Molecular Microbiology (2007) **64**(1), 83–93 doi:10.1111/j.1365-2958.2007.05642.x

Antibiotic-mediated recombination: ciprofloxacin stimulates SOS-independent recombination of divergent sequences in *Escherichia coli*

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Summary

The widespread use and abuse of antibiotics as therapeutic agents has produced a major challenge for bacteria, leading to the selection and spread of antibiotic resistant variants. However, antibiotics do not seem to be mere selectors of these variants. Here we show that the fluoroquinolone antibiotic ciprofloxacin, an inhibitor of type II DNA topoisomerases, stimulates intrachromosomal recombination of DNA sequences. The stimulation of recombination between divergent sequences occurs via either the RecBCD or RecFOR pathways and is, surprisingly, independent of SOS induction. Additionally, this stimulation also occurs in a hyperrecombinogenic mismatch repair *mutS* mutant. It is worth noting that ciprofloxacin also stimulates the conjugational recombination of an antibiotic resistance gene. Finally, we demonstrate that *Escherichia coli* is able to recover from treatments with recombination-stimulating concentrations of the antibiotic. Thus, fluoroquinolones can increase genetic variation by the stimulation of the recombinogenic capability of treated bacteria (via an SOS-independent mechanism) and consequently may favour the acquisition, evolution and spread of antibiotic resistance determinants.

Introduction

Bacteria must be capable of adapting to the ever-changing environment in order to successfully colonize ecological niches. Three major natural strategies are involved in the spontaneous generation of genetic variability in bacteria giving rise to diverse populations where the fittest variants are selected. These strategies are: (i) small local changes in the nucleotide sequence of the genome, (ii) intragenomic reshuffling of genomic sequences and (iii) the acquisition of DNA sequences from other organisms via horizontal gene transfer (HGT).

DNA damage is one of the most common types of stress in nature. It triggers the SOS response which involves the induction of *recA* transcription. Contact with single-stranded DNA (ss-DNA) activates the coprotease activity of the RecA protein promoting the self-cleavage of LexA, the SOS transcriptional repressor, leading to the SOS response (Little *et al*., 1980; Luo *et al*., 2001). The autogenous control of *lexA* transcription supports a cellular response that is exquisitely proportional to the DNA damage level and prevents false triggering of the SOS response (Camas *et al*., 2006). RecA has multiple functions that affect different cellular processes, such as the rescue of stalled replication forks (Lusetti and Cox, 2002; Courcelle and Hanawalt, 2003) and coprotease action involved in the autocleavage of the LexA and UmuD proteins needed for both SOS induction and the formation of an active error-prone DNA polymerase V respectively. Finally, mutagenesis increases as a result of SOS induction (Friedberg *et al*., 2005). It has been stated that some antibiotics, such as fluoroquinolones, increase the frequency of mutants by inducing the SOS response in bacteria (Ysern *et al*., 1990). Consequently, the phenomenon of SOS mutagenesis may also influence the appearance of antibiotic resistant bacteria (Cirz *et al*., 2005; Cirz and Romesberg, 2006).

Another function of RecA is to promote homologous recombination between complementary DNA strands (Kowalczykowski *et al*., 1994) which is crucial for the survival and evolution of bacterial cells. Intragenomic recombination helps to repair collapsed replication forks (Michel *et al*., 2004) and may also produce gene or operon rearrangements. The recombination of divergent yet related sequences present in the same chromosome may produce short cuts in the development of novel activities. In addition, the strategy of acquiring novel DNA sequences by HGT allows microorganisms to share the evolutionary success of others. This strategy is very

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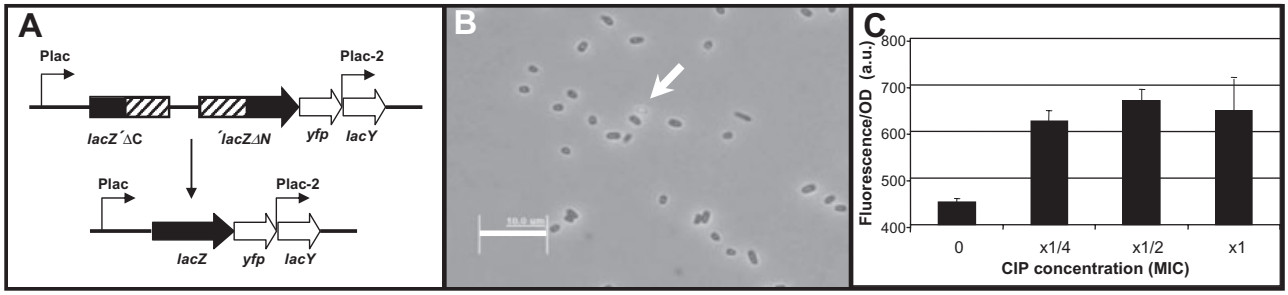

Fig. 1. A. Schematic representation of the Lac region in the strains used to detect recombination events. *lacZ′ΔC* and *′lacZΔN* (black boxes) are two non-functional *lacZ* alleles with C-terminal or N-terminal deletion respectively. The two alleles share an overlapping DNA region of 1.3 kb (dashed boxes) and are separated by a 569 bp region. One recombination event between the two *lacZ* alleles restores a functional *lacZ* gene. The two *lacZ* alleles are either 100% identical in strain ME12 or 96% identical in strain ME12C. The genes *yfp*, encoding a yellow fluorescent protein, and *lacY,* encoding the lactose permease LacY, are shown as white boxes. The vertical lines with an arrow indicate the position and direction of transcription from *lac* promoters (P*lac*).
B. In addition to the production of Lac+ colonies (used in this work), recombinant cells (indicated by an arrow) can be visualized as bright cells under fluorescence microscopy.
C. Effect of CIP treatment on homologous recombination as detected by yellow fluorescence. For details see text.

efficient and can result in essential novel abilities in a single step. However, to provide sufficient novelty the incoming DNA must unavoidably be different (i.e. divergent). Homologous recombination *in vivo* relies upon the nearly perfect homology between the two complementary DNA strands and on the activity of specialized proteins (Petit *et al*., 1991). As sequence divergence increases, the frequency of recombination decreases exponentially (Matic *et al*., 1995; 2000). Apart from plasmids which can replicate autonomously and transposable elements that do not require homologous recombination to be installed in the chromosome, the horizontally transferred DNA must integrate in the bacterial genome via recombination. Recombination of divergent sequences is thus a major driving force in bacterial evolution (Guttman and Dykhuizen, 1994; Spratt *et al*., 2001).

