Chapter 4

Effect of Silver Species on the Cell Machinery of *Escherichia coli* K12

4.1 Introduction

Silver is known since long time as the best antibacterial agent among various other metals like copper, zinc, gold, etc. (Alexander, 2009). Various silver species viz. metallic, ionic, and nano forms are known to show antibacterial activity (Alexander, 2009; Mijnendonckx et al., 2013). Different forms of silver have different mode of antibacterial action. The metallic form of silver upon oxidation releases a small amount of silver ions, which makes it a slow antibacterial agent. On the other hand, ionic form of silver directly shows efficient antibacterial activity. Although, silver ions used to possess such efficient antibacterial potential and smooth mode of action, these are not accepted in medicinal applications because the direct exposure of silver ions in high concentration has been reported to show toxic effects on mammalian cells (Chernousova and Epple, 2013). Silver ions are microbicidal at very low concentration and cause adverse effects on humans at such low concentrations (Panáček et al., 2018). On the other side, the nano form of silver provides more surface area to volume ratio in comparison to metal. It is noteworthy that silver nanoparticles (Ag NPs) can act as reservoir and result in continuous release of silver ions (Le Ouay and Stellacci, 2015), which can result in development of bacterial resistance to Ag NPs (Graves jr. et al., 2015). Ag NPs with proper physico-chemical properties (size and surface capping) can lead to a controlled and constant release of silver ions, which can strongly fight with pathogen, yet projecting lower risk to the host cells (Pareek et al., 2018; Bhargava et al., 2018). Systematic and in-depth mechanistic studies can help in developing an efficient Ag NPs based antimicrobial system.

As discussed in previous chapters, the chemically and biologically synthesized Ag NPs with varied physico-chemical properties (size and surface capping) were evaluated for their antibacterial activity (MIC) against *E. coli* K12. Based on the best antibacterial activity among all the tested Ag NPs types, lysozyme coated Ag NPs (L-Ag NPs) of ~ 5 nm size were selected for further studies on the mechanistic insights of their antibacterial activity in comparison to silver ions.

Although the exact molecular mechanism behind the antibacterial activity of Ag NPs can be understood by deeply analysing the transcriptomic profile of bacterial cells upon exposure to NPs, it is imperative to initially study the effect of NPs exposure at biochemical level (McQuillan et al., 2012). In order to get an initial idea about the possible mechanism behind the antibacterial activity of L-Ag NPs, biochemical and selective differential gene expression studies were performed by exposing the bacterial cells at concentrations below the MIC values of silver ions [6 µg (Ag) mL^{-1}] and L-Ag NPs [9 µg (Ag) mL^{-1}]. Considering the fact that MIC

is the lowest concentration of antimicrobial agent that completely inhibit the bacterial growth (Lambert and Pearson, 2000; CLSI, 2015) we have chosen sub-MIC values to unveil the mechanism behind the antibacterial action of silver ions and L-Ag NPs. There are many known biochemical mechanisms in bacteria which can take place under the stress of Ag NPs viz. generation of ROS, membrane damage, energy depletion, DNA damage, protein denaturation, etc. (Yun'an Qing et al., 2018). Among all these different mechanisms, ROS generation and membrane damage are the two most destructive and most studied mechanisms (Pareek et al., 2018). Hence, to study the preliminary antibacterial action of L-Ag NPs, ROS generation and membrane damage analysis was performed by 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) and malondialdehyde (MDA) assay, respectively (Jain et al., 2015; Bhargava et al., 2018). Biochemical studies were followed by differential gene expression studies. It is well known that bacterial growth at different time points makes significant difference in the bacterial gene expression profiling (Klumpp et al., 2009; Klumpp and Hwa, 2014). In order to understand the regulation of gene expression upon L-Ag NPs exposure at different time intervals during the growth of *E. coli* K12, we selected genes from the important physio-biochemical pathways, which play a vital role under the oxidative stress, i.e. stress sensing, efflux machinery, homeostasis maintenance, DNA damage repair, protein damage, metabolic inactivation of a bacterial cell, etc. (McQuillan et al., 2012; Ivask et al., 2013; McQuillan and Shaw, 2014; Sun et al., 2017). *E. coli* K12 cells were exposed to sub-MIC concentrations of silver ions and L-Ag NPs for 5, 30 and 60 min., followed by the quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The concentrations and time points were selected based on the results obtained during biochemical assays and the doubling time of bacteria (~20 min.), respectively (Chandler et al., 1975).

4.2 Materials and methods

4.2.1 Materials

All chemicals used were of analytical grade and purchased from Sigma Aldrich (USA) unless otherwise stated. Primers were synthesized by Integrated DNA Technologies, Inc., and Eurofins scientific (USA). SYBR green dye used for the qRT-PCR was procured from Bio-Rad Laboratories (USA). Milli-Q water was acquired from a Milli-Q Biocel water purification system manufactured by Merck Millipore (Merck KGaA, Darmstadt, Germany).

4.2.2 Preparation of bacterial culture

E. coli K12 was revived from the glycerol stock and cultured in MLB broth medium (casein enzyme hydrolysate 10 g L^{-1} and yeast extract 5 g L^{-1}, pH 7.2 ± 0.2) for 12 h at 37°C under shaking (150 rpm). For preparation of inoculum, overnight grown bacterial culture was inoculated in freshly prepared MLB medium and allowed to raise till the log phase (OD_{600} - 0.5). The experiments were done in both technical and biological duplicates.

4.2.3 Biochemical studies

The preliminary antibacterial action of L-Ag NPs was analysed by measuring the ROS generation and membrane damage in comparison to silver ions.

