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ORIGINAL ARTICLE

Subinhibitory concentrations of ciprofloxacin induce SOS response and mutations of antibiotic resistance in bacteria

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Abstract In this work, *Salmonella typhimurium* strains (Sal94) containing plasmid-borne fusions of *Vibrio fischeri lux* to the *recA* promoter was used to test the SOS response induced by sub-minimum inhibitory concentration (sub-MIC) of ciprofloxacin. The SOS response of Sal94 strain was rapidly increased during 20 min when supplemented with sub-MIC ciprofloxacin, but came down thereafter. The induction level of 1/2 MIC was higher than that of 1/4 MIC, showing some dose-dependency. Both mutation frequencies (MF) of anti-ciprofloxacin resistance and transformation frequency of pMD18-T Vector (*lacZ* ori Amp^r) caused by the treatment of 1/4 and 1/2 MIC ciprofloxacin in *Escherichia coli* AB1157 strain (wild-type *recA*, SOS inducible) were markedly increased, but there were no or only slight changes in *Escherichia coli* IC400 strain (*recA* mutant, deficient in SOS response). These results, combining the positive relationship between the induction factor and the MF of anti-ciprofloxacin induced by sub-MIC ciprofloxacin in AB1157 strain, indicated that SOS response played an important role in the acquirement of antibiotic resistance resulting from sub-MIC ciprofloxacin treatment.

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Keywords Antibiotic resistance · SOS response · Ciprofloxacin · Sub-inhibitory concentration · Mutation

Abbreviations

MIC	Minimum inhibitory concentration
Sub-MIC	Subminimum inhibitory concentration

Introduction

The application of penicillin as a therapeutic agent in 1942 ushered in the era of antimicrobial chemotherapy and marks a historic milestone in medicine. Many additional classes of antibiotics spanning a broad range of chemical structures and targets soon followed, forming the foundation of the current armory of antibiotics (Lipsitch and Samore 2002; Peter and Romesberg 2007; Rice 2006). But in recent years, the use of antibiotics is continually being challenged by the emergence of resistant strains of bacteria, resulting in an worldwide medical, social and economic problem (Diekema et al. 2004; Smolinski et al. 2003), especially in the developing countries where the inappropriate or over use of anti-biotics is comparatively prevalence.

Antibiotic resistance has been mainly considered a consequence of errors (spontaneous and induced muta-tions) that accumulate during replication of the bacterial genome. Chemists at the Scripps Research Institute and the University of Wisconsin have uncovered evidence that spontaneous mutations are not the only way in which bacteria acquire resistance to antibiotics (Cirz et al. 2005; Cirz and Romesberg 2006). The rapid rate at which bacteria develop antibiotic resistance is due in large part to mutations arising during stress-induced DNA repair

and during the lateral transfer of genes between organisms (Foster 2005; Hersh et al. 2004; Matic et al. 2004). Previous studies showed that the SOS response resulting from antibiotic therapy played an important role in the acquirement of antibiotic resistance in bacteria (Beaber et al. 2004; Cirz et al. 2005; Cirz and Romesberg 2006; Hastings et al. 2004).

Customarily, antibiotic therapy is based on achieving and exceeding a minimum inhibitory concentration (MIC) for a sufficient amount of time in infected tissues. But there are some conditions that bacteria face in sub-MICs of the antibiotic: inappropriate antibiotic use, therapeutic option of sub-MIC of the antibiotic, in wild environments, the antibiotics use in farm animals, etc. When an antimicrobial is used inappropriately—for too short a time, at too low a dose, at inadequate potency—or for the wrong disease, microbes are more likely to develop resistance to that drug. Therefore, choosing and using the right drug at the right dose is an important way to combat drug resistance. The use of antibiotics for growth promotion has arisen with the intensification of livestock farming, but evidence strongly suggests that it results in the development of drug-resistant microbes in those animals. Drug resistance in animals may lead to drug resistance in humans, because the drug-resistant bacteria can be transmitted from animals to humans (Teuber 1999, 2001; Witte 1998). Sub-MIC antibiotic therapies can also lead to treatment failure and antibiotic resistance (Roe and Pillai 2003). Furthermore, bacteria in wild niches frequently meet sub-MICs of various antibiotics. Therefore, careful evaluation of sub-MIC effects on bacterial physiology is needed prior to therapeutic use of sub-MICs and animal growth promotion.

