Antibiotic-induced recombination in Gram-negative bacteria requires the formation of double-strand breaks

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Abstract

Recombination is an essential process in bacterial drug resistance evolution. Fluoroquinolones, an important class of antibiotics, have been reported to stimulate recombination in *Escherichia coli.* Bacteria's sublethal exposure to several other antibiotics from different families, including strong inducers of the SOS stress response and antimicrobial peptides, does not affect recombination rates. Here we ask why fluoroquinolones, and no other antimicrobials, increase recombination rates. We found that the fluoroquinolone ciprofloxacin stimulated recombination in *E. coli* not only via increased SOS induction but also by DNA double-strand breaks (DSBs). On this basis, we tested whether other antimicrobials, known for their ability to cause double-strand breaks, such as mitomycin C and bleomycin, also affect recombination rates. Indeed, both antibiotics also increased recombination frequency. We found a positive correlation between the number of DNA double-strand breaks and the recombination frequency. These results imply that recombination, at least in Gram-negative bacteria, is conservative, and even if the substrate of recombination is available, the basal recombination rate is low, and it is more intended to repair than to generate diversity. We also show that only antibiotics that cause DNA double-strand breaks can increase genetic diversity via recombination and increase the risk for antibiotic resistance.

Keywords**:** homologous recombination, antibiotic resistance, quinolone, double-strand breaks.

Introduction

Antimicrobial failure to kill bacterial pathogens is becoming a global problem due to the very rapid emergence of resistant strains [1]. According to a recent global report, antimicrobial resistance (AMR) led to an estimated 4.95 million deaths in 2019. The number of deaths varied between regions, with the highest toll being reported in regions with lower resource settings. *E. coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* were the six most significant pathogens causing antimicrobial resistance [1]. Resistance to fluoroquinolones and β-lactams, the first go-to antibiotics in most infections, contributed to at least 70% of AMR deaths [2]. Antibiotics themselves are a selective pressure, but they also enhance evolvability by elevating mutation rates and accounting for increasing recombination frequency and lateral gene transfer in bacteria [3].

An excellent example of an increasing issue with antibiotic resistance over the years is quinolones, whose resistance, along with other antibiotics, develops faster as soon as they are introduced [4,5]. The mechanism of quinolone action is mainly by inhibition of two essential bacterial type II topoisomerase enzymes, DNA gyrase and DNA topoisomerase IV [6]. Both of these enzymes are hetero-tetramers with two subunits. The activity of both enzymes causes the catalysis of a DNA double-strand break (DSB), passing another DNA strand through the break and then resealing it together to regulate the DNA supercoiling state. DNA strand passing occurs via domains localised in GyrA and ParC, whereas the ATPase activity is required for enzyme catalysis and is located in domains belonging to GyrB and ParE subunits [7].

The SOS stress pathway is activated when sub-lethal antibiotic treatments induce direct DNA damage. Consequently, error-prone alternative DNA polymerases repair the damage at the price of introducing mutations, a process called trans-lesion synthesis [8]. The general stress response controlled by the sigma factor RpoS in Gram-negative bacteria also increases mutation rates under antibiotic stress by indirectly down-regulating MutS, a key protein of the DNA mismatch-repair system [9]. Moreover, other classes of antibiotics such as sulphonamides increase mutagenesis directly due to the imbalance they produce in the nucleotide pool during replication [10]. All these direct or indirect mutagenic effects are common to many families of antimicrobials except for cationic antimicrobial peptides that do not increase the mutation rate or recombination [11,12].

Recombination is a fundamental driver of antimicrobial resistance by inducing, for example, gene duplication and amplification [13,14]. It is known that fluoroquinolones stimulate recombination in *E. coli* suggesting the potential for acquiring antibiotic resistance [15]. In pathogenic *E. coli* isolates, recombination rates can be significantly higher than in commensal strains [16]. However, the number of antibiotics that increase recombination in *E. coli* is limited to fluoroquinolones, suggesting this is a unique property of this family [17]. Also, the human pathogen *Streptococcus pneumoniae* displays higher recombination rates

in resistant strains when compared to sensitive ones showing a link between resistance and recombination [18].

