Effect of RecA inactivation and detoxification systems on the evolution of ciprofloxacin resistance in Escherichia coli

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Background: Suppression of SOS response and overproduction of reactive oxygen species (ROS) through detoxification system suppression enhance the activity of fluoroquinolones.

Objectives: To evaluate the role of both systems in the evolution of resistance to ciprofloxacin in an isogenic model of Escherichia coli.

Methods: Single-gene deletion mutants of E. coli BW25113 (wild-type) (ΔrecA, ΔkatG, ΔkatE, ΔsodA, ΔsodB), double-gene (ΔrecA-ΔkatG, ΔrecA-ΔkatE, ΔrecA-ΔsodA, ΔrecA-ΔsodB, ΔkatG-ΔkatE, ΔsodB-ΔsodA) and triple-gene (ΔrecA-ΔkatG-ΔkatE) mutants were included. The response to sudden high ciprofloxacin pressure was evaluated by mutant prevention concentration (MPC). The gradual antimicrobial pressure response was evaluated through experimental evolution and antibiotic resistance assays.

Results: For E. coli BW25113 strain, ΔkatE, ΔsodB and ΔsodA/ΔsodB mutants, MPC values were 0.25 mg/L. The ΔkatG, ΔsodA, ΔkatG/ΔkatE and ΔrecA mutants showed 2-fold reductions (0.125 mg/L). The ΔkatG/ΔrecA, ΔkatE/ΔrecA, ΔsodA/ΔrecA, ΔsodB/ΔrecA and ΔkatG/ΔkatE/ΔrecA strains showed 4–8-fold reductions (0.03–0.06 mg/L) relative to the wild-type. Gradual antimicrobial pressure increased growth capacity for ΔsodA and ΔsodB and ΔsodB/ΔsodA mutants (no growth in 4 mg/L) compared with the wild-type (no growth in the range of 0.5–2 mg/L). Accordingly, increased growth was observed with the mutants ΔrecA/ΔkatG (no growth in 2 mg/L), ΔrecA/ΔkatE (no growth in 2 mg/L), ΔrecA/ΔsodA (no growth in 0.06 mg/L), ΔrecA/ΔsodB (no growth in 0.25 mg/L) and ΔrecA/ΔkatG/ΔkatE (no growth in 0.5 mg/L) compared with ΔrecA (no growth in the range of 0.002–0.015 mg/L).

Conclusions: After RecA inactivation, gradual exposure to ciprofloxacin reduces the evolution of resistance. After suppression of RecA and detoxification systems, sudden high exposure to ciprofloxacin reduces the evolution of resistance in E. coli.

Introduction

The SOS response is a coordinated cellular response to genotoxic damage that can contribute to antimicrobial resistance evolution.1 Fluoroquinolones are potent inducers of the SOS response.2 When DNA synthesis inhibition and DNA damage occur (primary damage), RecA proteins form nucleofilaments around single-stranded DNA and promote self-cleavage of LexA,3,4 which causes activation of more than 50 genes, notably related to DNA repair and recombination.1,5,6 At the same time, bactericidal antimicrobials play an important role in reactive oxygen species (ROS) accumulation under aerobic conditions,7,8 inducing complex redox reactions that contribute to cellular damage and death (secondary damage).9,10 Bacteria have multiple oxidative detoxification systems to combat oxidative stress, such as three types of superoxide dismutase (SodA, SodB and SodC) and two types of catalase (KatG and KatE), which remove O2− and H2O2, respectively.11,12

Recently, we showed that suppression of both the SOS response (by recA gene deletion) and overproduction of ROS (by multiple detoxification system gene deletion) enhances the activity and
lethality of fluoroquinolones against *E. coli*. Previous studies have shown that antimicrobial cocktails can lead to the emergence of resistant bacteria due to SOS-induced mutagenesis or ROS production. In this study therefore, we used *E. coli* mutants with a recA gene deletion in combination with suppressed ROS detoxification system genes (ΔsodA, ΔsodB, ΔkatG and ΔkatE) to evaluate the interplay of DNA repair, recombination processes and detoxification systems in the evolution of resistance to ciprofloxacin under gradual or sudden antibiotic pressure.

