < 0.0015$) (with the exception of the 4-6 hour time block), but only at 8-10 hours in the 1:10 ratio ($p < 0.0015$ at 8-10 hours, but $p > 0.05$ at all other time points).

Temporal shifts in relative fitness varied depending on starting ratio (interaction between time and ratio, $F = 3.9$, $p = 0.0004$), and on whether the non-producer was in mixed or monoculture (interaction between time and sociality, $F = 13.1$, $p = 3.7\times10^{-9}$). However, there was no three-way interaction between time, ratio and sociality ($F = 1.6$, $p = 0.13$), although there was a three-way interaction between some

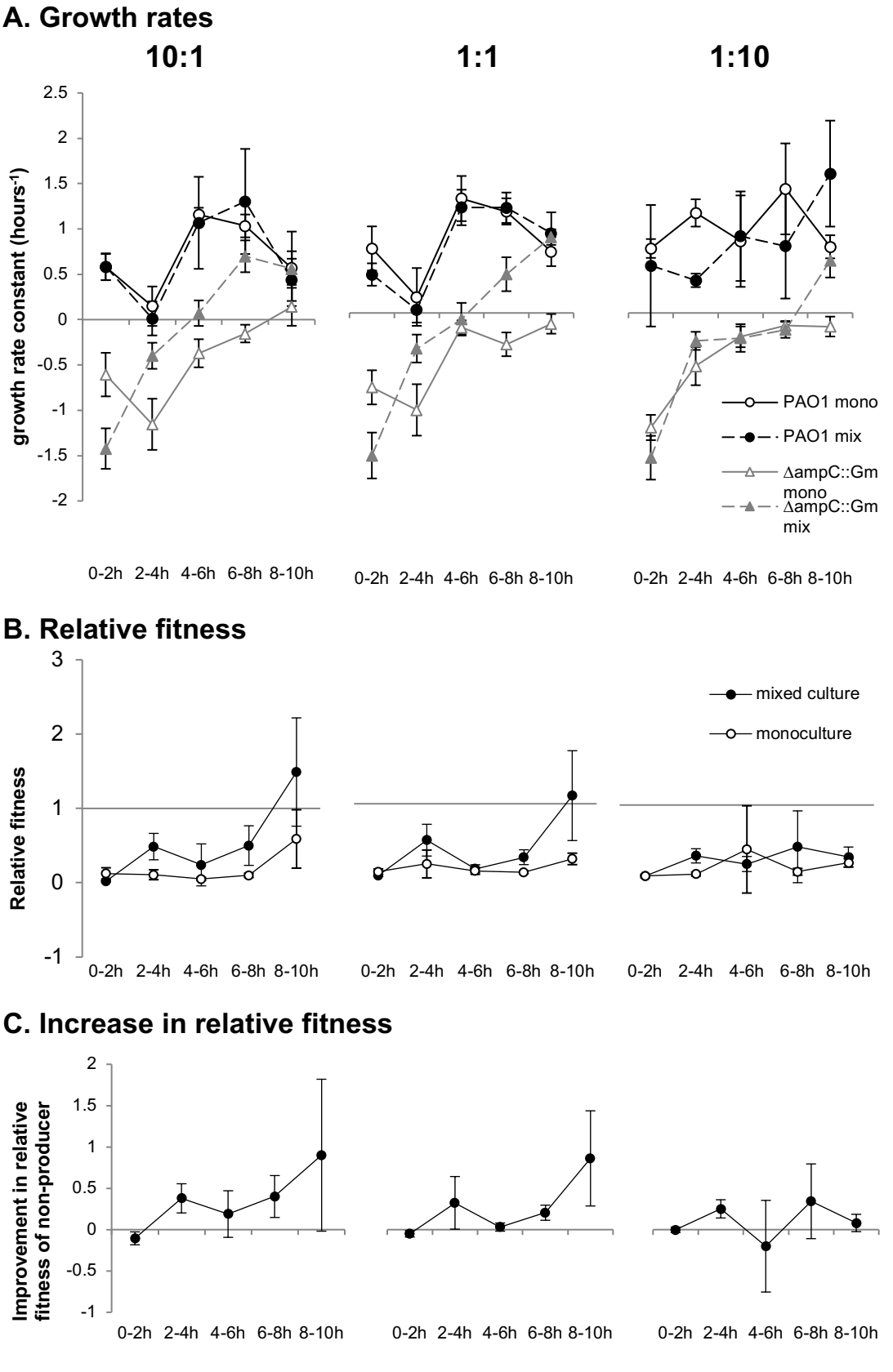
of the individual coefficients. I therefore proceeded to *post hoc* testing of pairwise interactions of the effect of sociality at each time point for each starting ratio.

At the 10:1 ratio, the non-producers have a higher relative fitness in mixed culture than monoculture in the 2-4 hour time block and again at 6-8 hours (Figure 4C), although at no point does the relative fitness significantly exceed one (Figure 4B). At the 1:1 ratio a similar pattern is seen, although the second period of improvement is delayed until the 8-10 hour time block. At the 1:10 ratio there is a small improvement in fitness at 2-4 hours, but no improvement in fitness later (see table in Figure 4 for full statistics).

The relative fitness benefit at 2-4 hours at all ratios coincides with the period when producers are making beta-lactamase at the highest rate (Figure 1A) and growing at the slowest rate (Figure 4A). Non-producer growth rate in this time block was increased in mixture relative to monoculture when there were at least 50% producers (*post hoc* pairwise comparison of estimated marginal means: 10:1 ratio, $p = 0.0015$; 1:1 ratio, $p = 0.0015$), although not by enough to make growth positive (Figure 4A).

The later period of non-producer benefit at 6-8 hours (1:10 ratio) or 8-10 hours (1:1 ratio) comes when the producers are making less beta-lactamase (Figure 1A). Despite this late improvement in relative fitness, the non-producers did not invade in mixed culture (Figure 4B, relative fitness not significantly different from one: one-tailed *t*-test at 8-10h: 10:1 ratio, $t = 1.32$, $df = 5$, $p = 0.12$; 1:1 ratio, $t = 0.35$, $df = 5$, $p = 0.37$). Instead, the non-producers are growing at the same rate as the producers (Figure 4A).

There was no difference to the significance of any results when reanalysed using selection rate rather than relative fitness.