It has been described that the antibiotic-induced SOS response promotes the transfer of pathogenicity island-encoded virulence factors (Ubeda *et al*., 2005) and the mobilization of integrating conjugative elements (ICEs) in *Vibrio cholerae* (Beaber *et al*., 2004), suggesting that mobile genetic elements respond to DNA damage by regulating their escape from damaged bacterial hosts. A recent report has demonstrated that fluoroquinolones induce DNA transformation, via competence, in the naturally competent *Streptococcus penumoniae* (Prudhomme *et al*., 2006). Fluoroquinolones are broad-spectrum antibiotics that block DNA replication by trapping DNA gyrase and DNA topoisomerase IV on DNA (Drlica and Hooper, 2003). Because this blockage induces the SOS response, it has been assumed that fluoroquinolones may act as promoters of homologous recombination in treated bacteria. However, to date, this is an unverified assumption and little or nothing is known about the effect of fluoroquinolone on genetic recombination of identical and divergent sequences. Moreover, there is no knowledge as to whether the stimulation of recombination, should this actually happen, relies upon SOS induction.

In this work we have explored whether (i) ciprofloxacin (CIP), a fluoroquinolone, affects homologous recombination of both identical and divergent DNA sequences, (ii) the induction of the SOS response is necessary for this effect and, if not, which molecular performers are responsible for this CIP-mediated effect, and (iii) cells can recover from the CIP challenge and subsequently benefit from this stress.

Results

Ciprofloxacin stimulates intrachromosomal genetic recombination of both identical and divergent DNA sequences

The SOS response is induced as a result of CIP activity on type-II DNA-topoisomerases (gyrase and topoisomerase IV) (Ysern *et al*., 1990; Maxwell and Critchlow, 1998). Therefore, we verified the effect of different CIP concentrations on *recA* transcription in the strain MG1655-Nal^R harbouring the pSC101-P*recA*::GFP plasmid (Ronen *et al*., 2002). This low copy number plasmid contains a *gfp* gene located downstream of the *recA* promoter. The level of *recA* transcription increased consistently upon CIP treatment (not shown).

To study the specific effect of CIP on homologous recombination we used a genetic assay that measures intrachromosomal recombination between two non-functional *lacZ* alleles, sharing an overlapping region of 1.3 kb. These alleles are separated by an unrelated region 569 bp long (Fig. 1A) (M. Elez and I. Matic, manuscript submitted). When recombination occurs, a functional *lacZ* gene is reconstructed and the resulting recombinant cells do acquire the ability to grow on

Table 1. Effect of CIP on recombination frequencies between identical and divergent repeats in different genetic backgrounds.

Genotype	Recombination frequencies (mean ± SD)	Fold difference[a]	N	MIC of CIP (μg ml^{-1})	Increase with CIP[b] (1/2 × MIC) ± SD	N
ME12 (100% identical)	$6.9 \times 10^{-3} \pm 2.0 \times 10^{-3}$	–	12	0.16	4.7 ± 0.35	6
ME12C (96% identical)	$6.2 \times 10^{-6} \pm 2.2 \times 10^{-6}$	1.00	12	0.16	5.6 ± 1.31	6
ME12C *recA*	$3.9 \times 10^{-7} \pm 2.0 \times 10^{-7}$	0.06	8	0.016	1.0 ± 0.49	3
ME12C *lexA1*	$5.3 \times 10^{-6} \pm 2.2 \times 10^{-6}$	0.85	12	0.016	4.4 ± 0.94	9
ME12C *mutS*	$3.8 \times 10^{-4} \pm 7.6 \times 10^{-5}$	61.17	6	0.16	5.0 ± 2.00	6
ME12C *recB*	$1.5 \times 10^{-6} \pm 6.1 \times 10^{-7}$	0.24	6	0.016	3.7 ± 0.82	6
ME12C *recF*	$2.2 \times 10^{-6} \pm 2.0 \times 10^{-7}$	0.36	6	0.16	7.1 ± 1.81	6
ME12C *recBrecF*	$5.1 \times 10^{-7} \pm 1.5 \times 10^{-7}$	0.08	6	0.016	1.2 ± 0.34	6

a. Fold difference indicates the increase/decrease with respect to the recombination frequency of the strain ME12C.
b. Increase with CIP refers to the increase in recombination with respect to that obtained without antibiotic challenge.
N, number of independent experiments.

minimal medium plates containing lactose as the sole carbon source. Thus, the production of Lac+ colonies indicates the number of recombination events. The presence of the *yfp* gene downstream of *lacZ* allowed us to follow the recombination events directly via the expression of the yellow fluorescent protein (YFP). When the *lacZ* gene is reconstructed by recombination the *yfp* expression increases (Fig. 1B). By measuring the change in fluorescence of the CIP-treated cultures we detected that CIP enhances the number of recombination events proportionally to its concentration (Fig. 1C).

Studies on the effect of CIP on genetic recombination should consider the fact that SOS response leads to impaired cell septation and the production of filaments (see below and Fig. 3). Filamentation reduces the number of colony-forming units while maintaining the number of putative recombinogenic nucleoids, thus leading to an over estimation of the recombination frequency. In our experiments, after the antibiotic-challenging period, the antibiotic is eliminated and the cells are allowed to recover in fresh broth before plating for viables and recombinants. In this way, filaments are resolved forming single cells from each nucleoid.

