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1 **Selective conditions for a multidrug resistance plasmid depend on the**
2 **sociality of antibiotic resistance**

3

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8

9 Running heading: Social selection of a MDR plasmid

10

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12

13

14 **ABSTRACT**

15 Multidrug resistance (MDR) plasmids frequently encode antibiotic resistance
16 genes conferring qualitatively different mechanisms of resistance. We show that
17 the antibiotic concentrations selecting for the RK2 plasmid in *Escherichia coli*
18 depend upon the sociality of the drug resistance: Selection for a selfish drug
19 resistance (efflux-pump) occurred at very low drug concentrations, just 1.3% of
20 the sensitive's MIC, whereas selection for a cooperative drug resistance
21 (modifying-enzyme) occurred at drug concentrations exceeding the MIC of the
22 plasmid-free strain.

23

24 TEXT

25 Antibiotics are critical to modern medicine, but their widespread use and misuse
26 has lead to the evolution of resistant strains to most commonly used antibiotics
27 (1, 2). Antibiotic resistance has become a major threat to global health, with
28 multi-drug resistant (MDR) bacteria observed globally (3). Environmental
29 antibiotic resistance genes (ARGs) are a major source of clinical resistance (4).
30 ARGs can be selected for at very low concentrations of antibiotic, far below the
31 minimum inhibitory concentration (MIC) of sensitive cells (5, 6), with antibiotic
32 contamination at sub-MIC concentrations being proposed as the main driving
33 force behind environmental selection for resistance (7–9). However, ARGs can
34 encode qualitatively different forms of resistance ranging from selfish to
35 cooperative. Selfish drug resistances only confer a benefit to the individual cell
36 harbouring it, for example efflux pumps, reduced membrane permeability and
37 alteration of antibiotic targets (10, 11). By contrast cooperative antibiotic
38 resistances benefit both the resistant cell and surrounding cells whether they are
39 resistant or not. For example, modifying enzymes such as β -lactamase inactivate
40 the antibiotic through hydrolysis, decreasing its environmental concentration.
41 Localisation of the β -lactamase enzyme in the periplasmic space may enhance
42 the share of the benefit for the resistant cell, but nevertheless, the decrease in
43 the overall environmental concentration of antibiotic will benefit both resistant and
44 sensitive cells (12). We hypothesised that the sociality of drug resistance could
45 alter the selective conditions for the spread of ARGs (13, 14). Specifically,
46 because the benefits of selfish drug resistance are directed solely to the resistant

47 cell, whereas the benefits of cooperative drug resistance are shared between
48 resistant and sensitive cells, we predict that selfish drug resistance should be
49 selected at lower relative drug concentrations (i.e. % of the sensitive MIC) than
50 cooperative resistance.

51

52 Multiple ARGs are frequently clustered together onto conjugative plasmids
53 including combinations of selfish and cooperative drug resistances (15). How
54 combinatorial antibiotic usage selects for MDR plasmids is not clear, especially
55 for combinations of antibiotics requiring qualitatively different modes of drug
56 resistance, such as selfish or cooperative drug resistances. Here we tested how
57 the sociality of drug resistance, and single versus combined antibiotic treatment,
58 altered the selective conditions for the MDR plasmid RK2 (16) in *Escherichia coli*
59 MG1655. RK2 encodes both cooperative ampicillin resistance, mediated by a β -
60 lactamase, and selfish tetracycline resistance, mediated by an efflux pump. We
61 report that the selfish drug resistance is selected for at far lower relative antibiotic
62 concentrations than the cooperative drug resistance, and that combined antibiotic
63 selection is additive, showing no interaction.

