# Selective conditions for a multidrug resistance plasmid depend on the sociality of antibiotic resistance

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Running heading: Social selection of a MDR plasmid

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ABSTRACT

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

TEXT

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



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

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

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

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

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

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

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

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

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

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

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

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REFERENCES

1. Laxminarayan R, Duse A, Wattal C, Zaidi AKM, Wertheim HFL, Sumpradit N, Vlieghe E, Hara GL, Gould IM, Goossens H, Greko C, So AD, Bigdeli M, Tomson G, Woodhouse W, Ombaka E, Peralta AQ, Qamar FN, Mir F, Kariuki S, Bhutta ZA, Coates A, Bergstrom R, Wright GD, Brown ED, Cars O. 2013. Antibiotic resistance—the need for global solutions. Lancet Infect Dis 13:1057–1098.

2. Bush K, Courvalin P, Dantas G, Davies J, Eisenstein B, Huovinen P, Jacoby GA, Kishony R, Kreiswirth BN, Kutter E, Lerner SA, Levy S, Lewis K, Lomovskaya O, Miller JH, Mobashery S, Piddock LJV, Projan S, Thomas CM, Tomasz A, Tulkens PM, Walsh TR, Watson JD, Witkowski J, Witte W, Wright G, Yeh P, Zgurskaya HI. 2011. Tackling antibiotic resistance. Nat Rev Microbiol 9:894–896.

3. World Health Organization. 2014. Antimicrobial resistance: global report on surveillance. World Health Organization.

4. Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F, Bürgmann H, Sørum H, Norström M, Pons M-N, Kreuzinger N, Huovinen P, Stefani S, Schwartz T, Kisand V, Baquero F, Martinez JL. 2015. Tackling antibiotic resistance: the environmental framework. Nat Rev Microbiol 13:310–317.

5. Gullberg E, Albrecht LM, Karlsson C, Sandegren L, Andersson DI. 2014. Selection of a Multidrug Resistance Plasmid by Sublethal Levels of Antibiotics and Heavy Metals. mBio 5:e01918–14.

6. Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L, Hughes D, Andersson DI. 2011. Selection of Resistant Bacteria at Very Low Antibiotic Concentrations. PLoS Pathog 7:e1002158.

7. Sandegren L. 2014. Selection of antibiotic resistance at very low antibiotic concentrations. Ups J Med Sci 119:103–107.

8. Andersson DI, Hughes D. 2014. Microbiological effects of sublethal levels of antibiotics. Nat Rev Microbiol 12:465–478.

9. Liu A, Fong A, Becket E, Yuan J, Tamae C, Medrano L, Maiz M, Wahba C, Lee C, Lee K, Tran KP, Yang H, Hoffman RM, Salih A, Miller JH. 2011. Selective Advantage of Resistant Strains at Trace Levels of Antibiotics: a Simple and Ultrasensitive Color Test for Detection of Antibiotics and Genotoxic Agents. Antimicrob Agents Chemother 55:1204–1210.

10. Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJV. 2015. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol 13:42–51.

11. Li X-Z, Plésiat P, Nikaido H. 2015. The Challenge of Efflux-Mediated Antibiotic Resistance in Gram-Negative Bacteria. Clin Microbiol Rev 28:337–418.

12. Vega NM, Gore J. 2014. Collective antibiotic resistance: mechanisms and implications. Curr Opin Microbiol 21:28–34.

13. Yurtsev EA, Chao HX, Datta MS, Artemova T, Gore J. 2013. Bacterial cheating drives the population dynamics of cooperative antibiotic resistance plasmids. Mol Syst Biol 9:683.

14. Conlin PL, Chandler JR, Kerr B. 2014. Games of life and death: antibiotic resistance and production through the lens of evolutionary game theory. Curr Opin Microbiol 21:35–44.

15. Carattoli A. 2013. Plasmids and the spread of resistance. Int J Med Microbiol 303:298–304.

16. Pansegrau W, Lanka E, Barth PT, Figurski DH, Guiney DG, Haas D, Helinski DR, Schwab H, Stanisich VA, Thomas CM. 1994. Complete Nucleotide Sequence of Birmingham IncPα Plasmids: Compilation and Comparative Analysis. J Mol Biol 239:623–663.

17. Hughes D, Andersson DI. 2012. Selection of resistance at lethal and non-lethal antibiotic concentrations. Curr Opin Microbiol 15:555–560.

18. Baltrus DA. 2013. Exploring the costs of horizontal gene transfer. Trends Ecol Evol 28:489–495.

19. Zhang T, Li B. 2011. Occurrence, Transformation, and Fate of Antibiotics in Municipal Wastewater Treatment Plants. Crit Rev Environ Sci Technol 41:951–998.

20. Batt AL, Bruce IB, Aga DS. 2006. Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. Environ Pollut 142:295–302.

FIG 1

Cell density ($OD_{600}$) 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.

FIG 2

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.

