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Research paper

Overexpression of SOS genes in ciprofloxacin resistant *Escherichia coli* mutants

Razieh Pourahmad Jaktaji [a,*], Shirin Pasand [b]

[a] Genetics at Faculty of Science, Biotechnology Center, University of Shahrekord, Iran
[b] Genetics, University of Shahrekord, Iran

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ABSTRACT

Introduction: Fluoroquinolones are important antibiotics for the treatment of urinary tract infections caused by *Escherichia coli*. Mutational studies have shown that ciprofloxacin, a member of fluoroquinolones induces SOS response and mutagenesis in pathogenic bacteria which in turn develop antibiotic resistance. However, inhibition of SOS response can increase recombination activity which in turn leads to genetic variation.
Objective: The aim of this study was to measure 5 SOS genes expressions in nine *E. coli* mutants with different MICs for ciprofloxacin following exposure to ciprofloxacin.
Methods: Gene expression was assessed by quantitative real time PCR. Gene alteration assessment was conducted by PCR amplification and DNA sequencing.
Results: Results showed that the expression of *recA* was increased in 5 mutants. This overexpression is not related to gene alteration, and enhances the expression of *polB* and *umuCD* genes encoding nonmutagenic and mutagenic polymerases, respectively. The direct relationship between the level of SOS expression and the level of resistance to ciprofloxacin was also indicated.
Conclusion: It was concluded that novel therapeutic strategy that inhibits RecA activity would enhance the efficiency of common antibiotics against pathogenic bacteria.

1. Introduction

Pathogenic *Escherichia coli* strains cause health problems, such as urinary tract infections (UTIs). UTIs can be treated with fluoroquinolone members, including ciprofloxacin and norfloxacin (Lindgren et al., 2003). Fluoroquinolones' first target in Gram negative bacteria is DNA gyrase. This protein consists of two subunits, GyrA and GyrB, encoded by *gyrA* and *gyrB* genes, respectively (Drlica and Zhan, 1997; Ruiz, 2003). These antibiotics reversibly bind to gyrase-bridged DNA double strand break (DSB) and interfere with gyrase activity. Dissociation of an antibiotic–gyrase complex without religation of DSB would be lethal unless homologous recombination repairs DSB (Drlica and Zhan, 1997). Meanwhile the triple complex antibiotic–gyrase–DNA would block replication forks and produce DSB after fork collapse. This DSB triggers the SOS response. This response promotes the transcription of approximately 30 genes (Cirz et al., 2007; Lopez et al., 2007). Most of these genes participate in repair, bypass and tolerance mechanisms, including *recA*, *lexA*, *uvrAB*, *umuDC*, *polB*, *sulA* and *dinB* (Friedberg et al., 2006; Janion, 2008). Normally, this regulon is repressed by LexA repressor, but under stress conditions, like exposure to antibiotics, this repressor is self-cleaved with the help of RecA. RecA also promotes cleavage of UmuD to form UmuD′. UmuD′ binds to UmuC to form an active UmuD′$_2$UmuC complex (DNA polymerase V) conducting error-prone translesion replication of blocked fork in the process called SOS mutagenesis (Friedberg et al., 2006; Hare et al., 2006). Thus, DNA polymerase V (Pol V) activates later following SOS induction, and participates in the generation of base substitution mutations during polymerization of damaged DNA (Friedberg et al., 2006; Yeiser et al., 2002). However, DNA polymerase II (Pol II) acting early after SOS induction catalyzes error-free replication restart. Both polymerases activate in dividing cells (Friedberg et al., 2006; Yeiser et al., 2002). It was shown that inhibition of SOS induction in an *E. coli* mutant that is unable to cleave LexA prevents the development of ciprofloxacin resistance (Cirz et al., 2005).

Moreover, it was demonstrated that fluoroquinolones can increase genetic variation by the stimulation of the recombination activity in the SOS deficient LexA mutant (Lopez et al., 2007).

Mutations in *gyrA* and *gyrB* genes especially in the quinolone resistance determining region (QRDR), the binding site of antibiotics, cause resistance (Yoshida et al., 1990). Meanwhile overactivation of the endogenous transmembrane efflux pumps mainly AcrAB-TolC reduces

Abbreviations: E. coli, *Escherichia coli*; DSB, double strand break; NER, nucleotide excision repairs; Pol V, DNA polymerase V; QRDR, quinolone resistance determining region; MIC, minimum inhibitory concentration; qRT PCR, real time PCR; OD, optical density.

* Corresponding author at: Department of Genetics, Faculty of Science, University of Shahrekord, Shahrekord, Iran.
E-mail addresses: Razieh_Jaktaji@yahoo.com (R. Pourahmad Jaktaji), pasandshirin@yahoo.com (S. Pasand).

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the concentration of the fluoroquinolones in the bacterial cytoplasm (Swick et al., 2011). Experimental studies with *E. coli* shows that low-level and high-level of fluoroquinolone resistance are acquired from single and multiple mutations, respectively. Multiple mutations include both target and efflux-related mutations and correlated with a higher mutation rate in resistant strains (Lindgren et al., 2003).

The importance of SOS induction in the development of ciprofloxacin resistance in *E. coli* strains was shown with mutational analyses (Cirz et al., 2005; Dorr et al., 2009; Lopez et al., 2007), but not with gene expression analyses. In this study the transcriptional level of main SOS regulon genes, including *recA*, *lexA*, *polB* and *umuCD* after exposure to ciprofloxacin in *E. coli* mutants with different MIC for ciprofloxacin was reported.