64

65 Conventionally, ARGs are thought to be positively selected at antibiotic
66 concentrations exceeding the MIC of sensitive cells in monoculture (17) (i.e. the
67 conventional selective window, Fig 1). To determine whether the sociality of
68 resistance affected the selection window for the RK2 MDR plasmid, we estimated
69 the relative fitness of plasmid bearing versus isogenic plasmid free cells by direct

70 competition following standard methodology (see supplementary material). In the
71 absence of antibiotics the plasmid imposed a significant cost of carriage,
72 decreasing the fitness of *E. coli* by 19% (Fig. 1A/B, t test, $p < 0.001$, $t = -9.8674$,
73 $df = 23$). An intrinsic cost is often associated with plasmid carriage when
74 accessory traits are not under positive selection due to cellular disruption and
75 increase transcriptional load (18). Cooperative ampicillin resistance was
76 positively selected at ampicillin concentrations exceeding the MIC of sensitive *E.*
77 *coli* (Fig. 2A). Importantly, sensitive cells were able to maintain positive growth in
78 mixed cultures at ampicillin concentrations that completely inhibited their growth
79 in monoculture ($>8\mu\text{g/ml}$; cf. Fig. 1A & Fig. S4), justifying the assignment of
80 ampicillin resistance as cooperative. Thus cooperative resistance permits
81 persistence of a sensitive subpopulation beyond the sensitive MIC due to the
82 inactivation of the antibiotic, potentially allowing reinvasion by sensitive cells
83 once the antibiotic concentration is sufficiently reduced by the action of resistant
84 cells.

85

86 In contrast, selfish tetracycline resistance was positively selected at tetracycline
87 concentrations of just 1.3% of the MIC of sensitive *E. coli* (Fig. 2B). Indeed, at
88 concentrations of tetracycline above 10% of the MIC of sensitive *E. coli*, the
89 resistant plasmid bearers competitively excluded the plasmid-free bacteria, with
90 no plasmid-free cells observable (Fig. S1). This is despite the fact that plasmid-
91 free *E. coli* could survive at these tetracycline concentrations when grown alone
92 (Fig. 1B). Our data suggest that selfish tetracycline resistance is positively

93 selected in the sub-MIC selective window at very low tetracycline concentrations,
94 similar to those observed in the natural environment (19).

95

96 When ampicillin and tetracycline were applied in combination there was no
97 significant interaction ($F_{1,68} = 0.2395$, $p = 0.6261$) indicating that when these two
98 antibiotics were used in combination their selective effects were independent and
99 additive (Fig. 2C). This means that very low concentrations of tetracycline were
100 sufficient to completely mask the population-level effects of cooperative ampicillin
101 resistance. With increasing tetracycline concentrations, the ampicillin
102 concentration positively selecting for the MDR plasmid shifted to lower and lower
103 sub MIC levels, reducing the window of selective conditions where sensitive cells
104 could persist (Fig. 2D).

105

106 Residues of multiple antibiotics are commonly found contaminating the same
107 environments at low concentrations (19, 20). These combinations, and
108 particularly the presence in the environment of antibiotics like tetracycline
109 targeted by selfish efflux-mediated resistance, will select for the spread of MDR
110 plasmids and competitive exclusion of sensitive cells. This is despite being
111 present at concentrations far below the level required to positively select
112 resistance individually. This adds further evidence that ARGs, whether
113 chromosomal or plasmid encoded, can be positively selected at antibiotic
114 concentrations far below the MIC of sensitive strains (5, 6, 9).

115

116 Our study has a number of possible limitations: First, it is possible that other
117 factors, in addition to sociality, may have contributed to differences in the fitness
118 reaction norms of the antibiotics, including the contrasting effects of sub-MIC
119 concentrations on monoculture densities and the fact that ampicillin is
120 bacteriocidal whereas tetracycline is bacteriostatic. Second, we use exemplars of
121 cooperative and selfish resistance but more research will be required to test the
122 importance of sociality on the selective conditions for other resistance
123 mechanisms.