Appropriate dilutions of treated and recovered cultures were plated onto minimal M9-Lac agar. We found that CIP promoted recombination between identical DNA sequences at concentrations close to the CIP MIC (from 0.02 to 0.08 μg ml^{-1}), with the highest increase (4.7-fold) upon treatment with 1/2 × MIC of CIP (Table 1).

As stated before, recombination of divergent sequences (already carrying novel functions) is one of the main sources of genetic innovation. Therefore, we decided to study the effect of CIP on the recombination of divergent DNA sequences. For this purpose we used a derivative of the genetic system described above, in which the sequences of the two *lacZ* alleles are 4% divergent (strain ME12C). Cultures of ME12C were treated with CIP as previously described for ME12. CIP also promoted recombination between divergent DNA sequences with the highest effect (5.6-fold increase) at a concentration of 1/2 × MIC (Table 1). To study the effect of RecA on this stimulation of recombination the response of the ME12C *recA* strain was investigated. The MIC of CIP for this strain is 0.016 μg ml^{-1} (10-fold below that of the wild-type strain ME12C) (Table 1). Thus, the recombination experiments were performed with different concentrations of CIP (doubling concentrations from 0.002 to 0.064 μg ml^{-1}). None of these concentrations produced a significant increase in recombination (data not shown) including 1/2 × MIC (Table 1). Therefore, as expected, the effect of CIP on recombination depends on RecA activity. It is also interesting to note that in the absence of antibiotic challenge 94% of recombinational events producing functional *lacZ* genes are *recA*-dependent (Table 1), while the remaining deletions (6%) are *recA*-independent, probably produced via DNA-polymerase slippage.

The SOS induction is not required for the CIP-mediated stimulation of genetic recombination

As stated above, one consequence of SOS induction is an elevated concentration of the RecA protein. This could be the main cause of the observed increase in recombination (Matic *et al*., 1995; Delmas and Matic, 2005; Lanzov *et al*., 2005). To know whether this CIP-mediated stimulation of recombination requires the SOS induction, we performed the experiments with the ME12C *lexA*1(ind$^-$) strain. The *lexA*1(ind$^-$) allele carries a mutant cleavage-resistant LexA protein that prevents the induction of the SOS response. At a concentration of 1/2 × MIC of CIP (and also other concentrations; not shown) prevention of SOS induction has a small effect on homologous recombination. The CIP-mediated increase in recombination was 4.4-fold in the *lexA*1(ind$^-$) strain (Table 1), thereby clearly indicating that SOS response is unnecessary for the CIP-mediated stimulation of recombination

between divergent DNA sequences. The slight difference in the CIP-mediated increase between the wild type and the *lexA*1(ind$^-$) variant (5.6 vs. 4.4 respectively) may indicate a small effect due to the induction of the SOS response, because an elevated RecA dose can produce a further increase in homologous recombination (Matic *et al*., 1995).

CIP-dependent stimulation of recombination occurs via either the RecBCD or RecFOR pathways

It has been stated that quinolones induce the SOS response via a mechanism requiring the RecBCD nuclease/helicase (Newmark *et al*., 2005). In addition, RecA/RecBC-mediated recombination seems to be important for cell viability in the presence of CIP (Cirz *et al*., 2005). To study the specific requirements of CIP-mediated stimulation of homologous recombination, we performed the induction experiments with the mutant strains ME12C *recB*, ME12C *recF* and the double mutant ME12C *recB recF*. In the absence of CIP, the inactivation of either *recB* or *recF* reduces homologous recombination between 4% divergent sequences (Table 1). This result is in accordance with other authors (Kowalczykowski *et al*., 1994; M. Elez and I. Matic, manuscript submitted). These single *recB* or *recF* mutations have apparently no effect on the increased recombination of 4% divergent DNA sequences produced by CIP (Table 1). The slight difference in the recombination increase observed in the *recB* background with respect to the wild type (3.7 vs. 5.6), as in the case of the *lexA*1 mutant, may be due to the fact that SOS induction does not occur in this background (both LexA and RecB are needed for the CIP-mediated induction) (Newmark *et al*., 2005). Although this induction is not essential, an increase in RecA concentration may be a plus. Moreover, CIP does not increase recombination in the *recB recF* double mutant (Table 1). All together these results strongly suggest that CIP-induced recombination of divergent DNA sequences may follow either the RecBCD or RecFOR recombination pathways.

CIP-mediated induction of recombination is not affected by the mismatch repair system

The mismatch repair system (MMR) inhibits recombination between non-identical DNA sequences (Rayssiguier *et al*., 1989; Matic *et al*., 1995). Therefore, we tested the effect of MutS on the CIP-induced recombination events. The inactivation of *mutS* increased the basal rate of recombination events 61-fold (Table 1). However, the absence of an active MMR system (strain ME12C *mutS*) does not impede an additional fivefold increase in recombination frequency (Table 1). This is a particularly significant result because it indicates that recombination frequency can be further increased, even in a stable hypermutator such as a *mutS* strain. Specifically, there is a 300-fold increase in recombination frequency versus that of the untreated wild type (ME12C) when the *mutS* mutant is treated with CIP.