4.2.3.1 ROS generation analysis

To check the bacterial ROS generation under the exposure of silver species (silver ions and L-Ag NPs), DCFH-DA assay was performed following the protocol of Wang and Joseph (1999) with some modifications. It is generally used to determine the generation of overall ROS using the light sensitive and cell permeable DCFH- DA (non-fluorescent) dye. Briefly, the fresh culture of bacterial cells (~3×10^7 CFU mL^{-1}) was obtained and washed thrice with phosphate buffered saline (PBS) (pH 7.2) followed by its suspension in freshly prepared MLB broth medium. DCFH-DA (prepared in PBS) was added to bacterial cell suspension at a final concentration of 10 µM, followed by incubation at 37°C for 1 h under dark conditions. After incubation period. The DCFH-DA loaded bacterial cells were separated by centrifugation at 8,000 rpm for 15 min., followed by washing with PBS (pH 7.2). Bacterial cells were then exposed to sub-MIC values of silver ions i.e. MIC_{25} [1.5 µg (Ag) mL^{-1}], MIC_{50} [3.0 µg (Ag) mL^{-1}], MIC_{75} [4.5 µg (Ag) mL^{-1}], & MIC_{100} [6.0 µg (Ag) mL^{-1}] and L-Ag NPs i.e. MIC_{25} [2.25 µg (Ag) mL^{-1}], MIC_{50} [4.5 µg (Ag) mL^{-1}], MIC_{75} [6.75 µg (Ag) mL^{-1}], & MIC_{100} [9.0 µg (Ag) mL^{-1}], in the freshly prepared MLB media for 30 min. at 37°C under shaking conditions (150 rpm). In living bacterial cells, DCFH-DA gets deacetylated by esterases into dichlorodihydrofluorescein (DCFH) which is non-fluorescent in nature and further gets oxidized to fluorescent 2'-7' dichloro fluorescein (DCF) in presence of ROS present in the bacterial cells. The intensity of fluorescence was detected by Perkin Elmer VICTOR X Multilabel Plate Reader (USA) at an excitation and emission wavelength of 485 nm and 535 nm, respectively. The bacterial cells without exposure to silver species were used as control.

4.2.3.2 Membrane damage analysis

The effect of silver species treatment on the bacterial cell envelope was examined by visualizing the treated bacterial cells by Scanning Electron Microscopy (SEM). In order to prepare sample for SEM analysis, the bacterial cells exposed to MIC_{75} of L-Ag NPs and silver ions were centrifuged at 8,000 rpm for 10 min. Subsequently, the supernatant was discarded and the bacterial pellet was washed twice with 1X PBS buffer followed by their fixation in 2.5% electron microscopy grade glutaraldehyde (prepared in 0.05 M sodium cacodylate buffer; pH 7.2) for 2 h at 4°C. After fixation, the cells were washed thrice with cacodylate buffer (without glutaraldehyde) and were resuspended in the same buffer. Then, the bacterial cells were drop-coated on glass slide followed by overnight vacuum drying and later viewed on Apreo SEM, Thermo fisher, USA. The cells unexposed to silver ions and L-Ag NPs were used as control.

The extent of membrane damage in the bacterial cells was further analysed in terms of lipid peroxidation measurement using malondialdehyde (MDA) assay (Buege and Aust, 1978). Lipid peroxidation is a process of oxidative degradation of bacterial membrane lipids in which the free radicals present in the cells withdraw an electron from the cell membrane lipids that leads to damage in the cell membrane. Estimation of MDA level in bacterial cells treated with different MIC values of silver ions and L-Ag NPs, i.e. MIC_{25}, MIC_{50}, MIC_{75}, and MIC_{100} was performed as per the protocol used during our previous study (Bhargava et al., 2018).

The membrane damage was also determined by quantifying the total sugar content using anthrone assay in the supernatant of treated bacterial cells. In anthrone assay, the carbohydrates hydrolysed into furfurals and hydroxyl-methyl furfurals which further reacts with anthrone reagent and form blue-green complex that was detected at 620 nm using Jasco V-630 UV visible spectrophotometer (USA) (Fales, 1951). All the biochemical experiments were performed using technical & biological duplicates and the results are expressed as mean ± SD.

4.2.4 Differential gene expression studies

Mechanistic pathways involved in the antibacterial action of silver ions and L-Ag NPs were evaluated by differential expression profile of selected genes using qRT-PCR assay. Based on the detailed literature survey, 10 genes were selected across various metabolic pathways of the bacterial genome to check the effect of silver species treatment on their expression in bacterial cells (McQuillan et al., 2012; McQuillan and Shaw, 2014; Sun et al.,

2017). The rationale (domain location and function) for the selected genes have been detailed in Figure 4.1 and Table 4.1.

Table 4.1: Domain location and function of selected *E. coli* K12 genes to decipher the antibacterial mechanism of silver species.

Domain	Gene	Function
Flagellar protein	*fliG*	fliG is a flagellar protein which forms a rotor mounted switch complex with fliM and fliN at the base of the basal body (C ring).
Membrane proteins	*wbbH*	wbbH is a O-antigen polymerase which helps in linking the subunits to form a long chain O- antigen polysaccharide.
	aqpZ	aqpZ is an aquaporin protein located in the plasma membrane.
	cusC	In *E. coli*, the CusCBA efflux complex renders resistance to toxic Cu (I) and Ag (I). The CusC channel is the final step in the Cu^{+}/ Ag^{+} ions efflux pathway.
	copA	copA gene product, a copper-translocating P-type ATPase, is the major protein found to be active in copper resistance in *E. coli.*
Redox stress	*sufB*	The SufBCD complex helps in the assembly or repair of oxygen radicals/ ROS-labile iron-sulfur clusters under oxidative stress.
	soxS	soxRS regulon- soxR protein activates the transcription of soxS, which in turn activates the transcription of RS regulon gene under redox stress.
Chaperons	*dnaK*	dnaK is a chaperone molecule which helps in protein folding and replication.
DNA damage	*recA*	recA-LexA system initiates different DNA damage repair mechanisms under stress conditions.
Apoptotic proteins	*mazF*	mazE-mazF is an antitoxin-toxin pair which releases activated mazF when the wide range of stresses are posed to the cell.

Table 4.2: Details of gene specific primers used for qRT-PCR.

