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Full Length Research Paper

Detection of QnrB alleles in Enterobacteriaceae and quinolone-resistance expression

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Plasmid-mediated resistance to quinolones in clinical isolates has been found. We have recently identified the types of the plasmid-mediated qnrB genes in *Klebsiella pneumoniae*, *Escherichia coli* and *Citrobacter freundii*. Through BLASTn analysis of qnrB alleles' characteristics, we had obtained qnrB5 gene and qnrB31 gene (GenBank accession number HQ418999) in plasmids of isolates of *K. pneumoniae*, qnrB9 and qnrB16 genes in plasmids of isolates of *E. coli*, and qnrB2, qnrB15 and qnrB18 genes from *C. freundii*. And the susceptibility testing showed that the main causes of resistance to quinolone were mediated by plasmid. The analysis of the structure of *qnrB* alignment showed that LexA-protein-binding site was the determining gene of fluoroquinolone resistance, and if the gene exist, then strains were sensitive to fluoroquinolone and vice versa.

Key words: Quinolone-resistance, QnrB, *Klebsiella pneumonia*, *Escherichia coli*, *Citrobacter freundii*.

INTRODUCTION

Plasmids carrying qnr gene have been found to transmit quinolone resistance (Martínez et al., 1998). These genes encode pentapeptide repeat protein that block the action of ciprofloxacin on bacterial DNA gyrase and topoisomerase IV (Tran and Jacoby, 2002; Tran et al., 2005), resulting in low-level quinolone resistance with an increase in Minimum inhibitory concentration (MIC) of ciprofloxacin for wild-type *Escherichia coli* J53 from 0.016 to 0.25 µg/ml. This reduced susceptibility is most likely important in that it facilitates the selection of mutants with higher-level resistance (Martínez et al., 1998).

The first plasmid-mediated quinolone resistance gene (qnr) was discovered in a *Klebsiella pneumoniaee* isolating from Birmingham, Alabama, 1994 (Martínez et al., 1998). It occurred in a multi resistance plasmid, pMG252, an integron-like structure near Orf513 (Tran and Jacoby, 2002). Qnr plasmids have been found in clinical isolates of *Citrobacter freundii*, *Enterobacter* spp, *E. coli, K. pneumoniaee*, *Providencia stuartii*, and *Salmonella* spp, from the United States, Europe, and the Near and Far East (Cheung et al., 2005; Nordmann and Poirel, 2005). Another qnr gene, qnrS, has also recently been found in a plasmid from a strain of *Shigella flexneri* which was isolated in Japan (Kim et al., 2010). qnrD has also been found in four *Salmonella enterica* isolates which were isolated from China (Cavaco et al., 2008). Since then, qnr alleles have been discovered in clinical strains of gram-negative bacilli around the world. Qnr proteins confer quinolone resistance, and belong to the pentapeptide repeat protein (PRP) family (Guo et al., 2010).

QnrB gene was found to be of most alleles in qnr families, up to now, there were 51 qnrB alleles that have been discovered in the world (see Lahey Clinic http://www.lahey.org/qnrstudies/). Recently, Thomas Guillard discovered qnrB25 (GenBank accession number HQ172108); Xia R, Guo X and Xu H discovered qnrB26; Shin JH discovered qnrB27, qnrB28, qnrB29, qnrB30, the GenBank accession number are HM439641, HM439643, HM439649, HM439650, respectively; and Wang D discovered qnrB31 (HQ418999) in *K. pneumonae* (http://www.lahey.org/qnrstudies/).

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Table 1. Primers used for PCR and sequencing.

Gene	Primer	Primer sequence (5'→3')	Reference Primer	Annealing temperature (°C)	Size of product (bp)
qnrB	Forward	CCTGAGCGGCACTGAATTTAT	DQ777878	57	681
qnrB	Reverse	GTTTGCTGCTCGCCAGTCGA			
qnrB (sequencing)	Forward	ATGACGCCATTACTGTATAAAAAA	DQ777878		
qnrB (sequencing)	Reverse	CTAGCCAATAATCGCGATGCCA			
qnrA	Forward	GCCGTATGGATATTATTGA	AY070235	57	657
qnrA	Reverse	CTAATCCGGCAGCACTAT			
qnrS	Forward	ATGGAAACCTACAATCATAC	AB178643	50	657
qnrS	Reverse	AAAAACACCTCGACTTAAGT			
qnrC	Forward	ACTGAGTTGGCTCATGTAGC	EU917444	50	666
qnrC	Reverse	CCATTAAGTGACCCGTTG			
qnrD	Forward	ACTAACTCGCCGTTTAACAT	EU917444	51	645
qnrD	Reverse	TACCACATTGGGGCATTAGG			

We have recently identified the types of the plasmid-mediated qnrB genes in *K. pneumoniae*, *E. coli* and *C. freundii*. This study was conducted in order to compare the characteristics and prevalence of the plasmid-mediated qnrB alleles gene among *K. pneumoniae*, *E. coli* and *C. freundii*, which were isolated from different specimens from 2008 to 2010 in Taizhou Municipal Hospital of China.

