Title: DNA breaks are key contributors to the cost of antibiotic resistance

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Abstract:

Antibiotic resistance often causes a fitness cost, which can lead to decreasing prevalence of resistance upon reducing antibiotics usage. The causes of the costs of resistance are, however, poorly understood, impairing our ability to manipulate its fate. Here we show that DNA breaks explain 73% of the variation in the cost of resistance mutations common in pathogens. We find that RNase HI is a key determinant of these costs and a novel target for antimicrobials specific against resistant bacteria. Indeed, lack of RNase HI rapidly drives *Escherichia coli* resistant clones to extinction in polymorphic populations with high resistance levels. These results provide a novel framework for the development of new strategies to lower the alarming levels of resistance in the human microbiome.

One Sentence Summary: DNA breaks drive the cost of antibiotic resistance, revealing specific targets against resistant bacteria.

Main Text:

Antibiotic resistance (AR) entails a large human and economic burden worldwide (*1*). Its dissemination depends on the rate at which resistances are acquired and on their effects on bacterial fitness (cost). The latter is influenced by the environment, by interactions between the resistances and their genetic background (epistasis) and by the subsequent acquisition of mutations compensating for fitness defects (compensatory evolution) (*2*). Despite its importance, the causes of this cost are not completely understood (*3*), and the identification of its causes has become the Holy Grail in the AR field. Resistance mutations often map to genes encoding the proteins targeted by the antibiotics. These are typically involved in essential functions, such as transcription, translation, DNA replication or cell wall biosynthesis. Resistance mutations cause alterations in the structure of the target protein, rendering it insensitive to the drug, but often also affecting its function (*4*, *2*). Rifampicin and streptomycin resistance mutations (Rif^R and Str^R) paradigmatically represent resistances to antibiotics targeting transcription and translation, respectively. Rif^R mutants often show cost (*5*), commonly associated with alterations in the rates of transcription initiation, elongation, slippage, and termination (*6*). Most Str^R mutations also cause a cost (*7*), generally linked to increased translation fidelity and reduced processivity (*8*). Thus, the costs of Rif^R and Str^R mutations are commonly attributed to defects in protein synthesis, either globally (*9*), or circumscribed to specific functions or regulons (*10*, *11*, *12*).

We recently showed that compensation for the cost of double resistance (Rif^R Str^R) can occur via overexpression of *nusG* and *nusE* (*13*), which encode the proteins that connect the RNA polymerase (RNAP) and the ribosome (*14*). This suggests that compensation involves reinforcing the coupling between transcription and translation, presumably disturbed by the

mutations. Both NusG and transcription-translation coupling prevent spontaneous RNAP backtracking (*15*, *16*), which cause double-strand DNA breaks (DSBs) (*17*). We thus hypothesize that perturbation of transcription-translation coupling caused by Rif^R and Str^R mutations generate DSBs, which drives their cost. To test this hypothesis, we developed a novel experimental system to simultaneously measure competitive fitness and activation of the SOS response, a well-known proxy for the occurrence of DSBs (*18*). We used *Escherichia coli* as model, and studied 16 strains: sensitive, Rif^R strains ($RpoB^{H526L}$, $RpoB^{H526Y}$, and $RpoB^{S531F}$), Str^R strains ($RpsL^{K43N}$, $RpsL^{K43T}$, and $RpsL^{K43R}$) and their double resistant combinations. Remarkably, 14 out of the 15 resistant strains show increased SOS activation (Fig. 1A), which strongly correlates with the cost of resistance, explaining 73% of its variation (Fig. 1B). To independently confirm the occurrence of DSBs in resistant bacteria, we directly visualized double-stranded DNA ends (*19*), combined with the SOS reporter, in a subset of resistant mutants. This corroborated that resistance indeed causes increased DSBs (Fig. S1, Table S1). Erythromycin targets the 50S ribosomal subunit, affecting translation and its coupling with transcription (*20*). Erythromycin resistance mutations (Erm^R) mapping in the genes *rplD* and *rplV* (encoding L4 and L22, respectively) are known to reduce translation elongation rate (*21*), credibly affecting transcription-translation coupling. We isolated Erm^R clones carrying either $RplD^{G66R}$ or $RplV^{\Delta 82\text{-}84}$ mutations and found that both mutants show increased SOS (Fig. 1C), demonstrating that mechanistically different perturbations of transcription-translation coupling cause DSBs as well.