Time:	0-2	2-4	4-6	6-8	8-10	0-2	2-4	4-6	6-8	8-10	0-2	2-4	4-6	6-8	8-10
t	-3.51	3.82	1.35	3.44	2.36	-1.65	3.31	0.76	2.96	3.05	-0.42	4.13	0.67	1.42	-0.32
p	**0.009**	**0.003**	1.0	**0.012**	0.299	1.0	**0.018**	1.0	**0.054**	**0.042**	1.0	**0.002**	1.0	1.0	1.0
mean diff	-0.11	0.379	0.189	0.401	0.900	-0.05	0.325	0.031	0.205	0.862	-0.00	0.251	-0.20	0.343	0.081

Figure 4 (FACING PAGE). A. Growth rates of producers (PAO1) and non-producers (Δ*ampC*::Gm) over time in mixed and monoculture. The points are the growth rate constant from the exponential growth rate equation for each two hour time block. **B. Relative fitness of non-producers in mixed and monoculture over time.** The points are the relative fitness of the non-producer in relation to the producer for each two hour time block. **C. The improvement in relative fitness of the low beta-lactamase producer in mixed culture over time.** Each point is the relative fitness in mixed culture minus the relative fitness in monoculture for each time block, showing the difference between the mixed and monoculture points in (B). For all graphs, each point is six replicates and error bars are 95% confidence intervals. The **statistics table** shows the results of *post hoc* pairwise comparisons (with Bonferroni correction) on the difference in relative fitness between mixed and monoculture in each time block for each starting ratio.

Durability of beta-lactamase

There was a statistically significant difference between the half-life of beta-lactamase in different treatments (one-way ANOVA $F_{3,11} = 4.8$, $p = 0.022$, Figure 5). In the absence of cells, beta-lactamase has a mean half-life of 12 hours at 37°C. This is significantly longer than any of the treatments with cells: 2.8 hours with PAO1 (difference from no cell treatment, $t = -3.3$, $p = 0.0073$), 2.9 hours with Δ*ampC* cells ($t = -3.2$, $p = 0.0080$) and 4.4 hours with Δ*lasR* (protease deficient) mutant ($t = -2.5$, $p = 0.028$). There was no significant difference between the half-life of beta-lactamase in any of the treatments with different cell types (PAO1 v. Δ*ampC*, $t = 0.05$, $p = 0.96$; PAO1 v. Δ*lasR*, $t = 0.51$, $p = 0.62$; Δ*ampC* v. Δ*lasR*, $t = 0.47$, $p = 0.65$). Removing one outlying point does not affect the significance of the results.

A linear model confirms the treatments have a different effect at different time points ($F_{18,66} = 8.1$, $p = 1.0\times10^{-10}$). This also reveals that, although I found no difference in the half-life between Δ*lasR* and the other treatments with cells, the beta-lactamase activity after four hours remains higher in the presence of Δ*lasR* cells than PAO1 cells ($t = -3.9$, $p = 0.0002$) or Δ*ampC* cells ($t = -3.6$, $p = 0.0007$), implying slower degradation by Δ*lasR* cells, despite the half-life as calculated not being statistically

significantly different. This model also shows that the no cell treatment still has higher beta-lactamase activity after 24 hours than the treatments with cells (no cells v. PAO1, t = -2.3, p = 0.03; no cells v. Δ*ampC*, t = -2.2, p = 0.03; no cells v. Δ*lasR*, t = -2.1, p = 0.04, Figure 6).

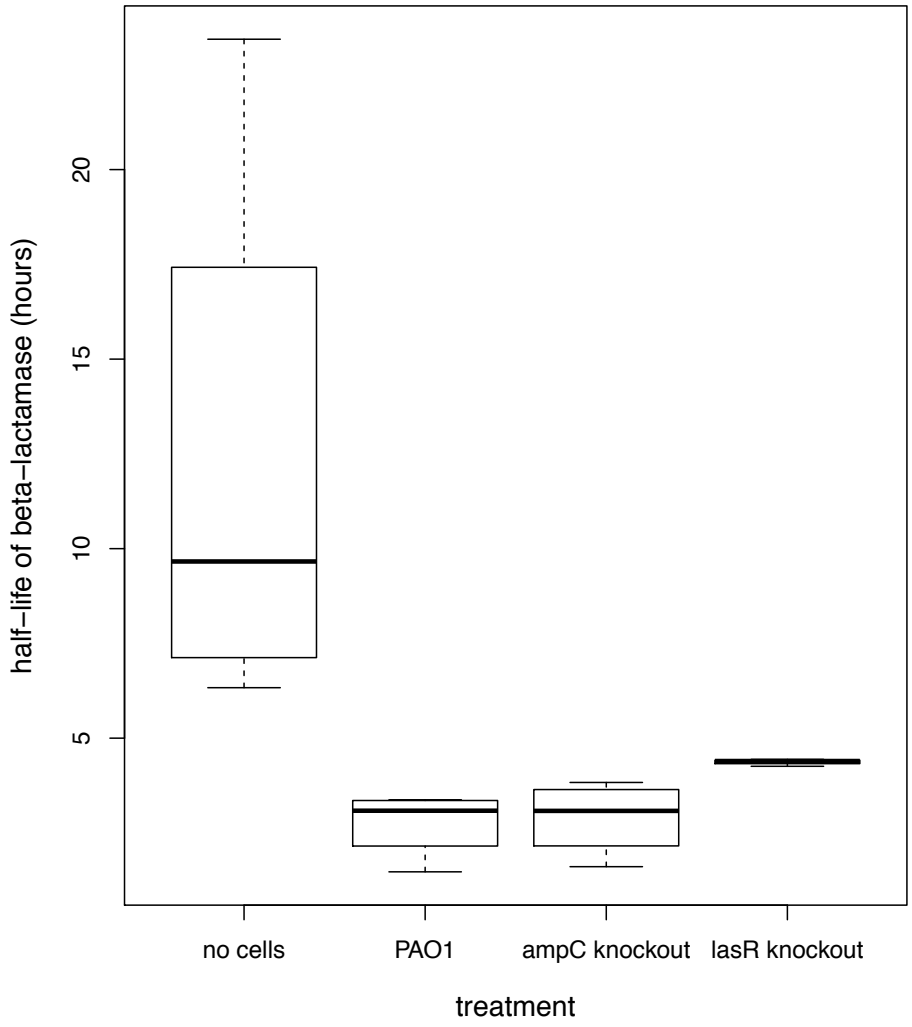


Figure 5. Half-life of beta-lactamase. The half-life of beta-lactamase in the absence of cells is significantly longer than in the presence of PAO1 (t = -3.3, p = 0.0073), Δ*ampC* (t = -3.2, p = 0.0080) or Δ*lasR* (t = -2.5, p = 0.028). Removing an outlier from the no cells treatment did not change the results. There was no difference between the half-life in any of the treatments with different cell types (PAO1 v. Δ*ampC*, t = 0.05, p = 0.96; PAO1 v. Δ*lasR*, t = 0.51, p = 0.62; Δ*ampC* v. Δ*lasR*, t = 0.47, p = 0.65).

