Cooperation, competition and antibiotic resistance in bacterial colonies

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Abstract

Bacteria commonly live in dense and genetically-diverse communities associated with surfaces. In these communities, competition for resources and space is intense, and yet we understand little of how this affects the spread of antibiotic resistant strains. Here we study interactions between antibiotic resistant and susceptible strains using *in vitro* competition experiments in the opportunistic pathogen *Pseudomonas aeruginosa* and *in silico* simulations. Selection for intracellular resistance to streptomycin is very strong in colonies, such that resistance is favoured at very low antibiotic doses. In contrast, selection for extracellular resistance to carbenicillin is weak in colonies, and high doses of antibiotic are required to select for resistance. Manipulating the density and spatial structure of colonies reveals that this difference is partly explained by the fact that the local degradation of carbenicillin by β-lactamase secreting cells protects neighbouring sensitive cells from carbenicillin. In addition, we discover a second unexpected effect: the inducible elongation of cells in response to carbenicillin allows sensitive cells to better compete for the rapidly growing colony edge. These combined effects mean that antibiotic treatment can select against antibiotic resistant strains, raising the possibility of treatment regimes that suppress sensitive strains while limiting the rise of antibiotic resistance. We argue that the detailed study of bacterial interactions will be fundamental to understanding and overcoming antibiotic resistance.

Introduction

Antibiotic resistance is a major concern that is threatening our ability to treat the most common of bacterial infections (Howard *et al.*, 2003). As well as attempting to find new drugs, we need to better understand the processes that promote, or inhibit, the spread of antibiotic resistant strains (Stewart and Costerton, 2001). There is a large literature on the evolution of antibiotic resistance that has primarily focused on understanding how bacteria respond to antibiotics in liquid culture (Hughes and Andersson, 2015; zur Wiesch *et al.*, 2011; MacLean *et al.*, 2010). While tractable, these conditions lie in stark contrast to the way that bacteria often grow. Both in the environment and in infections, bacteria commonly grow in dense and genetically-diverse communities, where competition for space and resources is intense (Nadell *et al.*, 2009; Stacy *et al.*, 2016; Hall-Stoodley *et al.*, 2004).

Growth of bacteria in these communities, often known as “biofilms” when on a surface (Vlamakis and Kolter, 2010), is well known to increase the phenotypic ability of bacteria to resist antibiotics (Stewart and Costerton, 2001; Ceri *et al.*, 1999). However, these observations are based on single strains and, to understand the rise and fall of resistance, we need to understand how genetically antibiotic-resistant strains compete with sensitive strains under these conditions. Growth in dense and diverse communities generally means that each cell can much more strongly influence the growth and survival of other cells (Costerton *et al.*, 1995; Parsek and Singh, 2003). This effect is known to promote the evolution of competitive phenotypes, including toxin secretion (Koch *et al.*, 2014), smothering polymers (Kim *et al.*, 2014) and adhesion (Schluter *et al.*, 2015). However, growth in dense communities can also lead to spatiogenetic structure and the evolution of

64 cooperative phenotypes, such as enzymes that digest complex molecules outside the
65 cell (Nadell *et al.*, 2009; Griffin *et al.*, 2004).

66 The key insight from these studies is that the details of how cells are
67 arranged and how they affect each other are critical to understanding natural
68 selection in dense colonies. How might colony growth impact selection for antibiotic
69 resistant strains? If the mechanism of resistance only benefits the cell that carries it,
70 intense competition in colonies is likely to lead to strong selection for resistance. By
71 contrast, if the resistance of one cell protects another, antibiotic resistance can
72 become a cooperative trait. In this case, resistant cells may protect sensitive
73 neighbours from antibiotics, weakening the strength of selection for resistance.
74 Recent work shows that it can be difficult to predict whether a particular mechanism
75 of resistance will have competitive or cooperative effects (Sorg *et al.*, 2016). Here we
76 use the term cooperation as it is widely used in social evolution theory, which is a
77 phenotype that provides a benefit to another individual and that evolved, at least in
78 part, because of this benefit (Rakoff-Nahoum *et al.*, 2016; West *et al.*, 2007, 2006).
79 Cooperation appears particularly likely for resistance to β-lactams (Yurtsev *et al.*,
80 2013; Dugatkin *et al.*, 2005), which often arises via the secretion of β-lactamase
81 enzymes that break down antibiotics both in the periplasm and extracellularly
82 (Iredell *et al.*, 2016; Ciofu *et al.*, 2000).

