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The Role of Some Transcription Factors in Expression of *GyrA* and *GyrB* Following Exposure to Ciprofloxacin

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Abstract

Background: *GyrA* and *gyrB* genes encode DNA gyrase subunits. This enzyme regulates DNA supercoiling. Inhibitors of this enzyme, such as ciprofloxacin, may change the level of supercoiling and the expression level of genes, including *gyrA* and *gyrB*.
Objectives: The aims of this research were first to select some transcription factors, which regulate the expression of *gyrA* and *gyrB*. Secondly, the effect of these transcription factors was investigated on the expression of these genes in *Escherichia coli* mutants with different levels of resistance to ciprofloxacin in the presence and absence of these transcription factors.
Methods: For this purpose, the online software called Promoter Analyzer in Virtual Footprint version 3 was used to find and select some transcription factors. The relative expression of genes was determined by quantitative real-time polymerase chain reaction (qRT-PCR).
Results: Theoretical results showed that CspA, FhlA, and SoxS transcription factors (with a score of match higher than 6), could be selected for further analysis. The expression of *gyrA* and *gyrB* genes remained unchanged in the presence and absence of CspA and FhlA transcription factors following exposure to the low amount of ciprofloxacin. However, SoxS transcription activator might have indirect effects on the expression of these genes, as *soxS* gene was overexpressed following treatment with a higher amount of ciprofloxacin.
Conclusions: It is concluded that overexpression of *gyrA* and *gyrB* genes is not dependent on CspA and FhlA transcription factors, but may be dependent indirectly on regulatory proteins involved in oxidative stress following exposure to ciprofloxacin.

Keywords: DNA gyrase, Transcription Factors, *Escherichia coli*, Ciprofloxacin Resistance, Gene Expression

1. Background

The chromosome of *Escherichia coli* is normally maintained in a negatively supercoiled state by the activity of DNA gyrase and topoisomerase I (1, 2). DNA gyrase is a heterotetramer enzyme that consists of two *GyrA* and two *GyrB* subunits (A2B2). Treatment of cells with antibiotics, which target genes encoding DNA gyrase subunits (*gyrA* and *gyrB*) leads to DNA relaxation in *E. coli* (1). These relaxing conditions are known to promote *gyrA* and *gyrB* gene expression. DNA gyrase is one of the important targets for antibacterial drug development. DNA gyrase inhibitors are classified into two groups based on their origin, including natural and synthetic drugs (2). Aminocoumarins, simocyclinone D8, and cyclothialidines are examples of natural drugs. Although they have potent activity, their clinical use is limited due to various reasons, including eukaryotic toxicity, poor efficiency against Gram-negative bacteria, poor penetration into bacterial cytoplasmic cell membrane (2).

On the other hand, synthetic inhibitors examples are microcin B17, CcdB, and fluoroquinolones (a group of quinolones). The first example is a weak inhibitor of DNA gyrase as compared with quinolones. The second one needs ATP for its activity (2). While fluoroquinolones are good and widely used drugs against Gram-negative and -positive bacteria. These antibiotics interact with both subunits of DNA gyrase, namely *GyrA* (N-terminal domain) and *GyrB* (C-terminal domain), and trap the enzyme attached to DNA break, thereby preventing DNA religation in a ternary complex (quinolone-DNA gyrase-DNA) (2). However, the development of quinolone resistance limits the clinical efficacy of these antibiotics.

Previous studies have shown that quinolone resistance arises from mutations in target genes (*gyrA* and *gyrB*), up-regulation of efflux pump, AcrAB-TolC and protection of *gyrase* with the plasmid-encoded protein QnR (3, 4). To eradicate the quinolone-resistant mutants, there is an urgent need to decrease the synthesis of DNA gyrase subunits at transcriptional levels. In the previous study, the ribonucle-

ase P (RNase P)-based external guide sequence (EGS) technique was used to downregulate *gyrA* expression (5). In order to design new drugs, it is important to find target proteins, which upregulate the expression of *gyrA* and *gyrB* at the logarithmic phase of growth at 37°C. There is no information about this matter. It was known that CspA as a transcription activator increases the synthesis of *GyrA* under cold shock conditions (10°C) through binding at specific sequence upstream of the *gyrA* gene (6).