Here we asked why fluoroquinolones, and no other antimicrobials, increase bacterial recombination rate. Many antibiotics induce the SOS system, up-regulating several recombination proteins such as RecA. Nevertheless, this up-regulation is not sufficient to increase recombination. Previous data suggest that the induction of DSB, a hallmark of fluoroquinolone-induced damage, could be responsible for boosting the recombination rate [15]. Therefore, we hypothesised that in order to stimulate recombination by antibiotics in Gram-negative bacteria such as *E. coli*, the induction of the SOS system alone is not sufficient and that the generation of DNA double-strand breaks is necessary. To test the generality of our findings, we compared ciprofloxacin to other antibiotics known for their capacity to generate DSBs, but that are not fluoroquinolones. Thus, we included other drugs that also cause DSBs, mitomycin C and bleomycin. Two soil bacteria produce these antibiotics: *Streptomyces caespitosus* produces mitomycin C [19], while bleomycin is produced by *Streptomyces verticillus* [20].

Results and Discussion

It has previously been shown that from a broad panel of antibiotics belonging to different families, only those from the fluoroquinolone family stimulate recombination in *E. coli* [17]. Since we are using a more sensitive plasmid-based system [21,22] than was previously used to measure recombination, we tested again many of those previously tested antimicrobials (refer to reference 16), after determining the minimal inhibitory concentration for each one (Table S1), covering diverse different antibiotic classes. This confirmed that only ciprofloxacin from the previously tested antibiotics increased recombination rates, which is in agreement with previous reports for ciprofloxacin [12,15,17].

We also found that the glycopeptide and the anti-tumour antibiotic mitomycin C changed recombination rates (Figure 1). In *E. coli* and enterobacteria in general, essential proteins of the recombination machinery are controlled by the SOS pathway [21,22]. One explanation for the variation in recombination rates could be that not all antibiotics activate the SOS response mechanisms. Besides fluoroquinolones, amongst the drugs we tested, β-lactams and sulphonamides are potent inducers of SOS response [23]. An additional and unique feature of quinolones is their ability to induce DSBs. Interestingly, we found that mitomycin C and bleomycin increased the recombination frequency by more than one order of magnitude compared with the controls' basal level and similar to ciprofloxacin (Figure 1).

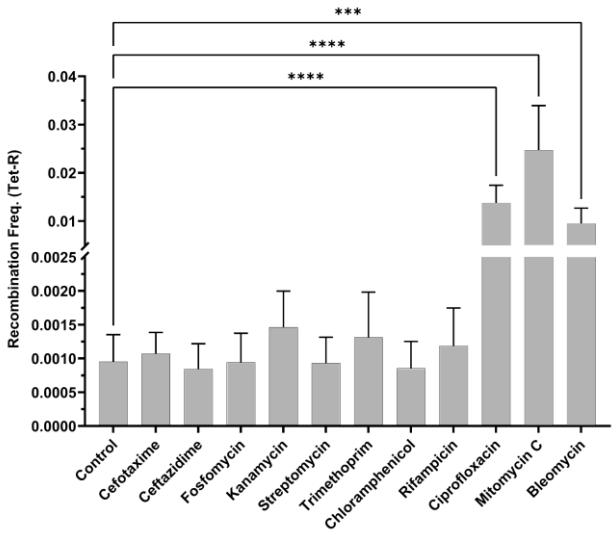

Figure 1. Recombination frequencies of *E. coli* MG1655 harbouring the plasmid pRHOMO (recombination construct) treated with antibiotics at their 1xMIC during three hours. Bars represent the mean (+ SD) values of recombination frequency of the bacteria exposed to half of the MIC for 4 h. Ciprofloxacin, mitomycin C and bleomycin had a significantly higher recombination frequency compared to the control group.

Mitomycin C and bleomycin also induce the SOS response as they cause direct DNA damage [24]. For ciprofloxacin, mitomycin C and bleomycin, the increase in recombination frequency was dependent on the recombination protein RecA (Figure 2). A RecA deficient mutant suppressed the recombination increase to

similar levels of wild type. This and the previous experiment suggest that the recombination stimulation probably requires SOS induction and DSBs. The activation of SOS is not always correlated with the presence of DSBs, but [25] DSBs seem always to trigger the transcription of SOS genes in our experiments.