124

125 Here we show that the extent to which an ARG is positively selected at sub-MIC
126 antibiotic concentrations depends upon the sociality of the mechanism of drug
127 resistance. Cooperative ampicillin resistance is positively selected at ampicillin
128 concentrations exceeding the MIC, whereas selfish tetracycline resistance is
129 positively selected at 100-fold lower relative drug concentrations. This striking
130 difference in the selective window for ARGs co-located on the same MDR
131 plasmid probably arises because of the population-level effects of the ARGs:
132 Cooperative ampicillin resistance allowed sensitive bacteria to survive past their
133 MIC by reducing the ampicillin concentration and sharing the benefits of
134 resistance, whereas, selfish tetracycline resistance drove complete competitive
135 exclusion of sensitive cells at >10% MIC due to the exclusively individual benefits
136 of efflux-mediated resistance. Combining the two antibiotics – at concentrations
137 that would not normally select for resistance individually – selects for both
138 resistances and spread of the MDR plasmid. Taken together these findings

139 suggest that selfish efflux-mediated drug resistances are likely to be especially
140 important for the selective maintenance and spread of MDR plasmids.

141

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149

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154

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- 219
- 220 FIG 1
- 221 Cell density (OD₆₀₀) of sensitive plasmid free bacteria (green line) and resistant
222 plasmid containing bacteria (blue line) as a function of **A** ampicillin concentration,
223 **B** tetracycline concentration after 24 hours growth in monoculture. Error bars

224 show SEM (n=6). Area shaded in green shows the sub-MIC selective window,
225 and the area shaded in blue shows the selective window conventionally thought
226 to select for resistance.

227

228 FIG 2

229 Fitness reaction norms as a function of antibiotic concentration during
230 competition experiments between *E. coli* harboring the RK2 plasmid and isogenic
231 plasmid free sensitive strains. Competitions in the presence of **A** ampicillin, **B**
232 tetracycline, red lines show fitted regression. **C/D** Fitness reaction norms of
233 combination treatments with both ampicillin and tetracycline during competition
234 experiments between RK2 harboring and plasmid free strains. There is no
235 significant interaction of antibiotic treatments upon the relative fitness ($F_{1,68} =$
236 0.2395, $p = 0.6261$) indicating treatments were non-interacting and additive. Error
237 bars show SEM (n=6), Antibiotic concentrations shown as percentages of
238 sensitive MIC.

239

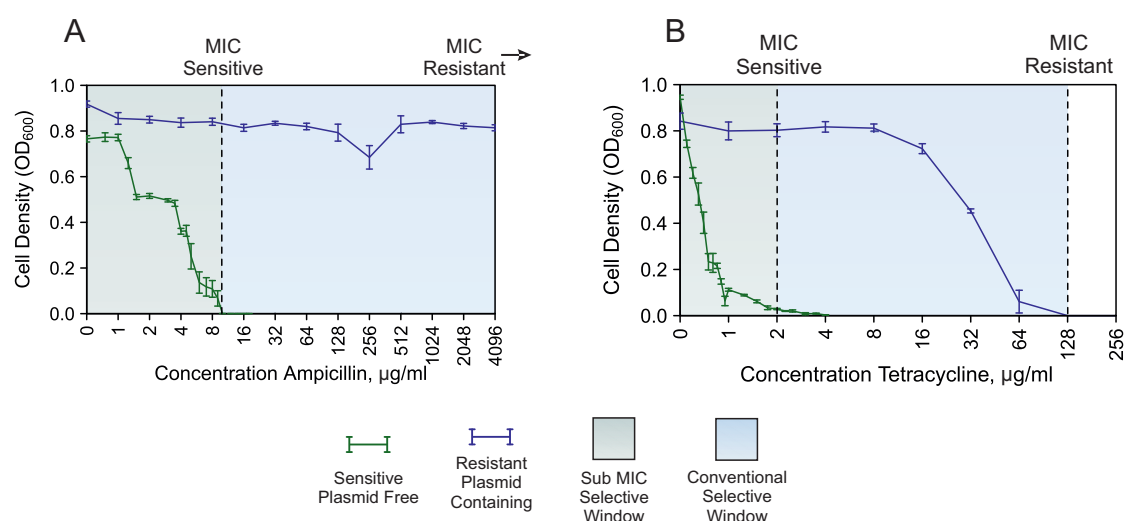


FIG 1 Cell density (OD₆₀₀) of sensitive plasmid free bacteria (green line) and resistant plasmid containing bacteria (blue line) as a function of A ampicillin concentration, B tetracycline concentration after 24 hours growth in monoculture. Error bars show SEM (n=6). Area shaded in green shows the sub-MIC selective window, and the area shaded in blue shows the selective window conventionally thought to select for resistance.