The mechanisms leading to the development and transmission of antibiotic resistance by sub-MICs of antibiotics are not fully understood. Recent researchers indicated that sub-MICs of various antibiotics changed the expression of many proteins in bacteria, especially proteins related to SOS response (Cirz et al. 2007; Nanduri et al. 2006, 2008). But the relationship of antibiotic resistance mutations and SOS response induced by sub-MICs of antibiotics, and/or whether sub-MICs of antibiotics exerting natural selection for resistance on these organisms is still not very clear. In this work, we used *Salmonella typhimurium* strains (Sal94) containing plasmid-borne fusions of *Vibrio fischeri lux* to the *recA* promoter (Mingli et al. 2009), directly examined the SOS induction factor induced by 1/2 and 1/4 MIC of ciprofloxacin in bacteria, and compared the MF of ciprofloxacin resistance at different induction times to evaluate the relationship of SOS response and mutations of resistance by sub-MICs of antibiotics.

Materials and methods

Bacterial strain and antimicrobial agents

All strains used in this work are listed in Table 1. *Salmonella typhimurium* strains (Sal94) containing plasmid-borne fusions of *Vibrio fischeri lux* to the *recA* promoter was used to test the SOS response induced by sub-minimum inhibitory concentration (sub-MIC) of ciprofloxacin. AB1157 and IC400 are *Escherichia coli* K12 strains, and they are different in the *recA* gene (wild-type in AB1157 and deficient in IC400).

The antimicrobial agents of ampicillin (Amp), kanamycia (Kan), tetracycline (TC), ciprofloxacin (Cip) were obtained as standard reference powders of known potency for laboratory use (National Institute for the Control of the Pharmaceutical and Biological Products, China)

Bacteria growth

Bacteria were cultured overnight in LB medium (Maniatis et al. 1982), in a shaking incubator with or without 50 μg/ml ampicillin and 30 μg/ml kanamycin, according to their needs for maintaining the plasmid. Cells were diluted 100-fold with fresh LB medium and grown to mid-log with shaking to a density of about 1×10^8 to 2×10^8 bacteria per ml, the samples were used for the induction of the SOS response and the mutations of antibiotic resistance by sub-MIC of ciprofloxacin. Plate cultures were grown on LB agar with or without antimicrobial according to their needs. Incubation temperatures were 37°C throughout, except for strain Sal94, which was grown at 26°C.

Susceptibility testing

Minimal inhibitory concentrations (MICs) of antimicrobial agents were determined by means of the Agar Dilution Method according to a standard procedure described by the Clinical and Laboratory Standards Institute/NCCLS (2005). The antimicrobial agents were incorporated into Mueller-Hinton Agar (Oxoid, UK) in serial twofold concentrations from 0.06 to 128 μg/ml. Quality control strains were *E. coli* ATCC 25922 and *E. coli* ATCC 35218 with every batch of clinical strains to ensure accurate and comparable performance of assays. The plates were incubated in ambient air at 35°C for 18 h. The inoculating concentration of bacteria was approximate 1.5×10^8 CFU/ml, equivalent to a 0.5 McFarland standard. An inoculum of 10^4 CFU per spot was delivered with a multipoint inoculator (AQS, UK).

Table 1 Strains used in this work (all are *Escherichia coli* K-12 strain except Sal94)

Strain/plasmid	Genotype	Source/reference
AB1157	*thr-1 araC14 leuB6*(Am)Δ *(gpt-proA)62 lacY1 tsx-33 supE44*(AS) *galK2*(Oc) *hisG4* (Oc) *rfbD1 mgl-51 rpoS396*(Am) *rpsL31*(Str^R) *kdgK51 xylA5 mtl-1 argE3*(Oc) *thi-1*	Our laboratory/(Bachmann 1972)
IC400	AB1157 *recA430*	E. Botello/(Grompone et al. 2003)
Sal94	*S. typhimurium* strain with *pRecA::LuxCDABE* $tolC^C Cm^R Amp^R$	S. Belkin/(Davidov et al. 2000)

Influence of the bacteria growth by sub-MIC of ciprofloxacin

Overnight cultured cells were diluted 100-fold with fresh LB medium, incubated with shaking with or without sub-MIC of ciprofloxacin. The cell density at 600 nm was examined sequentially with a Beckman DU640 spectrophotometer every 20 min.

