Collateral Sensitivity Associated with Antibiotic Resistance Plasmids

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Abstract (250 words)

Plasmids play a key role in the dissemination of antibiotic resistance (ABR) genes between bacterial pathogens. Plasmid-mediated ABR is particularly concerning in clinical settings, where bacteria carrying resistance and multi-resistance plasmids often produce life-threatening infections. Novel therapeutic strategies based on antibiotic combinations have been proposed to control the progression of ABR over the last years. One of the most promising approaches in this field are multi-drug strategies that exploit the collateral sensitivity (CS) effects associated with the acquisition of ABR mechanisms. A key limitation for the development of these strategies is that most of the data on CS come from ABR mutations in chromosomal genes, while CS associated with ABR plasmids remains largely unexplored. Here, we analyzed the collateral effects associated with the acquisition of six plasmids carrying clinically relevant ABR genes in *Escherichia coli* MG1655. Our results revealed that ABR plasmids, including the clinically important carbapenem-resistance conjugative plasmid pOXA-48, produced a moderate but significant number of CS events. Moreover, we analyzed the fitness effects of ABR plasmids in *E. coli* MG1655, and showed that plasmid fitness costs are not responsible for CS effects. This study provides the first evidence of CS associated with ABR plasmids, and paves the way for new studies aimed at understanding the molecular bases of these CS effects and at designing new antibiotic treatment strategies able to exploit plasmid-mediated CS.

Importance (150 words)

New therapeutic approaches are urgently needed to counteract the rising problem of antibiotic resistance (ABR) in bacteria. Evolution-informed strategies based on deploying multiple antibiotics simultaneously or sequentially represent a promising alternative approach. These therapies usually exploit CS effects, which occur when the acquisition of resistance to one antibiotic produces increased susceptibility to a second antibiotic. However, for these strategies to be widely applicable in the clinical practice they would have to be effective against the main resistance mechanisms available to bacteria. Recent works have focused on CS strategies designed against ABR mediated by chromosomal mutations. However, clinically relevant bacteria also acquire ABR

through horizontal gene transfer mediated by mobile genetic elements (mainly plasmids) carrying dedicated ABR genes. In this study, we analyzed, for the first time, the CS effects associated with the acquisition of complete ABR plasmids, setting up the bases for the development of CS-informed treatment strategies against plasmid-mediated ABR.

Body

The rapid evolution of antibiotic resistance (ABR) in bacteria reduces the utility of clinically relevant antibiotics, making ABR one of the main challenges facing public health (1, 2). In this context, there is an urgent need for alternative treatment strategies to fight against resistant pathogens (3). Recent works have shown that collateral sensitivity (CS), which occurs when acquiring resistance to one antibiotic causes increased susceptibility to a second antibiotic (4), can be exploited to design multi-drug strategies that select against ABR (5, 6). Specifically, treatments based on the sequential cycling of antibiotics which resistance mechanisms produce reciprocal CS may prevent, or even reverse, the evolution of resistance. Several studies have explored the CS networks emerging from ABR mutations in chromosomal genes [e.g. (5–9)], and a few studies have analyzed the CS effects produced by the horizontal acquisition of specific ABR genes (10, 11).

Mobile genetic elements, especially plasmids, play a crucial role in the dissemination of ABR genes between clinical pathogens (12). Despite the advantage conferred by plasmids in the presence of antibiotics, they usually produce physiological alterations in their bacterial hosts leading to fitness costs (13). Importantly, plasmids tend to impact the expression of metabolic genes, and a recent study showed that different plasmids produce common metabolic alterations in their host bacterium (14). We reasoned that the physiological alterations associated with the acquisition of ABR plasmids could lead to exploitable CS responses.

In this study, we analyzed the CS effects associated with the acquisition of 6 clinically relevant ABR plasmids in the host bacterium *Escherichia coli* MG1655 (Table 1, Supplementary Figure 1). Five of these plasmids had been described in previous works (15–19), while plasmid pCF12 and its genomic sequence, combining Illumina and PacBio technologies (see methods), is first described

69 in this study. All the plasmids in this study carried important ß-lactamase genes, including
70 extended-spectrum ß-lactamases (*bla*$_{SHV-12}$ and *bla*$_{VEB-9}$), AmpC-type ß-lactamase (*bla*$_{FOX-8}$), and
71 carbapenemases (*bla*$_{OXA-48}$, *bla*$_{VIM-1}$, *bla*$_{IMP-8}$, and *bla*$_{GES-5}$), as well as other relevant ABR genes
72 (Table 1). These plasmids belonged to a broad range of incompatibility groups, presented different
73 sizes, and included conjugative and non-conjugative specimens. We introduced each plasmid
74 independently in *E. coli* MG1655. The presence of the plasmids was confirmed by sequencing the
75 complete genomes of the transconjugant clones, which also revealed that no significant
76 chromosomal mutations accumulated during the process of plasmid acquisition (0-2 mutations
77 between plasmid-carrying and plasmid-free complete genomes, see Supplementary Table 1).