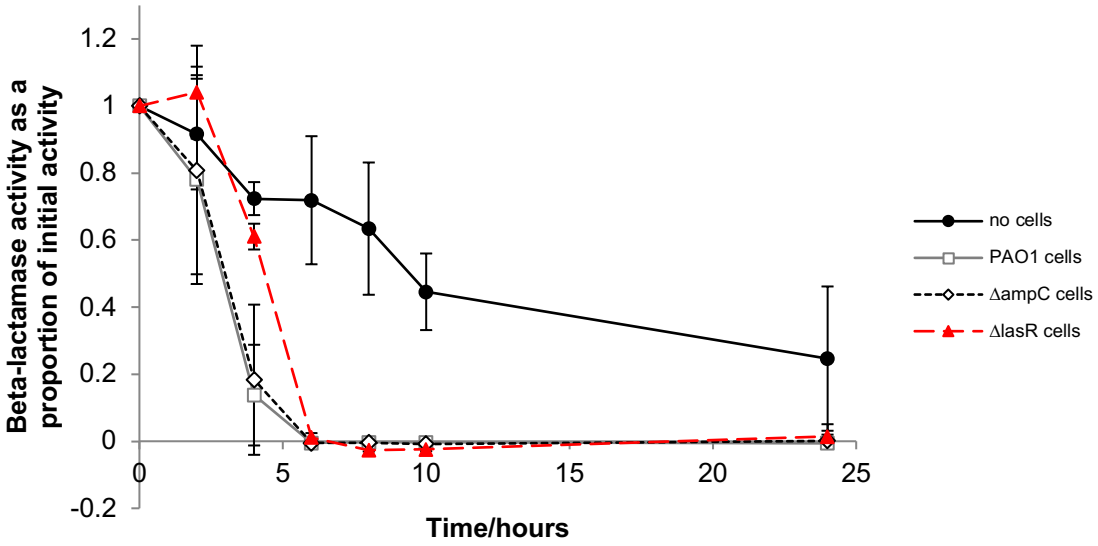


Figure 6. Durability of beta-lactamase. Initial beta-lactamase activity was set to 1, so the y-axis represents the proportion of original activity remaining. At 24 hours, beta-lactamase incubated in the absence of cells still shows more activity than if cells were present (no cells v. PAO1, t = -2.3, p = 0.03; no cells v. Δ*ampC*, t = -2.2, p = 0.03; no cells v. Δ*lasR*, t = -2.1, p = 0.04). At 4 hours, beta-lactamase incubated with Δ*lasR* shows higher activity than beta-lactamase incubated with either PAO1 (t = -3.9, p = 0.0002) or Δ*ampC* (t = -3.6, p = 0.0007). Error bars are 95% confidence intervals. There were four replicates for the no cell, PAO1 and Δ*ampC* treatments and three replicates for Δ*lasR*.

Discussion

The data presented in this chapter provide clear support for the hypothesis that beta-lactamase producers respond to the presence of cheats, and that they do so in a manner that leads to increased costs and consequent loss of fitness. The presence of potential cheats in mixed culture reduces the total amount of beta-lactamase available (Figure 2). This has a measurable impact on the behaviour of producing cells: producer cells increase beta-lactamase production as a likely strategy to compensate for reduced beta-lactamase detoxifying their surrounding environment (Figure 1), although the compensation is only partial (Figure 2). Non-producers have an increased relative fitness during the period of high beta-lactamase production (Figure

4C and Figure 1A) but have a further period of increased relative fitness once beta-lactamase production has dropped (Figure 4C and Figure 1A). It is likely this latter period is when the antibiotic concentration has become sub-inhibitory. I show that beta-lactamase has a half-life of approximately 12 hours, but that it is removed from the media by cells, reducing half-life to 3 hours (Figure 5). This may in part be due to breakdown of beta-lactamase by *lasR* encoded proteases (Figure 6). This suggests a further way of regulating extracellular beta-lactamase levels.

Partial compensation

The partial compensation for the presence of non-producers, through increased beta-lactamase production by producer cells, demonstrates a response to the social environment, with implications for the costs and benefits to producers and non-producers. Partial compensation is consistent with the findings shown in the public good pyoverdin (Harrison, 2013). More broadly it echoes cooperative behaviour in birds (Harrison *et al.*, 2009) and models of evolutionarily stable strategies (Houston & Davies, 1985; McNamara *et al.*, 2003). Partial compensation, as opposed to full compensation, reduces the amount of public good available to both producers and non-producers, limiting population growth. Ross-Gillespie *et al.* (2007) identified this as a cause of frequency-dependence, limiting the advantage to non-producers by reducing the period of growth over which they could gain an advantage. This prevents a tragedy of the commons scenario. Partial compensation may keep public good production at a level where growth is still possible. My results are a new example of phenotypic plasticity in response to the social environment in bacteria. Partial compensation could help explain the maintenance of beta-lactamase production in chronic infections shown in Chapter 2.

The mechanism of partial compensation is unclear. That there is any compensation at all could be through producers responding directly to the presence of non-producers, or indirectly to antibiotic concentration. There is no direct evidence for the former, although a role for quorum sensing in *ampC* expression has been shown (Zhao *et al.*, 2015). It is clear, however, that producers respond to antibiotic concentration (Nordström & Sykes, 1974; Livermore & Yang, 1987; Giwercman *et al.*, 1991; Bagge *et al.*, 2004b). This is controlled through a complex mechanism involving the regulator AmpR, in response to damage to the cell wall caused by the antibiotic (Jacoby, 2009). Beta-lactams disrupt the constant recycling of cell wall peptides (muropeptides), leading to an increase in the muropeptide anhMurNAc-tripeptide. This binds to and alters the conformation of AmpR, causing it to activate *ampC*, leading to an increase in AmpC beta-lactamase levels (Jacobs *et al.*, 1997). It therefore seems likely that producer cells will respond directly to antibiotic concentration. Antibiotic concentration will remain higher for longer when there are fewer beta-lactamase producers, hence there is stronger and more prolonged induction of beta-lactamase and compensation is seen.

That the compensation is only partial could be because producers have reached maximum levels of induction and cannot produce more beta-lactamase. This, however, seems unlikely here as producers in fact make less beta-lactamase per cell when there is a high burden of non-producers (1:10 producer:non-producer ratio) than when there is a lower burden (1:1 and 10:1 ratios) (Figure 1A). More likely is a response through one or both of the two mechanisms described as causes of compensation in the first place – i.e. either a form of direct response to the cells present, such as through quorum sensing, or a response to antibiotic concentration. In

terms of a response to other cells, compensation could be partial because producer cells are likely to be at a lower density for longer when there is a high burden of non-producers, because of slow growth caused by the cost of extra beta-lactamase production. Lower density would be likely to reduce any effect of quorum sensing on beta-lactamase induction. Alternatively, if the response were to antibiotic, it would have to be because antibiotic concentration were lower in mixed culture with many non-producer cells than in producer monoculture. This initially seems unlikely, but work by Snoussi *et al.* (2018) suggests sensitive cells (as the non-producers are) in effect use up antibiotic as they are killed. While this mechanism has not been shown with beta-lactams, it could lower antibiotic concentration and reduce induction of AmpC beta-lactamase when there are high numbers of non-producers. Either of these mechanisms could cause the compensation to be partial rather than full and thereby stabilize cooperation. Whether partial compensation is a selected strategy or simply a mechanistic by-product, the evolutionary outcome of stabilization of cooperation is the same.