MATERIALS AND METHODS

Strains

We have tested 90 cases of the qnr genes that were resistant to quinolone for *Enterobacteriaceae*, in which 36 isolates were *K. pneumoniae*, 34 isolates were *E. coli* and 20 isolates were *C. freundii*. The qnr gene were identified by amino acid sequence (Strahilevitz et al., 2009). All *K. pneumoniae*, *E. coli* and *C. freundii* were isolated from different specimens from 2008 to 2010 in Taizhou Municipal Hospital of China, which sufficient amount of bacteria could be obtained from blood for culturing. The samples were cultured directly on MacConkey agar (Difco) and were identified as *K. pneumoniae*, *E. coli* and *C. freundii* using biochemical procedures (Chen et al., 2004).

Conjugation and susceptibility testing

According to Wang et al. (2003), conjugation experiments were carried out in LB broth with *E. coli* J53 Az^R (resistance to sodium azide) as the recipients, polymerase chain reaction (PCR) positive strains as donor strains. Cultures of donor and recipient cells in logarithmic phase (0.5 ml of each) were added to 4 ml of fresh LB broth and incubated overnight without shaking. Transconjugants were selected on Trypticase soy agar (TSA) plates containing sodium azide (300 mg/L) and ciprofloxacin (0.03 mg/L), and then incubated to 18-24 h at 35°C. To determine if quinolone resistance was co-transferred, MICs for the donor, recipient, and transconjugant strains were compared (Kim et al., 2010). The MICs were determined by broth dilution and interpreted according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2011).

PCR-amplified and sequencing

To investigate the genetic characteristics of the *qnrB* allele gene in *K. pneumoniaee*, *E. coli*../../../../Program Files/Youdao/Dict4/resultui/queryresult.html, *C. freundii* and their *E. coli* transconjugants, PCR amplification was performed to analyze qnr genes used primers listed in Table 1. Corresponding sense and antisense strands were obtained through positive results of DNA sequencing, then sequencing results were assembled. The analysis of all genes was performed through BLASTn program (http://blast.ncbi.nlm.nih.gov/).

Plasmid analysis

To study the plasmids carrying qnrB2, qnrB5, qnrB9, qnrB15, qnrB16, qnrB18 and qnrB31 genes mapping, the plasmids DNA was extracted (Axygen kit, USA) and separated by 0.6% agarose gel electrophoresis (60 V, 90 min), and then the different sizes of plasmid DNA fragments were cut and recycled (Promega, USA). Used each of recycled plasmid DNA as template, PCR was conducted to amplify qnrB2, qnrB5, qnrB9, qnrB15, qnrB16, qnrB18 and qnrB31genes, where the initial position of all genes plasmids was determined. The primers used for qnrB were all listed in Table 1. The estimated size of plasmid DNA was referenced (Wang et al., 2003).

RESULTS AND DISCUSSION

Susceptibility testing

The MICs results were shown in Table 2. And we could see that the isolates of *K. pneumoniaee*, *E. coli*../../../../

Table 2. Characteristics of isolates for *K. pneumoniaee*, *E. coli*, *C. freundii* and their *E. coli* transconjugants.