Ciprofloxacin stimulates the conjugational recombination of an antibiotic resistance gene but not conjugation

We have shown that CIP increases the frequency of intrachromosomal recombination of both identical and divergent sequences. However, HGT is a major mechanism of diversification and antibiotic resistance acquisition in prokaryotes (see, for instance, De la Cruz and Davies, 2000 and references therein). To know whether CIP may also stimulate conjugational (inter-chromosomal) recombination and recombinational transfer of antibiotic resistance genes, we studied both identical and divergent conjugational recombination. Conjugations between two *Escherichia coli* strains (identical sequences) and between *E. coli* K12 and *Shigella flexneri* (approximately 5% divergence) (Vulic *et al*., 1997) were performed. For conjugational recombination between identical sequences, the recipient strain MG1655 Nal^R was treated for 4 h at different CIP concentrations. The treated recipient and the untreated donor strain *E. coli* ELE-1 [P4X Hfr (Δ*fhuD::*Kan)] were allowed to mate for 1 h. Increases in the frequency of recombination were observed at concentrations close to the MIC of CIP for the strain MG1655 Nal^R (Fig. 2A). This effect was not the result of an enhanced efficiency of the conjugative transfer, because the F′episome transfer (from *E. coli* MG1655 F′::Tn*10*) did not increase with the CIP treatment (Fig. 2B). A similar experiment was performed to study the conjugational recombination between divergent sequences, except that the recipient strain in this case was the *S. flexneri* strain MM1-Nal^R. The treated recipient and donor strain *E. coli* ELE-1 [P4X Hfr (Δ*fhuD::*Kan)] were mixed for 1 h. As in the case of recombination of identical sequences, the frequency of recombination but not of conjugation increased when the recipient strain was treated with CIP concentrations close to the MIC (Fig. 2A and B).

Thus, our results indicate that antibiotic treatment of the receptor strain also stimulates HGT by increasing DNA recombination in the recipient bacteria.

Biological relevance of responses to CIP

To gain insight on the biological relevance of the stimulatory effect of CIP on recombination we used different methods: DAPI staining, Live/Dead staining and viability measurement after CIP treatment, to analyse the continuity of DNA replication, the integrity of the cell wall during

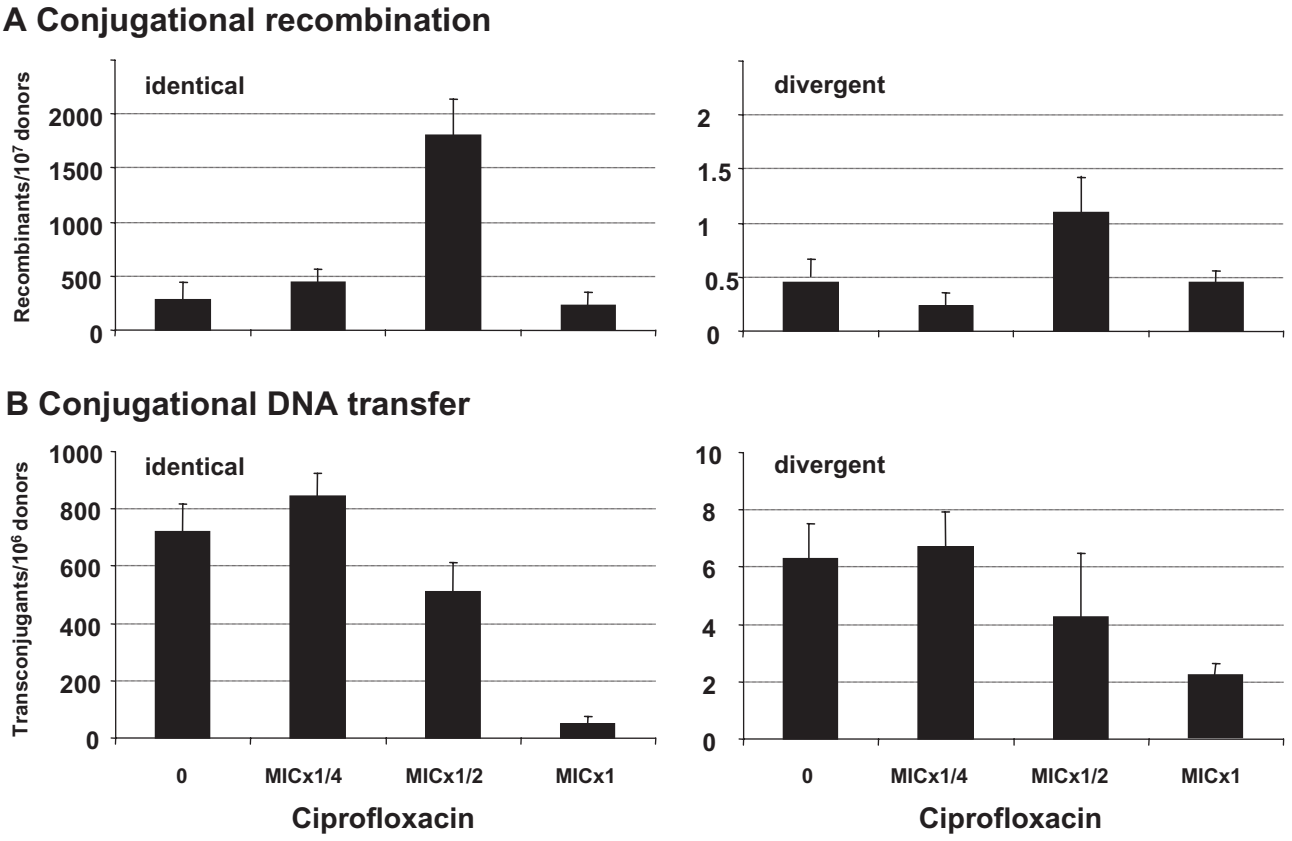

Fig. 2. A. Frequency of conjugational recombination after treatment of the recipient strains, MG1655 Nal^R (identical sequences) and *S. flexneri* (divergent sequences), for 4 h at different CIP concentrations and mating with donor strain *E. coli* ELE-1 (P4X Hfr (Δ*fhuD::kan*).
B. No increased conjugational transfer was observed with CIP pretreatment of both recipient strains. The donor was MG1655 Rif^R F′::Tn*10* [F′ *proAB lacI*^{q}ZΔ*M15* Tn*10* (Tet^R)] and the recipients were MG1655 Nal^R (identical sequences) and *S. flexneri* MM1Nal^R (divergent sequences). Error bars: standard deviation.