To examine the mechanism by which partial compensation may arise, the experiment ought to be repeated with measurement of antibiotic concentration at each stage. Manipulation of antibiotic concentration would help distinguish between the response to antibiotic concentration versus a direct response to non-producer cells. For example, dual-flask systems (Clark *et al.*, 2009) can be used to keep antibiotic concentration constant. If a compensatory effect were seen here, this would exclude an indirect response to antibiotic concentration.

Temporal dynamics of costs and benefits

The lack of synchrony observed between producer costs and non-producer benefits (Figure 4) could prevent a potential cheat from invading. The non-producer only had positive growth after the high producers downregulated beta-lactamase production – i.e. once the antibiotic concentration had fallen. At this point, producers and non-producers could potentially grow equally (Chapter 4, Figure 1, page 113), but the non-producer has no cost advantage and cannot invade. Invasion would be possible if a constitutive level of production remained (discussed below), or if there were a cost to carrying the beta-lactamase gene, as there often is for plasmid-borne genes (Marciano *et al.*, 2007; San Millan & MacLean, 2017). This concurs with work by Clark *et al.* (2009) where non-producers could only survive in mixed culture when beta-lactamase resistance was borne on a costly plasmid. It is also consistent with work by Ghoul *et al.* (2016) showing invasion was only possible during the period of public good production, and Kümmerli *et al.* (2009b), who showed that downregulating public good production once it is no longer needed reduces the relative fitness of non-producers. My results show the importance of the timing of the costs and benefits in the outcome of competition between cooperators and cheats, along with how facultative public good production can minimize benefit to the non-producers.

Durability of beta-lactamase

The durability of beta-lactamase, combined with the removal and likely recycling of beta-lactamase once it is no longer needed, are likely to minimize the ability of non-producers to gain a competitive advantage. Although the beta-lactamase half-life of

approximately 12 hours is less than that of pyoverdin, which has been shown to be fully functional after 48 hours at 37ºC (Kümmerli & Brown, 2010a), it is hard to know how meaningful a comparison of completely different molecules with different functions is. It does, however, suggest that the effect of beta-lactamase could last considerably beyond the peak total beta-lactamase activity at 8-10 hours (Figure 2), and that the period of production could be shorter than with a less durable molecule. A degree of durability, coupled with facultative control of production, is likely to reduce the fitness benefits available to non-producers, as in Kümmerli & Brown (2010). While Brown & Taddei (2007) showed the opposite effect, they assumed constitutive rather than facultative public good production. Furthermore, the removal of beta-lactamase from the environment by cells, including possible breakdown by proteases (Figures 5 and 6) is likely to enable the uptake and recycling of amino acids, in effect minimizing the net cost of production if the producer has preferential access to the amino acids, which is likely in a structured lung biofilm.

However, there are some potential difficulties in inferring the rate and cause of beta-lactamase degradation from these results. For example, loss of beta-lactamase activity in the presence of cells could be caused by other changes to the media as a result of the presence of cells, such as acidification of the media over time. However, such a dramatic drop in beta-lactamase activity in the first four hours due to pH would be surprising when the catalytic efficiency (specificity constant, k_{cat}/k_m) of AmpC beta-lactamase has been shown to be almost unchanged at pH 5.8 compared with pH 7.5, in contrast to certain other beta-lactamases (Ohsuka *et al.*, 1995).

Secondly, while slower degradation in the presence of the Δ*lasR* mutant suggests a role for proteases in AmpC breakdown since LasR regulates the expression of three major proteases (LasA elastase (Toder *et al.*, 1991), LasB elastase (Gambello & Iglewski, 1991) and alkaline protease (Gambello *et al.*, 1993)), LasR is an important transcriptional regulator in the LasR/LasI quorum sensing (QS) system, which in turn regulates the RhlR/RhlI QS system (Whitehead *et al.*, 2001). As such, the Las and Rhl systems together are thought to control the expression of 6-12% of chromosomal genes in *P. aeruginosa* (Heurlier *et al.*, 2005), including genes for toxins (Gambello *et al.*, 1993), motility (Glessner *et al.*, 1999), metabolic enzymes (Heurlier *et al.*, 2005) and siderophores (Stintzi *et al.*, 1998). Therefore it is hard to confirm if the prolonged half-life of beta-lactamase with Δ*lasR* mutants is caused by protease deficiency rather than another unknown effect. To confirm the role of proteases, this experiment should be repeated to see if addition of a protease inhibitor slows the degradation of beta-lactamase by *P. aeruginosa* cells.

Nevertheless, the possible removal of beta-lactamase from the extracellular environment by cells suggests a mechanism, in addition to production, which could influence total beta-lactamase levels. Though I used the results in Figure 1 as an estimate of beta-lactamase production per cell, they in fact represent the beta-lactamase present per cell, which is a function of both production and degradation. The cost of extra production could be mitigated in part by decreased degradation. Indeed, the failure to find a clear relationship between increasing the burden of non-producers and decreased producer growth could support this possibility (Figure 3). Whether this is a factor and how it is regulated requires further investigation.

Further considerations

Constitutive rather than facultative production of public goods could have a major effect on the three areas discussed in this chapter – the effect of the burden of cheats, the timing of the costs and benefits, and whether durability benefits producers or non-producers. Constitutive production may limit the ability of producers to respond to the presence of cheats, although 17 out of 22 constitutive producers found in my isolate collection can be induced to increase production to at least double their constitutive level (Chapter 2). The timing of the costs and benefits would change with constitutive production: the continuous cost of production paid by the producer increases the relative fitness of the non-producer and gives a longer time window for invasion. And as discussed above, Brown & Taddei's model (2007) of durability with constitutive production found durability benefited cheats, in contrast to models which assumed facultative production (Kümmerli & Brown, 2010a). It would therefore be interesting to repeat competition assays with constitutive producers to test these effects. Given the potential advantage of facultative production, it is interesting to consider why constitutive producers of beta-lactamase are seen at all in chronic infections, albeit rarely (Chapter 2, Figures 5, 8 and 15). An important reason is likely to be that not all antibiotics are inducers (Jacoby, 2009), and so constitutive production will have a selective advantage with exposure to non-inducing antibiotics.

Ultimately all the factors examined in this chapter are linked as they each have a bearing on the costs and benefits to the producers and non-producers, and so determine the outcome of competition. That the outcome of competition here is whether antibiotic resistance is selected or not is a pointed reminder of the importance of studying social factors in the evolution of antibiotic resistance.

Chapter 6. Discussion

The aim of this thesis is to examine the role social evolutionary dynamics might play in antibiotic resistance in infections. This was with the purpose of furthering understanding of social evolution in bacteria, and of providing greater understanding of evolutionary pressures involved in the very real problem of antibiotic resistance.

I provided a detailed discussion of the results within each chapter. Here I summarize the main results, drawing together the key points to build an overall picture of the role of social dynamics. I then consider the implications before suggesting directions for future research.

