Evaluation of gene expression and protein structural modeling involved in persister cell formation in *Salmonella* Typhimurium

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Abstract

Persisters are phenotypic variants of the bacterial population that survive against lethal doses of bactericidal antibiotics. These phenotypes are created in numerous bacterial species, including those of clinical significance, such as *Salmonella* Typhimurium. Since persister cells are associated with the failure of antibiotic treatment and infection recurrence, it is crucial to identify the mechanisms that influence the formation of these cells. The aim of this study is to investigate the persister cell formation and expression analysis as well as to predict the 3D structure of the genes involved in the production of persister cells. The presence of persisters in *S. Typhimurium* was determined by time dependent killing of different types of bactericidal antibiotics and expression of genes associated with persister cell formation which was assessed five hours after the addition of antibiotics by the qRT-PCR. Indeed, the 3D structural model of the proteins studied was predicted by performing several computational methods of retrieved primary protein sequences. The results of the study showed that the *S. Typhimurium* produced high levels of persister cells in the exposure of bactericidal antibiotics. Furthermore, qRT-PCR resulted in the fact that the expression of related genes was different depending on the type of antibiotic. Overall, this study provides information on the creation of persister cells and the role of different genes in the formation of these cells and structure of proteins involved in the production of persister cells in *S. Typhimurium*.

Keywords *Salmonella* Typhimurium · qRT-PCR · Persister cell · 3D structure

Introduction

Persister cells are defined as part of the bacterial population that survives against lethal doses of antibiotics [1]. These cells, with genetic and antibiotic minimal inhibitory concentration (MIC) values, are similar to antibiotic susceptible cells in the exposure of antibiotics by phenotypic switching which cause inactivation of physiological state, slow growing, and

dormancy and can tolerate high concentrations of antibiotics [2]. However, after the antibiotic removal, the persister cells regrow and form a heterogeneous population, including tolerant and susceptible subpopulations equivalent to the parental culture [3]. Multi-drug tolerant persister cells not only cause failure the antibiotic treatment, but also help the evolution of antibiotic resistance [4]. Therefore, persister cells are becoming a major global concern due to their clinical importance.

Since the first description of persister cells in 1944 by Joseph Bigger, bacterial persister cells have been identified in various microbial species, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Mycobacterium tuberculosis*. Most of these bacterial species contain persister cells in biofilm and studies have demonstrated that there is a direct link between the presence of these cells and recurrence of infections such as tuberculosis and cystic fibrosis pneumonia after antibiotic therapy [5, 6].

Persister cells which contribute to the failure of treatment and enhance drug resistance not only is in many pathogenic bacteria, but also in eukaryotic cells, such as yeasts and even human cancer cells [7]. Accordingly, a better understanding of

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the mechanisms associated with persister cell formation is important for developing treatment strategies against recurrent infections as well as improving our ability to cope with the continuing crisis of antibiotics [8].

Various mechanisms such as SOS response, oxidative stress response, stringent response, toxin antitoxin (TA) systems, and efflux pumps that can enable some cells in a bacterial population will become multi-drug tolerant persister cells [9].

The SOS response is an important mechanism of bacterial survival under stress conditions activated by DNA damage and maintains cell survival and cell integrity by repairing genetic material [10]. A network of genes was involved in the SOS response whose expression is regulated by a transcriptional repressor called LexA and the DNA binding activator protein, RecA [11]. The SOS gene network not only affects mechanisms such as recombination and repair of DNA, but also influences other cellular processes, including bacterial virulence, biofilm formation, and antibiotic tolerance [10]. Treatment with bactericidal antibiotics generates reactive oxygen species (ROS) containing superoxide and hydrogen peroxide and induces an oxidative stress response in bacterial cells [12]. In *Salmonella* Typhimurium, many genes are expressed during oxidative stress, whose expression is regulated by SoxRS and OxyR regulons involved in the formation of persister cells by inactivating ROS [13]. During environmental pressure such as amino acid starvation, the two enzymes RelA and SpoT are responsible for producing “alarmone” molecule guanosine tetraphosphate (ppGpp) and inducing the stringent response in the *S. Typhimurium* [14]. During the stringent response, ppGpp—having direct interactions with RNA polymerase until the cell condition is improved—reduces protein synthesis and modifies the transcriptional profile, thereby contributing to cell persistence [15]. Multi-drug efflux systems in the bacteria cause the removal of antibiotics from the bacterium and reduce the accumulation of antibiotics within the bacterial cell. Various studies have demonstrated that increased expression of these efflux systems by eliminating antibiotics results in the survival of the bacteria and also tolerance to different drugs within the bacterial population [16]. One of the best mechanisms studied in the formation of persister cells is toxin antitoxin (TA) system [17, 18]. TA systems consist of two genes on a single operon encoding toxin and cognate antitoxin [19, 20]. Under normal conditions, both genes are expressed and by forming a protein-protein complex, antitoxin inhibits the activity of the toxin, while under stress conditions, the proteases degrade the antitoxin, render the toxins by effecting on essential cellular processes (DNA replication and protein translation), cause growth inhibition and bacterial dormancy, and escape the effects of drugs or stress conditions [21, 22]. Universal stress proteins are found in bacteria, archaea, fungi, protozoa, and plants. UspA upregulates in response to a large number of different environmental stress such as oxidative, high temperature, starvation, and antibiotic stress. Overexpression of the *uspA* under stresses conditions by altering the expression of many genes