2. Objectives

The aims of this work were to select regulatory proteins, which could bind upstream region of *gyrA* and *gyrB* genes using online software and to investigate the expression of *gyrA* and *gyrB* genes in mutants, lacking these regulatory proteins.

3. Methods

3.1. Prediction of Transcription Factors (TFs)

TFs are regulatory proteins that bind to target DNA, usually located near promoters and regulate transcription from promoters through interaction with RNA polymerase (7). Approximately 500 nucleotides upstream of the translation start site of *gyrA* and *gyrB* genes were considered to find TFs. An online software, Virtual Footprint Promoter Analyzer version 3.0 (http://prodoric.tu-bs.de/vfp/vfp_promoter.php) was used to find TF of *gyrA*, *gyrB*, and dnaA genes. The TF sequence binding site with the score of match higher than 6 to given sequence (upstream of *gyrA*, *gyrB*, and *dnaA* genes) were selected for further consideration.

3.2. Bacterial Strains and Growth Condition

We used Lauria Bertani (LB; Merck, Darmstadt, Germany) as the liquid medium and LB agar as the solid medium, prepared by addition of agar (Merck, Darmstadt, Germany) to LB broth. Ciprofloxacin was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Stock concentration was 10 mg/mL. In addition, the parent strain, *E. coli* BW25113 and knockout mutants JW3525 (BW25113 Δ*cspA*::Kanr) and JW2701 (BW25113 Δ*fhlA*::Kanr) were obtained from the Keio collection (8). The minimum inhibitory concentration (MIC) of the strains was almost similar to the MIC of wild type strain (MG1655) for ciprofloxacin (35 ng/mL). Clones with higher resistance to ciprofloxacin were generated from JW3525 and JW2701 mutants after cultivation on LB broth-containing increasing amounts of ciprofloxacin (9). The resistant derivatives, as well as the MG1655 derivatives with their corresponding resistance, are presented in Table 1.

Table 1. Bacterial Strains and Mutants

Strain/Mutant	Genotype	MIC (μg/mL)	Source/Reference
MG1655	Wild type	0.035	A gift from Prof. R G Lloyd
W52	*cspA*$^+$ *gyrA* ($Ser_{83}\rightarrow$Leu)	0.3	(10)
C22	*cspA*$^+$ *gyrA* ($Ser_{83}\rightarrow$Leu) *marOR* (20 bp duplication)	2	(10)
M2	*cspA*$^+$ *gyrA* ($Ser_{83}\rightarrow$Leu) *marOR* (20 bp duplication) *acrAB* overexpression	100	(10)
JW3525	BW25113 Δ*cspA*::Kanr	0.05	Keio collection
HA2	cspA$^-$	0.3	This work
JW2701	BW25113 Δ*fhlA*::Kanr	0.045	Keio collection
HA1	*fhlA*$^-$	0.3	This work
HA3	*fhlA*$^-$	2	This work

3.3. 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 different concentrations of ciprofloxacin (0.1 × MIC and 0.5 × MIC) and grown to mid-logarithmic phase (OD_{600} of 0.5 - 0.6), as described previously (10). Then, RNA protect reagent (Qiagen, Hilden, Germany) was added to cultures and incubated for 5 minutes at room temperature. Cultures were centrifuged, and cell pellets were used for RNA extraction.