78

79 **Table 1. Plasmids used in this study.**

Plasmid	Incompatibility group	Mobility (MOB family)	Size (bp)	Resistance Genes	Relative fitness[1] (± SD)	Reference (Genbank Ac. No.)
pOXA-48_K8[2]	IncL	Conjugative (MOBP)	65,499	*bla*$_{OXA-48}$	0.78 ± 0.02	(19) (MT441554)
pKAZ3	IncA/C	Conjugative (MOBH)	147,957	*bla*$_{VEB-9}$, *qnrVC-1*, *sul1*, *tetA'*, *tetC*, *dfrA1*, *dfrA23*	0.73 ± 0.01	(15) (KR827392.1)
pKA2Q	IncQ	Mobilizable (MOBF)	8,789	*bla*$_{FOX-8}$	0.80 ± 0.02	(16) (MT720904)
pCF12	IncX3	Conjugative (MOBP)	43,704	*bla*$_{SHV-12}$, *qnrS1*	0.90 ± 0.02	This work (MT720906)
pCEMR	ColE-like	Mobilizable	14,478	*bla*$_{VIM-1}$, *aac(6')-Ib3* *sul1*, *aadA1,* *qacEΔ1*	0.76 ± 0.01	(17) (MG049738.1)
pKP-M1144	ColE-like	Non-transmissible	12,029	*bla*$_{BEL-1}$, *bla*$_{GES-5}$, *bla*$_{IMP-8}$, *aac(6')-Ib3*	0.73 ± 0.01	(18) (KF745070.2)

80 [1] Relative fitness of plasmid-carrying clones compared to plasmid-free parental *E. coli* MG1655.
81 [2]pOXA-48_K8 is a pOXA-48-like plasmid. For simplicity, we refer to this plasmid as pOXA-48
82 throughout the study.

83

84 We performed dose-response experiments using 13 different antibiotics belonging to 8 different
85 antibiotic families (Supplementary Table 2). Specifically, we determined the minimal inhibitory
86 concentration (MIC, see methods) for plasmid-free MG1655 strain and for each of the six plasmid-
87 carrying derivatives. We analyzed the collateral antibiotic susceptibility effects associated with the
88 presence of each plasmid, measured as the fold-change in the antibiotic MIC, between plasmid-
89 carrying and plasmid-free bacteria (Figure 1A). As expected from their genetic content, all plasmids
90 conferred resistance to ß-lactam antibiotics and most of them conferred additional resistance to
91 other antibiotics from different families (Supplementary Table 2). We identified 8 cases of plasmid-
92 induced CS, which we defined as a reduction in the MIC values of at least 2-fold (calculated as the
93 fold-change of the median from 4-5 independent MIC determinations). The plasmids that produced
94 CS effects were pOXA-48 (colistin and azithromycin), pKAZ3 (kanamycin and gentamicin), pKA2Q
95 (tetracycline), and pCF12 (kanamycin, azithromycin, and tetracycline).
96 One limitation of MIC measures is that they only provide information on end-point growth after
97 overnight exposure to the antibiotic. To corroborate MIC results, we conducted two further series of
98 experiments for the 8 cases of plasmid-induced CS: (i) complete growth curves under a range of
99 antibiotic concentrations (Figure 1B) and (ii) disk-diffusion antibiograms on agar plates (Figure 1C).
100 Growth curves qualitatively confirmed plasmid-mediated CS effects at different antibiotic
101 concentrations for five of the combinations (pOXA-48-azithromycin, pCF12-azithromycin, pOXA-
102 48-colistin, pCF12-kanamycin, and pKAZ3-kanamycin). For the three remaining cases, we
103 observed weaker differences in the complete growth dynamics between plasmid-carrying and
104 plasmid-free strains (pCF12-tetracycline, pKA2Q-tetracycline, and pKAZ3-gentamicin). Disk-
105 diffusion antibiogram results largely agreed with those from MIC and growth curves, with a general
106 trend towards an increase in the diameter of the antibiotic inhibition zones in plasmid-carrying
107 strains compared to the plasmid-free MG1655 (Figure 1C). These differences were statistically
108 significant for the cases of pCF12-kanamycin, pCF12-azithromycin, pOXA-48-azithromycin, pOXA-
109 48-colistin, pCF12-tetracycline and pKA2Q-tetracycline (Dunn's multiple comparisons test

110 corrected two-tailed t-test *P*< 0.001). Together, these results demonstrate that ABR plasmids

111 produce moderate but significant CS to different antibiotics.