CIP treatment and the capacity to recover after the CIP challenge. As expected, *E. coli* cells treated with CIP formed large filaments (Fig. 3A) and these filaments contained multiple nucleoids, as seen by DAPI staining (Fig. 3B), indicating that cell growth also involved chromosome replication. Treated cells were stained with the BacLight Live/Dead dyes showing that, according to generally accepted criteria, many of them were alive (i.e. they conserved membrane integrity) even after a 4 h treatment with CIP (Fig. 3C). CIP-treated cells retained their capacity to recover after the CIP challenge since once the antibiotic disappeared the filaments were resolved giving rise to single cells (Fig. 3D). Interestingly, filamentation was induced by CIP even when the treated strain lacked the *sulA* gene, indicating that SulA is not the only product able to inhibit cell septation (data not shown). Our results are in accordance with those published previously (Blázquez *et al*., 2006) where filaments of *Pseudomonas aeruginosa* treated with ceftazidime, a PBP3 inhibitor also inducing the SOS response, are resolved originating a number of single cells from the filaments, once the antibiotic disappears.

Finally, Fig. 4 shows that cells (ME12C, ME12C *recA* and ME12C *lexA*1) treated with 1/4 × MIC and 1/2 × MIC of CIP could recover viability at a similar level to the untreated controls after overnight growth in CIP-free medium. When cells were challenged with concentrations of 1 × MIC the number of viable failed to reach that of the untreated control after the incubation period in fresh Luria–Bertani (LB) (Fig. 4). When higher concentrations of CIP were used, viability experienced a sudden decline (not shown).

Discussion

The extended use of antibiotics over the past six decades has caused a major impact, leading to the selection and spread of resistant bacteria. The adaptation of bacterial pathogens to antibiotics is one of the most rapid and striking phenomena of biological evolution generated by mankind, thus paradoxically, antibiotic resistance may be regarded as the outcome of the success of antibiotic therapy.

Seminal studies have demonstrated that the exposure of bacteria to antibacterial agents results in the selection

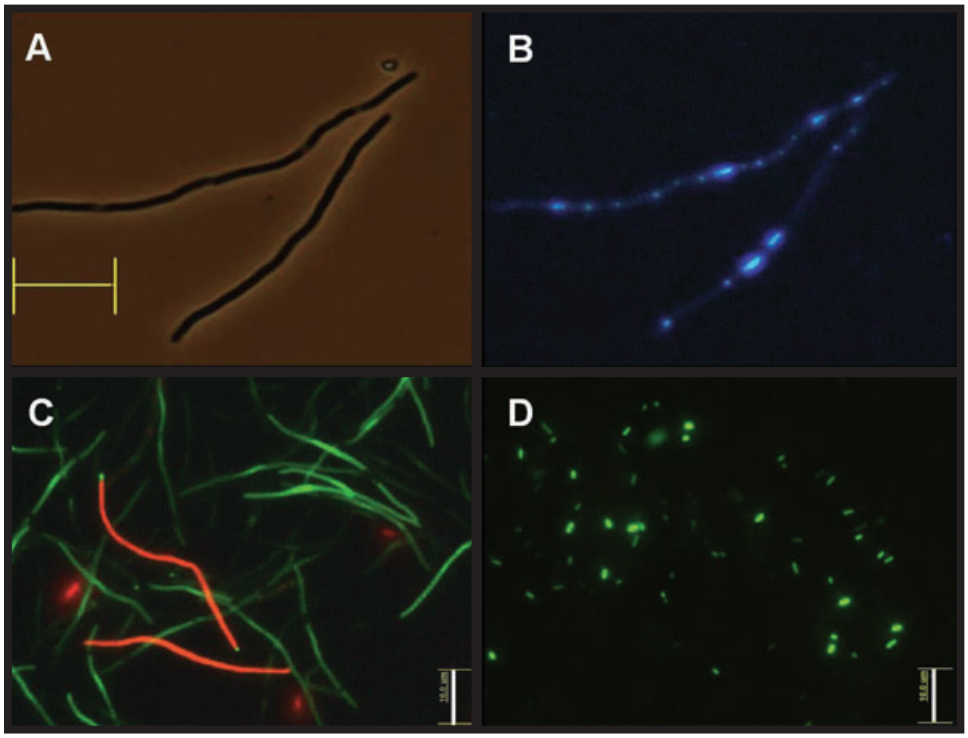

Fig. 3. Visualization of ME12 cells treated with 0.08 μg ml^{-1} (1/2 × MIC) of CIP for 4 h.
A. Typical cell filaments produced by CIP treatment.
B. DAPI staining of the same cell filaments where multiple nucleoids can be observed.
C. Live/dead staining of filaments after 4 h of treatment. Green and red staining indicates undamaged and damaged membrane, respectively (see *Experimental procedures* for details).
D. Filaments produce single cells after overnight growth in fresh LB medium. Scale: 10 μm.

of pre-existing resistant variants that ultimately become fixed in the population (Luria and Delbrück, 1943; Newcombe, 1949; Lederberg and Lederberg, 1952). Thus, for a long time it has been assumed that antibiotic treatments select for pre-existing 'lucky' antibiotic resistant variants. However, some studies have raised doubts as to whether bacteria are simply passive subjects along their process of evolution by mutation and natural selection (Rosenberg, 2001; Blázquez, 2003; Caporale, 2003). For instance, it has been described that fluoroquinolone antibiotics, by means of SOS induction, may stimulate two major bacterial strategies to produce genetic variation: small local changes in the nucleotide sequence (mutations) and the horizontal transfer of DNA sequences (Ysern *et al*., 1990; Beaber *et al*., 2004; Cirz *et al*., 2005; Ubeda *et al*., 2005). Interestingly, while this manuscript was being written, a report was published demonstrating that stress produced by fluoroquinolones induces transformability via competence in *S. penumoniae*, a naturally competent bacteria (Prudhomme *et al*., 2006). Antibiotics may also select for cells with increased frequency of mutation and recombination (hypermutators) (Mao *et al*., 1997). Therefore, the possibility of some antibiotics stimulating bacterial mutation may depict a disturbing prospect for antibiotic resistance induction and spread (Ysern *et al*., 1990; Power and Phillips, 1993; Pérez-Capilla *et al*., 2005; Blázquez *et al*., 2006).