3.4. RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Assay

Total RNA was extracted from all strains mentioned above using an RNeasy Mini kit (QIAGEN, Hilden, Germany), according to the protocol of the manufacturer. To remove DNA, RNA samples were treated with RNase-free DNase (Fermentas, Waltham, MA), according to the manufacture's protocol. DNase treated RNA was repurified using an RNeasy Mini kit (QIAGEN, Hilden, Germany). RNA samples were amplified by PCR to show the absence of DNA. Primers for *soxS* gene were used in PCR reaction (Table 2). The purity and concentration of samples were determined using the Biochrom™ Ultrospec 1100 spectrophotometer (Thermo Fisher Scientific, Ottawa, Ontario, Canada). Suitable RNA samples should have a ratio of A260/A280 more than 1.8. Then, they were used for the synthesis of cDNA. Reverse transcription was performed using the RevertAid Reverse Transcriptase kit (Thermo Fisher Scientific, Ottawa,

Table 2. List of Primers

Gene	Primer sequence (5′-3′)	Length of amplicon (bp)	Reference
gyrA	F: GCCATGAACGTACTAGGC	180	This work
	R: GGATATACACCTTGCCGC		
gyrB	F: AGAAATTATCGTCACCATTCACGC	278	(11)
	R: GTACACCGTGTTCGTAGATCT		
soxS	F: CCAGGTCCATTGCGATATCA	201	(9)
	R: CGCATGGATTGACGAGCATA		
gapA	F: ACTTACGAGCAGATCAAAGC	170	(9)
	R: AGTTTCACGAAGTTGTCGTT		

Abbreviations: F, forward; R, reserve.

Ontario, Canada), random hexamer and Purified total RNA (2 μg).

The cDNAs obtained from reverse transcription were amplified by PCR reaction with specific primers. Then, they were used to determine the level of gene expression by qRT-PCR using Rotor Gene 6,000 thermocycler (Corbet Research, Sydney, Australia) and the SYBR Green kit (TaKaRa, Otsu, Japan). Primers for *gyrB*, *soxS*, and *gapA* were described in previous studies and presented in Table 2 (9, 11). Primers for *gyrA* were designed by Primer3 V. 0.4.0 software (http://primer3.ut.ee). Thermal cycling conditions were selected based on previous research, except for the annealing temperature, which varied from one gene to another (10). Melting curve analysis (60 - 95°C) was conducted with continuous fluorescence readings. The relative gene expression was determined using the pfaffl method (ratio of target gene expression to *gapA* expression) (12). Data related to gene expression are presented as the average of duplicate analyses. Significant differences in gene expression were determined by Student's *t*-test (two-paired samples, with two-tailed distribution), using SPSS version 16 software (SPSS Inc., Chicago, IL).

4. Results

4.1. Prediction of TFs for gyrA and gyrB Genes

For prediction of TFs for the above genes, online software called Virtual Footprint Promoter Analyzer version 3.0 was used. Sixty-one segments with TF binding characteristics were found for *gyrA*. Twenty-seven segments were bound to 14 TFs with scores above six (Table 3). Among these TFs, CspA had a score of 10. There were three CCAAT sequences on the negative strand. These sequences are known to be the recognition elements for CspA protein (6) (Figure 1A). It was shown that the overproduction of CspA clearly increases the synthesis of *GyrA* following cold shock conditions. These conditions also enhance the synthesis of *GyrB* protein (6). *GyrB* is located on a four-gene operon (ordered *dnaA-dnaN-recF-gyrB*). In addition to general promoter, each of these genes has its own promoter(s) (13). There were three CCAAT sequences upstream of above operon (Figure 1B), and CspA might be an activator of this promoter.