83 Here we develop a simple experimental system to understand how
84 competition and cooperation influence the spread of antibiotic resistant strains in
85 bacterial colonies. Specifically, we directly compete antibiotic resistant and sensitive
86 strains of the pathogen *P. aeruginosa* against each other in dense colonies, that are
87 exposed to variable concentrations of antibiotics, that are broken down by the

resistant strain, either intracellularly or extracellularly. The resistant strain carries a clinical, non-conjugative, plasmid (Rms149 (Haines *et al.*, 2005)) that confers resistance to an aminoglycoside (streptomycin), which is adenylated in the cytoplasm, and a β-lactam (carbenicillin), which is thought to be hydrolysed both in the periplasm and extracellularly (Iredell *et al.*, 2016; Ciofu *et al.*, 2000). As we will show, this allows us to directly compare a purely competitive resistance mechanism, which protects only the cells that carry it, with one that functions cooperatively. This reveals that how cells compete, whether they cooperate, and how they are arranged are all fundamental to the rise, or fall, of resistant strains in bacterial colonies. Crucially, this approach allows us to identity antibiotic dose regimes that inhibit the growth of bacterial colonies without selecting for resistance *in vitro*.

Materials and Methods

In our experiments we compete susceptible and resistant strains of *P. aeruginosa* on LB media, either in the form of broth culture or on agar plates. Resistant strains are isogenic with susceptible strains with the exception that they have been transformed with a clinical, multidrug resistant plasmid, Rms149 (Haines *et al.*, 2005). Streptomycin resistance is conferred by the aadA5 gene, which encodes an aminoglycoside adenylyltransferase. The blaA gene provide carbenicillin resistance, in the form of a constitutive class A β-lactamase. Cells divisions are calculated using the following equation:

$$cell\ divisions = \log_2\left(\frac{cells_{\ final}}{cells_{\ initial}}\right)$$

Relative susceptible fitness is calculated using the following equation (Lenski *et al.*, 1991):

$$susceptible\ fitness = \frac{\log_2\left(\frac{cells_{susceptible,final}}{cells_{susceptible,initial}}\right)}{\log_2\left(\frac{cells_{resistant,final}}{cells_{resistant,initial}}\right)}$$

Further details of the experimental methods and the models used in this paper are provided in the Supplementary Materials and Methods.

Results

Low antibiotic doses select for resistant strains in liquid cultures

In order to establish our system, we first competed plasmid-bearing and plasmid-free strains against each other in well-mixed broth (Figures 1 and S1). Plasmid carriage reduced fitness by 3% (s.e. = 0.38%, supplementary experimental procedures) in the absence of antibiotics, demonstrating a cost to resistance (t_{21} = 9.7, $P < 0.001$). As expected, supplementing culture medium with either an aminoglycoside (streptomycin) or β-lactam (carbenicillin) caused the fitness of the antibiotic sensitive strain to rapidly decrease (Figure 1E and F), because antibiotics suppress the growth rate of the sensitive strain (Figure 1A and B), but not the resistant strain (Figure 1C and D). One way of quantifying this effect is to estimate the minimal concentration of antibiotic required to select for the resistant strain (Minimum Selective Concentration, MSC) as a fraction of the minimal inhibitory concentration required to fully suppress the sensitive strain (MIC). We estimate that the MSC was very low for both streptomycin (1.6 μg/ml; 9.7% MIC) and carbenicillin 2.3 μg/ml (7% MIC; Figure 1). Moreover, we saw no evidence of resistant cells

protecting susceptible cells for either antibiotic, such that neither form of resistance is functioning as a cooperative mechanism under these conditions. These finding are in line with previous results (Vogwill and MacLean, 2015; Gullberg *et al.*, 2014; Bottery *et al.*, 2016; Yurtsev *et al.*, 2016, 2013), and demonstrate that the mechanism of resistance has little, if any, impact on selection for resistance in mixed broth cultures.