The SOS response induced by sub-MIC of ciprofloxacin

Mid-log Sal94 cells were supplemented with sub-MIC of ciprofloxacin, and cultured to the desired times. The induced samples were diluted 100-fold with fresh LB medium and continually cultured for 1.5 h for the SOS expressing.

Luminescence assay: 1 ml of expression cells of induction or control were directly transferred to a 1.5-ml Eppendorf tube. The emitted luminescence (arbitrary relative light units, RLUs) was monitored using a luminometer (GLOMAX 20/20 Luminometer) and the cell density was measured using a Beckman DU640 spectrophotometer at 600 nm.

The SOS induction factor of control sample (no induction at 0 time) was defined as 1.0. The SOS induction factor of induced samples was calculated by dividing the mean luminescence ($RLUs/OD_{600}$) of an induced sample displayed at the different times by that of the non-induced sample at 0 time.

Induction of antibiotic resistance by sub-MIC of ciprofloxacin

Overnight culture cells of AB1157 and IC400 were diluted 100-fold with fresh LB medium and grown to mid-log with shaking to a density of about 1×10^8 to 2×10^8 bacteria per ml, then supplemented with ciprofloxacin at 1/4 or 1/2 MIC of these strains, and incubated continually with shaking. Samples at desired times were diluted to appropriate concentration with sterile water, then 0.1 ml was spread on the screen plate containing ciprofloxacin of 5-fold MIC and on the LB agar plate without ciprofloxacin for overnight culture. The colonies on the screen plate were ciprofloxacin-resistant mutants, and the colonies on the LB plate without antibiotic represented the total survival cells. The mutant frequencies were obtained by the mean of the mutant colonies divided by the mean of the survival colonies.

Determination of transformation frequency

IC400 and AB1157 strain were used as a recipient for pMD18-T Vector (*lacZ* ori Amp^r) (Takara, China). Cells of IC400 and AB1157 were cultured to logarithmic phase in Mueller-Hinton medium. Then 1/2 and 1/4 MICs of ciprofloxacin were added to the mixture, respectively, and incubated for 1–2 h with surveillance. After the calcium chloride treatment, pMD18-T Vector was introduced into the mixture, and incubation continued at 30°C for 30 min and then at 37°C for a further 2 h for transformation. Cells were plated on Mueller-Hinton agar supplemented with ampicillin (100 μg /ml) overnight for screening the transformer. The frequency of ampicillin-resistant transformants was determined by colonies on plate with and without ampicillin (100 μg /ml).

Results

Antimicrobial susceptibility

The MICs of bacteria strains in this work are shown in Table 2. AB1157 and IC400 are *E.coli* strains but different in *recA* gene (wild-type in AB1157 and deficient in IC400). The MIC of the IC400 strain is lower than that of the AB1157 strain.

Growth kinetics

The growth of strains Sal94, IC400, AB1157 affected by 1/4 and 1/2 MIC of ciprofloxacin are shown in Figs. 1, 2 and 3,

Table 2 The ciprofloxacin susceptibility of bacteria

Strain	MIC (μg ml^{-1})
AB1157	0.125
IC400	0.06
Sal94	0.5

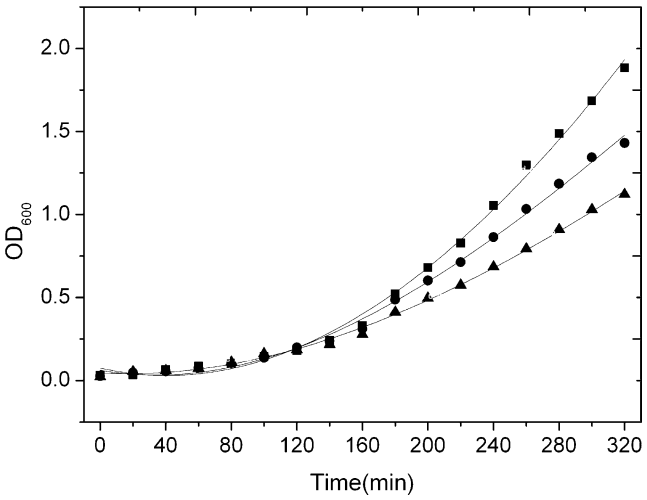

Fig. 1 The growth of *S. typhimurium* strain Sal94 affected by subinhibitory concentrations of ciprofloxacin. Sub-minimum inhibitory concentration doses used are based on MIC values in Table 2. Symbols: ■ Control, ● 1/4 MIC, ▲ 1/2 MIC