allows the bacterium to survive against stressors and persist in these conditions [23].

Based on the fact that the biological function of a protein is determined by its structure, as well as the structure of the protein can be determined which drugs and molecules can interact with the protein, which is important for drug development, protein structure prediction is one of the most important aspects of bioinformatics [24]. Protein structure can be predicted using X-ray crystallography and nuclear magnetic resonance (NMR). However, these techniques are very time-consuming and costly, so today, different reliable web-based bioinformatics software for predicting protein structure has been developed and computational methods supply a dependable alternative to experimental methods [25]. In this study, we aimed to investigate the persister formation ability of *S. Typhimurium* upon exposure to different types of antibiotics as well as to analyze the effects of lethal concentrations of selected antibiotics on the expression levels of genes that may be involved in persister cell formation. Additionally, to provide better comprehension concerning the structure and function of these genes, molecular modeling was performed.

Material and methods

Bacterial strain and growth conditions

S. Typhimurium ATCC 14028 was stored in brain heart infusion (BHI) broth containing 20% glycerol at -80°C and was grown in Luria–Bertani (LB) agar plates at 37°C .

Determination of Minimum Inhibitory Concentration (MIC)

The broth microdilution method was used to determine MICs of ampicillin, colistin, and ciprofloxacin against *S. Typhimurium* according to the Clinical and Laboratory Standards Institute (CLS M100 ED29, 2019). To determine the MIC of ampicillin, colistin, and ciprofloxacin (Sigma Aldrich, Taufkirchen, Germany), 100 μL of 0.5 McFarland overnight bacteria culture diluted 1:100 in Mueller-Hinton Broth (MHB) with serial two-fold dilution of each antibiotic in a range 128 to 0.25 $\mu\text{g}/\text{ml}$ for ampicillin and colistin and 8 to 0.0312 $\mu\text{g}/\text{ml}$ range for ciprofloxacin added to 96 well-microtiter plates.

The MIC value of each antibiotic was investigated independently three times.

Persister assay

Persister cells were isolated by time-dependent killing experiments. A single colony of *S. Typhimurium* inoculated into 5 ml LB broth for 24 h and an overnight culture was diluted 1:100 in LB broth and incubated at 37°C on a shaker at

200 rpm until the log phase optical density at 600 nm reached 0.25 and then these cultures were independently treated with 50 µg/ml ampicillin (100X MIC), 80 µg/ml colistin (40X MIC), and 5 µg/ml ciprofloxacin (80 X MIC). Subsequently, antibiotic treated cultures were washed three times with 0.85% sterile saline solution at different times (1, 2, 3, 5, 7, and 24 h) after adding antibiotics, and to determine the number of viable cells, serial dilution into phosphate-buffered saline (PBS) and plating onto LB agar for colony counting was performed.

Fig. 1 Expression level of genes involved in the formation of persister cells. **a** Ciprofloxacin. **b** Ampicillin. **c** Colistin. Error bars indicate standard deviations of three biological replicates ($P < 0.0001$ using one-way ANOVA)

Quantitative real-time PCR (qRT-PCR)

The relative expression levels of the SOS response (*recA*, *dnaK*), stringent response (*relA*, *spoT*), universal protein A (*uspA*), oxidative stress response (*soxS*), efflux pumps (*mdtD*, *acrA* and *tolC*), and TA systems (*GNAT/RHH*, *vapCB* and *relEB*) were assessed using qRT-PCR (Fig. 1).