Key findings

Extracellular beta-lactamase does not increase during chronic infection, but it does diverge (Chapter 2).

If selection for increased resistance were the main evolutionary pressure from treatment with beta-lactam antibiotics, a clear pattern of increasing beta-lactamase production during chronic infection would be expected. I did not find this. Instead I found that beta-lactamase production diverged within patients, and within clone types in patients, over time. This increase in variance positively correlated with beta-lactam treatment, suggesting that increased selection pressure from beta-lactam antibiotics

favours both high and low beta-lactamase production. This is consistent with social evolutionary dynamics, with selection for lower producing cheats, as well as high producers, during antibiotic treatment. I assess support for alternative hypotheses later on in this chapter.

Isolates produce high levels of extracellular beta-lactamase, and high and low producers coexist within patients' lungs (Chapter 2).

High levels of extracellular beta-lactamase (compared with laboratory wild-type PAO1) are likely to make indirect fitness effects more important in beta-lactamase evolution. Coexistence of high and low producers opens the possibility of social interactions between strains with different levels of production.

Beta-lactam resistance also diverges over time, while quinolone resistance does not (Chapter 3).

An alternative explanation to the finding in Chapter 2 that beta-lactamase diverges during an infection is that the divergent isolates inhabit spatially separated parts of the lung and evolve separately under different selection pressures. If this were the case, similar divergence should be seen in the resistance to antibiotics of all classes. This, however, is not the case. I found that resistance to beta-lactam antibiotics diverges, and that this divergence correlates with the intensity of beta-lactam treatment (mirroring the finding for beta-lactamase itself in Chapter 2). Aminoglycoside resistance also diverged, although a correlation with treatment intensity was only found within patients (and not clone types). Quinolone resistant did not diverge and there was no effect of treatment. This is not consistent with spatial separation driving

the divergence, but is consistent with social dynamics causing divergence in resistance when resistance mechanisms are cooperative, and not when resistance is solely private.

Low beta-lactamase producers benefit from beta-lactamase from high-producing clinical isolates (Chapter 4).

I conducted experiments to show that low-producing clinical isolates and an *ampC* knockout are protected from antibiotic by the supernatant from high-producing clinical isolates. I also show that the *ampC* knockout strain gains a benefit when grown in direct competition and can sometimes invade. An inhibitor of AmpC beta-lactamase abolishes the beneficial effect, confirming AmpC beta-lactamase is driving the fitness differences observed in my experiments.

High producing isolates partially compensate for the presence of non-producers (Chapter 5).

High producers make more beta-lactamase in the presence of non-producers, but only enough to partially compensate for the non-producers' presence. As discussed in Chapter 5, partial compensation stabilizes cooperation.

The timing of costs and benefits may make it difficult for non-producers to invade in some circumstances (Chapter 5).

Unlike other public goods such as pyoverdin, the private cost of producing beta-lactamase in not entirely synchronous with the public benefit of its action. While producers start making beta-lactamase in the first two hours after exposure to an

inducing antibiotic, non-producers in mixed culture are unable to grow until after six hours, which is likely to represent the point where the antibiotic concentration falls below its MIC. Producers thus get a 'head start'. When the concentration of an inducing antibiotic falls, producers downregulate their beta-lactamase production, so they are no longer paying the cost. This temporal disjunction between costs and benefits may limit the ability of non-producers to invade in certain circumstances.

Summary

The findings from Chapters 2 and 3 are consistent with social dynamics affecting beta-lactamase production in clinical infections; Chapter 4 confirms that AmpC beta-lactamase from the high-producing clinical isolates can act as a public good, protecting non-producers; Chapter 5 suggests some mechanisms which may stabilize cooperation, preventing a tragedy of the commons, despite some benefit to the non-producers.

Contribution to the literature

Though there is now a considerable body of work examining microbial social evolution, this thesis particularly contributes in three areas: first, it adds to the literature on social dynamics in natural populations (Chapters 2 to 4); second, while much previous work has focussed on the public good pyoverdin, this thesis gives a detailed study of social dynamics in a different public good, providing a useful comparison (Chapters 4 and 5). Finally, it is of considerable clinical relevance. I shall

consider the social evolutionary points first followed by a broader discussion of social evolutionary issues, before going on to consider the clinical implications.

Social evolution

Natural populations

The majority of social evolution studies have involved *in vitro* studies of laboratory strains, and it is unclear the extent to which these may reflect natural populations. Some work has found potential cheats in soil isolates (Bruce *et al.*, 2017), and a number of potentially social traits decline in chronic CF infections (Jiricny *et al.*, 2014). However, many have interpreted this latter example as adaptation to the lung environment (Marvig *et al.*, 2015; Schick & Kassen, 2018), although more recent work has clearly supported social dynamics as an explanation (Andersen *et al.*, 2015, 2018), and social dynamics are increasingly recognized as important (Winstanley *et al.*, 2016). It would be difficult to interpret the lack of increase in beta-lactamase producers in CF lungs (Chapter 2) as adaptation to the lung environment, given the high level of beta-lactam antibiotic treatment these patients receive. This thesis therefore adds to the small number of examples of social dynamics in natural populations.

Comparison with pyoverdin

Pyoverdin is the most studied bacterial public good (Griffin *et al.*, 2004; Ross-Gillespie *et al.*, 2007, 2009; Kümmerli *et al.*, 2009b, 2009a, 2015; Jiricny *et al.*, 2010;

Kümmerli & Brown, 2010b; Ghoul *et al.*, 2014b, 2016; Andersen *et al.*, 2015, 2018), but it is not necessarily typical of all public goods. My study with beta-lactamase therefore provides a useful comparison. Individual properties of a public good – including its purpose, its durability and recycling, the timing of the costs and benefits to producers and recipients – will all affect the outcome of cooperator-cheat dynamics. Chapter 5 looked at this in detail. In particular, I showed that the benefit to the non-producers (the ability to grow once antibiotic concentration had fallen) came after the period when producers were paying the cost of production – and as there appeared to be no inherent cost to carrying the gene for beta-lactamase, the non-producers were unable to invade. This is very different from the dynamic in pyoverdin, where non-producers can gain an immediate benefit once they take up a pyoverdin molecule. This is likely to make cheats more able to invade pyoverdin, as has been shown (Griffin *et al.*, 2004; Ross-Gillespie *et al.*, 2007; Jiricny *et al.*, 2010; Ghoul *et al.*, 2014b), whereas it has rarely been shown with beta-lactamase (Yurtsev *et al.*, 2013). Concurring with this, one study suggests an inherent cost to bearing the beta-lactamase gene is necessary if non-producers are to survive in the population (Clark *et al.*, 2009).