Colony growth has complex effects on selection for resistance

We next asked how life in a dense and genetically-diverse community impacts the fate of an antibiotic resistant strain. To do this, we repeated our competition experiments on agar plates (Figures 2, S2 and S3); known as the "colony biofilm" model (Vlamakis and Kolter, 2010). Our first observation was that, without antibiotics, the cost of plasmid carriage is significantly increased to 5.1% ($t_{17.8} = 2.7$, $P = 0.0157$). This increase is consistent with the finding that growth under biofilm conditions increases competition between strains. Cell division in mature colonies and biofilms only occurs at the very edge (Mitri *et al.*, 2015), and this generates strong competition and natural selection to reach the edge and gain sustained access to nutrients and space for proliferation (Nadell *et al.*, 2010; Mitri *et al.*, 2011; Van Dyken *et al.*, 2013; Nadell *et al.*, 2016; Kim *et al.*, 2014). This effect – competition for the growing edge - is expected to amplify the fitness benefit of rapid cell division because fast-growing strains have the added benefit of preferentially accessing the growing edge. While expected from a large body of previous work, this effect appears to have never been formalised. We, therefore, used an individual-

158 based model of colony growth in order to reconcile our empirical findings with this
159 intuitive effect (Figure S4).

160 Theoretical considerations suggest that the effect of colony growth on
161 selection for resistance should depend strongly on whether resistance has the
162 potential for cooperative effects. This is because growth in dense colonies should
163 increase the scope for cross protection, which protects susceptible cells and,
164 thereby, weakens selection for cooperating resistant strains (Nadell *et al.*, 2016).
165 Moreover, while not guaranteed (Sorg *et al.*, 2016; Nicoloff and Andersson, 2016),
166 mechanisms of resistance that occur intracellularly are expected to have weaker
167 effects on other cells than mechanisms that act outside the cell owing to the cell
168 membrane (Nadell *et al.*, 2016).

169 Consistent with these predictions, the MSC for streptomycin, where
170 resistance is conferred via an intracellular mechanism, was approximately an order
171 of magnitude (0.78 μg/ml; 2.4% MIC) less than the MSC for carbenicillin (20 μg/ml;
172 62% MIC) in bacterial colonies (Figure 2). Furthermore, we see no evidence for cross
173 protection of susceptible cells by resistant cells for streptomycin (Figure 2A). Rather
174 we see that susceptible cells do worse in direct competition with resistant cells than
175 growing on their own. This is again consistent with streptomycin acting as a non-
176 cooperative mechanism as we saw for the liquid culture experiments.

177 The carbenicillin data are qualitatively different between the liquid culture
178 and colony experiments. Theory predicts that selection for cooperative resistance
179 will be potentially weak in colonies because the secretion of detoxifying enzymes by
180 plasmid-carrying cells provides cross-protection to nearby sensitive cells (Nadell *et*
181 *al.*, 2016). This allows sensitive strains to benefit from reduced antibiotic exposure

without paying the fitness cost of resistance (Bottery *et al.*, 2016; Dugatkin *et al.*, 2005; Yurtsev *et al.*, 2013). Consistent with this explanation, the growth rate of the susceptible strain is dramatically increased by the presence of the resistant strain in colonies (Figure 2).