The first gene of the operon (*dnaA*) contained two promoters (14, 15). Based on the results, 83 segments were found for *dnaA*. Thirty-five segments were bound to 18 TFs, with scores above six (Table 4). One of these TFs is DnaA, which can also bind the upstream region of *gyrA* and *gyrB*. For *gyrB*, there were eight segments bound to seven TFs, with scores higher than 6 (Table 5). FhlA had a score of 10.7. There was also one FhlA binding site in the promoter region of the operon (Figure 1B). FhlA is generally a transcriptional activator of formate metabolism under anaerobic conditions (16). It is claimed that FhlA is not produced in the presence of *oxyS* mRNA (16). However, the role of FhlA in the expression of *gyrA* and *gyrB* genes is not clear. The transcription of *oxyS* is upregulated due to oxidative stress by OxyR. Both OxyRS and SoxRS are mediated by oxidative stress, induced by ciprofloxacin (17). Based on these theoretical findings, the expression of *gyrA* and *gyrB* in the presence and absence of CspA and FhlA, in addition to the expression of the *soxS* gene in ciprofloxacin-resistant *E. coli* mutants are described in the following sections.

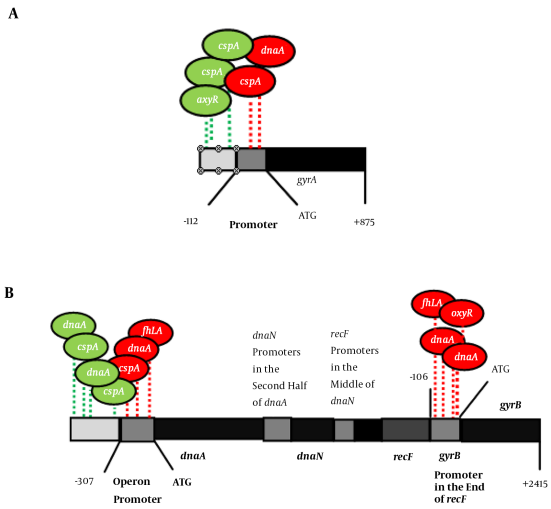

Figure 1. Several transcription factors (TF) involved in regulation of *gyrA* (A), *dnaA* and *gyrB* (B). Green and red ovals show TFs that assume to regulate genes positively and negatively, respectively.

Table 3. The List of Predicted Transcription Factors for *gyrA*

TF	Name	Start	End	Strand	Score	Sequence
ArcA	Aerobic respiration control protein	348	357	+	6.4	TGTTATAATT
ArcA		307	316	+	6.36	TGTGAATAAA
ArcA		131	140	+	6.16	GGTTAATGCG
ArgR	DNA arginine binding transcriptional	303	316	+	8.86	TGGATGTGAATAAA
ArgR		346	359	+	8.61	TGTGTTATAATTTG
Crp	cAMP receptor protein	209	230	+	6.17	CTTCGTGGTCTACGTTATGGTT
Crp		60	81	-	6	AAAGGTGCTCGATGTCGGTTGT
CspA	Cold shock protein	301	305	-	10	CCAAT
CspA		273	277	-	10	CCAAT
CspA		253	257	-	10	CCAAT
CytR	Regulator for deo operon	350	361	-	7.9	CGCAAATTATAA
CytR		192	203	-	7.84	GCTAAATTTGAA
CytR		192	203	+	7.58	TTCAAATTTAGC
DnaA	Initiation of chromosome replication	307	315	-	7.48	TTATTCACA
Fnr	Transcriptional regulation of respiration	322	335	-	7.16	TTGAGGTAAACCTA
Fnr		303	316	+	6.96	TGGATGTGAATAAA
IHF	Integration host factor	280	295	+	6.21	AGACAAACGAGTATAT
IHF		141	156	+	6.14	GTGCAGCGGTTTGAAC
IHF		123	138	+	6.10	ACGCAGCGGGTTAATG
MetJ	Methionine repressor	161	169	+	6.81	CTTCCAGAT
MetR	Regulator for metE and metH	309	315	+	9.17	TGAATAA
Mlc	Putative NAGC-like transcriptional regulator	241	246	-	6.39	CGAAAA
OmpR	Regulator transcription of ompC and ompF	239	245	-	8.7	GAAAAAT
OxyR	Positive regulator of hydrogen peroxide inducible activator	111	156	+	13.28	AATATAGCCCAGACGCAGCGGGTTAATGCGGTGCAGCGGTTTGAAC
PdhR	Transcriptional regulator for PDH	193	198	+	6.35	TCAAAT
PdhR		272	277	-	6.26	CCAATT
PdhR		332	337	+	6.20	TCAAAC