We then reasoned that, since DSBs appear to drive the cost of resistance, compensatory evolution of resistant strains should lead to lowering DSBs. Thus, we compared the cost and SOS induction in the $RpsL^{K43T}$ $RpoB^{H526Y}$ double mutant and in an isogenic strain additionally carrying a prevalent compensatory mutation: $RpoC^{Q1126K}$ (*13*). As hypothesized, the cost and SOS

induction are greatly reduced in the compensated strain (Fig. 2A). Furthermore, 9 compensated clones from 3 independently evolved populations of RpsL^{K43T} RpoB^{H526Y} double mutants show decreased SOS induction compared to their resistant ancestor (Fig. 2B), demonstrating that compensatory evolution widely targets a major cause of the cost: DSBs. The cost of specific Rif^{R} and Str^{R} mutations is reduced in minimal medium (*11*, *22*). Thus we reasoned that DSBs should be reduced in this environment. Indeed, resistant mutants show reduced DSBs in minimal medium (Fig. S2A), and a weaker correlation with their cost (Fig. S2B), further validating the association between DSBs and cost.

Generation of DSBs by transcription-translation uncoupling encompasses increased formation of R-loops (*17*). We thus hypothesize that deleting RNase HI, which specifically degrades R-loops (*23*), would increase DSBs in strains carrying Rif^{R} and Str^{R} mutations. Accordingly, both DSBs and the cost of resistance are greatly exacerbated in the *ΔrnhA* background (Fig. 3A, Fig. S3, Table S1, Fig. S4). Conversely, overproduction of RNase HI can ameliorate both phenotypes in a subset of mutants (Fig. S5); however, strong overproduction is toxic for the cell (*24*), irrespectively of its genotype (Fig. S6). These results underline the importance of RNase HI function for the cost of resistance. We then queried if targeting RNase HI function could be used to specifically select against resistant bacteria in polymorphic populations with high frequency of resistance. We tested the effect of a commercially available compound, RHI001, shown to inhibit the activity of the *E. coli* RNase HI protein *in vitro* (*25*), in competitions between sensitive and resistant bacteria. We observed that RHI001 increases the cost of resistant mutants (Fig. 3B, Fig. S7A). Chemical inhibition was not as effective as genetic removal (Fig. S7B), as may be expected, since the effectiveness of RHI001 *in vivo* is unknown,

and potentially suboptimal. Nevertheless, inhibition of RNase HI appears a plausible strategy to specifically select against resistant strains, as long as they fail to evolve adaptations that abrogate their extinction. To test this, we propagated a mixture of CFP-labeled sensitive bacteria competing against a pool of YFP-labeled resistant mutants ($RpsL^{K43N}$, $RpsL^{K43T}$, $RpoB^{H526L}$, $RpoB^{H526Y}$, and $RpoB^{S531F}$) during 15 days, in the absence of antibiotics. We studied the frequency dynamics of resistant clones under both strong bottlenecks (1:1500), where new adaptive mutations are less likely to spread, and weak bottlenecks (1:50), where propagation of adapted clones is more likely. In parallel, we performed identical propagations, but in strains lacking RNase HI, mimicking optimal inhibition of RNase HI. In the presence of RNase HI, sensitive bacteria initially outcompete resistant clones but, eventually, resistant bacteria increase in frequency (likely due to compensation (*2*)), reaching coexistence (Fig. 4, blue lines). Remarkably, in the absence of RNase HI, resistant bacteria went extinct by day 9 (Fig. 4, red lines), even under mild bottlenecks (Fig. S8). Altogether, these results show that targeting RNase HI is a promising strategy to selectively eliminate resistant bacteria.