S. No.	Genes	Primers 5` - 3`	Amplicon size (bp)
1.	***FliG***	Forward primer- CAGGCACCGATAAAAGCGTCA	139
		Reverse primer- TGCTTGTTGGAGATCTGCGTG	
2.	***WbbH***	Forward primer CTTTTTACGTGGACTGCCTGG	178
		Reverse primer- CAACGACATAGAGACTCCACC	
3.	***AqpZ***	Forward primer- CTGGCTTGTTTTTGGTGGCTG	186
		Reverse primer-5' CGTCCGCCAGCCCATAAACC 3'	
4.	***soxS****	Forward Primer CCCATCAGAAAATTATTCAGGA TCT	155
		Reverse primer ATCAGACGCTTGGCGATTAC	
5.	***recA***	Forward primer- AGATCCTCTACGGCGAAGGT	82
		Reverse primer- AAGCTGATCGAGAAAGCAGG	
6.	***dnaK***	Forward primer- AGATCCTCTACGGCGAAGGT	152
		Reverse primer- AAGCTGATCGAGAAAGCAGG	
7.	***sufB***	Forward primer- TGGTCAGACTCGTATGCC	92
		Reverse primer- GCAGCAGTACGTCTTTTACG	
8.	***cusC***	Forward primer- AACGCCAGCTTTGACCTC	158
		Reverse primer- GCATACGCCAGTTGCTGAT	

9.	***copA***	Forward primer- CTGTTAAATGAGCAACAGGT	190
		Reverse primer- TAAAGCGGGATATCGTCTGG	
10.	***mazF***	Forward primer- GGCGATCTGATTTGGGTTG	186
		Reverse primer- GCCATCACGTTCCTGACC	
11.	***rrsB** (16s rRNA)***	Forward primer- ACTCCTACGGGAGGCAGCAGT	198
		Reverse Primer- TATTACCGCGGCTGCTGG	

* denotes the sequence taken from McQuillan et al., (2012, 2014)

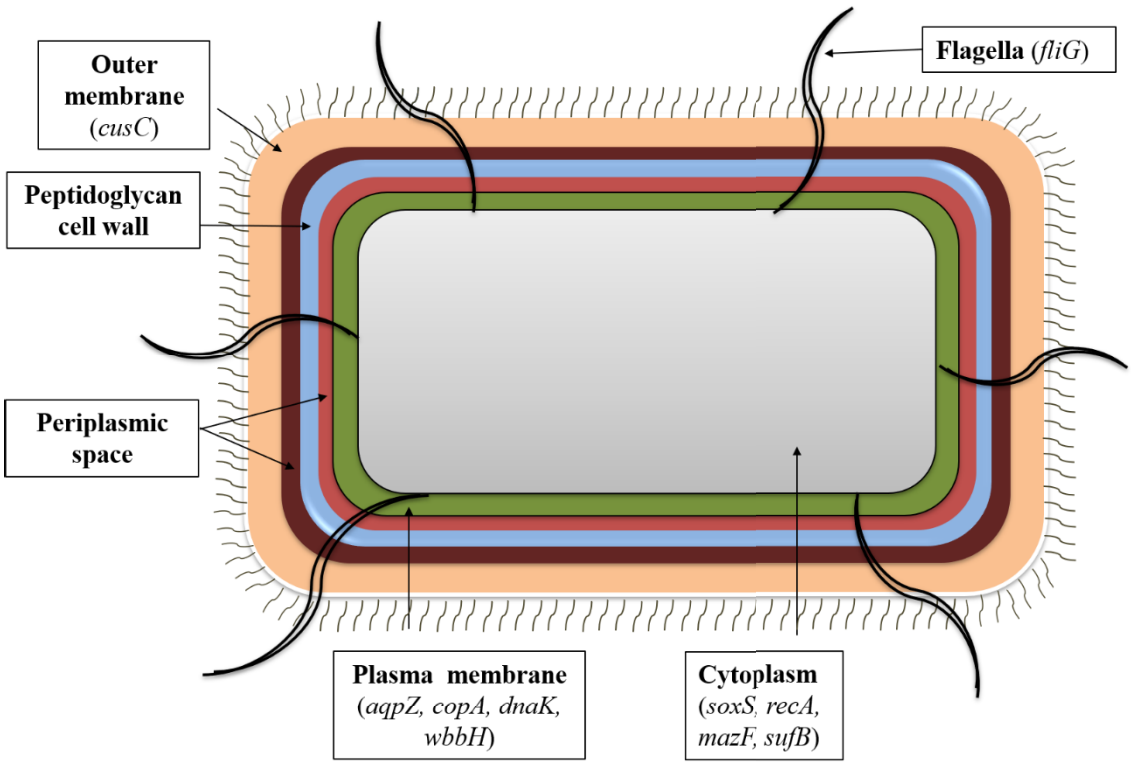


Figure 4.1: Location of selected genes in the bacterial cell.

For qRT-PCR analysis, freshly grown bacterial cells (~ 3×10^7 CFU mL^{-1}) were separately exposed to MIC_{50} and MIC_{75} of silver ions and L-Ag NPs for the duration of 5, 30, and 60 min. at 37°C in MLB broth. The concentrations and exposure time duration were selected based on the results of biochemical assays and doubling time of bacteria (~ 20 min.), respectively (Chandler et al., 1975). Bacterial cells without any treatment of silver ions/ L-Ag NPs were selected as a control for each time point. After treatment, the total RNA from each sample was isolated using Trizol reagent following standard protocol of manufacturer. Isolated RNA was quantified spectrophotometrically (SimpliNano, GE Healthcare Life Sciences, USA) and quality of RNA was assured by performing gel electrophoresis. One µg of total RNA was used for the synthesis of cDNA using Verso cDNA synthesis kit (Thermo Fisher Scientific, USA) as per manufacturer instructions. To analyze the gene expression profile, the primers of selected genes were designed using the Primer 3 online tool (http://bioinfo.ut.ee/primer3/) and subsequently selected according to the local alignment analyses with basic local alignment search tool (BLAST) (Table 4.2). The primers were synthesized by outsourcing to Integrated DNA Technology, (IDT) and Eurofins Genomics, India. The sequences of a few primers were taken from already available reports.

qRT-PCR was carried out with technical and biological duplicates using a 10 µl reaction volume on CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, USA). The 10 µL reaction mixture contained 5 µL of Bio-Rad super mix SYBR green, 1 µL of each forward and reverse primer (10 pmol μL^{-1}), 2 µL of nuclease-free water and 1 µL of 10 time diluted cDNA template. Thermal cycler operation was performed as per conditions given in Figure 4.2. The relative fold change in the mRNA expression level was determined according to the $2^{-\Delta\Delta Ct}$ method described by Livek and Schmittgen (2001) by keeping *rrsB* gene (16s rRNA) as an internal control. Relation between all the differentially expressed genes was analysed by the heat map analysis tool of Morpheus (https://software.broadinstitute.org/morpheus) (Adelman and Myles, 2018). The results are expressed as mean ± SD.