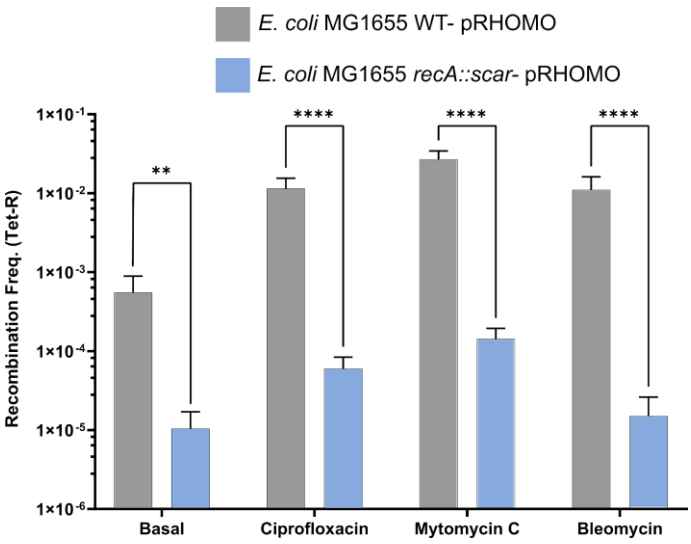

Figure 2. Antibiotic-stimulated recombination for mitomycin C, bleomycin, and ciprofloxacin (1xMIC during three hours) is mostly dependent on RecA protein repair activity since *E. coli* MG1655 *recA*::scar harbouring the plasmid pRHOMO suppressed the change in recombination rate observed in the wild-type strain when treated with DSB-inducer antimicrobials. Differences in recombination rates between strains were analysed with a two-way ANOVA and Šídák's multiple comparisons test, with individual variances computed for each comparison; Asterisks represent statistical differences (**** - $p<0.0001$, ** - $p=0.002$). Each experiment consisted of five replicates a non-treated controls was included (basal recombination rate value of the non-treated bacteria).

To test the hypothesis that DSBs are significant determinants of the induction of recombination, we correlated the number of DSBs lesions and the recombination rate. For that, we used ciprofloxacin at three different concentrations and determined the number of DSBs at each dose using caused by ciprofloxacin, mitomycin C and bleomycin. We use a reporter system that allows observing these lesions in real-time. The system consists of a green fluorescent protein (GFP) fusion containing bacteriophage GAM protein. GAM protein recognises the DNA ends of DSBs, while GFP allows the tagging and forms foci [26]. We found a positive correlation between the number of DSB lesions and recombination frequency (Figure 3). We repeated this analysis to check if there was a positive correlation between antibiotic concentration and recombination frequency. For that, we used ciprofloxacin at three different concentrations and determined the number of DSBs at each dose. We found that as the ciprofloxacin concentration increased, the recombination frequency also increased (Figure 4). Microscopic pictures of cells treated with growing concentrations of DSB-inducing antibiotics show the induction of DSBs by ciprofloxacin, mit0mycin C and bleomycin (Figure 5).

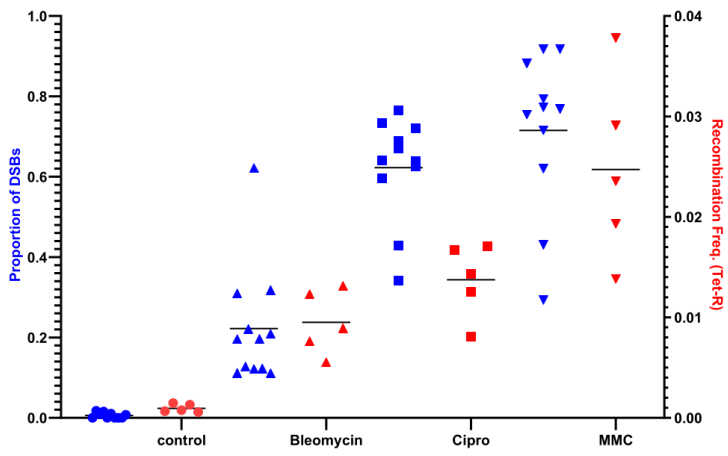

Figure 3. The graph shows a positive correlation (Spearman correlation coefficient r= 0.81, *p*=0.0034) between the number of DSBs lesions and increased recombination frequency when bacteria are treated with antibiotics that induce DSBs. Each experiment consisted of five replicates and had its non-treated control (basal recombination rate value of the non-treated bacteria). The positive control for DSBs (I-*SceI*) consisted of a unique restriction site in the bacterial chromosome that can be cut by inducing the expression of the restriction enzyme *SceI*.