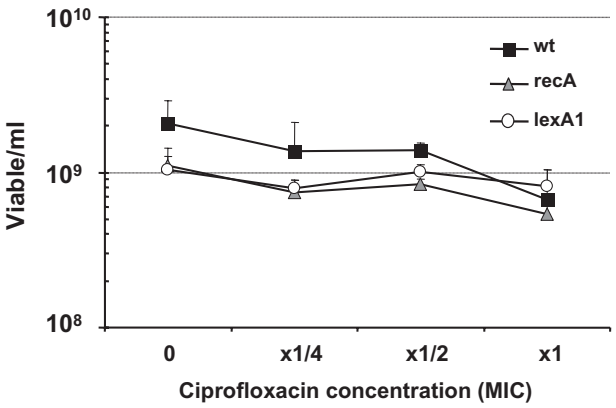

Fig. 4. Viability of ME12C (black squares) and its recA (grey triangles) and lexA1 (white circles) derivatives after 4 h of treatment at different concentrations of CIP and further overnight growth in fresh medium. Viable cells per millilitre of culture were calculated by plating appropriate dilutions on LB-agar plates and subsequently counting the number of colonies. Data are the mean values (±standard deviation) of at least three independent experiments.

In addition to mutation, HGT is considered the major diversification mechanism in prokaryotes (Guttman and Dykhuizen, 1994; Lawrence and Roth, 1996; Lawrence and Ochman, 1998). The impact of homologous and divergent recombination on bacterial evolution is evident (Groisman and Ochman, 1996; Katz, 1999; Ochman *et al*., 2000; Woese, 2000; Spratt *et al*., 2001). Recombination probably mediates genetic variation in all bacterial species and has possibly been crucial to allowing bacteria to evade immune response, distributing genes that increase virulence and providing increased resistance to antibiotics (De la Cruz and Davies, 2000). For instance, it has been estimated that any single nucleotide change is about 50 times more likely to have occurred by recombination (with a partner carrying such a nucleotide change) than by a *de novo* mutation for *E. coli* in nature (Guttman and Dykhuizen, 1994).

Because fluoroquinolones are known to induce SOS and produce double-strand breaks in DNA (Kreuzer, 2005) and because a higher level of RecA produces increased recombination frequency between divergent sequences (Dimpfl and Echols, 1989; Matic *et al*., 1995), it wouldn't be too surprising to find CIP stimulating recombination. In fact, it has been suggested that the prevention of LexA cleavage during antibiotic treatment may render bacteria unable to develop antibiotic resistance (Cirz *et al*., 2005). Here we show that, as expected, CIP also stimulates the intra- and inter-chromosomal recombinogenic ability of the challenged organism, even in bacteria without natural competence. Indeed, the recombination frequency of both identical and divergent DNA molecules is stimulated by CIP. However, the most striking result obtained in this work is that CIP-stimulated recombination does not depend on SOS induction, as this stimulation occurs in a *lexA*1 background. Thus, according to the results presented herewith, it appears to be more convenient to inhibit RecA rather than LexA to prevent antibiotic resistance development and spread.

The result indicating that the high rate of recombination can be further increased by CIP in an MMR-deficient mutant is of particular interest, because MMR-deficient strains are stable hypermutators present in natural environments at high frequency. These strains also have increased recombination rates between divergent sequences (Leclerc *et al*., 1996; Matic *et al*., 1997; Oliver *et al*., 2000; 2002; Bjorkholm *et al*., 2001; Richardson and Stojiljkovic, 2001; Watson *et al*., 2004). The stimulation of recombination can confer an additional advantage to the treated hypermutators.

The data obtained in *recB*, *recF* and double *recB recF* backgrounds indicate that this stimulation can occur via either the RecBCD or RecFOR pathways. Our results also suggest that at low doses of CIP there are few DNA lesions and that the constitutive concentration of Rec proteins can easily deal with them. This may be biologically relevant because the constitutive concentration of Rec proteins can help the cells to survive certain types of damage without requiring new protein synthesis via SOS triggering. In fact, this is one of the properties conferred by the autogenous regulation of LexA (Camas *et al*., 2006). This may be particularly important under conditions where the transcription needed for SOS induction is also inhibited, as can be expected upon CIP challenge (Willmott *et al*., 1994).

Finally, our results indicate that bacteria can survive long enough at considerable antibiotic doses to produce genomic rearrangements and, should the antibiotic be eliminated or sufficiently reduced, to take advantage of this stress situation.

As concerns the clinical use of antibiotics, the stimulatory effect of fluoroquinolones on genetic mutation, transfer and recombination may be considered too modest to exert any effect on bacterial evolution. However, in Biology small differences sometimes draw a fine dividing line between failure and success. For instance, modest changes in mutation frequency may greatly influence antibiotic resistance development (Denamur *et al*., 2005). Likewise, modest increases in recombination frequency may have similar effects. The problem may reach new dimensions when considering the vast amounts of bacteria challenged by antibiotic treatments. Although antibiotics are mainly used to combat pathogens they also challenge commensals collaterally. While an infection is usually produced by a relatively small number of cells (10^8–10^9), about 10^{14} prokaryotic cells belonging to hundreds of different species conform our commensal flora (Andremont, 2003) and these species have different intrinsic levels of antibiotic susceptibility (Zhanel *et al*., 2002). Likewise, variability of intraspecific susceptibility can be expected. Furthermore, due to different factors, a huge diversity of spatial and temporal antibiotic concentration gradients may occur in the human body (Baquero *et al*., 1998). Thus, any particular window of sub-MIC recombination-stimulating concentrations of fluoroquinolones should not be difficult to find. The fact that thousands of tons of fluoroquinolones are used every year to treat billions of human and veterinary infections and to promote animal growth, increases the probability of finding the suitable conditions for the stimulation of recombination.