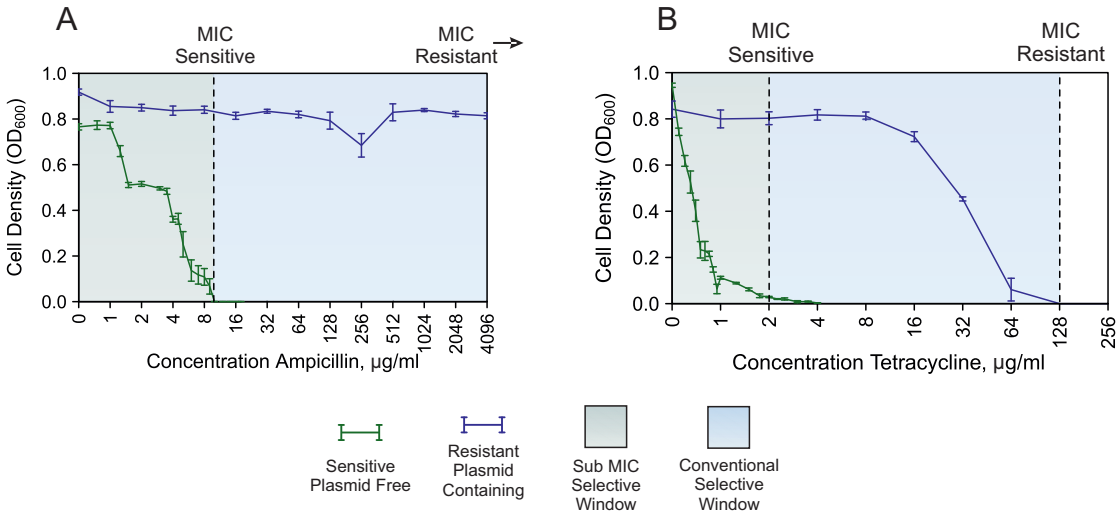


**FIG 1** Cell density (OD600) of sensitive plasmid free bacteria (green line) and resistant plasmid containing bacteria (blue line) as a function of A ampicillin concentration, B tetracycline concentration after 24 hours growth in monoculture. Error bars show SEM (n=6). Area shaded in green shows the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

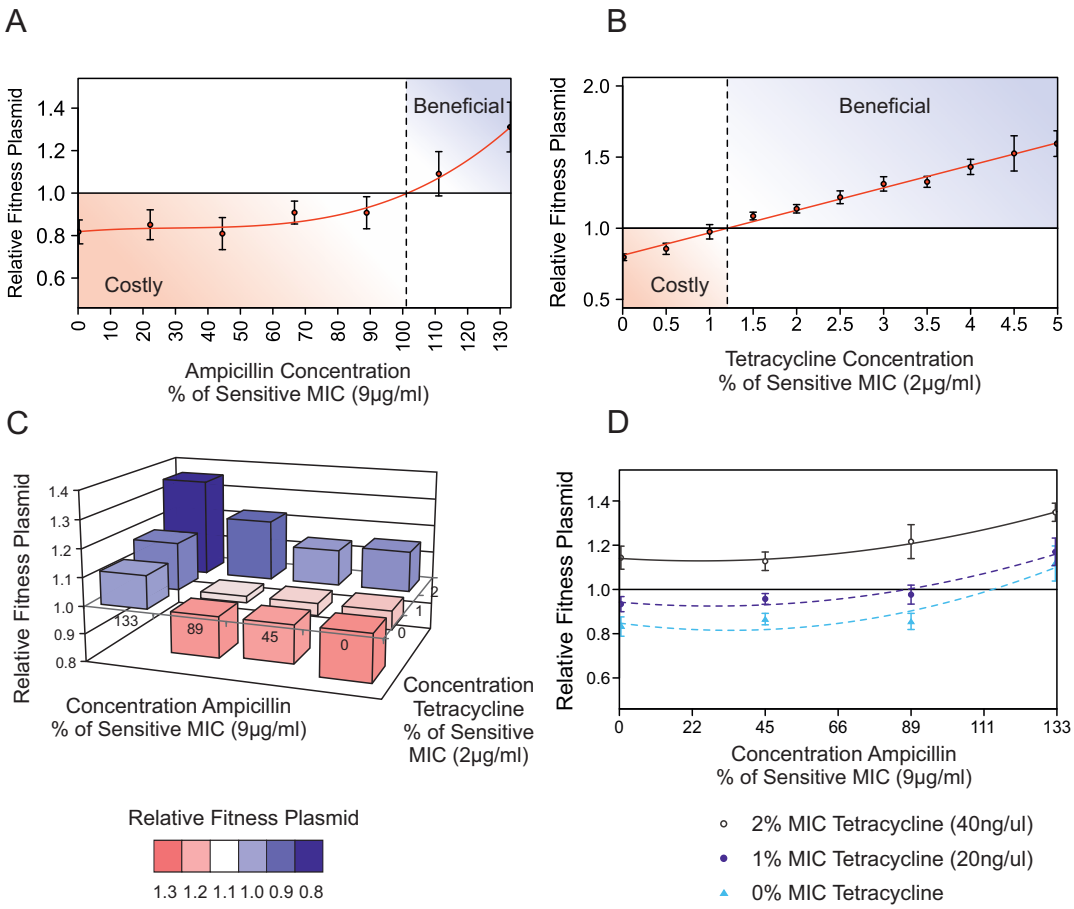


**FIG2** Fitness reaction norms as a function of antibiotic concentration during competition experiments between E. coli harboring the RK2 plasmid and isogenic plasmid free sensitive strains. Competitions in the presence of A ampicillin, B tetracycline, red lines show fitted regression. C/D Fitness reaction 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.