Further evidence of cross-protection comes from comparing the morphology of susceptible cells grown in the presence and absence of resistant cells. Carbenicillin is known to cause cell elongation and filamentation due to induction of the SOS response (Miller *et al.*, 2004; Blázquez *et al.*, 2006) and inhibition of cell wall synthesis (Kohanski *et al.*, 2010). Cell elongation occurs in susceptible cells both when they grow alone and when they are grown in combination with resistant cells. However, the presence of resistant cells markedly reduces the filamentation of susceptible cells, providing evidence of cross-protection at the single-cell level (Figure 3A, B). One possible effect of cell elongation is that colony forming units may underestimate the fitness of susceptible cells. This would occur if elongated cells immediately divide upon plating to make several smaller cells in a manner that non-elongated cells do not. However, this would only lead to an underestimation of the extent of cooperation and cross-protected in the experiments.

Genetic mixing weakens selection for carbenicillin resistance

The profound differences in selection for streptomycin and carbenicillin resistance observed in bacterial colonies suggest that local interactions between neighbouring cells are key to understanding selection for β-lactamase secretion in bacterial colonies. Theoretical work predicts that the spatial organisation of genotypes is fundamental for the evolution of cell-cell interactions in biofilms (Nadell *et al.*, 2010;

206 Oliveira *et al.*, 2014; Mitri *et al.*, 2011; Van Dyken *et al.*, 2013; Xavier and Foster,
207 2007; Nadell *et al.*, 2016). Specifically, it predicts that the formation of clonal
208 patches within colonies will limit cross-protection, which will favour resistant cells
209 over susceptible cells. We, therefore, tested the importance of spatial structure for
210 the competitiveness of our antibiotic resistant strain. We inoculated colonies with a
211 varying number of cells, at a concentration of carbenicillin that selected against
212 antibiotic resistance in our earlier experiments (Figure 2). Inoculating with fewer
213 cells leads to a greater degree of spatial structure in the form of larger clonal patches
214 (Figures 4 and S5) (van Gestel *et al.*, 2014). In line with our prediction, the fate of
215 the resistant cells was reversed under conditions of low inoculation density and they
216 were able to outcompete the susceptible cells (two-way ANOVA, $F_{1, 15} = 10.9$, $P = 0.005$),
217 0.005), (left hand data point, Figure 4E). By contrast, at higher inoculation densities,
218 we observe that susceptible cells can outcompete resistant cells, replicating our
219 earlier results. Moreover, the effect of density on competition is not seen until after
220 two days of growth, once the communities are fully confluent (Figure S5). This
221 suggests it is spatiogenetic structure within the colonies that is key to the observed
222 effects, not simply the fact susceptible cells are physically isolated for longer at a low
223 inoculation density (Figure 4).

224 If spatial structure within colonies is capable of causing resistant strains to
225 outcompete susceptible strains, then removing spatial structure should remove the
226 competitive advantage of resistant strains. In order to test this prediction, we mixed
227 one set of colonies daily using an inoculation loop (Kim *et al.*, 2014). When there was
228 no antibiotic present, this mixing had no effect on susceptible fitness (three-way
229 ANOVA, $F_{1, 31} = 0.978$, $P = 0.33$). However, as predicted, mixing the low density

230 treatment reversed the outcome of the competition such that the resistant strain
231 was no longer able to outcompete the susceptible strain (three-way ANOVA, $F_{1, 28}$ =
232 5.2, P = 0.03), (Figure 4E, G). Simply disordering a bacterial colony, therefore, can
233 remove the competitive advantage of an antibiotic resistant strain. In sum, our data
234 show that spatial structure in bacterial communities can be critical for the spread of
235 antibiotic resistance.
236
237 *An unexpected benefit to antibiotic susceptibility*
238 Our experiments demonstrate that density and spatial structure can be
239 fundamentally important for the rise of resistance to β-lactam antibiotics. Moreover,
240 our data generally support a simple model where resistant cells experience a growth
241 rate cost from carrying a plasmid, and will cross-protect susceptible cells when they
242 are nearby (high density and low spatiogenetic structure). However, there are a
243 number of intriguing patterns in our data, which all suggest that this simple view is
244 insufficient.
245 The first observation is that low doses of carbenicillin can actually benefit the
246 sensitive strain. Specifically, the fitness advantage of the susceptible strain doubles
247 from 5.5 % to 11 % upon the addition of low doses of carbenicillin (t_{16} = -2.9, P =
248 0.01). One explanation for this strong effect could be that carbenicillin promotes the
249 growth of susceptible cells. However, we see no evidence for growth promotion in
250 pure cultures (Figures 1B, 2B) and, more importantly, the increase in fitness in mixed
251 culture is due to a decrease in the growth rate of resistant cells, not an increase in
252 the susceptible cells (red line in Figure 2B and D). This effect on resistant cells is also
253 not a direct effect of the antibiotic, as this depression of growth rate is not seen in