**Materials and methods**

**Strains, growth conditions and antimicrobial agents**

Wild-type *E. coli* BW25113 was used as the starting strain (Table S1, available as Supplementary data at JAC Online). Single-gene deletion mutants of *E. coli* BW25113 (wild-type) (ΔrecA, ΔkatG, ΔkatE, ΔsodA, ΔsodB) were selected from the KEIO collection. The double-gene deletion mutants (ΔkatG/ΔrecA, ΔkatE/ΔrecA, ΔsodA/ΔrecA, ΔsodB/ΔrecA, ΔkatG/ΔkatE, ΔsodB/ΔsodA) and the triple-gene deletion mutant (ΔkatG/ΔkatE/ΔrecA) were generated by P1vir phage transduction. Some triple knockouts such as ΔsodA/ΔsodB/ΔrecA were planned at the start of the study, but proved not to be viable after several attempts.

Liquid or solid lysogeny broth (LB) and Mueller-Hinton broth (MHB) media were used. Strains were grown at 37°C. The antibiotic used for the different assays was ciprofloxacin (Sigma-Aldrich, Madrid, Spain).

**Minimum inhibitory concentrations**

MICs were determined in triplicate for each bacterial strain, using two different techniques: the gradient strip test and broth microdilution, following CLSI reference methods.

**Mutant prevention concentration (MPC)**

MPC was defined as the antibiotic concentration that prevents the growth of any resistant mutants following an inoculum of 10^{10} cells on LB plates containing dilutions of antibiotic, simulating an infectious focus. It was determined as previously described by Machuca et al. For each strain used in the study, a 0.5 McFarland culture density was obtained, a 10^{-5} dilution was performed (starting inoculum of ~10^4 cfu/mL) and incubated at 37°C overnight. One milliliter of overnight culture was inoculated into 100 mL of MHB, then incubated for ~6 h at 37°C with aeration until an OD_{540nm} of ~1.0 was reached (Genesys 20, Thermo, Barcelona, Spain), corresponding to ~10^4 cfu/mL. Cultures were then centrifuged at 4200 rpm for 20 min. The supernatant was discarded and the pellet containing ~10^{11} cells was resuspended in 1 mL of MHB. One hundred microliter (~10^{10} cells) was spread onto an MHA agar plate containing a specific concentration of quinolones. Each strain was tested against 2-fold-increasing concentrations of ciprofloxacin (ranging from 0.001 to 1 mg/L). Drug-free MH agar plates were inoculated with 100 μL of serial dilutions as an inoculum control. The plates were incubated for a total of 96 h at 37°C and examined every 24 h for the appearance of colonies. The MPC recorded was the lowest quinolone concentration at which no colonies grew on an agar plate at 96 h in at least three independent experiments. The time in hours when the MPC was determined (MPC time window) was also recorded, and the mutant selection window (MSW, defined as the antimicrobial concentration range extending from the minimum concentration required to block the growth of wild-type bacteria up to that required to inhibit the growth of the least susceptible single-step mutant) was calculated as the ratio of MIC to MPC.

**Experimental evolution of antibiotic resistance**

The experimental evolution study, as described by Escudero et al., started with the inoculation of 2 μL (~10^{9} cells) of the LB overnight culture into 96-well plates with 198 μL of LB containing a subinhibitory concentration of ciprofloxacin. For this approach, we started with 6.25 × 10^{-5} mg/L of ciprofloxacin, which is between 1/16 and 1/256 of the MIC (determined previously) for the selected strains. This approach maximizes the chances of populations acquiring resistance mutations. Plates were incubated for 20 h at 37°C, without agitation. After overnight culture, bacterial cultures were quantified daily by spectrophotometry at OD_{535nm} of each population, using a plate reader (Infinite 200 PRO plate reader; Tecan, Madrid, Spain). Each bacterial culture was then transferred to a new 96-well plate with double the antibiotic concentration of the day before. The plates were incubated again for 20 h at 37°C. In parallel, we performed control assays for each strain under the same conditions but in the absence of antibiotics. Optical density values <0.1 indicate extinction of the population. The percentage of cultures showing growth was calculated each day as the number of cultures showing OD values higher than 0.1. At least sixteen biological replicates were measured.

**Statistical analysis**

All statistical analyses were performed using Graphpad Prism 6 software (https://www.graphpad.com). The log-rank (Mantel-Cox) test was used for statistical evaluation. Differences were considered significant when P values were <0.05.