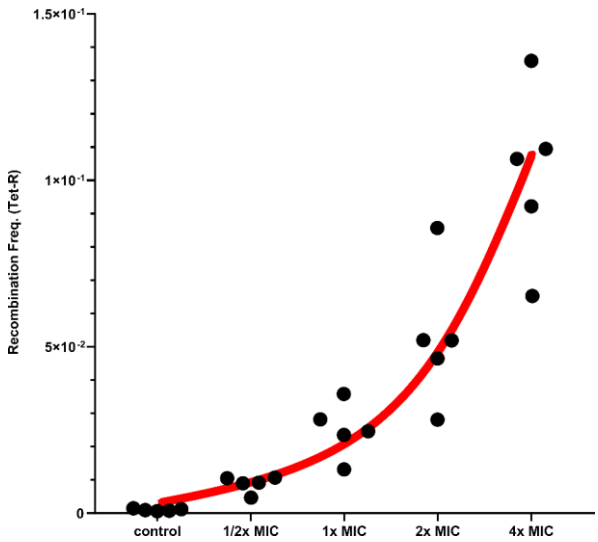

Figure 4. The graph shows a positive correlation between the recombination frequency and increasing dose of ciprofloxacin (Spearman correlation coefficient r=0.99, *p*=0.0012). Each experiment consisted of five replications and had its non-treated control (basal recombination rate value of the non-treated bacteria).

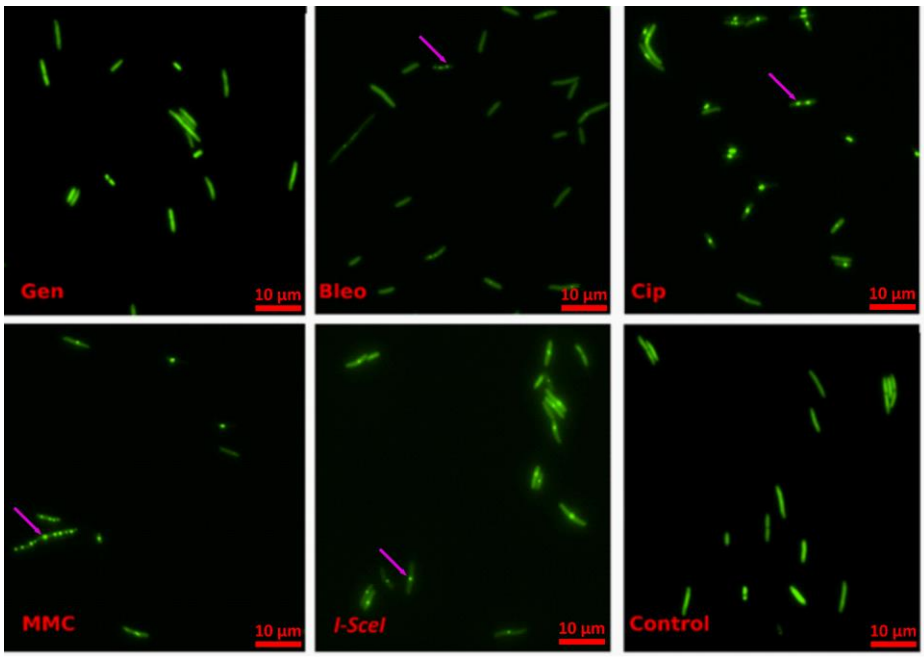

Figure 5. Fluorescent microscope images of antibiotic-generated double-strand breaks after treating the *E. coli* strain SMR14354 with gentamycin (Gen), bleomycin (Bleo) and ciprofloxacin (CIP), MIC of mitomycin C (MMC) with the minimal inhibitory concentration for 3 h. The green foci (indicated by magenta arrows) indicate the binding of the GFP-GAM fusion protein to free DNA ends and are only found at high frequency in treated bacteria with bloemycin, ciprofloxacin and mitomycin C. The foci formation (indicating the presence of DSBs) can be also observed in the positive control (I-*SceI*), that consisted of a unique restriction site in the bacterial chromosome that can be cut by inducing the expression of the restriction enzyme *SceI*. Gentamicin is used as a control antibiotic, which does not increase recombination frequency or the number of DSBs.