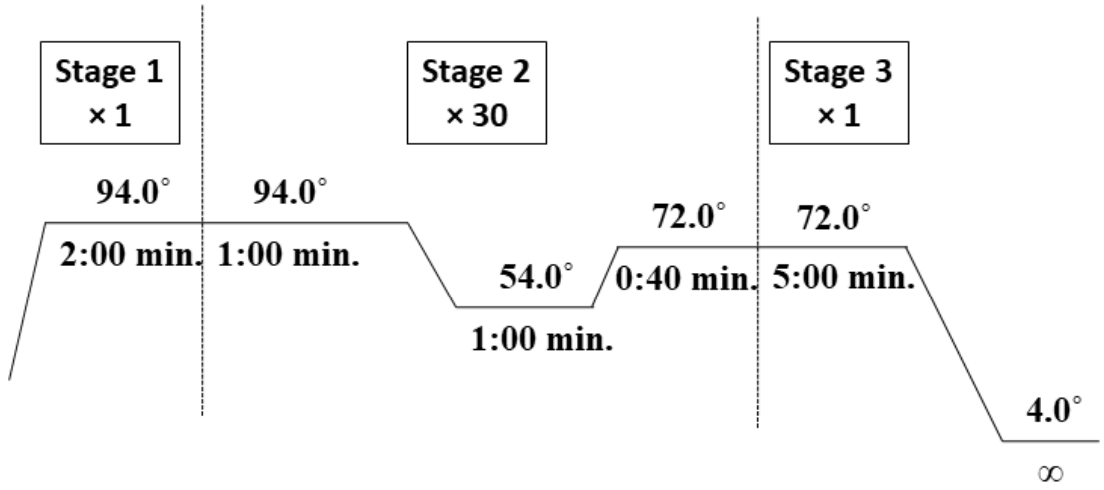


Figure 4.2: Real time PCR conditions.

4.2.5 Kinetics of cellular intake of silver ions

The kinetics of cellular intake of silver ions from the L-Ag NPs and direct exposure of silver ions, under as-defined conditions was determined by ICP-OES (Avio 200, PerkinElmer, USA). Freshly grown bacterial culture (~3×10^7 cfu mL^{-1}) was separately exposed to MIC_{75} of L-Ag NPs and silver ions, followed by incubation for 5, 30 and 60 min. in separate culture flasks at 37°C. After incubation, the bacterial cells were pelleted down by centrifugation at 8,000 rpm for 10 min. at 4°C followed by washing twice with phosphate buffer (pH 7.2). The dried bacterial cells were then digested in 1 ml mixture of H_2O_2/HNO_3 (50:50) for 2 h. After acid digestion, the final volume was made up to at 10 ml and filtered using 0.22 µ syringe filter. The filtrate was analysed for the presence of silver by ICP-OES (McQuillan et al., 2012). Bacterial cells without L-Ag NPs and silver ions treatment were taken as a control for the respective time points.

4.2.6 Statistical analysis

The results for the biochemical assay were analysed using unpaired Student t-test as appropriate for the data set. The qRT-PCR measurements data were statistically analysed using Prism software (Version 8.0; GraphPad Software Inc.) following the two-way analysis of variance. Boneferroni method was used to analyse the multiple comparisons (Motulsky, 1999). The symbol 'ns' used in the graphs shall be interpreted as statistically non-significant at $P > 0.05$. The asterisk symbols in the graphs can be interpreted as: * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$.

4.3 Results and discussions

4.3.1 Biochemical assays

The bacterial growth can be controlled by targeting and disturbing the oxidative phosphorylation of respiratory chain, membrane integrity, enzyme activity, etc. of some of the pivotal metabolic pathways (Jain et al., 2015; Yun'an Qing et al., 2018; Pareek et al., 2018). Among all these pathways, increase in the generation of ROS and damage to the bacterial cell membrane cause maximum harm to the bacterial cells (Park et al., 2009). Based on these facts, we decided to select and analyse these two major pathways in the present study.

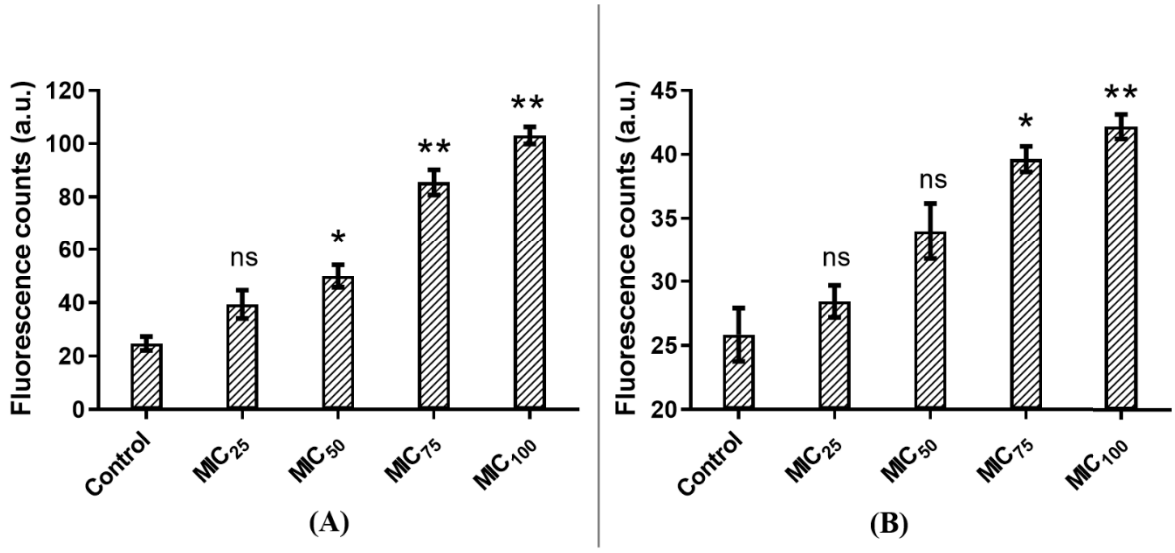


Figure 4.3: Analysis of ROS by DCFH-DA assay for (A) Silver ions and (B) L-Ag NPs. Vertical bars represent SD.