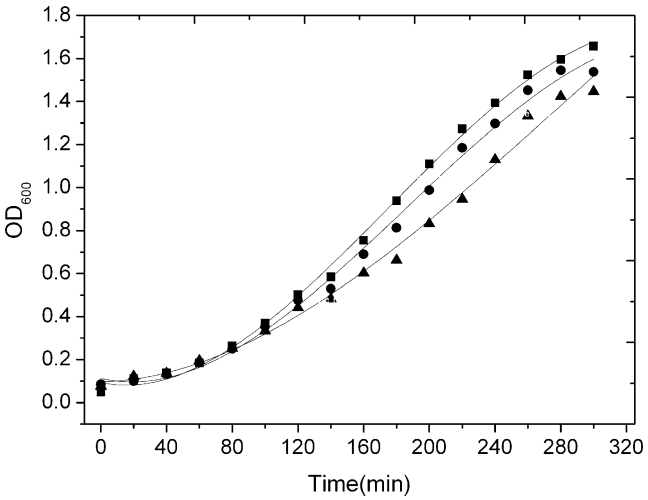

Fig. 3 The growth of AB1157 strain affected by subinhibitory concentrations of ciprofloxacin. Sub-minimum inhibitory concentration doses used are based on MIC values in Table 2. Symbols: ■ Control, ● 1/4 MIC, ▲ 1/2 MIC

respectively. When compared with the control, the growth of all strains was influenced by 1/4 and 1/2 MICs of ciprofloxacin treatments over 320 min. The data also showed a dose-dependent effect (the influence of 1/2 MIC of ciprofloxacin on the growth of all these strains was obviously more than that of 1/4 MIC of ciprofloxacin). Meanwhile, when the three strains were compared, the growth of strain IC400 with mutant *recA* gene was more impacted by sub-MIC of ciprofloxacin than that of strain Sal94 and AB1157 with wild-type *recA* gene.

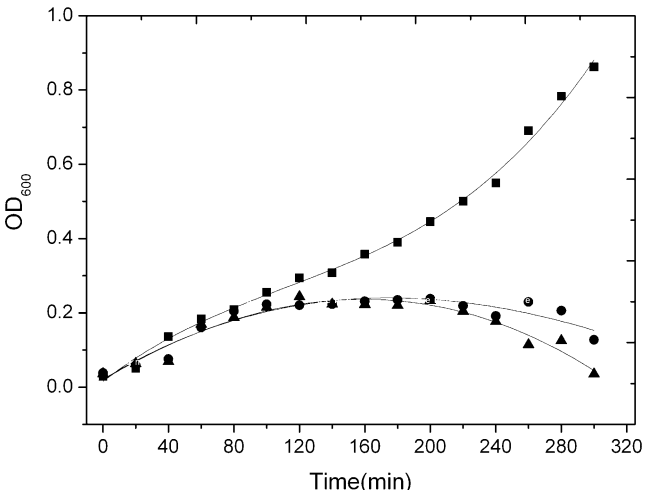

Fig. 2 The growth of IC400 strain affected by subinhibitory concentrations of ciprofloxacin. Sub-minimum inhibitory concentration doses used are based on MIC values in Table 2. Symbols: ■ Control, ● 1/4 MIC, ▲ 1/2 MIC

Induction factor induced by sub-MIC of ciprofloxacin

Bacteria Sal94 with plasmid p*RecALux*3 was induced by sub-MIC of ciprofloxacin, and the luminescence and cell density (arbitrary relative light units, RLUs) at different times were assayed. $RLUs/OD_{600}$ and the SOS induction factor was shown in Fig. 4. The data indicated that the SOS response gene *recA* could be induced rapidly by sub-MIC of ciprofloxacin in 10 min, but slowly came down to a definite level thereafter. The SOS response induced by 1/2 MIC of ciprofloxacin was relatively higher than that of 1/4 MIC of ciprofloxacin and displayed a dose-dependency.

Antibiotic resistance induced by sub-MIC of ciprofloxacin

Mutation frequencies (MF) of ciprofloxacin resistance in AB1157 strain induced by sub-MIC of ciprofloxacin were shown in Fig. 5. The MF induced by sub-MIC of ciprofloxacin was higher than that of spontaneous mutations, and its increase was time- and dose-dependent in this work (the MF increased with induced time, and 1/2 MIC of ciprofloxacin resulted in higher mutations than that of the 1/4 MIC of ciprofloxacin).