After 5 h of bacterial exposure to the lethal dose of ampicillin, colistin, and ciprofloxacin, these cultures were washed twice by PBS and RNA of antibiotic treated cultures and also control

cultures were extracted by high pure RNA isolation kit (Roche, Germany) according to the manufacturer's instructions. Total RNA was treated with DNaseI (Roche, Germany) according to the manufacturer's protocol to eliminate DNA contamination. The extracted RNA quality was evaluated by the NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis. Total RNA was converted to cDNA using the cDNA Synthesis Kit (Takara, Japan). The TA system genes in *S. Typhimurium* were identified by the Toxin Antitoxin Database (TADB) website and the primers used in this study were designed with the Primer 3 software (Table 1) [27, 28].

The qRT-PCR was performed in triplicate for each sample using Rotor-Gene thermal cycler. The *invA* gene was used as the control gene for normalization expression levels.

The qRT-PCR reactions contained 10 µL of 2 × SYBR green master mix (Ampliqon), 1 µL of sample cDNA, 1 µL of each primer, and 7 µL of deionized water. The reactions were performed according to an initial denaturation at 95 °C for 12 min,

followed by 40 cycles at 95 °C for 30 s and 60 °C for 45 s. The relative gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method [29].

Retrieval of the sequence, physicochemical characteristics, and secondary structure prediction

The RecA, DnaK, RelA, SpoT, UspA, SoxS, MdtD, AcrA, and TolC, protein sequences, and toxin/antitoxin systems (GNAT/RHH, VapCB and RelEB) of *S. Typhimurium* were retrieved from Uniprot and TADB [27, 30]. The physicochemical characteristics of protein sequence, including molecular weight, theoretical isoelectric point, extinction coefficient, instability index, aliphatic index, grand average hydropathicity, and number of negative and positive residues were computed by ProtParam bioinformatics tool (<http://web.expasy.org/protparam/>) and to predict the secondary structure predictions of the proteins studied, Self-optimized prediction method (SOPMA) was employed [31].

Table 1 Primers used for qRT-PCR

Primer name	Primer sequence	Product size (bp)	Reference
<i>recA</i>	F: TTCGCTTTACCCTGGCCAAT R: TTAAGCAGGCCGAGTTCCAG	148	This study
<i>dnaK</i>	F: TCGACGAAGTTGATGGCGAA R: TTGGCTTTTTCTGCGGCTTC	190	This study
<i>relA</i>	F: GCTACGCGATATCACCACCA R: GCATCAATCACATCCGGCAC	177	This study
<i>spoT</i>	F: CAACGCTTGACGATCTGCTG R: GGGATCGGACGACAACACTT	108	This study
<i>uspA</i>	F: GCCCTACAACGCGAAAATC R: CAGTGATAGGGTAGCCAGCG	106	This study
<i>soxS</i>	F: TCGGGCTACTCCAAGTGGTA R: TACACGCGAGAAGTTTGCT	183	This study
<i>mdtD</i>	F: AACCAGATTTCAGCAGCGA R: CGATCCCAGGCTTCTACGAC	152	This study
<i>acrA</i>	F: TCAATCCGTCTAGTCACCAGC R: AACCGACGGCATTACTGGTT	156	This study
<i>tolC</i>	F: TATGGCACGTAACGCCAACT R: TGCATCAGGTTCTCTGCCTG	186	This study
<i>GNAT</i>	F: TGTTATCTGCCGAAAGCGT R: TGGTACTGCGCCAAATCCTT	119	This study
<i>RHH</i>	F: AGCAAACCAACCTGACGGAT R: GCGCTCCGTCAGATATACCC	95	This study
<i>vapC</i>	F: CGAAATCATTGCGGTTGGCA R: TCCTGTACTGCGGGTTGTTC	126	This study
<i>vapB</i>	F: AACACCTGTCTGGGCCTTATG R: ATGCGTTCAAATTCGCGGAG	96	This study
<i>relE</i>	F: CGCTTCTGATTACCAACCT R: TCGCAGGCATTCATCCGTTA	140	This study
<i>relB</i>	F: ATGTCCTGGCTGAAATGGGG R: ATCAACGCCAGCTTCGCTAT	149	This study
<i>invA</i>	F: AGCGTACTGGAAAGGGAAAG R: ATACCGCCAATAAAGTTCACAAAG	116	[26]

Protein structural modeling and model evaluation

The modeling of the 3D structure of the proteins studied was performed by using the Phyre2 [32] server and refined by the Galaxy WEB server [33]. The validation of the refined models was evaluated by several structure validation tools including ProSA [34], RAMPAGE [35], ERRAT [36], QMEAN 6 [37], and PROQ server [38]. Furthermore, predicted 3D structures of proteins were viewed using Pymol software (Table 4).