4.2. RNA and cDNA Samples Quality

To ensure that RNA samples were not contaminated with DNA, they were amplified by the PCR method. Figure 2 shows the result of gel electrophoresis of PCR amplification. As can be seen from Figure 2, RNA samples were not contaminated with DNA. The ratio of A260/A280 of RNA samples was more than 1.8. These samples were used for cDNA synthesis. Moreover, cDNAs were used to amplify *gyrA*, *gyrB*, and *soxS* genes. PCR products were electrophoresed on the agarose gel. Figure 3 shows cDNAs were suitable for measuring gene expression by qRT-PCR.

4.3. Expression of gyrA and gyrB in the Presence and Absence of CspA and FhlA

The expression of *gyrA* gene was measured in low ciprofloxacin-resistant mutants ($cspA^+$ and $cspA^-$) in the presence of ciprofloxacin (0.1 × MIC). The expression of *gyrA* in a $cspA^-$ clone was nearly the same as $cspA^+$ one ($P > 0.5$) (Table 6). Therefore, the elimination of *cspA* did not affect the expression of *gyrA*. The same result was obtained for the expression of *gyrB* in a $cspA^-$ and $cspA^+$ clones ($P > 0.5$). Inactivation of *fhlA* did not significantly change the level of *gyrB* expression in $fhlA^-$ clones compared to $fhlA^+$ clones ($P > 0.5$) in the presence of ciprofloxacin (0.1 × MIC) (Table 6).

4.4. Expression of gyrA, gyrB, and soxS in the High Ciprofloxacin-Resistant Mutant (M2)

After exposure to high (0.5 × MIC) and low (0.1 × MIC) amounts of ciprofloxacin, the expression of *soxS*, *gyrA*, and *gyrB* increased in the mutant resistant to a high concentration of ciprofloxacin ($P < 0.5$), but not in mutants showing resistance to low and intermediate concentrations of ciprofloxacin ($P < 0.5$) (Table 6). This might indicate that at higher concentrations of ciprofloxacin, SoxS could act as a transcriptional activator of *gyrA* and *gyrB*.

Table 4. The List of Predicted Transcription Factors for *dnaA*

TF	Name	Start	End	Strand	Score	Sequence
ArgR	DNA arginine binding transcriptional	381	394	+	8.53	TGGATCTTTATTAG
CpxR		260	275	-	12.36	GGAAAAGCGCGGTAAA
Crp	cAMP receptor protein	291	312	+	6.82	GAAAATGTACGACCTCACACCA
Crp		64	85	-	6.45	AAGCGCAACCGTTCTCACGGCT
Crp		130	151	+	6.37	AAACTTGAATAAATTCAATGGC
CspA	Cold shock protein	434	438	+	10	CCAAT
CspA		405	409	+	10	CCAAT
CspA		155	158	-	10	CCAAT
CytR	Regulator for deo operon	424	435	-	7.65	GGAAAATTTAAT
CytR		254	265	-	7.54	GGTAAATAAGGA
CytR		403	414	+	7.48	CAAAAATTGGCT
DnaA	Initiation of chromosome replication	504	512	-	7.52	TTATCCACA
DnaA		270	278	+	6.44	TTTTCCGCA
DnaA		133	141	-	6.01	TTATTCAAG
FhlA	Transcriptional activator for fdhf	648	654	+	10.14	TTTTCGA
Fnr	Transcriptional regulation of respiration	134	147	-	7.40	TTGAATTTATTCAA
Fnr		134	147	+	7.26	TTGAATAAATTCAA
Fnr		651	664	-	6.57	TTGACGTACGTCGA
GcvA	Positive regulator of gcv operon	390	394	-	10	CTAAT
GlpR	Glycerol-3-phosphate regulon repressor	294	313	+	6.19	AATGTACGACCTCACACCAG
GlpR		586	605	-	6.14	TATGCCCGATCAAGATCCTG
GlpR		353	372	-	6.02	CTTGCGCTTTACCCATCAGC
IHF	Integration host factor	142	157	+	6.72	ATTCAATGGCTTTATT
IHF		88	103	+	6.02	GTACAGACGGTTGAA
MalT	Positive regulator of mal regulon	339	344	+	8.45	GGAGGA
MalT		375	380	-	7.86	GGACGA
MalT		716	721	-	7.82	GGCGGA
MetJ	Repressor of met genes	161	169	+	6.81	CTTCCAGAT
MetR	Regulator for metE and metH	309	315	+	9.17	TGAATAA
Mlc	Putative NAGC-like transcriptional regulator	241	246	-	6.39	CGAAAA
OmpR	Regulator transcription of ompC and ompF	239	245	-	8.7	GAAAAAT
OxyR	Positive regulator of hydrogen peroxide inducible activator	111	156	+	13.28	AATATAGCCCAGACGCAGCGGGTTAATGCGGTGCAGCGGTTTGAAC
PdhR	Transcriptional regulator for PDH	193	198	+	6.35	TCAAAT
PdhR		272	277	-	6.26	CCAATT
PdhR		332	337	+	6.20	TCAAAC