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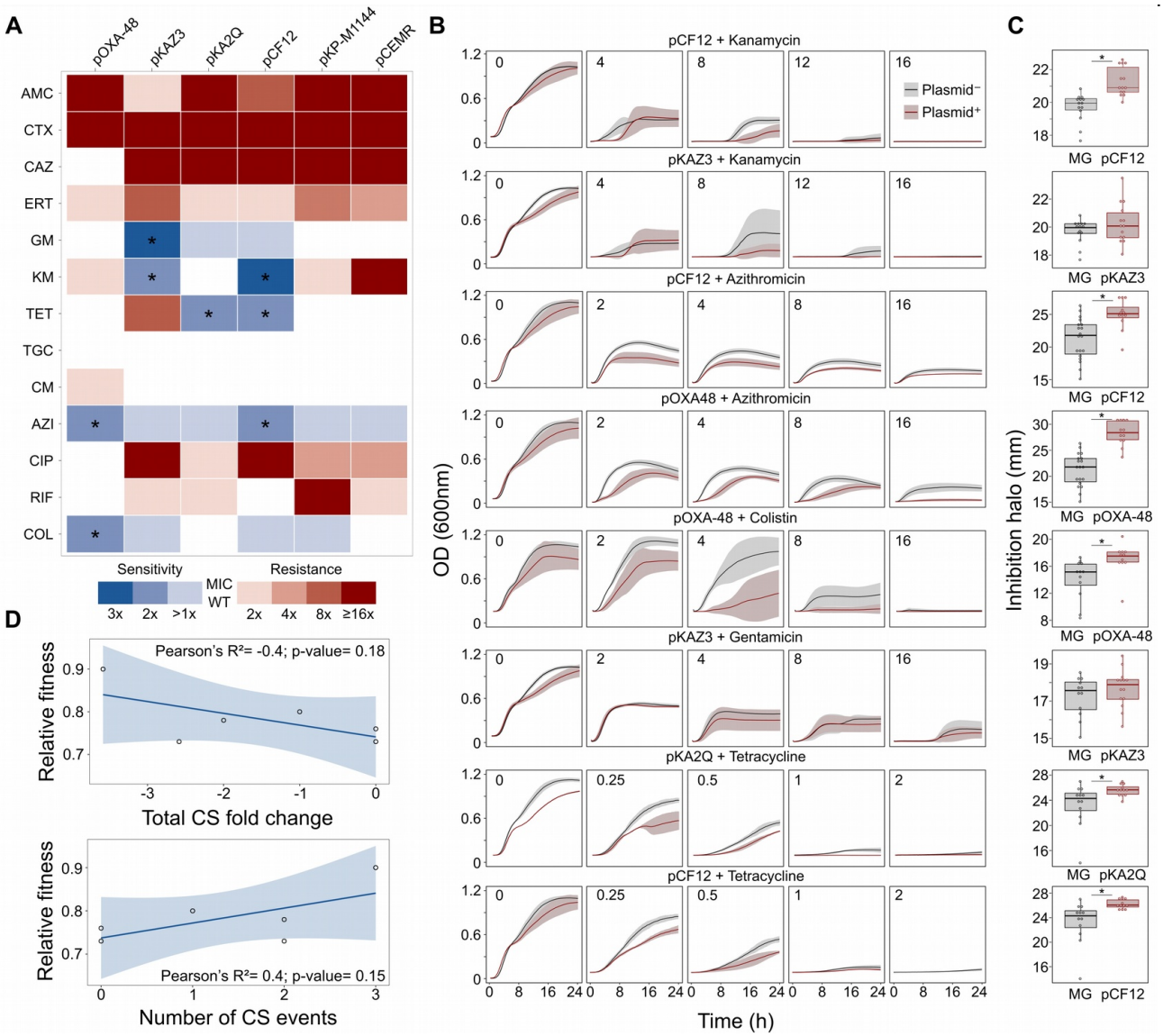

113 **Figure 1. Collateral sensitivity and fitness effects associated with ABR plasmids.** (A)

114 Heatmap representing collateral responses to antibiotics associated with plasmid acquisition. The

115 color code represents the fold change of MIC in plasmid-carrying derivatives compared to plasmid-

116 free MG1655 (see legend), obtained from the median of 4-5 independent MIC determinations.