Results

Identification of the persister in *S. Typhimurium* population

Since persister cells are capable of tolerating much higher concentrations of antibiotic MICs, we first determined the MICs of ampicillin, colistin, and ciprofloxacin for *S. Typhimurium* ATCC 14028 using a broth microdilution method. The MIC values for the ampicillin, colistin, and ciprofloxacin were 0.5 µg/ml, 2 µg/ml, and 0.0625 µg/ml, respectively.

As shown in Fig. 2, the bacterial exposure to colistin caused less reduction in the number of cells in the early hours than the other two antibiotics, but after 5 h, we observed a significant decrease in the number of bacteria so that at 5th hour compared to 3rd hour, we observed 2.3 log decrease in the number of bacteria cells. Also, after 24 h of exposure to colistin, with regard to the number of living cells compared to the other two antibiotics, it was the lowest, although a persister cell population of cells by about 10^3 cfu/ml was survived. The bulk of the bacterial

population was killed rapidly upon ampicillin and ciprofloxacin exposure within the first hour. There was also a slight decrease in the number of bacteria in ampicillin treatment after 24 h, whereas the number of viable cells in the ciprofloxacin treatment was almost constant over a 24 h period. These results indicate that *S. Typhimurium* is capable of producing persister cells in the exposure to lethal doses of the antibiotics.

Persister cells formation gene expression in *S. Typhimurium*

The qRT-PCR results indicate that the expression of the studied genes was different with variant antibiotics. In the presence of ciprofloxacin, the expression level of all genes was increased, although the expression levels of *soxS* and TA system genes (*GNAT/RHH*, *vapCB*) were significantly higher than those of other genes (Fig. 1a). In the presence of ampicillin, all of the studied genes were up regulated, whereas TA system genes *GNAT/RHH* and *soxS* respectively with 18.3, 26.8, and 18.2-fold increase showed the highest expression levels (Fig. 1b). In the presence of colistin, all the genes increased expression except *soxS* and *uspA*. In addition, the *relEB* of TA system genes with 3.5 and 9.7-fold increases had the highest expression level (Fig. 1c).

Sequence characteristics and secondary structure assessment

The UniProt IDs' for all the selected proteins of *S. Typhimurium* are provided in Table 2. The primary structures

Fig. 2 Time-dependent killing of *S. Typhimurium* exposed to different antibiotics. Exponential grown *S. Typhimurium* ATCC 14028 was treated with 100-fold MIC of ampicillin, 80-fold MIC of ciprofloxacin, and 40-fold MIC of colistin over a 24-h period. The bacterial culture without any antibiotic treatment served as a control. The values are means of three biological replicates and error bars indicate the standard deviation

Table 2 Physico-chemical parameters determined using ExPASy's ProtParam program

Gene name	Uniprot Id	Number of amino acids	MW	PI	EC	Ii	Ai	GRAVY	−R	+R
<i>recA</i>	A0A0F6B5N7	353	37,944.30	5.08	21,555	29.17	93.20	−0.159	49	40
<i>dnaK</i>	A0A0D6HKW9	638	69,258.06	4.83	17,420	34.38	88.71	−0.433	105	75
<i>relA</i>	A0A0F6B630	744	84,113.86	6.31	93,905	46.23	94.64	−0.359	103	93
<i>spoT</i>	A0A0F6B8P3	703	79,515.66	8.81	55,280	42.26	92.55	−0.325	86	94
<i>uspA</i>	A0A0F6B860	144	16,080.40	5.08	14,440	37.04	107.57	−0.053	21	12
<i>soxS</i>	A0A0F6BAB4	107	12,969.86	9.51	18,450	55.87	89.25	−0.538	13	16
<i>mdtD</i>	A0A0F6B3I2	470	50,792.21	10.27	57,535	42.35	120.13	−0.853	13	28
<i>acrA</i>	A0A0F6AXV5	397	42,234.88	8.41	18,910	33.40	92.87	−0.241	40	42
<i>tolC</i>	A0A0F6B6W5	489	53,426.34	5.42	38,280	29.08	86.09	−0.445	47	40
<i>GNAT</i>	A0A0F6B5X4	175	19,069.91	9.35	20,065	28.74	91.49	−0.094	13	19
<i>RHH</i>	A0A3Z6NX71	96	10,678.57	8.01	1490	43.34	107.81	−0.037	12	13
<i>vapC</i>	A0A0F6B6C5	132	14,938.30	6.83	10,095	22.72	96.06	−0.124	16	16
<i>vapB</i>	Q7CPV2	75	8514.51	5.21	11,000	55.19	64.93	−0.419	11	8
<i>relE</i>	A0A3V8MPN3	91	10,739.34	8.74	12,950	50.96	79.34	−0.679	12	14
<i>relB</i>	A0A0D6I8D2	86	9446.80	4.70		27.67	113.60	−0.100	16	11