We showed that transcription-translation uncoupling caused by resistance mutations generates DSBs, which explain 73% of the variation in their cost (Fig. 1, Fig. S1, Table S1). Consistently, the mutants showing the highest cost and DSBs ($RpsL^{K43N}$ $RpoB^{H526Y}$ and $RpsL^{K43T}$ $RpoB^{H526Y}$) combine alleles causing increased transcription elongation rate (*26*) and decreased translation rate (*7*), arguably resulting in maximized uncoupling. Agreeingly, mutations causing uncoupling by affecting a different ribosomal subunit generate DSBs as well (Fig. 1C). We also found that uncoupling mediated by resistance mutations generates R-loops (Fig. 3, Fig. S3, Table S1, Fig. S4, Fig. S5), as does uncoupling caused by antibiotics affecting translation (*27*, *17*).

Extensive uncoupling might render RNase HI insufficent, as costly resistances (*28*) show great DSBs even in its presence (Fig. S9A). Overall, these results suggest that combining antibiotics and RNase HI inhibitors could increase the effectiveness of antimicrobial treatments.

DSBs induced by uncoupling involve increased replication-transcription conflicts (*17*). We observed greater DSBs in rich than in minimal media (Fig. 1, Fig. S2), which might be linked to the fact that replication-transcription conflicts are maximized during fast replication (*29*) (Fig. S9B-C). Supporting the occurrence of replication-transcription conflicts, we observed that, although the costs of Rif^R and Str^R mutations are similar at 4h and 24h (Fig. S9D), the costs are generated in the first 4 hours (comprising exponential growth), while resistant bacteria show advantage afterwards (Fig. S9E). Coherently, Rif^R mutants can outcompete sensitive bacteria in aging colonies (*30*). Notably, both lack of RNase HI and conditions that activate SOS can induce initiation of DNA replication from sites different than *OriC* (constitutive stable DNA replication, cSDR) (*31*). Thus, Rif^R and Str^R mutations could favor replication-transcription conflicts via induction of cSDR, causing a feed-forward loop of synergistically deleterious effects, further enhanced by the downregulation of *rnhA* caused by SOS induction (*32*). Interestingly, this detrimental loop and the environmental effect on the cost could potentially be targeted, via concurrent chemical and dietary interventions, in order to maximize the cost of resistance.

We revealed RNase HI as a novel target specific against resistant bacteria (Fig. 3, Fig. 3, Table S1, Fig. S4, Fig. S7), and showed that lack of RNase HI favors extinction of resistant clones and prevents compensatory evolution (Fig. 4, Fig. S8). This is specially important for pathogens that acquire AR exclusively through mutation, such as *Mycobacterium tuberculosis*,

which often carries Rif^R and Str^R mutations (*33*). Interestingly, RNase HI has been proposed as an antimycobacterial target due to its essentiality in mycobacteria (*34*). The results presented here support the plausibility of this strategy.

Understanding the determinants of AR dissemination is urgently needed (*1*). Here we show that DNA breaks are key contributors to the cost of resistance and reveal the RNase HI as a novel antimicrobial target specific against resistant bacteria. Overall, our results unveil key effects of resistances on bacterial physiology, which provide novel strategies for the global challenge of AR.

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analyzed to obtain the data shown in Table S1, and those represented in Fig. S1, and Fig. S3 are available in Zenodo (DOI: 10.5281/zenodo.3381746).

Supplementary Materials:

Materials and Methods

Figures S1-S10

Table S1

References (*35-44*)

Auxiliary Supplementary materials:

Data S1 and S2

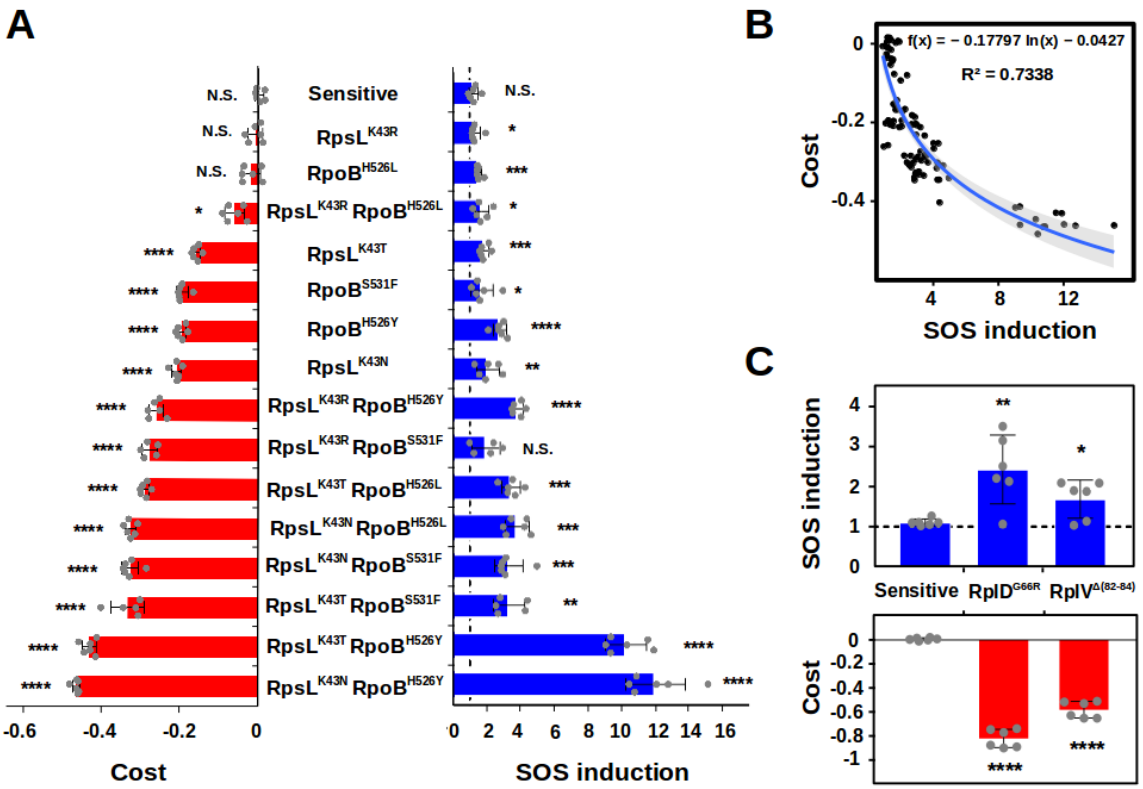

Fig. 1. The cost of resistance correlates with the SOS induction. A. Fitness cost (red bars) and SOS induction (blue bars) of sensitive bacteria and Str^R and Rif^R mutants in LB broth at 4 hours. The strains are ordered from lower to higher fitness cost (top to bottom). The dashed line indicates no SOS induction. Error bars represent mean ± standard deviation of independent biological replicates (n≥5). N.S. non-significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001 (two-tailed Student's *t* test). **B.** Correlation between the fitness cost (y axis) and the SOS induction (x axis) representing all the data points from A. The blue line represents the logarithmic regression line, and the grey area represents the 95% confidence interval. **C.** SOS induction (blue bars) and fitness cost (red bars) of sensitive bacteria and Erm^R mutants in LB broth at 4 hours. The dashed line indicates no SOS induction. Error bars represent mean ±

standard deviation of independent biological replicates (n=6). N.S. non-significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ (two-tailed Student's *t* test).