To investigate the generation of free radicals in the bacterial cells exposed to silver ion and L-Ag NPs, DCFH-DA assay was performed, which measures the signal intensity of fluorescent product namely dichloro fluorescein (DCF) formed due to oxidation of non-fluorescent compound DCFH in presence of intracellular ROS (Rastogi et al., 2010). Figure 4.3 shows the extent of free radicals generated from the bacterial cells treated with various concentrations of silver ions and L-Ag NPs by considering untreated bacterial cells as control. The induction of ROS was found to be directly proportional to the concentration of treatment (silver ions and L-Ag NPs). Bacteria treated with MIC_{100} of silver ions and L-Ag NPs produced ~312 and ~61 times higher ROS than their respective controls. The level of ROS generation was found to be 145 times higher in bacterial cells exposed to MIC_{100} of silver ions in comparison to L-Ag NPs. This could be due to the direct penetration of silver ions into bacterial cells. Whereas presence of lysozyme coating on L-Ag NPs could result in constant and

controlled release of silver ions from L-Ag NPs. Similarly, Al-sharqi et al., (2019) also reported the antibacterial efficiency of Ag NPs by measuring the ROS generation by DCFH-DA assay. Likewise, our research group also reported the use of DCFH-DA assay for the estimation of ROS generation from bacteria under the stress of Ag NPs (Jain et al., 2015; Bhargava et al., 2018).

Generation of ROS within the bacterial cell disturb the integrity of the membrane. SEM measurements were carried out to determine the morphology of bacterial cells after treatment with silver species (silver ions and L-Ag NPs). SEM micrographs of representative bacterial cells are depicted in Figure 4.4. Treatment with silver ions showed significant membrane damage resulting in release of cytoplasm. In contrast, lysozyme coated Ag NPs showed very less damage on membrane in comparison to the control.

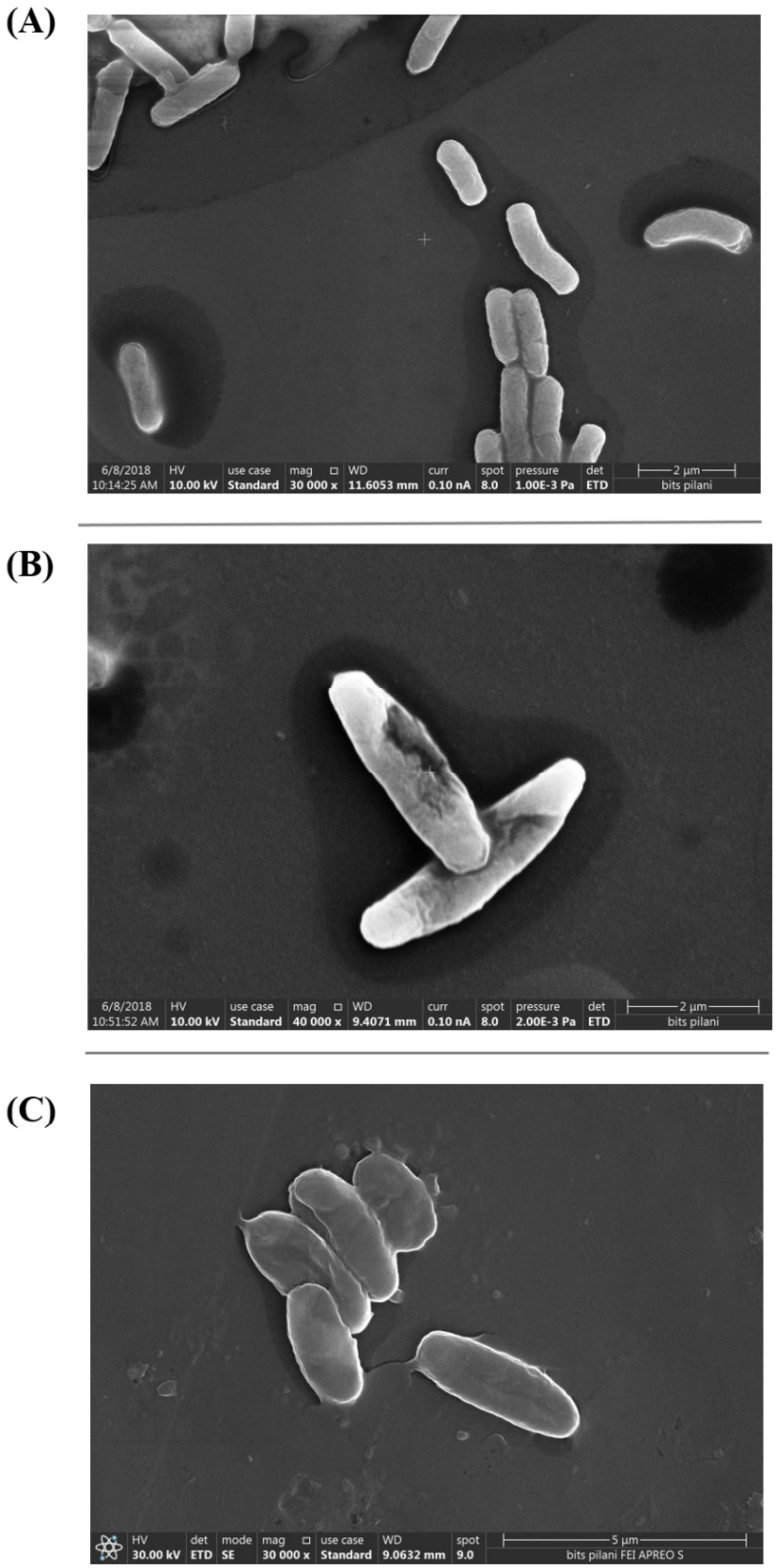


Figure 4.4: SEM micrograph of *E. coli* K12 cells showing the membrane damage in the *E. coli* K12 treated with (A) Untreated/ control, (B) MIC_{75} of silver ions, and (C) MIC_{75} of L-Ag NPs.

It is well known that once ROS begins to accumulate in bacterial cells, the oxidative stress in cells could damage the membrane integrity (Bhargava et al., 2018). Thus, we checked the extent of membrane damage by measuring the lipid peroxidation of the bacterial membrane using MDA analysis. A concentration-dependent significant increase in the level of lipid peroxidation was observed for both silver ions and L-Ag NPs treated cells in comparison to control, which suggests the damage/ leakage in the bacterial membrane (Figure 4.5 A and B). As expected, a higher MDA content was observed in case of silver ions as compared to L-Ag NPs treated bacterial cell. Recently, our research group has worked on the chemical synthesis of Ag NPs and analysed their antibacterial potential by various methods including ROS and MDA analysis, where concentration dependent increase was observed in both ROS generation and lipid peroxidation (Bhargava et al., 2018).