To determine whether there is a causal relationship between DSBs and increases in antibiotic-induced recombination, we devised an experiment to directly test whether DSBs caused by antibiotics are the trigger for antibiotic-induced recombination. One of the antibiotics that produce DSBs, bleomycin, requires iron as a cofactor to induce DSBs [24,27]. In the experiments described so far, we used lysogeny broth, an iron-rich medium [28]. Therefore, we reduced the iron content of the medium by suppressing iron (by adding 2–2′ bipyridyl) during the bleomycin treatment. If DSBs cause changes in recombination, suppressing the iron content significantly decreases the recombination frequency, and iron supplementation significantly increases the recombination frequency (Figure 6). Under low oxygen tension, the actual damage induced by bleomycin consists of forming oxidised abasic sites, while in aerobic environments, single and double-strand breaks predominate [29]. Our results entail an important trait of recombination, at least in Gram-negative bacteria. The canonical recombination machinery of *E. coli* in the absence of stress is extremely conservative, and even in the presence of substrate for recombination, the frequency of recombination events is low, and it works more towards repair processes.

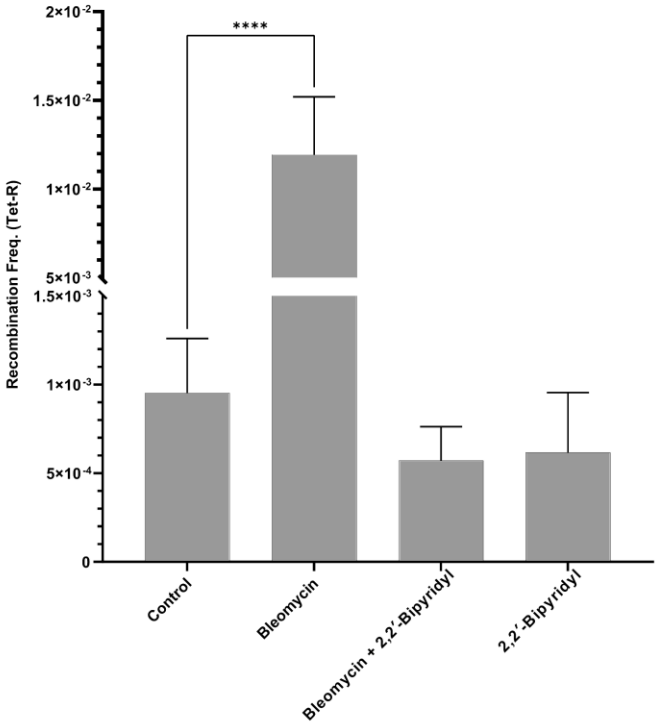

Figure 6. Impact of iron chelation by 2–2′ bipyridyl on recombination frequency of *E. coli* MG1655-pRHOMO treated with 1xMIC of bleomycin. LB medium contains between 15 to 20 uM of iron [28] which potentiate the capacity of bleomycin to induce DSBs [24]. A significant decrease of recombination rate was observed when an iron-titrating level of2–2′ bipyridyl (final concentration of 50µM) was added altogether with bloemycin. The experiment consisted of five independent replicates. Bars represent the mean and error bars represent the standard deviation.

One evidence of this notion is the need to use helper recombination systems (e.g., λ red recombinase) to increase the probability of DNA integration into the chromosome via homologous recombination [30,31]. An additional implication of our result is that the induction of double-strand breaks and hence recombination by antibiotics, can increase genetic diversity and enhance the risk for antimicrobial resistance. It is not a coincidence that two different soil bacteria produce bleomycin and mitomycin C [19] [20]. There are some other antibiotics, such as novobiocin and nitrofurantoin, which damage DNA and are produced by soil microorganisms [32]; pointing to the fact that this could be a ubiquitous phenomenon in environmental scenarios. Our study also reinforces the idea of controlling the pollution with other DNA damaging antibiotics such as fluoroquinolones that are extremely stable and have been shown to accumulate in the environment [33].