Donor bacteria	Amino acid point mutations[a]	qnr gene	MICs(μg/ml)[b]			
			NAL	OFL	LVN	CIP
***K. pneumoniaee* (36)**						
3 (3/36=8.3%)	Ala(N)2→Thr(T), Iie(I)20→Val(V), Ser(S)79→Val(V), Iie(I)142→Met(M), Iie(I)144→Thr(T), Asn(N)198→Ser(S)	qnrB5	>256	8-16	4-8	2
1 (1/36=2.8%)	Arn(N)27→Leu(L), Ser(S)79→Ala(A), Arg(R)87→Ser(S), Gly(G)188→Arg(R), Val(V)212→Iie(I)	qnrB31	>256	16	8	4
***E. coli* (34)**						
2 (2/34=5.9%)	Ser(S)79→Ala(A), Iie(I)142→Met(M), Val(V)212→Iie(I)	qnrB9	>256	16-32	8-16	2-4
1 (1/34=2.9%)	Ser(S)79→Ala(A), Iie(I)142→Met(M), Ala(A)144→Thr(T), Val(V)212→Iie(I)	qnrB16	>256	8-16	4-8	2-4
***C. freundii* (20)**						
1 (1/20=5.0%)	Asp(D)11→Ala(N), Ser(S)79→Ala(A), Iie(I)142→Met(M),Gly(G)188→Arg(R),Val(V)212→Iie(I)	qnrB2	>256	8-16	4-8	2-4
1 (1/20=5.0%)	Glu(E)20→Asp(D), Ser(S)79→Ala(A), Iie(I)142→Met(M)	qnrB15	>256	8-16	4-8	2-4
1 (1/20=5.0%)	Asp(D)11→Ala(N), Ser(S)79→Ala(A), Iie(I)142→Met(M),Gly(G)188→Arg(R), Val(V)212→Iie(I)	qnrB18	>256	8-32	4-8	2-4
*E.coliJ53*AZ^R			2	0.0039	0.0019	0.0019
Transconjugants						
KP1-*E.coli*J53		qnrB5	8-16	0.25- 0.50	0.064-0.128	0.064-0.128
KP2-*E.coli*J53		qnrB31	8	0.25	0.064	0.064
EC1-*E.coli*J53		qnrB9	8-16	0.25-0.50	0.064-0.128	0.064-0.128
EC2-*E.coli*J53		qnrB16	16	0.50	0.128	0.128
FC1-*E.coli*J53		qnrB2	16	0.25	0.064	0.064
FC2-*E.coli*J53		qnrB15	16	0.50	0.128	0.128
FC3-*E.coli*J53		qnrB18	8	0.25	0.064	0.064

[a]Amino acid point mutations were compared with qnrB1 gene (http://www.lahey.org/qnrstudies/). [b]NAL, nalidixic acid, OFX, ofloxacin, LVX, levofloxacin, CIP, ciprofloxacin.

Program Files/Youdao/Dict4/resultui/query-result.html and *C. freundii* showed resistance to ofloxacin, levofloxacin, ciprofloxacin, and nalidixic acid.

Furthermore, the MICs of plasmid transconjugantswere significantly higher than *E. coli J53* Az^R, the tests of transconjugants were successful, it illustrated that the main causes of resistance to quinolone were mediated by plasmid.

The comparison and analysis of variable sites in qnrB alleles

QnrB2, qnrB5, qnrB9, qnrB15, qnrB16, qnrB18 and QnrB31 that we had achieved were compared with other qnrB alleles, and the amino acid sequence diagram was made as Table 3. From Table 3, we could clearly identify the qnrB variable sites and variable

Table 3. Amino acid substitutions in qnrB1 to qnrB31[a].

Allele	Amino acid change at position																																				
	2	**7**	**11**	**18**	**20**	**21**	**22**	**27**	**35**	**36**	**55**	**60**	**69**	**74**	**79**	**80**	**87**	**94**	**118**	**129**	**142**	**144**	**147**	**151**	**162**	**163**	**168**	**171**	**186**	**188**	**198**	**202**	**204**	**205**	**212**	**213**
qnrB1	A	G	D	E	I	E	N	N	L	S	N	M	C	A	S	S	R	A	N	V	I	A	L	F	S	T	A	F	I	G	N	S	L	M	V	I
qnrB2			N												A						M									R					I	
qnrB3											K										M															
qnrB4	T				V							N			I	N		S			M	T			S		V			S			L		M
qnrB5	T				V										V						M	T								S				I	
qnrB6															A						M															
qnrB7															A						M				T										I	
qnrB8	T				V							I			V					A	M	T		I		S	T					A				
qnrB9															A						M														I	
qnrB10	T				V										V						M	T														
qnrB11	T			A	V							I			V			S			M	T				S			V		S		I	L		M
qnrB12	T			A	V							I			V			S			M	T				S			V		S		I	I		
qnrB13															A						M								R						I	
qnrB14						D									A						M													T	I	
qnrB15							S								A	N					M														I	
qnrB16															A						M	T													I	
qnrB17																					M															
qnrB18						D									A						M															
qnrB19	T				V										V						M	T								S						
qnrB20			N												A						M								R							
qnrB21	T				V							I			V					A	M	T		L		S	T			S	A					
qnrB22	T				V					C		N			I	N		S			M	T				S		V		V	S			I		M
qnrB23								Y							A						M														I	
qnrB24									M					V	A						M															
qnrB25					V							I			V				S	A	M	T		L		S	T				S	A				I	
qnrB27	T	S			V										A			S			M	T	A			A					S	A				
qnrB28	T	S			V										V			S			M	T	A			A					S	A				
qnrB29														V	A						M															
qnrB30													S		A						M															
qnrB31								L							A		S				M								R						I	

[a]Variations from the qnrB1 sequence numbered from the second potential ATG initiation codon are shown (http://www.lahey.org/qnrstudies/).