Apart from lipid peroxidation, the level of membrane damage was also analyzed by measuring the released intracellular carbohydrate from bacterial cells after treatment with silver species. Similar to ROS generation and MDA assay results, a concentration-dependent increase was observed for the carbohydrates released outside the bacterial cells due to membrane damage (Figure 4.5 C and D). It is well evident that damage to bacterial membrane can result in release of intracellular compounds (Singh et al., 2016; Miller and Salama, 2018). Previous report from our group also deals with the anthrone assay to disclose the damage/leakage in the bacterial membrane upon the treatment of Ag NPs where, biologically synthesized Ag NPs were tested for their antibacterial potential (Jain et al., 2015).

The obtained biochemical analysis results confirmed the antibacterial activity of silver ions and L-Ag NPs. The level of damage was found to be higher in bacterial cells treated with silver ions as compared to L-Ag NP's. This could be due to the controlled release of silver ions from L-Ag NPs. Careful analysis of results obtained in the biochemical assays showed that less significant changes were observed with the treatment of silver ions and L-Ag NPs at MIC_{25} concentration as compared to control. As MIC_{100} is the concentration of silver ions and L-Ag NPs which completely inhibit the bacterial growth, we have chosen MIC_{50} and MIC_{75} concentrations for further studies on differential gene expression which would unveil the time and concentration kinetics based antibacterial mechanism of silver ions in comparison to L-Ag NPs.

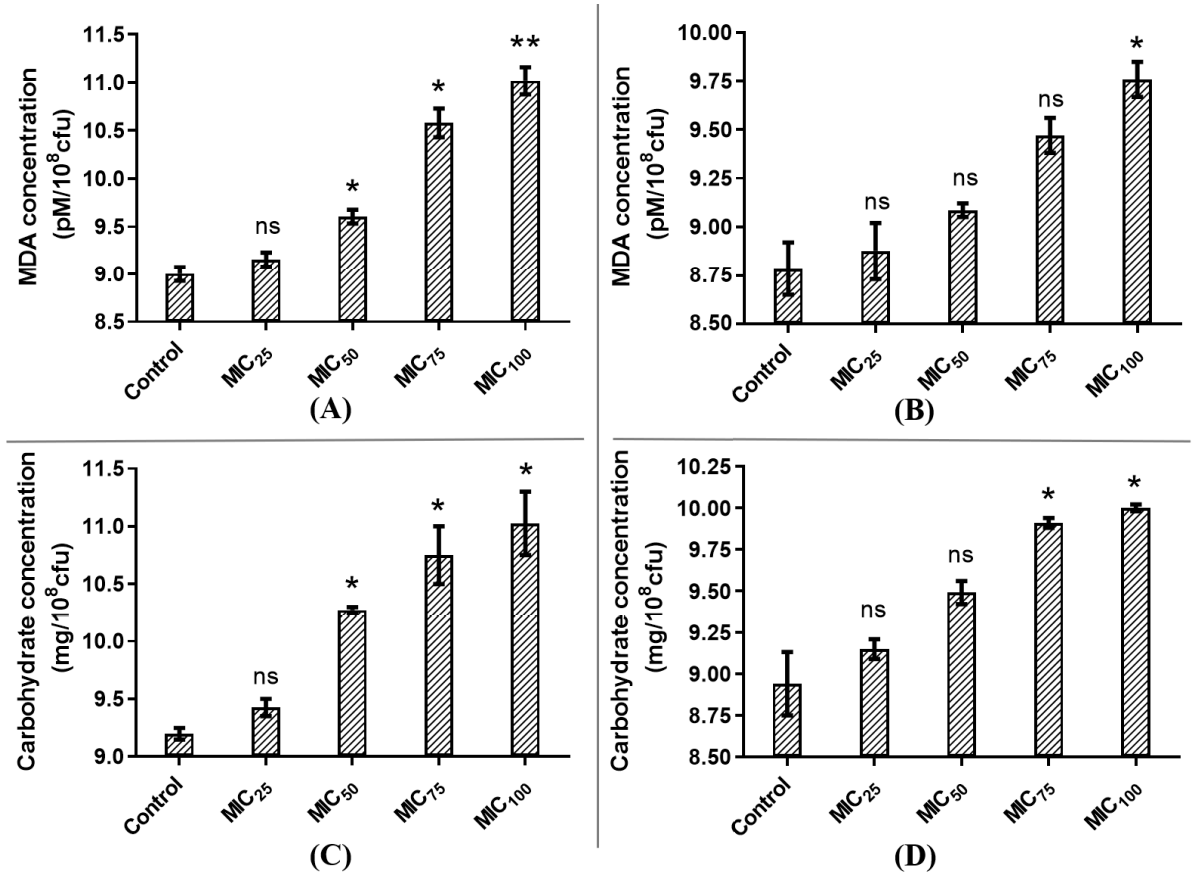


Figure 4.5: Analysis of membrane damage by MDA assay for (A) silver ions & (B) L-Ag NPs; and anthrone assay for (C) silver ions & (D) L-Ag NPs. Vertical bars represent SD.

4.3.2 Differential gene expression studies

Differential expression profile of 10 selected genes was evaluated to find out the detailed mechanism behind antibacterial action of silver species (silver ions and L-Ag NPs) using qRT-PCR analysis. The selection of genes was based on the molecular machinery of bacterial cells as depicted in Figure 4.6. The bacterial treatment concentrations of silver ions [MIC_{50} (3 µg mL^{-1}) and MIC_{75} (4.5 µg mL^{-1})] and L-Ag NPs [MIC_{50} (4.5 µg mL^{-1}) and MIC_{75} (6.75 µg mL^{-1})] were selected on the basis of results obtained in biochemical analysis. Considering the replication time of *E. coli*, three different time points were selected for the qRT-PCR analysis: (a) 5 min., when the first replication cycle is yet to be completed; (b) 30 min.- after the completion of first replication cycle, and (c) 60 min. when at least 2 replication cycle have been completed (Chandler et al., 1975; Skarstad et al., 1986). Differential expression analysis of all the selected genes was performed by using the $2^{-\Delta\Delta Ct}$ method by keeping *rrsB* (16s rRNA gene) as an internal control (Livak and Schmittgen, 2001; Franchini and Egli, 2006).