Taken together, our results provide strong evidence that antibiotics that induce DSBs can increase the basal level of recombination in *E. coli*. This conclusion answers our central question on how ciprofloxacin could increase the recombination rate, at least in *E. coli*. Ciprofloxacin is a broad-spectrum antibiotic with the exact killing mechanism across diverse bacterial species [7,34]. Because the three antibiotics in the focus of this work are broad-spectrum and produce similar damage in other bacterial species, we can foresee these results being potentially extendable to other microbes.

Our results might have some implications for cancer. Since bleomycin and mitomycin C are frequently used as antitumor drugs [29,35], it would be worthwhile to explore if our results apply to eukaryotic cells [36]. Future work will

uncover whether an increase in recombination rate is also possible in tumour cells, leading to chemotherapy resistance.
[37]

Our results also have implications for bacterial evolution. It has been shown that recombination facilitates adaptive evolution, particularly in fluctuating environments that require complex genomic changes [38]. For example, work in *E. coli* has shown that under doxycycline and erythromycin, costly adaptive evolution is facilitated by recombination [37]. Recent work in *pneumococci* demonstrated the importance of interspecies recombination in acquiring resistance against two antibiotics, tetracycline and macrolides [39]. Moreover, bleomycin and mitomycin C are two of many natural antibiotics produced by soil bacteria [20,40]. Therefore, bacteria in the soil are exposed to different dosages of several natural and synthetic antibiotics that can induce recombination and DSBs.

Recombination in bacteria occurs via two major mechanisms. On the one hand, an allelic exchange can buffer genetic variability within bacterial populations as gene exchange mediates selective sweeps. On the other hand, incorporating new genes via horizontal gene transfer leads to an increase in the size of the genome [41]. Horizontal gene transfer, which frequently entails recombination events, is additionally a significant contributor to antibiotic resistance [8]. Given the role of recombination in antibiotic resistance evolution, increasing recombination rates by administering drugs like those studied here, will almost certainly accelerate resistance emergence.

Material and methods

Bacteria and growth conditions. The main bacterial strain in this study was the *Escherichia coli* MG1655 transformed with the plasmid pRHOMO recombination system. We also used a *recA* deficient derivative of MG1655 [7]. To determine the impact of antimicrobials on the generation of DSBs, the *E. coli* strain SMR14354 was used [20]. The *E. coli* strain DH-5 alpha [*fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*] was used to maintain the plasmid of recombination system pRHOMO [11]. All strains were routinely cultured in Lysogeny Broth (LB medium, Carl Roth, Germany), supplemented with antibiotics when appropriate. For solid cultures, we added bacteriological agar to LB at a final concentration of 1.6 %.

Minimal Inhibitory Concentration. The minimal inhibitory concentration (MIC) was performed by broth microdilution methods according to EUCAST guidelines, except that LB medium was used instead of Mueller-Hinton. The antibiotics used were ciprofloxacin, mitomycin C, bleomycin, trimethoprim, streptomycin, kanamycin, chloramphenicol, ceftazidime, cefotaxime, fosfomycin and rifampicin.

Recombination frequency. A genetic assay was carried out as previously described to estimate the impact of antimicrobials on recombination frequency [11]. The system consisted of a plasmid harbouring two truncated *tetA* alleles separated by an antibiotic resistance cassette (*aacC1*, conferring gentamicin resistance). Recombination restores the functional *tetA* gene, thereby conferring tetracycline resistance, which can be selected for [7,11]. Thus, this assay allows quantification of the recombination frequency with high sensitivity due to the elevated copy number of the plasmid. We exposed 1 ml of culture containing approximately 1-2x10^7 bacteria [dilution of 1/10 of mid-exponential culture containing 1x10^8 colony-forming units per millilitre (CFU/ml)] to different concentrations of different antimicrobials (to match for all of them the MIC) during three hours similarly to a previously described experiment [6]. After incubation with gentle shaking, we added 9 ml of fresh LB to the cultures and centrifuged at 4000 x g for 10 minutes to reduce and remove the antimicrobials. The pellets were washed twice with 2 ml of LB, and cells were allowed to recover for one hour before adding ampicillin (to maintain the plasmid) to a final concentration of 50 µg/ml and incubated overnight with shaking at 37°C. The next day, appropriate dilutions were plated in LB agar containing 50 µg/ml of ampicillin to estimate the viability and LB agar containing 30 µg/ml of tetracycline to estimate the number of recombinants. Each experiment consisted of five replicas and was repeated twice. Recombinant frequencies were expressed as the ratio of means of recombinant number by the number of viable bacteria.