MW molecular weight (g/mol), *pI* isoelectric point, *EC* extinction coefficient ($M^{-1} \text{ cm}^{-1}$), *Ii* instability index, *Ai* aliphatic index, *GRAVY* grand average hydropathicity, *−R* number of negative residues, *+R* number of positive residue

were presented in Table 2. The predictions of the secondary structure of the studied proteins are shown in Table 3.

Structural modeling and evaluation of the 3D structure

The three-dimensional structure of the proteins and their refined are shown in Fig. 3. Quality analysis of the predicted models was evaluated using several validation servers. Ramachandran

plot was used to evaluate the quality of the models and showed that in all predicted models above 94% of total amino acids residues were observed in the most favored regions. In addition, the quality of the 3D models of all proteins was analyzed by dark gray zone through QMEAN and QMEAN scores were 0.96 (*RecA*), -0.67 (*DnaK*), -2.97 (*RelA*), -1.33 (*Spot*), 0.03 (*UspA*), -0.04 (*SoxS*), -2.67 (*MdtD*), -2.67 (*AcrA*), -0.57 (*TolC*), 0.05 (*GNAT*), -0.79 (*RHH*), -1.71 (*VapC*), -1.62 (*VapB*), 0.04 (*RelE*), and -0.33 (*RelB*). Therefore, these

Table 3 Secondary structure elements calculated using SOPMA tool

Gene name	Alpha helix (%)	Extended strands (%)	Beta turn (%)	Random coil (%)
<i>recA</i>	43.06	20.68	7.37	28.90
<i>dnaK</i>	24.76	20.53	8.62	24.76
<i>relA</i>	55.78	11.56	5.51	27.15
<i>spoT</i>	51.78	14.94	5.69	27.60
<i>uspA</i>	46.53	20.83	4.86	27.78
<i>soxS</i>	66.36	4.67	6.54	22.43
<i>mdtD</i>	55.11	14.04	4.04	26.81
<i>acrA</i>	30.48	22.67	7.30	39.55
<i>tolC</i>	61.76	4.91	2.04	31.29
<i>GNAT</i>	38.86	17.14	9.71	34.29
<i>RHH</i>	57.29	7.29	5.21	30.21
<i>vapC</i>	50.76	14.39	8.33	26.52
<i>vapB</i>	26.67	21.33	6.67	45.33
<i>relE</i>	27.47	15.38	8.79	48.35
<i>relB</i>	69.77	6.98	6.98	16.28

Fig. 3 The three dimensional structure by Phyre2 and refined by Galaxy web servers

analyses and other validation analyses indicated that the predicted 3D models have good accuracy (Table 4).

Discussion

In this study, we examined the persister cells existence in populations of *S. Typhimurium* ATCC 14028. The persistence to different bactericidal antibiotics that is commonly used for clinical treatment of infections by *S. Typhimurium* with the killing curves was characterized. We further evaluated the effect of *S. Typhimurium* exposure to the lethal dose of the studied antibiotics on the expression level of genes involved in the production of persister cells. Also, since a better understanding of the physicochemical and structural properties of proteins helps to identify the precise role of proteins and also according to the fact that little information was available on the structure of the proteins studied, consequently, we used several bioinformatics tools to predict the three-dimensional structure of these proteins. In a study by Mohammadzadeh et al., after protein modeling of three TA systems and prediction their interaction in *Listeria monocytogenes*, they designed in silico-derived peptides to inhibit TA systems and the proteins involved in formation persister cells [17]. In another study, considering the importance of LuxS protein in the bio-synthesis of autoinducer-2, which plays a significant role in bacterial communication and in particular in the induction of quorum sensing, in silico prediction and modeling of the LuxS in *Aeromonas hydrophila* was performed and then, the suitable inhibitor for inhibition of AI-2 was identified [39]. These studies provide new knowledge for developing novel therapeutic strategies against bacterial infections. Therefore, the results of protein modeling of our study and the evaluation of protein modeling with validated software suggest that these predicted structures can be used in future studies to investigate the interaction of these proteins and as suitable drug targets.