This difference in the temporal dynamics of pyoverdin and beta-lactamase also highlights the different purpose of these public goods. Pyoverdin is used for nutrient acquisition and the amount needed is a function of iron availability and the number of cells; by contrast, beta-lactamase is for detoxifying the environment, and so the amount needed is set by the environment and not the number of cells. How this affects cooperator-cheat dynamics is unclear. A further difference relates to how essential the public good is. For example, pyoverdin is needed for growth, but beta-

lactamase is needed for survival in the face of antibiotics. Again it is unclear how this might affect social dynamics, but work by Bottery *et al.* (2016) suggests that non-producers can only invade when the public good is not essential – i.e. when the antibiotic concentration is below the MIC of the non-producer. Theoretical modelling of the essentiality of a public good may prove informative. Non-producers are much less likely to be able to invade if the public good is essential for survival and where there is a time lag before the benefit accrues.

It is worth considering the mechanism by which beta-lactamase becomes extracellular. Pyoverdin is known to be secreted through efflux pumps (Schalk & Guillon, 2013). Beta-lactamase has been shown to be secreted in vesicles (Ciofu *et al.*, 2000). However, it is possible that lysis or deformation of cells under high antibiotic pressure could contribute to extracellular beta-lactamase as well, and a number of studies have speculated about this with no definitive conclusion (Giwercman *et al.*, 1992; Matsumura *et al.*, 2000; Høiby *et al.*, 2010). In the experiments I conducted, lysis of cells is comparatively unlikely as the antibiotic concentrations used were sub-inhibitory for the producer cells. It does, however, remain a theoretical possibility. While the temptation might be to suggest that lysis would imply any public good effect is a mere by-product rather than a selected strategy, this is not the case. First, it is the costs and benefits of the behaviour to the actor and recipient which is important in classifying a social behaviour, not the mechanism of the behaviour. Second, lysis, rather like programmed cell death in multicellular eukaryotes, can be a highly controlled behaviour in response to the environment (Bayles, 2014; McFarland *et al.*, 2015). It would be interesting to investigate the mechanism further, but is not crucial to the social interpretation of

results. Some preliminary work suggests lysis is not an important mechanism by which beta-lactamase becomes extracellular (Spragge, pers. comm).

Defining social terms

Terminology has proved a minefield in social evolution (West *et al.*, 2007b). Defining what is meant by a 'cheat' has proved particularly controversial and care is required in using this term to describe loss-of-function mutants in general. Cheating has been defined by Ghoul *et al.* (2014a) as '*(i) a trait that is beneficial to a cheat and costly to a cooperator in terms of inclusive fitness*' and '*(ii) when these benefits and costs arise from the actor directing a cooperative behaviour toward the cheat, rather than the intended recipient*'. I found a benefit to the non-producers in terms of improved growth rate when grown with producer cells relative to when grown alone, even though this is not always sufficient to allow the non-producer to invade (Chapter 4, Figures 7-14). And the experiments I describe in Chapter 5 show clear evidence for a cost to the producer from growing in the presence of non-producers. This fulfils the first part of the definition. The second part is fulfilled because I show that the benefit to non-producers arises from the cooperative behaviour being directed towards the potential cheat, rather than the intended recipient (which are cells related to the cooperator) (Chapter 4, Figures 7-14). For this reason, I refer to non-production of beta-lactamase as a cheating strategy where appropriate. Cheating is context-dependent, and it is important to note that this applies to my specific experimental set-up, and in the case of the non-producers in the lung, it is more correct to refer to them as 'potential cheats' to allow for the fact that the exact costs and benefits I impose in the laboratory will not be identical to the lung environment (discussed further below).

Potential non-social explanations for beta-lactamase production dynamics in the CF lung

(i) Spatial structure: As discussed in Chapters 2 and 3, the human lung is a highly structured and heterogeneous environment (Jorth *et al.*, 2015; Winstanley *et al.*, 2016), which could result in varying antibiotic concentration in different parts of the lung. This would mean cells in different parts of the lung were exposed to different concentrations of beta-lactam antibiotic and consequently to differing strengths of selection for beta-lactamase production. This could account for the divergence in production (Chapter 2, Figures 9 and 10) and in resistance levels (Chapter 3, Figure 8) I observe, while there is no increase in production on average (Chapter 2, Figure 6). However, this divergence would be predicted across all classes of antibiotics if spatial structure were the driving force, but results from Chapter 3 are not consistent with this hypothesis. Instead, I observed no increase in variance (Chapter 3, Figure 8) and a significant increase over time in private resistance mechanisms in a quinolone (Chapter 3, Figure 5 - all quinolone resistance is mediated by private efflux or binding site mutations (Poole, 2011)), which is the pattern expected in antibiotics resisted by private mechanisms.

(ii) Switch to alternative resistance mechanisms: loss of beta-lactamase could be caused by a shift from antibiotic class-specific mechanisms, such as beta-lactamase or aminoglycoside modifying enzymes, to more general mechanisms of resistance, such as efflux pumps. The efflux pumps MexAB-OprM and MexCD-OprJ resist both beta-lactams and quinolones, while MexXY-OprM resists aminoglycosides as well (Poole, 2011). Indeed, Vestergaard *et al.* (2016) show in particular that combination therapy (use of different classes of antibiotics) selects for efflux pumps. A difficulty here is

that generalized mechanisms of resistance such as efflux or decreased membrane permeability are private, making it hard to distinguish between a shift to private mechanisms or a shift to generalized mechanisms. Experimental evolution could be useful here to compare loss of beta-lactamase when treated by beta-lactams only, versus loss of beta-lactamase when treated with the combined therapy of multiple antibiotic classes. However, while generalization could explain loss of beta-lactamase, it would not provide an explanation for increasing variance, as all isolates should be under equal selection pressure to shift to non-specific resistance mechanisms. That there was an increase in beta-lactamase variance (Chapter 2, Figures 9-12) and that it correlated with beta-lactam treatment (Chapter 2, Figure 13) tends to favour a social explanation. Examining the exact antibiotic selection pressure may help clarify these mechanisms.

(iii) Collateral sensitivity: that selection for resistance to one class of antibiotics can lead to increased sensitivity to other antibiotic classes was raised in Chapter 1 as a potential cause of loss of antibiotic resistance. Previous work suggests that changes in efflux pump regulation are the main cause of this phenomenon in *P. aeruginosa* in CF (Imamovic *et al.*, 2018). Therefore the findings in Chapter 2 of considerable variation in beta-lactamase production itself are unlikely to be related to collateral sensitivity.

(iv) Late invasion of low beta-lactamase producing colonizers: Increased variance could result if later infections were more vulnerable to invasion by low-producing colonizing strains from the environment. A phylogenetic analysis of the isolates in this study (Ghoul, in prep) show clearly that low producers are without exception from resident strain, so this hypothesis is not supported.

Clinical implications

The potential importance of these results is in understanding and thereby limiting the harmful effects of the evolution of antibiotic resistance. In a simple practical sense, understanding that chronic infections are social communities made up of strains with varying levels of resistance, and where resistant strains may protect sensitive ones, may be helpful for optimizing treatment. High rates of treatment failure in chronic cystic fibrosis infections (Smith *et al.*, 2003; Parkins *et al.*, 2012) could be caused by cross-protection. While treatment failure because of cross-protection between species has been observed (Brook, 1985), the problem may be much wider and include intraspecies cross-protection, as suggested in this thesis.