Influence of antimicrobials in the generation of double-strand breaks. The strain SMR14354 was used to measure the number of double-strand breaks when the strain is incubated with different concentrations of tested antimicrobials. This strain carries a reporter fusion of the protein Gam, able to detect double DNA ends and a green fluorescent protein (GFP). The strain also harbours a unique I-*SceI*

restriction site in the chromosome and carries the gene for the restriction enzyme *SceI* whose expression is regulated by an arabinose inducible promoter [20]. Mid exponential cultures containing $0.5x10^8$ CFU/ml were exposed to different concentrations of selected antibiotics for four hours. Then, 5 µl drops of the cultures were added to LB agar blocks containing 1 µM Propidium Iodide (PI, Sigma, Germany) and the covered bacterial area of the blocks were placed on a circular bottom glass open cultivation system II (Pecom, Germany). This device consists of a metallic holder of metal, 0.17 mm round bottom glass and a glass lid that limits evaporation. The device was placed on a robotic inverted microscope Nikon Eclipse T2 (Nikon, Japan). Several fields of each sample were taken automatically using the red and green channels to capture the signal of PI (as viability criteria) and the signal of Gam-GFP fusion to detect DSBs, respectively. Three independent overnight cultures were analysed per antimicrobial per concentration.

Influence of iron on the level of double strand-beak and recombination frequency of bleomycin treated cells. Double-strand breaks induced by bleomycin depends on intracellular iron concentration [19]. To understand the relationship between double-strand breaks and recombination frequency, bleomycin treated cells were subjected to an iron depletion. The *E. coli* MG1655 harbouring the pRHOMO construct cultures treated with 1/2 MIC of bleomycin alone and bleomycin plus 2-2′ bipyridyl (50 µM) were examined for changes in recombination rate and the number of double-strand breaks determined as described elsewhere in this section.

Statistical analysis. To compare experimental groups with their control, a Welch test was performed. To avoid biases introduced by multiple corrections due to comparison against control, each antimicrobial experiment had its non-treated control. In addition, covariance analysis was carried out to estimate the co-occurrence of double-strand breaks and increase of recombination rate induced by different antibiotics. P-values less than or equal to 0.05 were considered statistically significant. Statistical tests and graph plotting were performed with R [42] and GraphPad Prism version 9.00 (GraphPad Software, Inc., San Diego, CA) as indicated.

Funding. ARR, AN and JR were supported SFB 973 (Deutsche Forschungsgemeinschaft), project C5).

Acknowledgements

We are especially grateful to Sophie Armitage (Freie Universität Berlin) for critical comments and proofreading. We are also thankful to Jesús Blázquez and Jerónimo Rodríguez-Beltrán from the Spanish National Centre for Biotechnology (Spanish National Research Council, CSIC), for providing us with the construct to determine recombination frequency in *E. coli* and Susan M. Rosenberg from Baylor College of Medicine (Texas, USA) for the strain of *E. coli* SMR14354 to determine double-strand breaks visually. We are grateful to Dr. Rees Kassen (University of Ottawa) for a scientific discussion about recombination in bacteria and antibiotics, which allowed us to develop the central hypothesis of the current study.

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Supplementary material

Table S1. Minimal inhibitory concentrations (MIC) values for *E. coli* MG1655 for different antimicrobials used in this work.

Antibiotics	**MIC (µg/ml)**
bleomycin	4
cefotaxime	0.125
ceftazidime	0.25
chloramphenicol	2
ciprofloxacin	0.125
fosfomycin	2
kanamycin	1
mitomycin C	2
rifampicin	4
streptomycin	4
trimethoprim	1