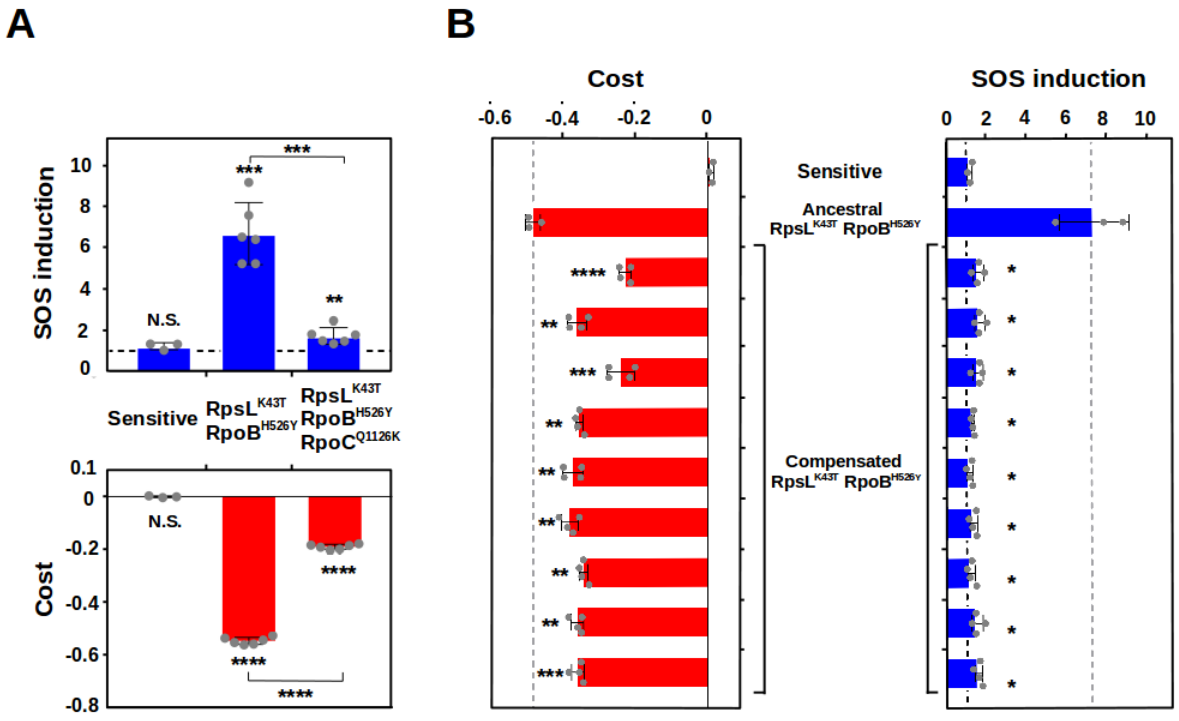

Fig. 2. Compensated strains show reduced SOS response. A. SOS induction (blue bars) and fitness cost (red bars), of sensitive (left) double resistant (center) and double resistant carrying a compensatory mutation (right) bacteria in LB broth at 4 hours. The dashed line indicates no SOS induction. Error bars represent mean ± standard deviation of independent biological replicates (n≥3). N.S. non-significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001 (two-tailed Student's *t* test). **B.** Fitness cost (red bars), and SOS induction (blue bars) of sensitive, ancestral $RpsL^{K43T}$ $RpoB^{H526Y}$ and nine compensated $RpsL^{K43T}$ $RpoB^{H526Y}$ clones in LB broth at 4 hours. The black dashed line indicates no SOS induction. The grey dashed lines mark the cost/SOS of the ancestral double mutant. Error bars represent mean ± standard deviation of independent biological replicates (n≥3). N.S. non-significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001 (two-tailed Student's *t* test).