Figure 6 compares the spontaneous mutation frequency of anti-ciprofloxacin resistance with that induced by sub-MIC of ciprofloxacin in strains AB1157 and IC400. The anti-ciprofloxacin resistance mutations produced by 1/4 and 1/2 MICs of ciprofloxacin treatment were remarkably increased in the AB1157 strain ($p<0.05$), but in the IC400 strain these changes were not very distinct ($p>0.05$).

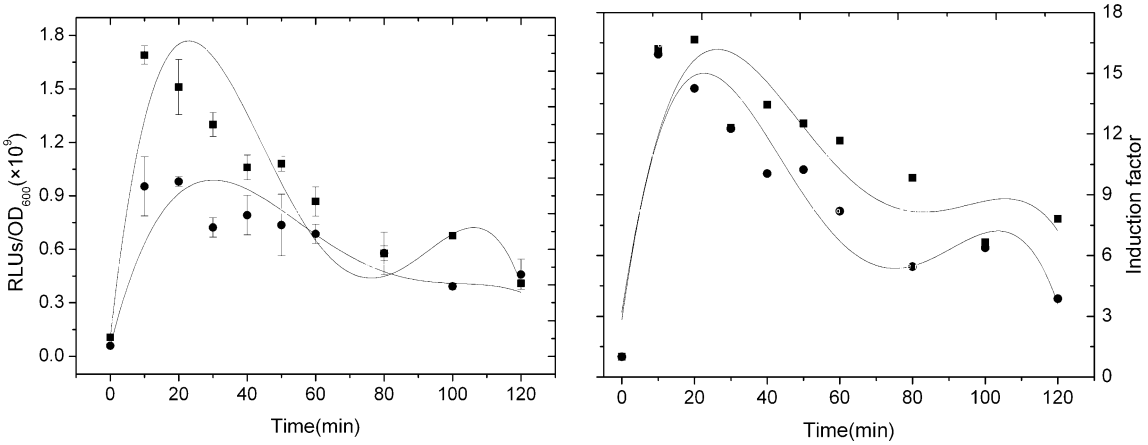

Fig. 4 The expression (*right*) and induction factor (*left*) of the *recA* in *S. typhimurium* strain Sal94 induced by subinhibitory concentrations of ciprofloxacin. RLUs/OD_{600} are expressed as the mean ± SD of at least three repeated experiments, and induction factors are expressed as mean values of the experiments. Sub-minimum inhibitory concentration doses used are based on MIC values in Table 2. Symbols: ■1/2 MIC, ● 1/4MIC

Transformation frequency induced by sub-MIC ciprofloxacin

The pMD18-T Vector with ampicillin resistance gene was transformed into AB1157 and IC400 strains which were induced by sub-MIC of ciprofloxacin. Treatment of sub-MIC of ciprofloxacin significantly increased the transformation frequency in the AB1157 strain, but barely changed the MF of transformation in the IC400 strain (Fig. 7).

Discussion

Bacteria often encounter sub-MICs of antibiotics in wild niches and therapeutic conditions, and many aspects of the bacteria response to these conditions have been investigated in recent years (Davies et al. 2006; Fajardo and Martínez 2008; Nanduri et al. 2006; Henderson-Begg et al. 2006). The data of growth kinetics in this work showed that sub-MIC of ciprofloxacin impaired the bacteria growth in all