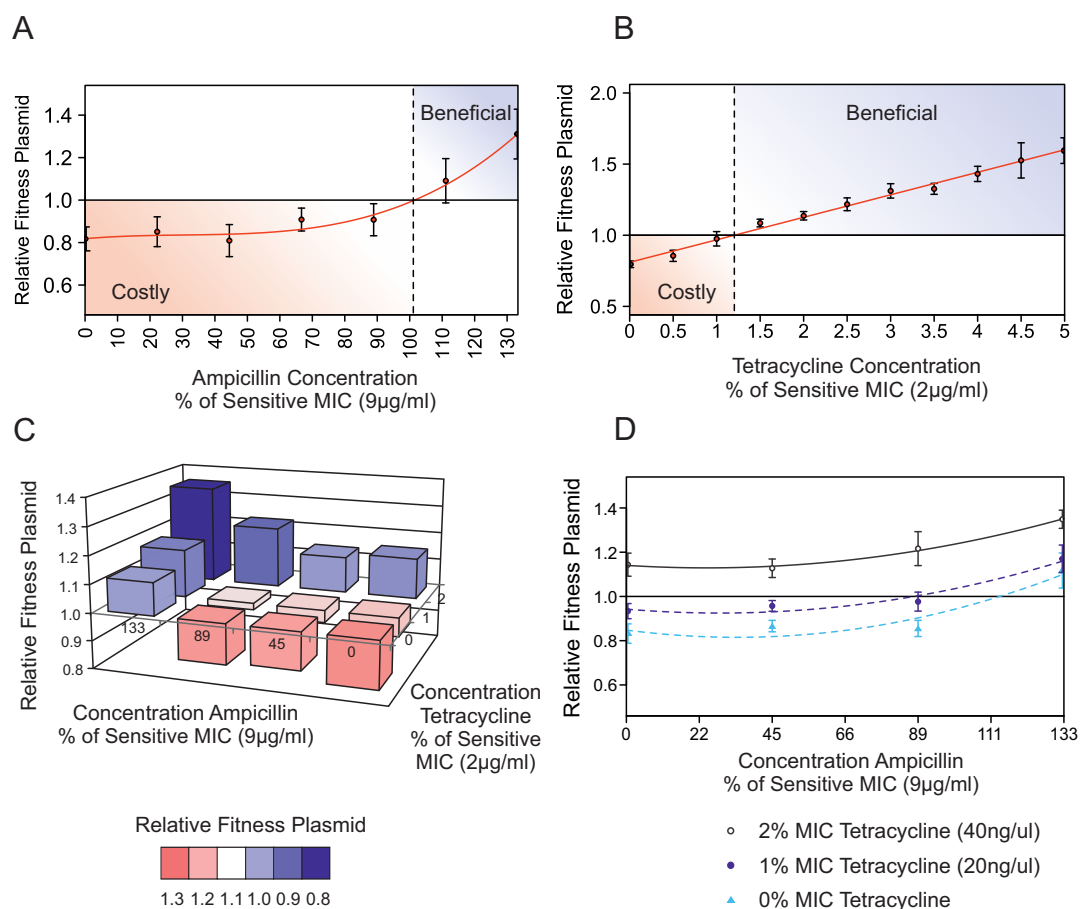


FIG2 Fitness reaction norms as a function of antibiotic concentration during competition experiments between *E. coli* harboring the RK2 plasmid and isogenic plasmid free sensitive strains. Competitions in the presence of A ampicillin, B tetracycline, red lines show fitted regression. C/D Fitness reaction norms of combination treatments with both ampicillin and tetracycline during competition experiments between RK2 harboring and plasmid free strains. There is no significant interaction of antibiotic treatments upon the relative fitness ($F_{1,68} = 0.2395$, $p = 0.6261$) indicating treatments were non-interacting and additive. Error bars show SEM ($n=6$), Antibiotic concentrations shown as percentages of sensitive MIC.