5. Discussion

One of the essential enzymes for DNA replication is DNA gyrase, which consists of two subunits, *GyrA* and *GyrB*. This enzyme is a proper candidate for the development of antibacterial drugs. Among current antibacterial drugs, ciprofloxacin is a suitable antibiotic against Gram-negative bacteria, such as *E. coli* (2). However, the emergence of ciprofloxacin-resistant *E. coli* mutants has limited the clinical use of this antibiotic. To overcome this prob-

Table 5. The List of Predicted Transcription Factors for *GyrB*

TF	Name	Start	End	Strand	Score	Sequence
Crp	cAMP receptor protein	9	30	+	6.5	CTTTGTCAGCGCGATCAGTGCT
CytR	Regulator for deo operon	54	65	+	7.78	CGAAAATTCGAA
DnaA	Initiation of chromosome replication	75	83	-	6.51	TTTTCCACG
DnaA		83	91	-	6.02	TTTTACCCT
FhlA	Transcriptional activator for fdhf	53	59	-	10.7	TTTTCGT
GlpR	Glycerol-3-phosphate regulon repressor	18	37	-	6.22	CGTGTTCAGCACTGATCGCG
OmpR	Regulator transcription of ompC and ompF	55	65	+	7.92	GAAAATT
OxyR	Positive regulator of hydrogen peroxide inducible activator	83	128	-	12.59	ACGTTTCTCGCTCATTTATACTTGGGTTAATCCGTTATTTTACCCT

Table 6. Relative Expression of Genes in Mutants as Determined by Real-Time PCR[a]

Gene	Wild Type		Mutants				
		W52	C22	M2	HA1	HA2	HA3
gyrA	1.0	0.8	1.3	2.6	-	1.7	-
gyrB	1.0	1	1.2	2.5	1.5	1.5	1.6
soxS	1.0	0.8	0.9	4.8			

[a]Values represent fold-change (mean of two sets with three samples) in comparison to the wild type strain (MG1655). In all cases, the standard deviation was less than 10% of the mean. Values more than 2 were considered overexpression.

lem, it is necessary to inhibit the expression of antibiotic target genes, encoding two enzyme subunits of *gyrA* and *gyrB*. Therefore, the identification of TFs, which upregulate the expression of these genes, is needed to design new drugs against these regulatory proteins. In the present study, it was theoretically proposed that CspA and FhlA might influence the expression of *gyrA* and *gyrB*. However, based on experimental findings, they do not play any roles in the regulation of these genes following the treatment with ciprofloxacin.