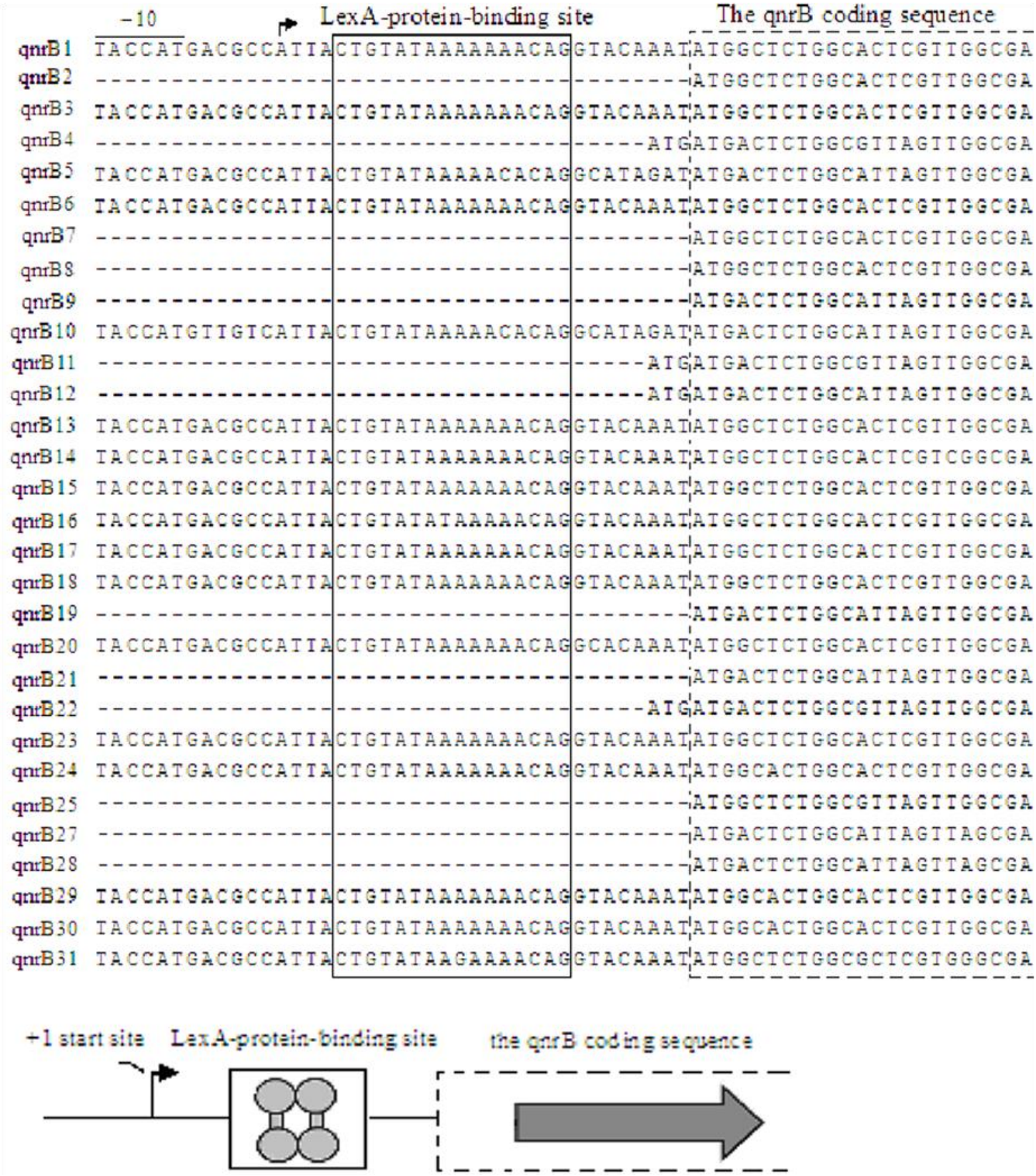

Figure 1. (a) Sequence alignment of the qnrB promoter and qnrB alleles. The –10 promoter elements are indicated; the +1 start site is represented by an arrow; the start of the qnrB coding sequence is indicated by a dashed-open frame and the consensus sequence of the LexA-protein-binding site is boxed. Sequence accession numbers DQ351241, DQ351242, DQ303920, DQ303921, DQ303919, EF520349, EU043311, EU043312, EF526508, DQ631414, EF653270, AM774474, EU273755, EU273757, EU302865, EU136183, AM919398, AM919399, EU432277, AB379831, FJ611948, FJ981621, FJ981622, HM192542, HQ172108, HM439641, HM439643, HM439649, HM439650 and HQ418999 for sequences with promoter regions for qnrB1, qnrB2, qnrB3, qnrB4, qnrB5, qnrB6, qnrB7, qnrB8, qnrB9, qnrB10, qnrB11, qnrB12, qnrB13, qnrB14, qnrB15, qnrB16, qnrB17, qnrB18, qnrB19, qnrB20, qnrB21, qnrB22, qnrB23, qnrB24, qnrB25, qnrB27, qnrB28,, qnrB29, qnrB30 and qnrB31 (to date Dec. 2010) and (b) Diagrammatic drawing of qnrB allele sequences.

composition in qnrB alleles. Although variable sites were fixed relatively in qnrB alleles and the base composition was different in variable sites, the expression of the amino acid composition was largely identical only with minor differences. It illustrated the bases of qnr alleles existed certain numbers of "silent" mutations, which should arouse people's attentions. Based on plasmid analysis (Figure 1), there were two or three different length plasmids in isolates. The qnrB2, qnrB5, qnrB9, qnrB15, qnrB16, qnrB18 and qnrB31genes located in about 23.1 kb length plasmids, respectively.

Prevalent distribution of qnr alleles

After BLASTn through detection of qnrB alleles for 36 isolates of *K. pneumoniae* which were resistant to quinolones, we had achieved qnrB5 gene and qnrB31 gene (GenBank accession number HQ418999) in plasmids of 3 isolates of *K. pneumoniae* and plasmid of 1 isolate of *K. pneumoniae*. The positive rates of qnrB5 and qnrB31 genes in 36 isolates of *K. pneumonia* were accounted for 8.3 and 2.8%.

After BLASTn through detection of qnrB alleles for 34 isolates of *E. coli* which were resistant to quinolones, we had achieved qnrB9 and qnrB16 genes in plasmids of 2 isolates of *E. coli* and plasmid of 1 isolate of *E. coli*. The positive rates of qnrB9 and qnrB16 genes were accounted for 5.9 and 2.9%.