To study the silver resistance mechanism in *E. coli*, McQuillan et al. (2012) also exposed the bacterial cells to sub-lethal concentrations of silver ions and Ag NPs. However, they isolated the RNA sample only at one time point i.e. 10 min. Although this study deals with the antibacterial mechanism of silver species but the major drawback of this study is that they used very large sized Ag NPs (142 ± 20 nm) and analysed the gene expression at only a single time point. In addition, they studied the transcription level of only 4 genes (*copA*, *cueO*, *cusA*, and *cusR*), which could not decipher the complete mechanism of antibacterial activity.

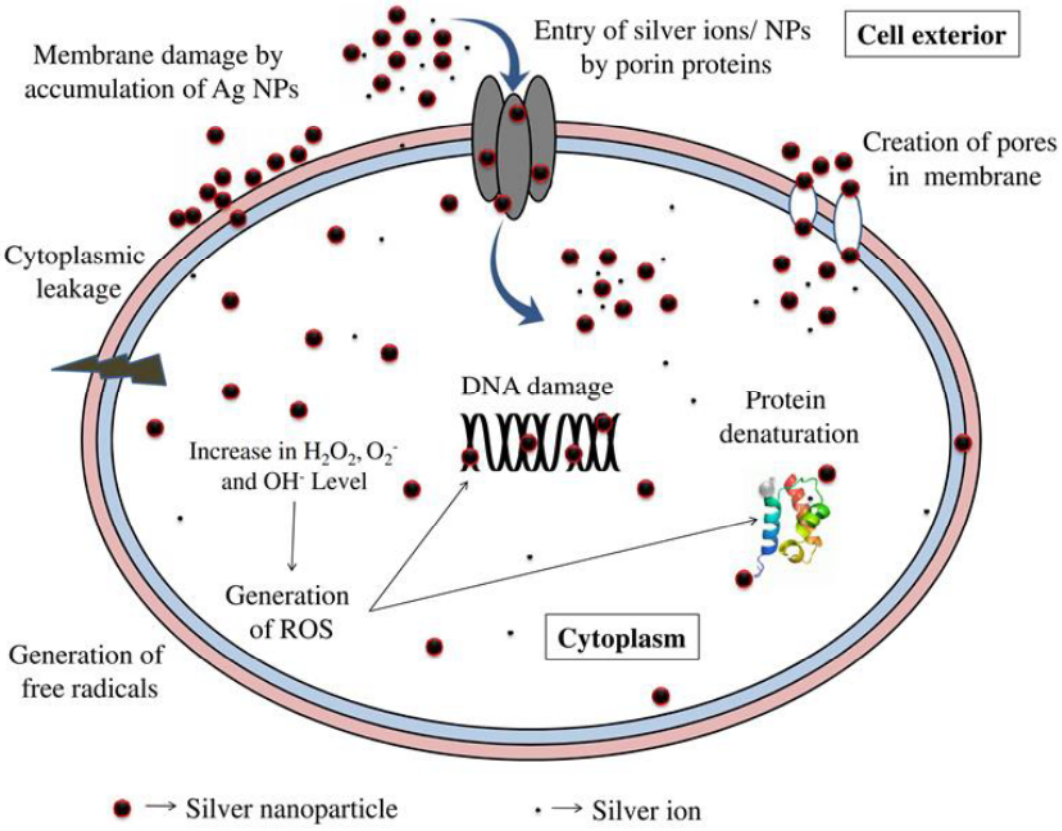


Figure 4.6: Effects of Ag species on the molecular machinery of the bacterial cell (Pareek et al., 2018).

In present study, based on the vast literature survey and considering the sequence of events which can occur on exposure of bacterial cells to silver ions and Ag NPs, we planned to systematically study the transcription levels of 10 selected genes (Table 4.1, Figure 4.1 and 4.6). The results of differential expression of these genes has been discussed below:

Upon exposure, initially the silver species encounter the outer membrane of gram negative bacterial cells. There are two probable pathways by which silver species can enter in the bacterial cells: (1) by damaging the bacterial outer membrane or (2) through porin proteins

present in the membrane. To investigate this, we selected appropriate probable genes related to the outer membrane. It is well known that the outer most layer of the outer membrane is called lipopolysaccharide (LPS) or lipoglycan, which is made up of lipid and polysaccharides (O-antigens) (Maldonado et al., 2016). For the assembly of LPS layer, a number of genes are required. Considering the structure of LPS, we selected a gene which code for the outer most part of LPS, i.e. the O-antigen polysaccharide. *wbbH* gene (also known as *rfc* gene) encode for the O-antigen polymerase, which helps in polymerization of O-antigen to form a fully functional LPS in the Gram-negative bacteria. In case of membrane mediated entry of silver ions/L-Ag NPs, changes in the expression of *wbbH* gene can be studied to find out the damage in the LPS layer of bacteria.

Differential expression analysis results of *wbbH* gene showed that at the initial stage of silver ions treatment (5 min.), no significant change was observed. However, as the time of exposure increased, silver ions started damaging the bacterial outer membrane, which is reflected at 30 min. time point in the form of concentration-dependent down-regulation in *wbbH* expression. In order to survive under oxidative stress, *E. coli* K12 activates the alternative sigma factor (σ^E) to direct RNA polymerase towards their specific promotors, hence inducing a set of genes or regulon(s) to combat the stress response (Kazmierczak et al., 2005). In *E. coli* K12, σ^E basically regulates the envelope stress response which is coherent with the synthesis, assembly and homeostasis of LPS and outer membrane porins (Rhodius et al., 2005). Expression of σ^E leads to the downregulation of genes related to the outer membrane (as observed for *wbbH*) and increase in the synthesis of molecular chaperones to assist the protein folding (discussed in detail in the later part of this chapter) which essentially helps in the survival of bacteria under stress condition by reducing the consumption of energy (Rhodius et al., 2005; Rollauer et al., 2015; Reniere, 2018). At 60 min. time point, reduction in the down-regulation of gene was observed, which might be due to the activation of bacterial defence system by that time.