CspA is a member of cold shock proteins (including CspA, CspB, CspE, CspG, etc.), which are found in Gram-positive and -negative bacteria. They may have overlapping functions, and at least one of these proteins is necessary for bacterial growth. It was shown that *cspA* transcripts are more abundant in the mid-log phase, while they decrease in the stationary phase to nearly undetectable levels in *E. coli* in both minimal (M9) and rich (LB) media at 37°C (18). During cold shock, CspA acts as a transcriptional activator both at transcription and translation levels of genes, such as *gyrA* (18). Our findings, which showed that elimination of *cspA* did not affect the expression of *gyrA* and *gyrB* might imply that regulation of these genes is not controlled by CspA in the presence of ciprofloxacin. It was reported that CspE negatively regulates *cspA* transcription (19). Therefore, CspE may act as an active cold shock protein in the presence of ciprofloxacin.

Moreover, *fhlA* gene belongs to *hyp* operon (formate hydrogenlyase), and its encoded protein, FhlA is involved in formate metabolism as a transcriptional activator under anaerobic condition (20). Our findings, which revealed that inactivation of *fhlA* did not change the expression of *gyrB* might indicate that induction of oxidative stress via either OxyRS or SoxRS inactivates FhlA. In addition, low expression of *fhlA* at a low concentration of ciprofloxacin (3 ng/mL) has been previously reported in a wild type strain (MG1655) (21). Therefore, under aerobic conditions, FhlA does not act as a regulatory protein to enhance the expression of *gyrA* and *gyrB* in the presence of ciprofloxacin. Oxidative response is mediated by the OxyR and SoxRS proteins.

SoxRS is closely related to a multiple antibiotic resistance regulator protein (MarA). MarA is upregulated following exposure to ciprofloxacin and activates the AcrAB-TolC pump (22). Ciprofloxacin, in turn, stimulates the production of reactive oxygen species (ROS) in bacterial cells (17). SoxS as a regulatory protein can also enhance the activation of the AcrAB-TolC pump. Our findings that indicating the overexpression of *soxS*, *gyrA* and *gyrB* in the high ciprofloxacin-resistant mutant, indirectly suggest that FhlA may be inactive in the high ciprofloxacin-resistant mutant and that SoxS may play an indirect role in the expression of *gyrA* and *gyrB* in the event of oxidative stress.

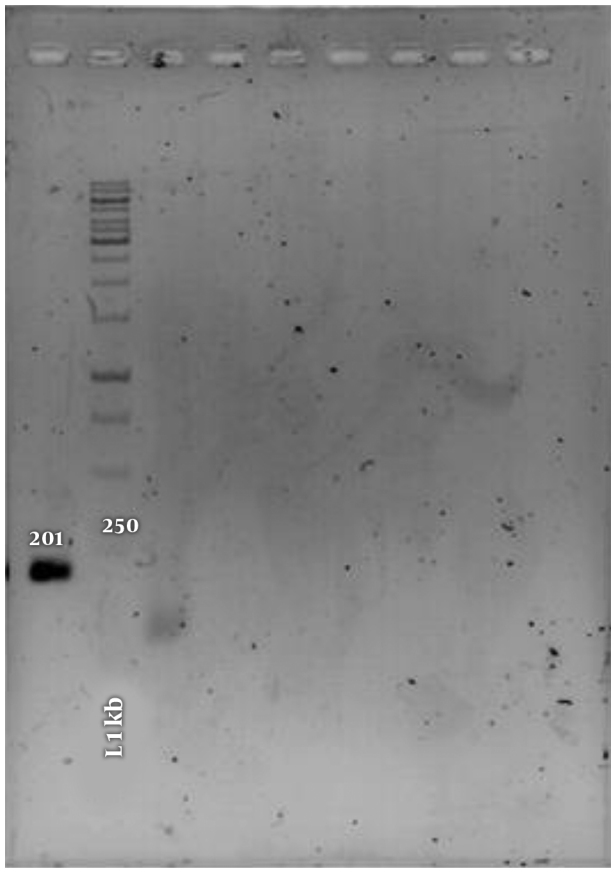

Figure 2. Gel electrophoresis of PCR products of RNA samples. First lane from left shows positive control for PCR reaction, which represents PCR product of soxS gene. Second lane shows size marker (1 kb). Remaining lanes represent PCR products of RNA samples.