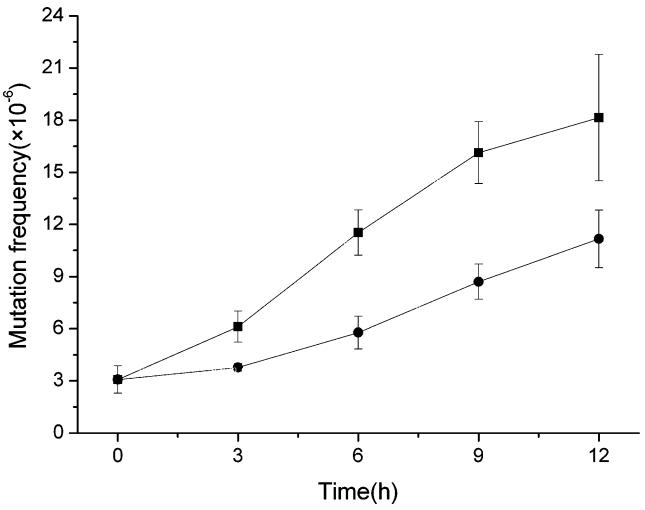

Fig. 5 Mutation frequencies of ciprofloxacin resistance induced by subinhibitory concentrations of ciprofloxacin in AB1157. Mid-log cells of AB1157 were induced by 1/4 and 1/2 MIC of ciprofloxacin, anti-ciprofloxacin mutants at desired times were selected on 5-fold MIC of ciprofloxacin plates. Data were at least three independent experiments. Sub-minimum inhibitory concentration doses used are based on MIC values in Table 2. Symbols: ■ 1/2 MIC, ● 1/4 MIC

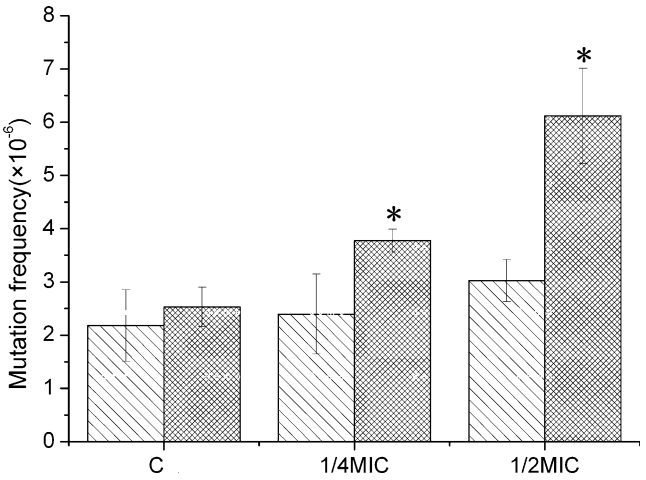

Fig. 6 Camparison of the mutation frequency of the AB1157 and IC400 strains induced by subinhibitory concentrations of ciprofloxacin. Mid-log cells of AB1157 and IC400 were incubated with shaking without (C) or with 1/4 and 1/2 MICs of ciprofloxacin for 2 h, anti-ciprofloxacin mutants were selected on 5-fold MIC ciprofloxacin plates. Data were at least three independent experiments. Sub-minimum inhibitory concentration doses used are based on MIC values in Table 2. Symbols: insert IC400, in the two square AB1157. *Statistically significant difference from those of spontaneous mutations ($p<0.05$)

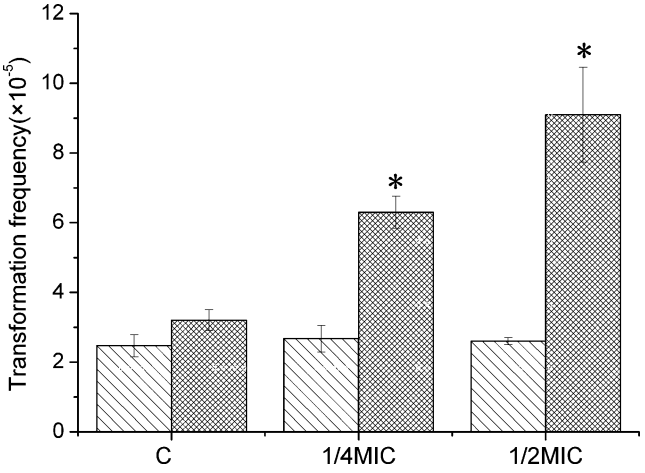

Fig. 7 Camparison of the transformation frequency of the AB1157 and IC400 strains induced by subinhibitory concentrations of ciprofloxacin. Data were at least three independent experiments. *C* Control groups, and sub-minimum inhibitory concentration doses are based on MIC values in Table 2. Symbols: IC400, AB1157. *Statistically significant difference from those of spontaneous mutations (p <0.05)

strains AB1157, IC400, and Sal94 compared to the control, and the influence level was positively correlated with the concentration (Figs. 1, 2 and 3). These were consistent with the results obtained in bacteria by sub-MICs of antibiotics (Lorian 1975; Reeks et al. 2005). The growth of strain IC400 with mutant *recA* gene was markedly reduced compared with those of AB1157 and Sal94 strains with wild-type *recA* gene by sub-MIC of ciprofloxacin. The possible reason is that the IC400 cells cannot repair the damage induced by sub-MICs of antibiotics because of the deficient *recA* gene, which results in more cell death than those with the wild-type *recA* gene.