When determining MICs in clinical laboratories, the sociality of the mechanism may affect the result. Two examples of this include the 'inoculum effect' (Brook, 1989) and the effect of biofilm growth (Moskowitz *et al.*, 2005). The inoculum effect, which has typically been observed in beta-lactamase producers but not in quinolones (Brook, 1989), is where higher initial cell density of the culture increases the MIC (Brook, 1989; Artemova *et al.*, 2015). Biofilm growth can have a similar effect on MIC measurements: for example, Moskowitz *et al.* (2005) showed in a test of sputum cultures from 40 CF patients that while conventional broth dilution suggested beta-lactams were suitable to treat all the isolates in the study, this figure reduced to only 43% when the MIC was measured in biofilms. Notably, the reverse pattern was observed in quinolones, which were more able to treat isolates in biofilms. These two observations are consistent with a social view of resistance. However, accounting for these observations has not made it into clinical practice: standard MIC measurements

involve inoculating only at one density (Andrews, 2001), and UK guidelines suggest there is no evidence to support the routine use of biofilm testing in CF lung samples (UK Cystic Fibrosis Trust, 2010).

Social considerations could help design future treatment regimes. Antibiotics never completely eradicate chronic *P.aeruginosa* infections (Folkesson *et al.*, 2012). Given this starting point, having at least some sensitive non-producers survive in the mix is potentially advantageous. After a course of antibiotics, if sensitive non-producers have survived they will contribute to repopulating the lung, making future treatment easier than if only resistant bacteria repopulate the lung. Indeed, in the absence of antibiotics between treatments, low producers would have a competitive advantage against constitutive beta-lactamase producers as they do not have to pay the cost of ongoing beta-lactamase production. This could explain the scarcity of constitutive producers (Chapter 2, Figures 4A and 5). Given that complete eradication from the CF lung has proved difficult, finding the optimal antibiotic regime which curtails infective exacerbations by limiting the growth of all bacteria while allowing some sensitive non-producers to survive in order to repopulate the lung is a possible goal. Such an approach might, for example, find an intermediate concentration of antibiotic more beneficial in a chronic infection rather than a very high dose which might kill low producers while still failing to eradicate high producers. While highly speculative, a social approach offers novel potential avenues for antibiotic resistance research.

Overall, an understanding of social evolution offers guiding principles to when social dynamics may be relevant, and hence when such strategies discussed above may be

successful. For example, social dynamics might be more important in chronic infections, both because greater time allows more mutants to arise, and because under certain conditions cheats have been shown to have a higher relative fitness after longer periods of competition (Ross-Gillespie *et al.*, 2007). Social interactions are also more likely in infections in a single organ cavity such as the lung – but even more so in the less structured gut or bladder – where the mixing of cells occurs, potentially allowing non-producers to gain an advantage. By contrast, social dynamics are unlikely to be relevant to intracellular pathogens or in acute infections lasting only a few days. Social interactions will of course be less important if private mechanisms of resistance are dominant in the bacteria of interest.

Future directions

Some references to future work have been referred to above, including modelling and experimental work examining the effect of the 'essentiality' of a public good, and experimental work to examine the mechanism of beta-lactamase secretion, and work to distinguish between social dynamics and selection for more generalized resistance mechanisms. Here I discuss some further avenues of research.

Privatization or generalization?

Following on from the discussion on whether loss of beta-lactamase could represent a move to private mechanisms or general mechanisms of resistance, it would first be necessary to clarify if there is a move away from beta-lactamase towards efflux pumps during chronic infection. Such a shift is implied by the finding that there was

no increase in beta-lactamase in chronic infection (Chapter 2) while there is an increase in beta-lactam resistance (Chapter 3). A shift in mechanisms could be estimated by taking the same collection of clinical isolates used in Chapters 2 and 3 and measuring the MIC and IC_{50} in isolates with and without a beta-lactamase inhibitor. The proportion of resistance due to beta-lactamase (rather than efflux or permeability changes) could then be calculated to show how this proportion changes during infection, although a complicating factor is that the effect of resistance mechanisms is not necessarily additive – as reflected by the need for both beta-lactamase and low permeability for carbapenem resistance (Livermore, 1992). If a shift in mechanisms were seen, further work to clarify if the selection pressure is for generalization or privatization would need to be done. This could include similar 'proportion of resistance' studies in aminoglycosides and quinolones, and the experimental evolution suggested above.

Evolutionary trajectories

Relating my phenotypic findings in Chapters 2 and 3 to genetic changes will help in understanding evolutionary trajectories and attempt to identify the genes responsible. For example, when high and low producers coexist, it could be that low producers have lost beta-lactamase, or that low producers never produced high amounts of beta-lactamase. Loss of beta-lactamase would provide stronger support for social dynamics. Some preliminary work I did suggested this may be the case (Figure 1), and further work by Ghoul (in prep) confirms this.