**Results**

Sensitization was confirmed first using MIC values (Table S1). No reductions in ciprofloxacin MIC were observed when any of the detoxification system genes were inactivated, either alone (ΔkatG, ΔkatE, ΔsodA and ΔsodB) or in combination (ΔkatG/ΔkatE, ΔsodB/ΔsodA), compared with wild-type BW25113. For the ΔrecA mutant, ciprofloxacin MICs were 3.75-fold lower than for wild-type BW25113. Finally, for the combinations (ΔrecA gene with detoxification system deletion), sensitization increased 7.5–15-fold relative to the wild-type and 2–4-fold relative to ΔrecA mutant. This confirmed our previous results on synergistic sensitization to ciprofloxacin by recA gene suppression in combination with inactivated detoxification system genes.

In this study, two different approaches were used. First, bacterial populations were exposed to a single antibiotic pressure that was constant and high (MPC above the MIC), referred to as sudden high ciprofloxacin pressure, and second, to incremental exposures extending from the minimum concentration required to block the growth of the least susceptible single-step mutant to the highest concentration that prevented the growth of the least susceptible single-step mutant. The MPC time window (MSW, defined as the antimicrobial concentration range extending from the minimum concentration required to block the growth of wild-type bacteria up to that required to inhibit the growth of the least susceptible single-step mutant) was calculated as the ratio of MIC to MPC.
ΔsodA double mutant, 17; in the ΔkatG and ΔsodA single mutants and the ΔkatG/ΔsodA double mutant, 8; in the ΔrecA single mutant, 31; in the ΔkatE/ΔrecA double mutant, 15; and in the ΔkatG/ΔrecA, ΔsodA/ΔrecA, ΔsodB/ΔrecA double mutants and the ΔkatG/ΔkatE/ΔrecA triple mutant, the MSW was 60.

These results indicate that suppression of the SOS response and the detoxification systems (both separately and, more markedly, in combination), reduces the MPC of ciprofloxacin under sudden high antimicrobial pressure. The MPC reduction was always greater when the SOS response was suppressed. Additionally, due to the initial reductions in MIC values, the MSW was wider and the values higher when the recA gene, alone or in combination with one or two detoxification systems, was suppressed.

In order to evaluate the response to gradual antimicrobial pressure, experimental evolution of was also performed and measured using antibiotic resistance assays (the aim of this approach was to simulate ecological conditions under environmental antibi-otic pressure). Experimental evolution of antibiotic resistance (Figures 2 and 3), in which ciprofloxacin concentrations were gradually increased on a daily basis, showed that all mutants, except for the ΔrecA mutant, grew at concentrations above the MIC of the ΔkatG and ΔsodA single mutants and the ΔkatG/ΔsodA double mutant, 8; in the ΔrecA single mutant, 31; in the ΔkatE/ΔrecA double mutant, 15; and in the ΔkatG/ΔrecA, ΔsodA/ΔrecA, ΔsodB/ΔrecA double mutants and the ΔkatG/ΔkatE/ΔrecA triple mutant, the MSW was 60.

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The second group included ΔkatG (40%), ΔkatE (20%), ΔkatE/ΔrecA (25%), ΔkatG/ΔrecA (15%) and ΔsodB/ΔrecA (20%) strains. The third group included ΔsodA/ΔrecA (5%), ΔkatG/ΔkatE/ΔrecA (5%) and ΔrecA (0%).

**Discussion**

Antibiotic-induced bacterial mutagenesis is considered to be partly SOS response dependent. When DNA damage occurs as a result of antimicrobials, genes in the SOS regulon related to DNA repair (including translesion synthesis) are expressed, contributing to mutagenesis. When the SOS response is suppressed, bacteria are unable to evolve resistance beyond the MIC (Figures 2 and 3). One explanation for this effect is that the SOS response is not activated and thus that mutagenesis is drastically reduced. Furthermore, the recA gene is also involved in double-strand break repair, caused by the inhibition of DNA gyrase activity, through homologous recombination, so that when the recA gene is suppressed, recombination processes do not occur via this pathway. On the other hand, mutagenesis is also ROS-dependent when low doses of antibiotics are used. Nevertheless, ROS have a killing effect at higher antibiotic doses (and hence also a bacteriostatic effect), characterized by DNA base oxidation, causing lethal DNA breaks. Accordingly, changes in MPC values could be related to deletions of detoxification system genes and ROS accumulation.