The results herewith add another twist to the putative side-effects of antibiotics such as mutagenicity, increased DNA transfer from treated donor bacteria, increased genetic transformability in naturally competent species and now, moreover, the increased genetic recombination of both identical and divergent DNA sequences. An increased intragenomic recombination may accelerate genetic variation because larger evolutive distances can

Table 2. Strains and plasmids.

Strain	Genotype/relevant phenotype	Source/construction
E. coli		
MG1655	Wild type	Laboratory stock
MG1655 Nal^R	Spontaneous Nal^R mutant	This work
MG1655 Rif^R	Spontaneous Rif^R mutant	This work
NEC222 (scavenger)	As MG1655 but Δ*lacZ*::cat	Laboratory stock
JW148	Δ*fhuD*::Kan	NARA Institut[a]
P4X Hfr	Hfr	Delmas and Matic (2005)
Ele1	P4X Hfr *fhuD*::Kan	P1 (JW0148) × P4X
XL1Blue MRF′	*recA1 endA1 gyrA96 thi-1 hsdR17supE44 relA1 lac*$^-$ [F′ *proAB lac*IqZ?*M15* Tn*10* (Tet^R)]	Stratagen, USA
MG1655 Rif^R F′::Tn*10*	[F′ *proAB lac*IqZ?*M15* Tn*10* (Tet^R)]	This work
ME12	MG1655lacZΔT-lacZΔP-yfp	Elez and Matic, submitted
ME12C	ME12 but *lacZΔT* is from *E. coli* CFT073	Elez and Matic, submitted
ME12C *recA*	ME12C but *recA938*::Tn*9*–200	Elez and Matic, submitted
ME12C *lexA*1	ME12C *lexA*1*malB::Tn9*	Elez and Matic, submitted
ME12C *mutS*	ME12C but *mutS::cat*	Elez and Matic, submitted
ME12C *recB*	ME12C *recB::phleo*	Elez and Matic, submitted
ME12C *recF*	ME12C *recF::Tn5*	Elez and Matic, submitted
ME12C *recB recF*	ME12C *recB::phleo recF::Tn5*	Elez and Matic, submitted
Shigella flexneri		
S. flexneri MM1	Clinical isolate	M. Morosini
S. flexneri MM1 Nal^R	Spontaneous Nal^R mutant	This work
Plasmids		
pSC101-Pless::GFP	Kan^R	Ronen *et al.* (2002)
pSC101-*recA*::GFP	Kan^R	Ronen *et al.* (2002)

a. Obtained from Genobase: http://ecoli.naist.jp

be traversed with respect to random mutation. In fact, variants created by recombination have a significantly higher probability of retaining their function than those generated by random mutation (Drummond *et al.*, 2005 and references therein). Fluoroquinolones may also accelerate genetic exchange in treated bacteria and furthermore raise the probability of developing new antibiotic resistance by increasing recombination. For instance, extended-spectrum β-lactamases are the result of combining a reduced number of mutations (Blázquez *et al.*, 1995; 2000). Bacteria producing multiple β-lactamases simultaneously are frequently isolated (Szabo *et al.*, 2005). Thus, fluoroquinolones may accelerate the evolution of new extended-spectrum variants by stimulating the recombination between single-mutants instead of accumulating successive mutations (Crameri *et al.*, 1998).

Nonetheless, despite the above considerations, the dangerous effect of fluoroquinolones on the evolution and spread of antibiotic resistance is still an open question and requires carefully conducted *in vivo* studies to be clearly established.

Experimental procedures

Bacterial strains, plasmids and media

Strains, plasmids and their origins are described in Table 2. MG1655 Rif^R F′::Tn*10* [F′ *proAB lac*IqZΔ*M15* Tn*10* (Tet^R)] was constructed by conjugation with strain XL1Blue MRF (Stratagene) selecting for transfer of the F′::Tn*10* episome with rifampicin and tetracycline. ME12 and ME12C *recA, lexA*1*, recB, recF, recBrecF* and *mutS* derivatives were constructed by P1 transduction from strains harbouring the desired mutations (M. Elez and I. Matic, manuscript submitted). Both *recA*- and *lexA*1 phenotypes were verified by measuring UV sensitivity. LB and minimal M9 with lactose as the sole carbon source were prepared according to Miller (Miller, 1972). MICs of CIP for ME12, ME12C and their mutant derivates, were determined according to NCCLS recommendations (NCCLS, 1999), except that the bacterial inocula were identical to those used in all subsequent recombination, conjugation and mutagenesis experiments. The antibiotics and concentrations used were: nalidixic acid, Nal (40 μg ml^{-1}), kanamycin, Kan (50 μg ml^{-1}), tetracycline, Tet (20 μg ml^{-1}), rifampicin, Rif (100 μg ml^{-1}), and chloramphenicol, Cm (20 μg ml^{-1}). CIP was used at different concentrations (see text). 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (Xgal) and Isopropyl-B-D-Thiogalactopyranoside (IPTG) were used at concentrations of 40 μg ml^{-1} and 1 mM respectively.

Live/dead and DAPI staining

Samples were stained with Live/dead® BacLight bacterial viability kit L-7012 following the supplier's instructions (Molecular Probes) which contains two fluorescent nucleic acid stains of different colours. The SYTO 9, green fluorescent, labels nucleic acids in all cells in a culture, and the red fluorescent nucleic acid stain propidium iodide only penetrates cells with damaged membranes. When both dyes are used, undamaged/living cells will stain green and damaged/dead cells will stain red (the SYTO 9 levels are reduced in

these cells) under the fluorescence microscope. Similarly, cells were stained with DAPI, as described elsewhere.