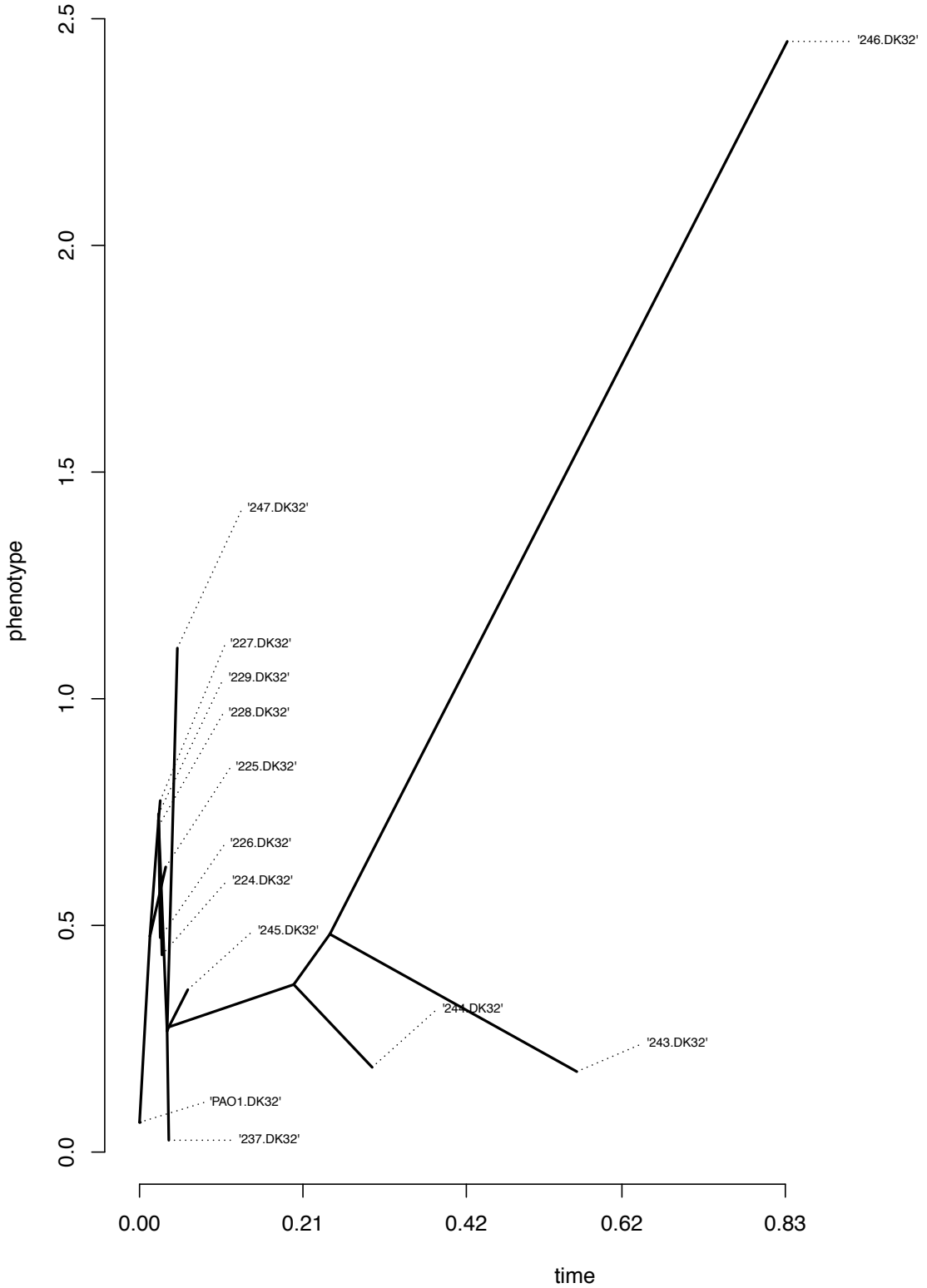


Figure 1. Example traitgram showing induced beta-lactamase production ('phenotype') superimposed on the phylogeny of isolates from patient P8203. 'Time' on the x-axis represents evolutionary distance rather than the timing of isolate sampling. Wherever there is a downwards gradient to the right, it suggests loss of beta-lactamase. This is preliminary work only for one patient to illustrate the type of phylogenetic analysis required across all patients. The traitgram was constructed using the 'phytools' package in R for ancestral state reconstruction.

Biofilms

Investigating further the effect of growth in biofilms on sociality will help relate my results to clinical infections (over 60% of which involve biofilm growth (Lewis, 2001)). All my work – both MIC measurements in Chapter 3 and competition experiments in Chapters 4 and 5 – was done in liquid media. The effect of this was discussed to some extent in Chapter 4. Work by Bagge *et al.* (2004) and Høiby *et al.* (2010) suggests that beta-lactamase being held in the biofilm may be important in the mechanism of resistance, potentially protecting cells deeper in the biofilm from exposure to antibiotics. It is unclear how this may affect cooperator-cheat dynamics: reducing diffusion of the public good may reduce cheating by allowing the benefit to accrue to relatives (Kümmerli *et al.*, 2009a), but it is equally possible that building up a dense layer of beta-lactamase in the biofilm could allow non-producers deep in the biofilm to benefit (similar to the effect of keeping density high, as in Ross-Gillespie *et al.* (2009) and Yurtsev *et al.* (2013)). Conducting competition experiments in a more structured environment will help clarify if the effect of viscosity or the effect of density is more important. Artificial sputum medium has been developed to mimic CF sputum in terms of nutrients (Palmer *et al.*, 2007) and sometimes also viscosity (Fung *et al.*, 2010). *P. aeruginosa* phenotype and gene expression profiles are similar in CF sputum and in the artificial media (Palmer *et al.*, 2007), and the media can be used for biofilm growth without the need for a flow cell (which does not mimic lung conditions) (Sriramulu *et al.*, 2005). Since Frost *et al.* (2018) showed that non-producers could gain a benefit on agar plates but not in liquid cultures, it seems likely that growth in artificial sputum could increase cheat survival compared with liquid cultures, supporting the suggestion that cooperator-cheat dynamics are likely to be

important in the CF lung. This is further supported by a recent paper by Amanatidou *et al.* (2019) showing that biofilms facilitate cheating, in contrast to liquid media.

Quorum sensing

Many cooperative microbial behaviours are controlled by quorum sensing (QS), but it is unclear if QS has a role in beta-lactamase regulation. A small body of work suggests some co-regulation between beta-lactamase and QS genes (Balasubramanian *et al.*, 2011; Zhao *et al.*, 2015). Since the benefit of a public good is affected by density (Ross-Gillespie *et al.*, 2009; Darch *et al.*, 2012), it would not be surprising if QS systems regulated beta-lactamase production at different densities. If there were QS-regulated density-dependent expression of beta-lactamase, it could explain an unusual result in Chapter 5, where the lowest density of producer cells produced the lowest amount of beta-lactamase per cell (Chapter 5, Figure 1A), even though the opposite may have been expected if the amount of antibiotic present per cell were the determining factor in production.

Degradation

Other factors involved in the regulation of beta-lactamase ought to be investigated. Chapter 5 suggests that beta-lactamase is degraded when it is not needed. It is therefore apparent that the amount of beta-lactamase present depends both on production and degradation. The balance between these mechanisms will affect both the cost of production and the period of benefit from the public good, thereby influencing the outcome of social interactions.

Role of relatedness

Returning to Hamilton's inclusive fitness theory as outlined in Chapter 1, fundamental to the whole concept of the sociality of antibiotic resistance mechanisms is the role of relatedness. High relatedness should favour cooperative resistance mechanisms while low relatedness should favour private resistance mechanisms. A selection experiment on resistance to beta-lactam antibiotics by beta-lactamase, compared with quinolone resistance by efflux pumps, while varying the relatedness between interacting individuals (as previously done by Griffin *et al.* (2004) for pyoverdin production) would confirm the role of relatedness. Such a study would underline the social concept of resistance, which could prove a valuable tool in tackling the problem of antibiotic resistance that was set out in Chapter 1.

Final word

What emerges out of this thesis is a picture where beta-lactamase clearly can act as public good in natural isolates (Chapter 4), that change in beta-lactamase in chronic infections is consistent with social dynamics and not with selection for higher resistance (Chapters 2 and 3), but that mechanisms exist which might limit the ability of cheats to invade, resulting in mixed populations (Chapter 5). The further work outlined above will help clarify some of my results and help build the concept of a sociality of resistance mechanisms. Clinically, this understanding may help design new treatment strategies by an awareness of when social dynamics may be relevant, and by allowing the design of treatment regimes in chronic infections which promote survival of sensitive cheats, thereby enabling future treatment. Fleming was correct to

raise his early concerns about resistance: understanding the evolutionary pressures involved will be vital to tackling this problem.

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