When ROS are produced at low antibiotic doses, the detoxification systems reduce the oxidative stress. When these systems are suppressed and bacteria are exposed to ciprofloxacin gradually, bacteria such as the wild-type are able to grow at concentrations above their MIC (Figures 2 and 3). This effect could be the consequence of mutagenesis due to ROS production, which leads to the appearance of ciprofloxacin resistance mutations. Of note, there are differences in behaviour between catalase system suppression (ΔkatG and ΔkatE) and superoxide dismutase system suppression (ΔsodA and ΔsodB), which may be the consequence of the different modes of action of the two systems, since superoxide dismutases remove O\(_2^-\) and catalases remove H\(_2\)O\(_2\). Furthermore, when both the SOS response and detoxication systems are suppressed, mutants are able to grow in ciprofloxacin concentrations above their MIC, which is different from what happens when the SOS response alone is suppressed. In this case, ROS production may be sufficient to lead to mutagenesis even though the SOS response is suppressed. A possible explanation for this effect is the activation of DNA damage repair systems independent of the SOS response. There are many systems described in the literature that are involved in DNA damage repair processes, such as the activities described for the Adaptive Response or the ‘GO system’.

It is noteworthy that even though BW25113 and the ΔrecA mutant showed different capacities for growth in the inter-day assays, the overall tendency was similar (Figures 2 and 3). It is also important to mention that there were differences between the two methodologies used. In the experimental evolution assays, bacteria were exposed to gradually increasing concentrations of ciprofloxacin, in which the initial ciprofloxacin concentrations simulated those found in the environment (3 × 10\(^{-5}\)-1.031 mg/L) which could also facilitate antibiotic-induced mutagenesis. By way of contrast, sudden high antimicrobial pressure limited adaptation and evolution (as measured by determination of the MPC). Finally, both methodologies were designed to analyse the behaviour of...
populations capable of active growth (colonies in the MPC assays and turbidity in the experimental evolution assays). In neither case were the populations of non-replicating surviving bacteria analysed for tolerance or persistence.

In conclusion, suppression of the SOS response, through the deletion of recA gene and detoxification systems, helps to reduce the evolution of resistance in *E. coli* after sudden exposure to ciprofloxacin. Suppression of the SOS response helps to reduce the evolution of resistance after gradual exposure to ciprofloxacin. In contrast, under this latter condition, detoxification systems, alone or in combination with SOS response suppression, could favour mutagenesis and the evolution of ciprofloxacin resistance.

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**Supplementary data**

Table S1 is available as Supplementary data at JAC Online.