Several studies have reported that the levels of persister cell formation in the exposure of various antibiotics were different [40]. A recent study demonstrated that *Acinetobacter baumannii* in the presence of colistin produced the lowest levels of persister cells compared with two other antibiotics: rifampicin and amikacin. [41]. In our study, the time-dependent killing curves of the studied three antibiotics were different and the lowest persister cell formation was also in the presence of colistin and the highest was in the presence of ciprofloxacin probably due to differences in their mechanism of antibiotic action. Bacterial persisters infections can occur in different tissues within the host and may be associated with the failure of antibiotic treatment. *M. tuberculosis* and *S. Typhimurium* both form persister cells in macrophages [42, 43]. Also, persistence has a role in the recurrence of infections. SNPs analysis of *S. Typhimurium* isolates in recurrent non-typhoidal salmonellosis demonstrated

Table 4 Validation parameters for built 3D protein structures determined using ERRAT, RAMPAGE, PROQ, and QMEAN server

Gene name	ERRAT analysis	RAMPAGE analysis			PROQ analysis		QMEAN score	Z score
		Overall quality factor	Number of residues in favored region (%)	Number of residues in allowed region (%)	Number of residues in outlier region (%)	LG score	Max sub	
<i>recA</i>	97.377	97.0	2.1	0.9	4.450	0.402	0.96	− 7.83
<i>dnaK</i>	69.7952	95.2	2.8	2.0	4.846	0.335	− 0.67	− 11.33
<i>relA</i>	95.5056	96.1	3.0	0.9	3.041	0.291	− 2.97	− 7.3
<i>spoT</i>	90.1099	97.9	1.5	0.6	3.626	0.311	− 1.33	− 8.24
<i>uspA</i>	100	99.3	0.7	0.0	4.766	0.485	0.03	− 7.42
<i>soxS</i>	98.9796	98.1	1.9	0.0	1.933	0.268	− 0.04	− 6.03
<i>mdtD</i>	97.9798	96.6	2.6	0.8	3.294	0.291	− 2.67	− 3.45
<i>acrA</i>	87.8981	97.6	1.5	0.9	2.472	0.307	− 0.57	− 9.42
<i>tolC</i>	96.6581	98.8	0.9	0.2	1.517	0.390	0.17	− 7.92
<i>GNAT</i>	95.5975	98.2	1.2	0.6	4.456	0.559	0.05	− 6.43
<i>RHH</i>	100	95.4	3.9	0.7	1.931	0.261	− 0.79	− 1.05
<i>vapC</i>	83.3333	100.0	0.0	0.0	0.607	0.079	− 1.71	− 1.16
<i>vapB</i>	91.0569	96.9	3.1	0.0	5.358	0.570	− 1.62	− 6.49
<i>relE</i>	50	94.3	4.6	1.1	2.288	0.380	0.04	− 4.47
<i>relB</i>	98.5294	100.0	0.0	0.0	1.752	0.305	− 0.33	− 1.09

that a high percentage of the isolates that were identified at recurrence fever was produced by the same genovar as the initial infection. These results show that the bacteria producing these infections were never removed during treatment with antibiotics in the host [43].