For a growing number of bacteria, the SOS response has been recognized as a critical component of the response to environmental stress, in particular to antibiotics such as ciprofloxacin (Cirz et al. 2007; Miller et al. 2004; Power and Phillips 1992). Ciprofloxacin induces double-stranded DNA breaks and stalled replication forks, both of which are processed to single-stranded DNA. *RecA* forms filaments on the single-stranded DNA, and these nucleoprotein filaments facilitate recombination repair as well as bind the SOS gene repressor *LexA*, stimulating its auto-proteolysis. This cleavage inactivates the *LexA* repressor and results in the induction of the SOS genes. In this work, sub-MIC of ciprofloxacin markedly induced SOS response in Sal94 strain in 10 min (Fig. 4), but slowly came down thereafter. The induction factor resulting from 1/2 MIC was comparatively higher than that of the 1/4 MIC. These results indicated that even sub-MIC of ciprofloxacin could rapidly induce double-stranded DNA breaks and stall replication forks, resulting in SOS response in bacteria. But this induction was relatively weak because of a lower concentration, and some of the subsequent damage might be rehabilitated by the bacteria repair system stimulated by the response.

The mutations of ciprofloxacin resistance of the AB1157 strain in this work were dose-dependent (Fig. 5); the MF of 1/2 MIC induced was comparatively higher than that of 1/4 MIC. Previous studies have shown antibiotics with different concentrations have different effects on bacteria: high concentrations of antibiotics (equal or above inhibitory concentrations) mainly deal with cell growth inhibition (or death), while low concentrations may act as signaling molecules modulating expression of specific genes (Fajardo and Martínez 2008). The data of this work reflected that the different intensity or a different kind of effect also existed below inhibitory concentrations. Combining the data of the induction factor resulting from the 1/2 and 1/4 MICs of ciprofloxacin (Fig. 4), a positive relationship between the SOS response and the mutations of anti-ciprofloxacin resistance was found with co-culture of sub-MIC of ciprofloxacin in the AB1157 strain. Further evidence for this positive relationship was obtained by comparing the MF of anti-ciprofloxacin resistance induced by sub-MIC of ciprofloxacin in the *recA* mutant strain IC400 (deficient strain in SOS response) with the *recA* wild strain AB1157 (SOS inducible strain) (Fig. 6); the MF of sub-MIC ciprofloxacin induction was significantly higher than the spontaneous mutations in the AB1157 strain (p<0.05), but only a slight higher in the IC400 strain (p>0.05). The SOS response which stimulated the bacteria error prone repair system has been considered the main reason for the mutations of antibiotic resistance induced by antibiotics, especially the type of beta-lactams and quinolones (Lewin et al. 1989; Miller et al. 2004). The evidence of this work indicated that SOS response was one of the main reasons for mutations of antibiotic resistance induced by sub-MIC of ciprofloxacin.

The mutations were time dependent with the supplement of sub-MIC of ciprofloxacin in AB1157 (Fig. 5). It is essential that the high concentrations of antibiotics have selective effects for mutations of antibiotic resistance, because only the mutants can grow in this condition. The data of Fig. 3 showed that cells of AB1157 could be slightly inhibited when sub-MIC of ciprofloxacin was supplemented. However, it was uncertain the increases of mutations were resulting from the different growth with and without sub-MIC of ciprofloxacin supplement.

Gene transfer is an important way for the acquirement of antibiotic resistance (Beaber et al. 2004; Hastings et al. 2004; Lewin et al. 1989). Generally, antibiotic resistance genes exist in plasmid, transposon, integron, and other transposable elements. In this work, the impact of antibiotics on transformation frequency was investigated for the

AB1157 and IC400 strains. The transformation frequency of pMD18-T Vector was significantly increased in the presence of subinhibitory concentrations of ciprofloxacin in the AB1157 strain, but there was almost no change in the IC400 strain (Fig. 7). These results were similar to the mutations induced by sub-MIC of ciprofloxacin in these strains (Fig. 6), and suggested that the *recA* gene played an important role for the transformation of plasmid in bacteria impacted by the stress of antibiotics.

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