2. Material and methods

2.1. Bacterial strains and growth

The strains used in this study are listed in Table 1. The liquid medium was LB broth (Merck). The solid medium was LB agar prepared by the addition of agar (Merck) to LB broth. Ciprofloxacin was obtained from Bayer Corporation. Stock concentration was 10 mg/ml and used at the concentration of 40 ng/ml. All bacteria were grown aerobically at 37 °C with shaking (180 rpm).

2.2. Sample preparation for transcriptional analysis

For each strain, three fresh colonies were inoculated into separate LB broth tubes and incubated overnight at 37 °C with shaking (180 rpm). They were diluted 1:100 in fresh LB broth containing ciprofloxacin and grown to a mid-logarithmic phase (OD_{600} of 0.5–0.6), as described previously (Lau and Zgurskaya, 2005; Pohlhaus et al., 2008). Appropriate volumes from each of the three cultures per strain were pooled and added to 2 volumes of RNA protect reagent (Qiagen). Cultures were centrifuged and cell pellets were used for RNA extraction. Total RNA was extracted using an RNeasy Mini Kit (Qiagen). This procedure was repeated twice.

During the experiment, OD_{600} was measured every 10 min to determine the doubling time of mutants and samples of logarithmic phase bacteria were Gram stained to assess the morphology of mutants.

2.3. Real time PCR (qRT-PCR)

RNA samples were treated with RNase-free DNase (fermentas) according to the manufacturer's protocol. DNase treated RNA was repurified using an RNeasy Mini Kit (Qiagen). No contaminating DNA was detected by PCR. The purity of protein concentration was determined using the Ultrospec 1100 spectrophotometer. Then, they were used for cDNA synthesis. Reverse transcription was performed using the RevertAid Reverse Transcriptase Kit (Fermentas), random hexamer and purified total RNA (2 μg). The cDNAs obtained from reverse transcription were amplified by PCR reaction with specific primers to first verify the presence of a single band at the expected size and second to find the best annealing temperature for real time PCR. Then, diluted cDNA (2 μl of 1:10), obtained from reverse transcriptase, was used to quantify the level of SOS genes, including *recA*, *lexA*, *polB*, *umuC* and *umuD* as well as *gapA* with specific primers as mentioned in Table 2 by real time PCR in a Rotor Gene 6000 thermocycler (Corbett Research) using an SYBR Green Kit (Takara). Thermal cycling conditions were described previously unless for annealing temperature, which differs from one gene to another (Viveiros et al., 2007). Melting curve analysis at 60–95 °C with continuous fluorescence reading was conducted. Relative gene expression was calculated using the efficiency method of Pfaffl (ratio of SOS gene expression to *gapA* expression) (Pfaffl et al., 2002). All data on SOS gene expressions are the average of duplicate analyses.

2.4. Statistical analysis

Statistically significant differences in gene expression were determined by Student's *t* test (two paired samples, with two tailed distribution), using SPSS version 17 software.

2.5. PCR amplification and DNA sequencing

To determine whether SOS induction might be related to nucleotide alteration within *recA* gene or its upstream regulatory region, including SOS box in mutant strains, two couples of primers were designed to amplify whole *recA* and its upstream sequence. The sizes of amplified samples were 734 and 585 base pairs. The first one started from the upstream region containing an SOS box and promoter region, to the nearly middle of the gene. The second one started from the middle to the end of the gene. Then, amplified samples were sequenced.

3. Results

3.1. SOS response in E. coli mutants

Nine genotypically and phenotypically different mutants were used in this study ranging from one with a single mutation in *gyrA* to others with double mutations, including *gyrA marR* and *gyrA acrR* with or without overexpression of *acrAB*. The doubling time of mutants was increased by the level of resistance to antibiotics from 25 to 40 min.

Table 1
Bacterial strain and mutants.

Strains	Genotype			MIC		Source/Reference
	gyrA	*marR*	*acrR*	Cip (ng/ml)	Tet (μg/ml)	
MG1655	Wild type			35	3	A gift from Prof. R. G. Lloyd
W29	*gyrA* (Ser_{83} → Leu)	*marR* (Met_{74} → Thr)		75	4	Pourahmad Jaktaji et al. (2012)
W30	*gyrA* (Ser_{83} → Leu)		*acrR* (Arg_{45} → Cys)	75	4	Pourahmad Jaktaji and Jazayeri (2013)
W31	*gyrA* (Ser_{83} → Leu)			75	3	Pourahmad Jaktaji and Mohiti (2010)
W49	*gyrA* (Ser_{83} → Leu)	*marOR* (20 bp duplication in operator)		625	4	Pourahmad Jaktaji et al. (2012)
C6	Derived from W29, *gyrA* (Ser_{83} → Leu)	*marR* (Met_{74} → Thr)		1000	45	Pourahmad Jaktaji et al. (2012)
C14	Derived from W30, *gyrA* (Ser_{83} → Leu)	*acrR* (Arg_{45} → Cys)		1000	30	Pourahmad Jaktaji and Jazayeri (2013)
C17	Derived from W49, *gyrA* (Ser_{83} → Leu)	*marOR* (20 bp duplication in operator)		1000	30	Pourahmad Jaktaji et al. (2012)
M1	Derived from C14, *gyrA* (Ser_{83} → Leu)	*acrR* (Arg_{45} → Cys) *acrAB* overexpression		2000	120	Unpublished data
M2	Derived from C17, *gyrA* (Ser_{83} → Leu)	*marOR* (20 bp duplication in operator) *acrAB* overexpression		2000	120	Unpublished data