254 pure resistant cultures (grey line in Figure 2D). Instead, the antibiotic is somehow
255 shifting the balance of competition in mixed culture toward the susceptible strain
256 and allowing it to suppress the growth of the resistant strain.

257 Competition within colonies is intense and a large body of theoretical and
258 empirical work has shown that the effects of natural selection occur primarily at the
259 growing edges (Mitri *et al.*, 2016; Korolev *et al.*, 2011; Kim *et al.*, 2014; van
260 Ditmarsch *et al.*, 2013). In particular, this shows that it is the lateral growing edge -
261 forming the circumference of a colony - that is most critical (upwards growth is much
262 more limited than lateral growth). This led us to hypothesise that susceptible cells
263 were somehow able to obtain better access to the lateral growing edge, allowing
264 them to grow outwards and inhibit the growth of resistant cells. All of our frequency
265 data so far has been from measurements of the whole colony (Figures 1, 2, 4).
266 Focussing in on the growing edges of colonies does indeed reveal that the edge
267 becomes increasingly dominated by susceptible cells over time (Figure 3B, middle
268 row). However, this might happen simply because susceptible cells divide faster
269 (above, Figure S4) and does not demonstrate an additional mechanism that allows
270 them to dominate at the edge. To assess the importance of positioning, we need to
271 experimentally test whether susceptible cells do better with antibiotic because they
272 can influence spatial structure.

273 This test was inadvertently performed in the experiment in Figure 4, which
274 examines whether increases or decreases in genetic mixing affect cross-protection.
275 As discussed above, mixing up the colonies enabled cross-protection and benefited
276 susceptible cells in the low density treatment. However, there was a second and
277 unexpected effect of mixing. In high density inoculations, where cells start off well-

mixed, physically mixing colonies *reduces* susceptible fitness (Figure 4E right hand points). Moreover, this effect is caused by increasing the resistant cell abundance (Figure 4C). Disrupting spatial structure in colonies where susceptible cells are already winning, therefore, reduces susceptible fitness. This is exactly what is predicted from a model where antibiotic treatment somehow helps susceptible cells to obtain a better position in the colony. This model also predicts that the effect of mixing is not seen in the absence of antibiotics, which is also the case (Figure 4F).

Cell shape and the increased fitness of susceptible cells

How does antibiotic exposure allow susceptible cells to find more favourable positions in the colony? Inspection of the colony edge gave a clear candidate: susceptible cells in mixed colonies change shape upon the addition of antibiotics (Figure 3). If shape is important, then a key prediction is that changes in cell shape should proceed any increase in the frequency of susceptible cells. To test this prediction we tracked the aspect ratio of cells during competitions and compared this with the frequency of susceptible and resistant cells at each time point. Consistent with the shape-drives-fitness model, the elongation of susceptible cells occurs prior to their competitive gains against resistant cells (Figure 5).