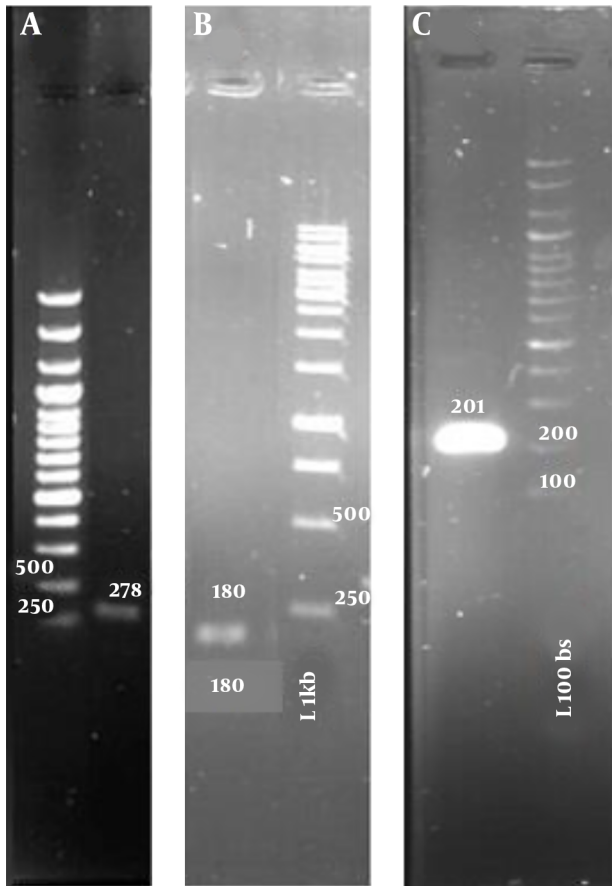

Figure 3. Gel electrophoresis of PCR products of the cDNA sample. Parts A, B, and C show PCR amplification of *gyrB*, *gyrA* and soxS genes, respectively. L 1 kb and L 100 bs represent size marker 1 kb and 100 bs, respectively.

Furthermore, the finding regarding the overexpression of *gyrA* and *gyrB* is consistent with a previous study, which suggested that transcription of topoisomerase genes was sensitive to supercoiling changes and enhanced following the treatment of *E. coli* cells with quinolone (norfloxacin) and coumarin (novobiocin) (23). However, we could not find any information about the expression of soxS in the mentioned study; this implies that the role of SoxS cannot be excluded. Since there are many DNA binding sites upstream of *gyrA* and *gyrB* for regulatory proteins, changes in DNA supercoiling may provide an opportunity for attachment of some regulatory proteins other than CspA and FhlA.

5.1. Conclusions

It can be concluded that under aerobic conditions, the production of ROS, especially by ciprofloxacin, induces SoxS regulon in bacteria, which acts as a strong defense against this antibiotic by activation of AcrAB-TolC efflux pump. It is recommended to use higher concentrations of ciprofloxacin with more caution against Gram-negative bacteria, such as *E. coli*.

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Footnotes

Authors' Contribution: Razieh Pourahmad Jaktaji conceived and designed the study and analyzed the data. Razieh Pourahmad Jaktaji and Seyed Mohammad Lesani conducted bioinformatic analyses. Razieh Pourahmad Jaktaji,

Seyed Mohammad Lesani, Hoda Akhavan, and Maryam Tanhaei performed the experiments. Razieh Pourahmad Jaktaji wrote the manuscript. All of the authors have read and approved the final manuscript.

Conflict of Interests: The authors declare that there is no conflict of interest.

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