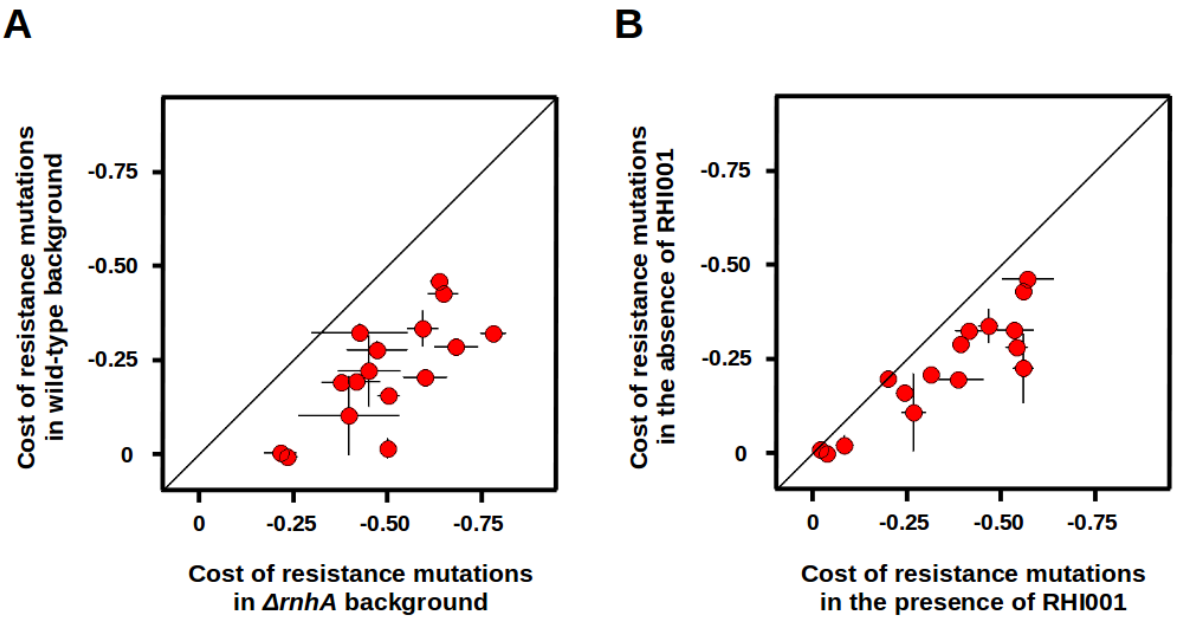

Fig. 3. Lack of RNase HI function increases the cost of resistance. A. Correlation between the fitness cost of resistance mutations in wild-type (y axis) or *ΔrnhA* backgrounds (x axis). The black line represents the linear regression if the costs were identical. **B.** Correlation between the fitness cost of resistance mutations in the absence of the RNase HI inhibitor (y axis) or in its presence (x axis). The black line represents the linear regression if the costs were identical. The values corresponding to the wild-type background in panel A and to absence of RHI001 in panel B are those shown in Fig. 1A. Error bars represent mean ± standard deviation of independent biological replicates (n≥3).

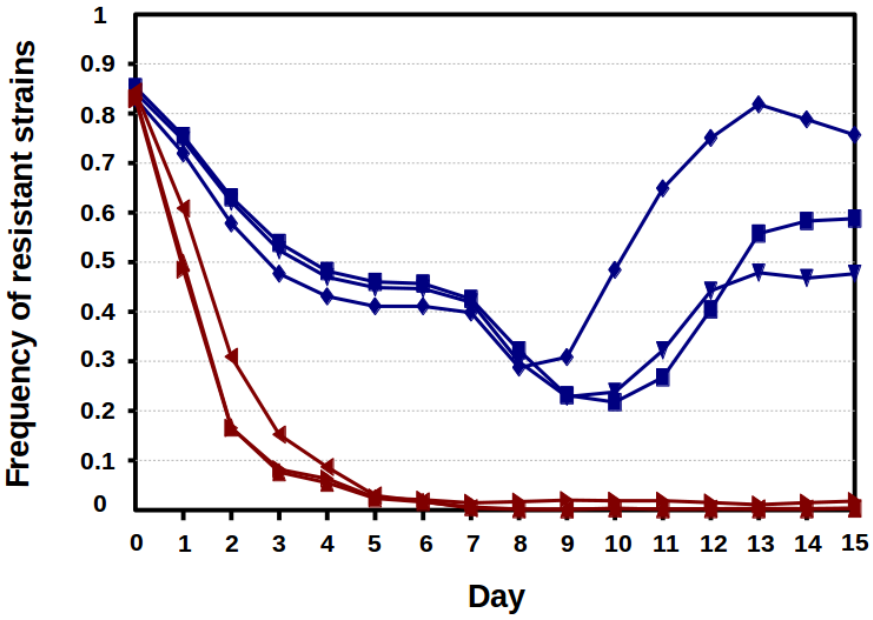

Fig. 4. Lack of RNase HI favors outcompetition of resistant mutants by sensitive bacteria. Frequency of single resistant mutants during three independent long-term competitions against sensitive bacteria either in a genetic background including RNase HI (blue lines) or in a *ΔrnhA* background (red lines), imposing a strong bottleneck (1:1500 dilutions).