Time-lapse imaging of the colony edge highlighted a candidate mechanism for the advantage of elongated cells. We see sets of elongated cells that align orthogonally to the edge of the colony and push themselves outwards. This enables cells to grow ahead of the resistant strain and cut it off from the growing edge (Figure 6A). The clearest support for the effect of cell shape would come from manipulating the shape of susceptible cells to ask how shape affects the ability to

spatially organise and compete. We do not know of any route to prevent susceptible cells from elongating under antibiotic treatment without strong pleiotropic effects on growth rate and other phenotypes. However, a recent study that combined individual-based modelling with the study of cell shape mutants in *Escherichia coli* also suggests that cell shape can be important for positioning in bacterial biofilms (Smith *et al.*, 2016). Moreover, filamentation of *Bacillus subtilis* can increase migration away from the colony (van Gestel *et al.*, 2015).

We, therefore, extended the simulation models of Smith *et al.* (2016) in order to study the effect of cell shape on positioning at the edge of a colony (Figure 6 and S6). We focused on the growing edge because this is the critical area of competition in an expanding colony and the region where we see strong enrichment for elongated susceptible cells. As we observe in the time-lapse imaging (Figure 6A and Movie S1), the individual based model shows how long cells can align themselves orthogonally to the colony edge and push outwards occluding shorter cells from the edge. This gives them a competitive advantage as they can spread outwards, allowing them to align with the direction of growth ahead of resistant strains and hence to dominate the growing edge (Figures 6B and S6).

To summarise, the combination of competition data, imaging and our individual based simulations all support a model where carbenicillin drives cell elongation and this provides an unexpected, but strong, fitness advantage to susceptible cells during biofilm growth. In combination with the effects of cross-protection, this means that sub-MIC doses of carbenicillin can inhibit bacterial growth while selecting against an antibiotic resistant strain.

Discussion

Understanding the spread of antibiotic resistant strains is a major goal for both science and society. In well-mixed broth cultures, we find that selection for resistance is strong, irrespective of the mechanism of resistance. In contrast, we find that selection for resistance in colonies depends critically on the mechanism of resistance, the spatiogenetic structure of colonies, and the regulatory responses of sensitive cells to antibiotic exposure. Although bacterial colonies are a simplification of natural communities, we find they are sufficient to show that structured communities have both complex and surprising effects on selection for antibiotic resistance.

Consistent with theory ((Nadell *et al.*, 2010; Mitri *et al.*, 2011; Van Dyken *et al.*, 2013; Nadell *et al.*, 2016; Kim *et al.*, 2014), Figure S4), we see that growth in colonies greatly increases the strength of competition between strains, because rapidly growing bacteria preferentially gain access to the nutrient-rich edge of the colony. All else being equal, this should increase the strength of selection for resistance in colonies. It is difficult to directly compare the results of broth and colony competition experiments with each other, because there are many differences between these two modes of growth other than spatial structure. However, it is clear from our colony competition experiments that selection for carbenicillin resistance was notably weak in colonies. Indeed, of the four conditions we consider (two growth conditions, two antibiotics), we only see evidence for cooperative resistance for colony growth on carbenicillin. In the other three conditions, antibiotic resistance serves only to protect the cells that carry it.

Our experiments, which included disrupting spatiogenetic structure, are consistent with extracellular β-lactamase production protecting the sensitive strain under colony growth. Indeed, this protection can be so strong that the susceptible strain outcompetes and outnumbers the resistant strain in the colony (Figures 3 and 4), as predicted by evolutionary theory for costly cooperative traits (West *et al.*, 2006; Mitri and Foster, 2013). Furthermore, by focussing on the growing edge, where natural selection is at its most powerful (Hallatschek and Nelson, 2010; Nadell *et al.*, 2010), we discovered a second unexpected effect. Our data and theory suggest that exposure to low levels of carbenicillin leads to cell elongation, which allows susceptible bacteria to preferentially access the growing edge of treated colonies (Figures 5 and 6).