117 Asterisks indicate cases of ≥ 2-fold decrease in MIC, which we defined as significant CS. AMC:

118 amoxicillin-clavulanic acid, CTX: cefotaxime, CAZ: ceftazidime, ERT: ertapenem, GM: gentamicin,

KM: kanamycin, TET: tetracycline, TGC: tigecycline, CM: chloramphenicol, AZI: azithromycin, CIP: ciprofloxacin, RIF: rifampicin, and COL: colistin (B) Growth curves of MG1655 and plasmid-carrying MG1655 strains under increasing antibiotic concentrations. The antibiotic concentration, in mg/L, is indicated in the top left corner of each panel. The lines represent the average of six biological replicates and the shaded area indicates standard deviation. (C) Representation of the inhibition zones diameters, in mm, obtained from disk-diffusion antibiograms of plasmid-free and plasmid-carrying MG1655 as boxplots. Antibiotic disk content were as follows: gentamicin 10 µg, azithromycin 10 µg, kanamycin 30 µg, colistin 10 µg, and tetracycline 30 µg. Horizontal lines inside boxes indicate median values, the upper and lower hinges correspond to the 25th and 75th percentiles, and whiskers extend to observations within 1.5 times the interquartile range, individual data points are also represented (11-15 biological replicates). Asterisks indicate statistically significant differences (Dunn's multiple comparisons test corrected two-tailed t-test $P < 0.001$). (D) Correlations between relative fitness and CS level. CS level is represented as the aggregated fold change in MIC for antibiotics showing CS effects (upper panel), and the number of antibiotics to which the plasmid produces any CS effects (lower panel).

Interestingly, one of the cases of CS observed in our study involved plasmid pKAZ3 and the kanamycin and gentamicin aminoglycoside antibiotics. Plasmid pKAZ3 encodes the tetracycline resistance efflux pump TetA, which is known to increase aminoglycoside uptake and susceptibility (11). This likely explains the pKAZ3-induced CS to aminoglycoside antibiotics and confirms that our experimental design is able to recapitulate known instances of CS.

Beyond the case of pKAZ3 and aminoglycoside antibiotics, one simple and general explanation for plasmid-mediated CS effects would be that CS is produced by the fitness costs imposed by plasmids. If a plasmid reduces bacterial growth, one may expect the effects of antibiotics to be more dramatic in the less fit plasmid-carrying clone than in the plasmid-free one, which would translate into a CS effect. To test this possibility, we performed competition assays to determine the relative fitness of the plasmid-carrying clones compared to the plasmid-free MG1655 strain. Our results revealed that all six plasmids imposed a fitness cost in the host bacterium MG1655,

146 which ranged from a 10% to a 27% reduction in relative fitness (Table 1). We hypothesized that if
147 fitness costs are responsible for CS, these two variables should be positively correlated. We used
148 two different metrics to capture the general CS level of each plasmid-carrying strain: number of
149 antibiotics affected by CS effects and the aggregated fold change of CS effects (see methods). We
150 observed no significant correlation between plasmids' fitness effects and CS level (Pearson's rank
151 correlation, $P > 0.15$, Figure 1E). In fact, plasmid pCF12 produced both the lowest fitness cost in
152 MG1655 and the greatest number of significant cases of CS. These results suggested that the
153 general reduction in fitness imposed by plasmids is not responsible for the observed CS effects.

154 **Concluding remarks**

155 In this study we investigated, for the first time, the CS associated with the acquisition of clinically-
156 relevant ABR plasmids. Our results revealed that ABR plasmids are involved in a moderate but
157 significant number of CS events, comparable to those generated by ABR chromosomal mutations
158 in *E. coli* (5–7). CS responses are variable among plasmids, with some plasmids producing no
159 significant CS and others increasing the susceptibility to up to three different antibiotics. Our study
160 set the bases for further work focused on at least two different research lines: (i) understanding the
161 molecular bases underlying the plasmid-specific CS responses and (ii) exploring if plasmid-
162 mediated CS could be exploited therapeutically for clinically relevant enterobacteria.