After BLASTn through detection of qnrB alleles for 20 isolates of *C. freundii* which were resistant to quinolones, we had achieved qnrB2, qnrB15 and qnrB18 genes in plasmids of 3 isolates of *C. freundii*. The positive rate of qnrB2, qnrB15 and qnrB18 genes were accounted for 5.0, 5.0 and 5.0%, respectively.

We did not detect qnrA, qnrS, qnrC and qnrD genes, but obtained the corresponding qnrB alleles by transconjugant with *E. coli J53* Az^R (Table 2). It showed that the plasmid-mediated qnrB genes were prevalent in our region, and we should pay more attention.

The structure of qnrB alignment and quinolone-resistance expression

Until now, a total of 51 qnrB alleles have been found. According to previous report (Da Re et al., 2009), we have analysed the qnrB gene as described in Table 3. A complete qnrB gene sequences consists of three parts sequence of different meanings: promoter sequence from -35 to -10 regions, the consensus sequence of the LexA-protein-binding site and the qnrB coding sequence. The consensus sequence of the LexA-protein-binding site is the most key sequence for quinolone-resistance (Da Re et al., 2009; Wang et al., 2009). If any kind of quinolone antibiotic could make the consensus sequence of the LexA-protein-binding site open and truncated, and only leave the promoter sequence and the qnrB coding sequence in qnrB alignment, the isolates would express quinolone-resistance (Figure 1). The isolates of *K. pneumoniaee*, *E. coli*../../../../Program Files/Youdao/Dict4-/resultui/queryresult.html and *C. freundii* showed resistance to ofloxacin, levofloxacin, ciprofloxacin, and nalidixic acid in our experiments (Table 2), suggesting that the LexA-protein-binding site had been made open and truncated. On induction of the SOS response, taking ciprofloxacin for example, single stranded DNA (ssDNA) is produced and the co-protease activity of the RecA protein is activated by binding to ssDNA. As described to Da Re et al. (2009), the interaction between LexA and the nucleoprotein filament RecA/ssDNA results in autoproteolytic cleavage of LexA, and subsequently leading to qnrB derepression. Induced expression of qnrB leads to an increase in the ciprofloxacin minimal inhibitory concentration.

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