Importantly, these two factors are also associated with greatly weakened natural selection for antibiotic resistance. Specifically, the dose of carbenicillin required to generate selection for resistance is over an order of magnitude higher than for streptomycin that is chemically modified inside the cell (20 µg/ml; 62% MIC versus 0.78 µg/ml; 2.4% MIC); an effect not seen in the liquid experiments (Table S2). The broader consequence of this is that natural selection for a multi-drug resistant strain may be reduced by treatment with an antibiotic that targets a cooperative resistance mechanism. More generally, it suggests that drugs with strong extracellular effects, like β-lactamases, may be particularly amenable to treatment strategies intended to slow the rate of resistance evolution. However, this requires the identification of doses that are high enough to limit bacterial growth but low enough to allow susceptible strains to remain competitive relative to resistant

strains. We have identified these conditions in our experiments, but additional work is needed to see if they can be harnessed as part of a treatment strategy.

In conclusion, our work cautions against the focus on liquid culture assays for understanding antibiotic resistance evolution. Few pathogenic bacteria live like this and we have shown that competitive outcomes in dense communities can be very different. Encouragingly, this shift in perspective may help to identify exposure regimes that suppress the growth of sensitive strains without favouring resistant strains (Read *et al.*, 2011; Kouyos *et al.*, 2014; Drusano, 2004).

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519 protection mutualism.

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521

522 **Figure Legends**

523 **Figure 1. Natural selection for antibiotic resistance in liquid culture.** Susceptible and
524 resistant strains were competed in liquid culture supplemented with carbenicillin
525 (extracellular resistance) or streptomycin (intracellular resistance). Both
526 streptomycin (A) and carbenicillin (B) suppress the growth rate of the susceptible
527 strain (red line shows the susceptible growth in the presence of the resistant strain,
528 black line shows the susceptible growth alone). In contrast, the growth rate of the
529 resistant strain is not altered by antibiotic exposure (C, D, red line shows the
530 resistant growth in the presence of the susceptible strain, grey line shows the
531 resistant growth alone). As a result, all antibiotic concentrations select for the
532 resistant strain (E, F). Please note that we use different axis ranges for the
533 susceptible (A and B) and resistant (C and D) cell division data, because the data
534 have very different ranges. The mean and standard error of two experiments, each
535 with n = 6 for co-cultures and n = 3 for monocultures, are shown. See also Figure S1.

536

537 **Figure 2**. **Natural selection for resistance in bacterial colonies.** Susceptible and
538 resistant strains were competed over seven days on solid agar with either
539 streptomycin (intracellular resistance) or carbenicillin (extracellular resistance). On

streptomycin, resistant strains outcompete susceptible strains at all concentrations of antibiotic (A, red line denotes susceptible growth in the presence of the resistant strain, black line denotes susceptible growth alone), where skulls denote that no colony forming units of the susceptible strain were observed. However, the resistant strain increased the growth rate of the sensitive strain in the presence of carbenicillin, demonstrating cross-protection (B). Resistant growth increases in the presence of streptomycin when the susceptible strain is present (C, red line denotes resistant growth in the presence of the susceptible strain, grey line denotes resistant growth alone) but is suppressed by the presence of the susceptible strain on carbenicillin (D, red line denotes resistant growth in the presence of the susceptible strain, grey line denotes resistant growth alone). This relative increase in resistant growth, on streptomycin, with the susceptible strain, is a competition effect. It is due to the resistant strain outcompeting the susceptible strain for resources more effectively than it does against another resistant strain. Streptomycin always selects for resistance (E) whereas low doses of carbenicillin select for the sensitive strain (F). Representative images of the colonies in which yellow resistant and red susceptible strains are competed over 6 days, also demonstrate this result (G, H) while control colonies, in which both colours are susceptible, show the effect of these concentrations of antibiotic on non-resistant cells (I, J). The mean and standard error of two experiments, each with n = 6 for co-cultures and n = 3 for monocultures, are shown. See also Figures S2 and S3.