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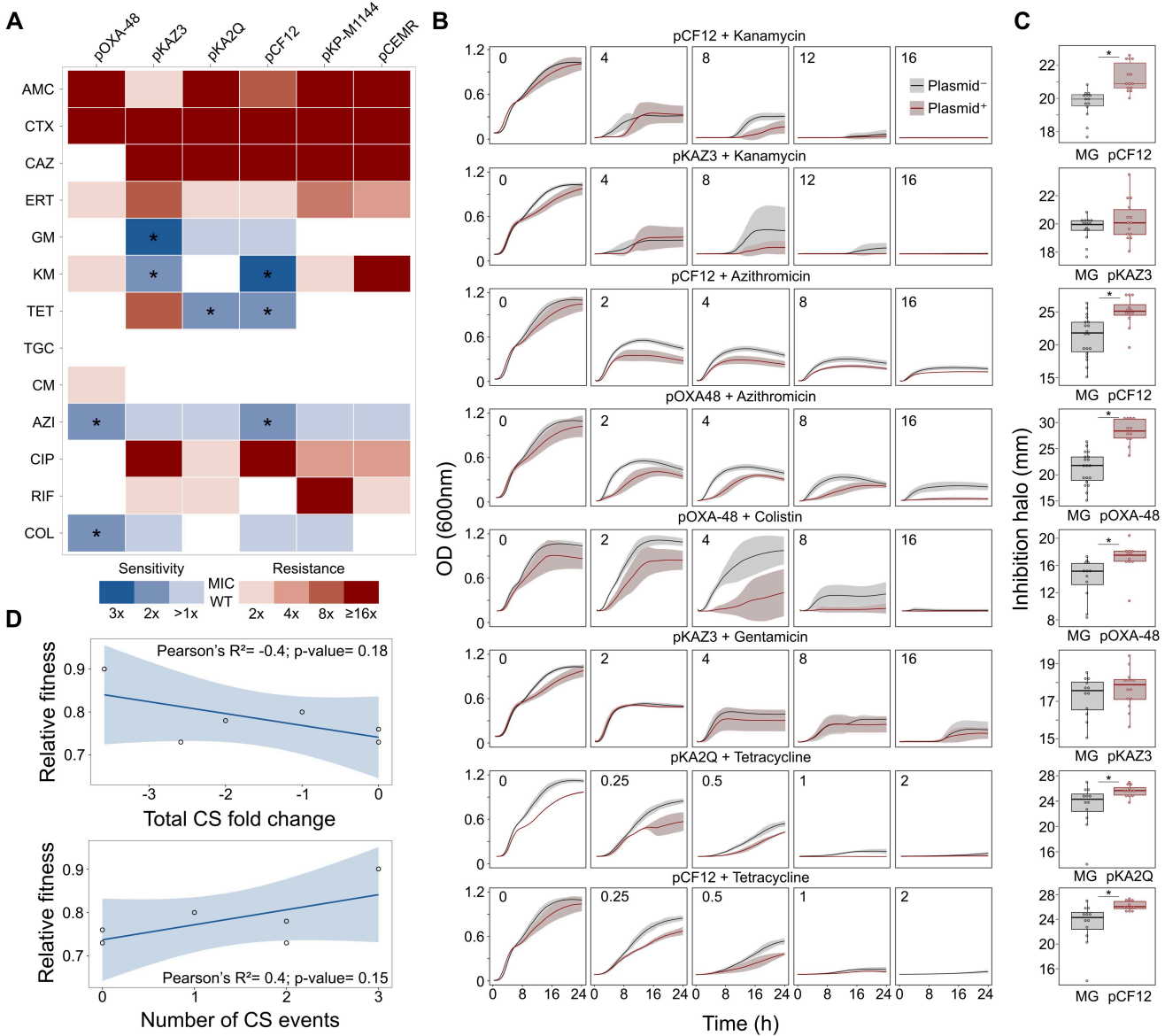
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pOXA-48
pKAZ3
pKA2Q
pCF12
pKP-M1144
pCEMR
AMC
CTX
CAZ
ERT
GM
KM
TET
TGC
CM
AZI
CIP
RIF
COL
Sensitivity
Resistance
MIC
WT
3x
2x
>1x
2x
4x
8x
≥16x
B
pCF12 + Kanamycin
pKAZ3 + Kanamycin
pCF12 + Azithromicin
pOXA48 + Azithromicin
pOXA-48 + Colistin
pKAZ3 + Gentamicin
pKA2Q + Tetracycline
pCF12 + Tetracycline
Plasmid⁻
Plasmid⁺
OD (600nm)
Time (h)
C
Inhibition halo (mm)
MG
pCF12
pKAZ3
pOXA-48
pKA2Q
D
Pearson's R²= -0.4; p-value= 0.18
Relative fitness
Total CS fold change
Pearson's R²= 0.4; p-value= 0.15
Number of CS events