**References**

1. Baharoglu Z, Mazel D. SOS, the formidable strategy of bacteria against aggressions. FEMS Microbiol Rev 2014; 38: 1126–45.
2. Blázquez J, Rodríguez-Beltrán J, Matic I. Antibiotic-induced genetic variation: how it arises and how it can be prevented. Annu Rev Microbiol 2018; 72: 209–30.
3. Little JW, Edmiston SH, Pocelli LZ et al. Cleavage of the Escherichia coli lexA protein by the recA protease. Proc Natl Acad Sci USA 1980; 77: 3225–9.
4. Luo Y, Pfuetzner RA, Mosimann S et al. Crystal structure of LexA: a conformerional switch for regulation of self-cleavage. Cell 2001; 106: 585–94.
5. Fernández De Henestrosa AR, Ogi T, Aoyagi S et al. Identification of additional genes belonging to the LexA regulon in Escherichia coli. Mol Microbiol 2000; 35: 1560–72.
6. Cohen SE, Foti JJ, Simmons LA et al. The SOS regulatory network. EcoSal Plus 2008; 3. doi:10.1128/ecosalplus.s5.4.3.
7. Hoeskstra M, Brul S, Ter Kuile SAH. Influence of reactive oxygen species on de novo acquisition of resistance to bactericidal antibiotics. Antimicrob Agents Chemother 2018; 62: 1–8.
8. Drlica K, Zhao X. Bacterial death from treatment with fluoroquinolones and other lethal stressors. Expert Rev Anti Infect Ther 2021; 19: 601–18.
9. Kohanski MA, DePristo MA, Collins JJ. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Mol Cell 2010; 37: 311–20.
10. Dwyer DJ, Belenky PA, Yang JH et al. Antibiotics induce redox-related physiological alterations as part of their lethality. Proc Natl Acad Sci USA 2014; 111: E2100–9.
11. Ezraty B, Gennarini A, Barras F et al. Oxidative stress, protein damage and repair in bacteria. Nat Rev Microbiol 2017; 15: 385–96.
12. Van Acker H, Coekey T. The role of reactive oxygen species in antibiotic-mediated killing of bacteria. Trends Microbiol 2017; 25: 456–66.
13. Díaz-Díaz S, Recacch E, Machuca J et al. Synergistic quinolone sensitization by targeting the recA SOS response gene and oxidative stress. Antimicrob Agents Chemother 2021; 65: e02004–20.
14. Cirz RT, Chin JK, Andes DR et al. Inhibition of mutation and combating the evolution of antibiotic resistance. PLoS Biol 2005; 3: 1024–33.
15. Cirz RT, Romnesberg FE. Induction and inhibition of ciprofloxacin resistance-conferring mutations in hypermutator bacteria. Antimicrob Agents Chemother 2006; 50: 220–5.
16. Blázquez J. Hypermutation as a factor contributing to the acquisition of antibiotic resistance. Clin Infect Dis 2003; 37: 1201–9.
17. Dwyer DJ, Kohanski MA, Collins JJ. Role of reactive oxygen species in antibiotic action and resistance. Curr Opin Microbiol 2009; 12: 482–9.
18. Baba T, Ara T, Hasegawa M et al. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2006; 2: 006.0008.
19. Thomason LC, Costantino N, Court DL. E. coli Genome Manipulation by P1 Transduction. Curr Protoc Mol Biol 2007; 79: 1.17.1–8.
20. Datensen KA, Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci USA 2000; 97: 6640–5.
21. CLSI. Performance Standards for Antimicrobial Susceptibility Testing—Twentieth Edition: M100. 2010.
22. Drlica K. The mutant selection window and antimicrobial resistance. J Antimicrob Chemother 2003; 52: 11–7.
23. Machuca J, Briales A, Labrador G et al. Interplay between plasmid-mediated and chromosomal-mediated fluoroquinolone resistance and bacterial fitness in Escherichia coli. J Antimicrob Chemother 2014; 69: 3203–15.
24. Escudero JA, MacLean RC, San Millan A. Testing the role of multicopy plasmids in the evolution of antibiotic resistance. J Vis Exp 2018; 2018: 57386.
25. Rodriguez-Rosado AI, Valencia EY, Rodriguez-Rojas A et al. Reactive oxygen species are major contributors to SOS-mediated mutagenesis induced by fluoroquinolones. bioRxiv 2018; 428961.
26. Do Thi T, López E, Rodriguez-Rojas A et al. Effect of recA inactivation on mutagenesis of Escherichia coli exposed to sublethal concentrations of antimicrobials. J Antimicrob Chemother 2011; 66: 531–8.
27. Kohanski MA, Dwyer DJ, Hoyete B et al. A common mechanism of cellular death induced by bactericidal antibiotics. Cell 2007; 130: 797–810.
28. Foti JJ, Devadoss B, Winkler JA et al. Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics. Science 2012; 336: 315–9.
29. Kreuzer KN. DNA damage responses in prokaryotes: regulating gene expression, modulating growth patterns, and manipulating replication forks. Cold Spring Harb Perspect Biol 2013; 5: a012674.
30. Li Y, Zhu G, Ng WJ et al. A review on removing pharmaceutical contaminants from wastewater by constructed wetlands: design, performance and mechanism. Sci Total Environ 2014; 468–469: 908–32.
31. Frade VMF, Dias M, Teixeira ACSC et al. Environmental contamination by fluoroquinolones. Braz J Pharm Sci 2014; 50: 41–54.
32. Gao L, Shi Y, Li W et al. Occurrence of antibiotics in eight sewage treatment plants in Beijing, China. C hemosphere 2012; 66: 665–71.
33. Wąglik M, Kuninska J, Stolte S et al. Development of sensitive and reliable LC-MS/MS methods for the determination of three fluoroquinolones in water and fish tissue samples and preliminary environmental risk assessment of their presence in two rivers in northern Poland. Sci Total Environ 2014; 493: 1006–13.