Measurement of the recA *transcription*

CIP-mediated induction of transcription from the *recA* promoter was measured by using the plasmids pSC101-P*recA*::GFP and pSC101-Pless::GFP, a promoterless GFP vector (Ronen *et al.*, 2002). Strain MG1655 Nal^R containing either pSC101-P*recA*::GFP or pSC101-Pless::GFP reporter plasmids was incubated overnight. Cultures were diluted to an OD_{600} of 0.1 in fresh LB-kanamycin medium on a flat-bottom 96-well plate and incubated for 4 h at 37°C with shaking. Absorbance at 595-nm and fluorescence (filters 485 nm, 535 nm) was measured at 37°C on an Infinite M200 multiwell fluorimeter (Tecan, Switzerland). Background fluorescence of cells bearing a promoterless GFP vector was subtracted from the data of cells harbouring plasmid pSC101-P*recA*::GFP. This avoided the possible undesirable effects of SOS induction such as an increase in plasmid copy number.

Construction of chromosomal LacZ diploid strains

Chromosomal LacZ diploid strains were constructed as described (M. Elez and I. Matic, manuscript submitted). Briefly, two non-functional *lacZ* alleles were cloned in close proximity (569 bp) in the *E. coli* chromosome. The first *lacZ* gene contains a C-terminal deletion (*lacZ′ ΔC*), whereas the second one contains an N-terminal deletion (*′lacZ ΔN*). The two alleles share an overlapping DNA region of 1.3 kb which is 100% (ME12) or 96% (ME12C) identical at the sequence level. The functional *lacZ* gene can be reconstituted by a single recombination event between the two gene fragments cited (Fig. 1A). Because *yfp* insertion in the lactose operon interfered with the *lacY* gene expression, a P*lac* promoter (Plac-2) was introduced in the *yfp/lacY* intervening region in both ME12 and ME12C strains (M. Elez and I. Matic, manuscript submitted). The expression of the YFP was very low, probably due to the presence of the long DNA region between the promoter Plac-1 and the structural *yfp* gene. When the LacZ gene is reconstructed by recombination, the *yfp* expression is increased. Recombinant individual cells can be visualized as bright cells under fluorescence microscopy (Fig. 1B). The time-course of the recombination induction can be followed by measuring the fluorescence of treated cultures.

Recombination experiments

Overnight cultures were diluted to an OD_{600} of 0.1 in fresh LB medium and grown for 1 h at 37°C without shaking. For fluorescence measurements, 150 µl of culture were inoculated in the wells of a 96-microwell plate containing different concentrations of CIP and covered with 50 µl of mineral oil. Plates were incubated at 37°C with agitation and fluorescence (excitation 500 nm and emission 530 nm), and optical density (595 nm) was measured after 4 h of incubation (Infinite M200 fluorimeter).

For the Lac+ recombinants measurement, 2 ml of recipient cells were treated with increasing concentrations of CIP and incubated for 4 h at 37°C with shaking (250 r.p.m.). One millilitre of these cultures was centrifuged 10 min at 6000 r.p.m. and the pellet was resuspended in 2 ml of fresh LB medium and incubated overnight at 37°C with shaking. This step is necessary to resolve the filaments formed after CIP treatment. Scavenger cells were grown to saturation in LB medium with the appropriate antibiotic. The resulting culture was diluted and a new culture started in fresh LB medium. When this culture reached saturation, about 10^8 scavenger cells were washed three times with 10 mM $MgSO_4$ (to clear any contaminating sources of sugar) and were inoculated in 4 ml of soft M9 minimal medium agar containing Xgal and IPTG. Cells were spread on M9 minimal medium agar plates containing Xgal, IPTG and lactose as the sole carbon source. After 1 h at room temperature, appropriate dilutions of the antibiotic-treated cultures, washed with 10 mM $MgSO_4$, were inoculated in 4 ml of soft M9 minimal medium agar containing Xgal and IPTG and spread over the scavenger cells. Plates were incubated 48 h at 37°C. Recombination events were measured as the production of Lac+ colonies. The frequency of recombination was calculated as the number of Lac+ colonies per viable cell.

Conjugation experiments

Experiments with CIP were conducted by diluting overnight cultures of donor and recipient cells to an OD_{600} of 0.1 in fresh LB medium and grown for 1 h at 37°C without shaking. Recipient cells were treated with increasing concentrations of CIP and incubated for 4 h at 37°C with shaking (250 r.p.m.). Cells were washed with 10 mM $MgSO_4$ to eliminate the antibiotic and resuspended in fresh LB. At this stage, appropriate dilutions were plated to perform a viable count. 5×10^7–1×10^8 donor cells (as deduced from previous experiments) were mixed 1:1 with cells of the CIP-treated recipient strain in a tube and incubated for 1 h at 37°C without shaking. The conjugation process was finished with vortexing. After incubation in ice for 10 min, the exconjugants were plated on rich medium agar with Nal and Kan for Hfr crosses and with Nal and Tet for F′::Tn*10* crosses and were incubated overnight at 37°C. For the interspecies (*E. coli* × *S. flexneri* MM1) experiments the process was identical, except that the number of mating cells was increased 10-fold. Conjugation and conjugational recombination frequencies were calculated by dividing the number of exconjugants/recombinants by that of the donors.

Acknowledgements

We thank U. Alon for kindly providing the plasmids pSC101-PrecA::GFP and pSC101-Pless::GFP, M. Morosini for *S. flexneri* MM1, Nara Institut for strain JW148, J. Poyatos, J.M. Gómez-Gómez and J.C. Alonso for useful discussions and Elizabeth Osobliwa for proofreading the manuscript. We are grateful to two anonymous referees for their constructive comments. This work was supported by Grant BFU2004-00879 from the Spanish Ministerio de Educación y Ciencia and by Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Spanish Network for the Research in Infectius Diseases (REIPI RD06/0008) and by Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Spanish Network for the Research in Infectious Diseases (REIPI RD06/0008).

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