Figure 3. Cross-protection of susceptible cells by resistant cells is supported by the degree of cell filamentation. Confocal images of the colony edge after two days

show carbenicillin causes susceptible cells to filament, when grown alone on increasing concentrations of antibiotic (A, moving horizontally across the first row, both red and yellow cells are susceptible). The presence of the resistant strain causes susceptible cells to filament less (B, moving across second row, susceptible cells in yellow, resistant cells in red) than when they are grown alone at the same concentration of antibiotic (A, top row). At intermediate concentrations of carbenicillin there is enrichment at the edge for the susceptible strain when in competition with the resistant strain (B, centre panels, susceptible strain in yellow, resistant strain in red). When the resistant cells are grown alone on the antibiotic (C, both colours are resistant) they do not filament at these concentrations of carbenicillin. The MIC (minimum inhibitory concentration) is the lowest concentration of antibiotic that completely inhibits growth of susceptible cells (here measured in a colony) (Table S1).

Figure 4. Spatiogenetic structure and the evolution of antibiotic resistance. Susceptible (red) and resistant (yellow) strains were co-inoculated onto plates containing either no antibiotic (black line) or 12 μg/ml carbenicillin (red line) (A - D). Final cell counts are shown here, as opposed to cell divisions (Figures 1 and 2), because the use of different densities itself changes the number of cell divisions that occur (low cell density treatments experienced reduced nutrient competition and increased cell division). The final cell count removes this effect and is an easier metric to interpret. The growth data is shown in the Supplementary Information (Figure S5). From this data the relative susceptible fitness was calculated (E, F). The extent of separation between susceptible and resistant strains in a colony can be

588 varied with the number of inoculating cells, as can be seen in images taken after 6
589 days (G, H). Mixing (dotted line, bottom row of G and H) decreases the fitness of the
590 resistant strain in colonies with a low initial density (colonies in LHS row in Figure 4G
591 go redder upon mixing (bottom row)) and increases fitness in colonies with a high
592 initial density (colonies in middle and RHS row go yellower upon mixing, bottom
593 row). The mean and standard error of two experiments, each with n = 3, are shown.
594 See also Figure S5.
595
596 **Figure 5. Susceptible cells increase in aspect ratio before outcompeting resistant**
597 **cells in carbenicillin treated colonies.** Yellow susceptible and red resistant cells were
598 competed on either 12 μg/ml carbenicillin (red) or no antibiotic (black). The edge of
599 these colonies was then imaged during growth to measure cell length and relative
600 cell numbers (A, B). The bottom images are micrographs of the colony edge under
601 the different conditions showing the shifts in aspect ratio in susceptible cells, in the
602 presence of resistant cells, during carbenicillin treatment (C). The mean and
603 standard error of two experiments, each with n = 3, are shown.
604
605 **Figure 6**. **Cell shape is important for competition at the edge of colonies.**
606 Micrographs show longer susceptible cells (yellow) extending from the colony edge
607 and occluding shorter resistant cells (red). This is shown in time-lapse images of
608 growth at the edge of a representative colony after 10 hours, growing on 12 μg/ml
609 carbenicillin (A). These images differ in quality from earlier images (e.g. Figure 5)
610 because we had to use a lower fluorescence intensity to avoid bleaching during
611 time-lapse imaging. An individual-based model shows the same effect (B). In the

612 model, we can alter cell shape alone and follow its effects on cell sorting. Starting
613 from a random configuration of Susceptible (S) and Resistant (R) cells in a 1:1
614 mixture, at t1, we observe that long cells extend from the colony edge (t2, black
615 arrows). This improves their resource access and, as in our experiments, allows them
616 to occlude the shorter cells from the edge. This, in turn stimulates further growth
617 (t3, black arrows). By t4, S cells have become enriched at the colony edge through
618 positive feedback. Histograms of cell x-coordinates track the growth of the colony
619 edge and quantify the shape-driven enrichment process in the individual-based
620 model, taken at the same 4 time points as in B (C). p(x) shows the probability of
621 finding a given strain at a given point in the colony. Data merged from a sample of 20
622 simulations; times t1-t4 correspond to 12, 24, 36 and 56h of growth respectively.
623 The colour brightness intensity of each cell corresponds to its growth rate, with
624 brighter cells indicating faster growth. See also Figure S6.