Multiple mechanisms have been identified that affect the formation of persister cells [44]. Although DNA is damaged by agents such as various antibiotics, oxidizing elements, and the induction of SOS response, studies have demonstrated that the antibiotic effect on SOS-mediated mutagenesis depends on the type of antibiotics and bacterial species [45]. In our study, when *S. Typhimurium* was exposed to various antibiotic families, the slight increase in *recA* expression was shown, which may indicate a low effect of this gene on the persistence of *S. Typhimurium* against the antibiotics studied. DnaK is a chaperone that plays an important role in the maintenance of persister cells in *E. coli* and *S. aureus* [46, 47]. However, a recent study evaluated the expression of *dnaK* in the presence of the colistin in *A. baumannii* and found that it did not have a significant effect on bacterial persistence [41]. In our study, expression of *dnaK* in the presence of ampicillin, ciprofloxacin, and colistin increased by 8.2, 6.5, and 1.7-fold, respectively. The universal stress protein (UspA) expression level of *E. coli* is increased in response to various stress conditions, including nutrient starvation and exposure to heat, oxidants, ethanol, and antibiotic [48]. Also, UspA is induced by the metabolic, oxidative, and thermal stresses in *S. Typhimurium* and inactivation of *uspA* leads to increased

susceptibility to these stress conditions [23]. However, it does not appear to be important in the formation of persister cells in *S. Typhimurium* because there was no change in its expression level after exposure to the antibiotics studied. Stringent response and ppGpp formation is an intracellular stress response produced by RelA and SpoT [14]. Stringent response affects the survival of various bacteria such as *Klebsiella pneumoniae* and *Enterococcus faecalis* under stress conditions and can produce a subset of persister cells capable of tolerating antibiotics [49, 50]. The RelA protein also seems to be crucial for the survival of *Helicobacter pylori* inside macrophages during phagocytosis [51]. In a study by Brooks and colleagues, it was shown stringent response in *E. coli* does not increase the tolerance of this bacterium to kanamycin, tetracycline, and ampicillin [52]. But another study demonstrated that RelA and SpoT increased the tolerance of *P. aeruginosa* to the ofloxacin and ciprofloxacin [53]. In this effort, the expression of *relA* and *spoT* did not change significantly in the presence of colistin and ampicillin; it only increased in the presence of ciprofloxacin by 5.1-fold. These findings may indicate that RelA and SpoT have different effects on bacterial viability depending on the type of antibiotic and its mechanism of action.

Increased expression of multi-drug efflux pumps in *E. coli* maintains the persistence of this bacterium by decreasing the concentration of intracellular antibiotics [16]. The AcrAB efflux pump plays an important role in the development of antibiotic resistance process. This pump facilitates the growth of *S. Typhimurium* in macrophages [54], promotes the development

of pneumonia with *K. pneumoniae* [55], and is also resistant to bile salts and survival of *Shigella* during gastrointestinal infection [56]. Also, studies have shown that upregulation of the multi-drug efflux systems *mdtABCD* caused increase resistance to β -lactams, novobiocin, and deoxycholate in *S. Typhimurium* [57]. In our study, *mdtA* with the increase of expression level of 8.9 and 3.7 in the presence of ampicillin and ciprofloxacin, respectively, showed the highest increase in the efflux pumps studied. Increased SoxS level activates important antioxidant genes and can be related to antibiotic resistance in bacterial infection. Presence of *soxRS* regulon in *Klebsiella aerogenes* demonstrated increased resistance to nalidixic acid, chloramphenicol, tetracycline, and kanamycin [58]. *soxS* expression was also increased 18.2-fold in the presence of ampicillin and 19.52-fold in the exposure of ciprofloxacin, which may indicate the role of this gene in the production of persister cells. One of the mechanisms that most studies have linked to the formation of persister cells is TA systems [59, 60]. In *E. coli*, the MazF toxin with its RNase properties, breaks down cellular RNAs and cause inhibiting cell growth and promoting persistence [61]. Cheverton and colleagues discovered that TacT of a type II TA system by acetylation of the primary amine group of charged tRNA caused blocking translation and was associated with the formation of intracellular *Salmonella* persisters [62]. In this study, among the type II of TA systems investigated, the GNAT/RHH system had the highest expression in the presence of ampicillin and ciprofloxacin, indicating the importance of these systems in producing bacterial viability. Also, in the presence of colistin, the RelEB system showed the highest expression which may indicate that RelEB system contributes to the survival of bacteria in the presence of colistin.

Our study showed that various classes of antibiotics can have different effects on the formation of persister cells and on the expression level of genes involved in producing persister cells. The observed differences in the expression of the studied genes may indicate the diverse effect of these genes in the formation of persister cells against different antibiotics.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest and this study was financially supported by a research grant (No.16354) and Code of Ethics (IR.IUMS.FMD.REC.1399.077) in Iran University of Medical Sciences (Tehran, Iran).

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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