In case of L-Ag NP, non-significant change in the expression profile of *wbbH* was observed at the initial time points of treatment (5 and 30 min.). We believe that this may be due to the slow release of silver ions from L-Ag NPs, which were not enough to interrupt the bacterial membrane integrity. But as the time passes, the concentration of silver ions released from L-Ag NPs was enough to decrease the functioning of O-antigen polysaccharides at a very low extent which resulted in the down-regulation of *wbbH*. But the extant of down-regulation was very less in comparison to the silver ions. This further suggests the controlled release of

silver ions from L-Ag NPs, which do not cause drastic changes in the bacterial membrane (Figure 4.7). These results are in accordance to the results of SEM analysis.

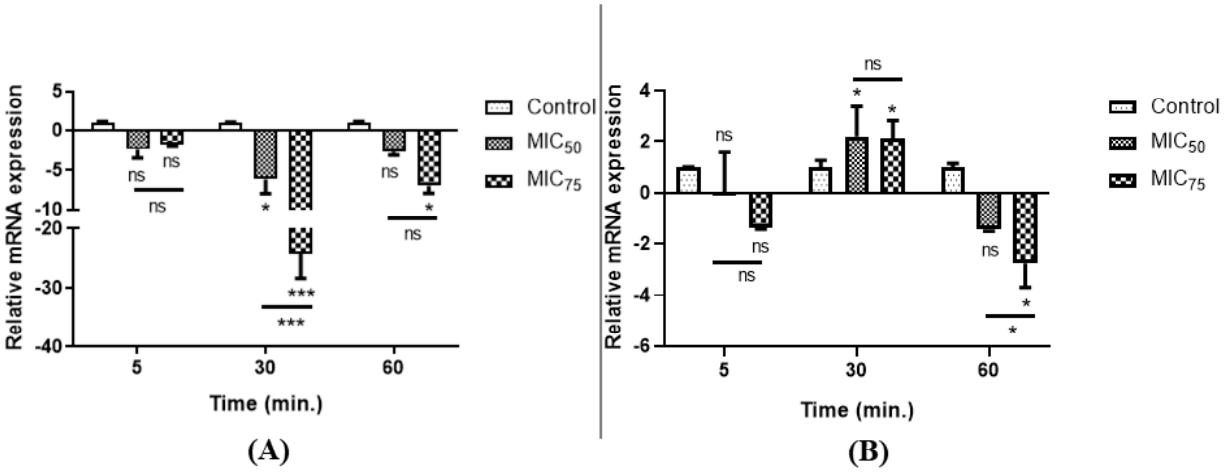


Figure 4.7: Differential expression of *wbbH* gene upon exposure of *E. coli* to (A) silver ion and (B) L-Ag NPs.

To investigate another pathway for the entry of silver ion/ L-Ag NPs entry into the bacteria, we have selected *aqpZ* gene which encodes for porin protein, i.e. aquaporin in *E. coli* (Calamita, 2000). Calamita et al. (1998) reported that expression of *aqpZ* gene regulation depends on the bacterial growth phase as well as osmolarity of medium. They reported that its expression increases at mid-log phase of growth and decline during late stationary phase. In contrast, Soupene et al. (2002) reported higher expression of *aqpZ* gene during transition into stationary growth phase. As per the report of Calamita et al. (1998), expression of *aqpZ* gene significantly increased in hypo-osmotic conditions, but greatly reduced in hyper-osmolar media. In the present study, differential expression analysis of *aqpZ* gene showed that at initial time points (5 and 30 min.) non-significant changes were observed in case of silver ion treatment. However, at 60 min. time point a significant down-regulation was observed in the bacterial cells exposed to silver ions (MIC_{75} concentrations). This may be to reduce the entry of silver ions inside the cells to maintain the cell's osmolarity. However, due to lesser release of silver ions from L-Ag NPs, non-significant down-regulation was observed at 60 min. time point in bacterial cells exposed to L-Ag NPs (Figure 4.8). Moreover, at later time points, the increased stress conditions could activate the bacterial defence system due to which bacteria down regulate the expression of *aqpZ* gene to conserve the energy as a survival strategy (Figure 4.8) (Kazmierczak et al., 2005; Rhodius et al., 2005; Reniere, 2018).

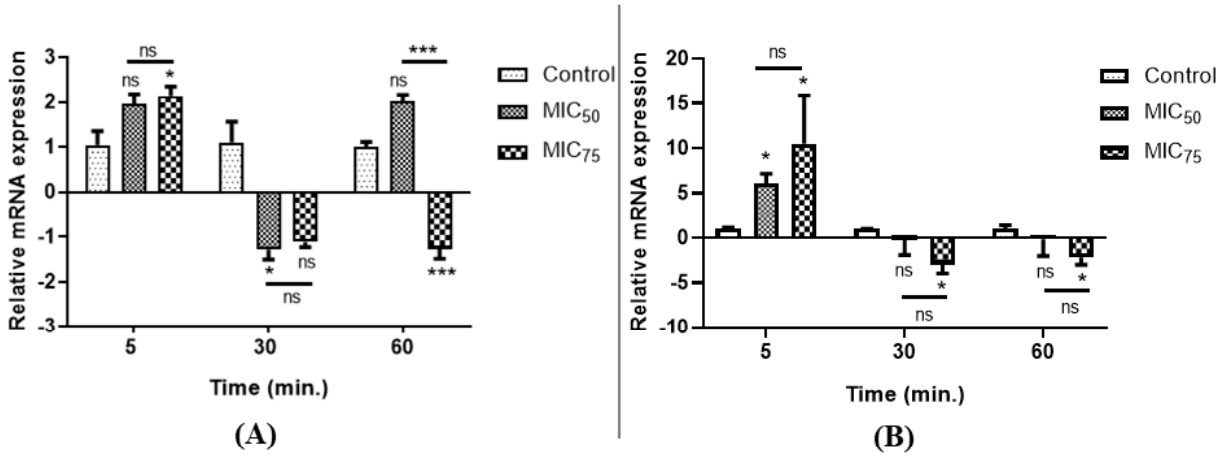


Figure 4.8: Differential expression of *aqpZ* gene upon exposure of *E. coli* to (A) silver ion and (B) L-Ag NPs.

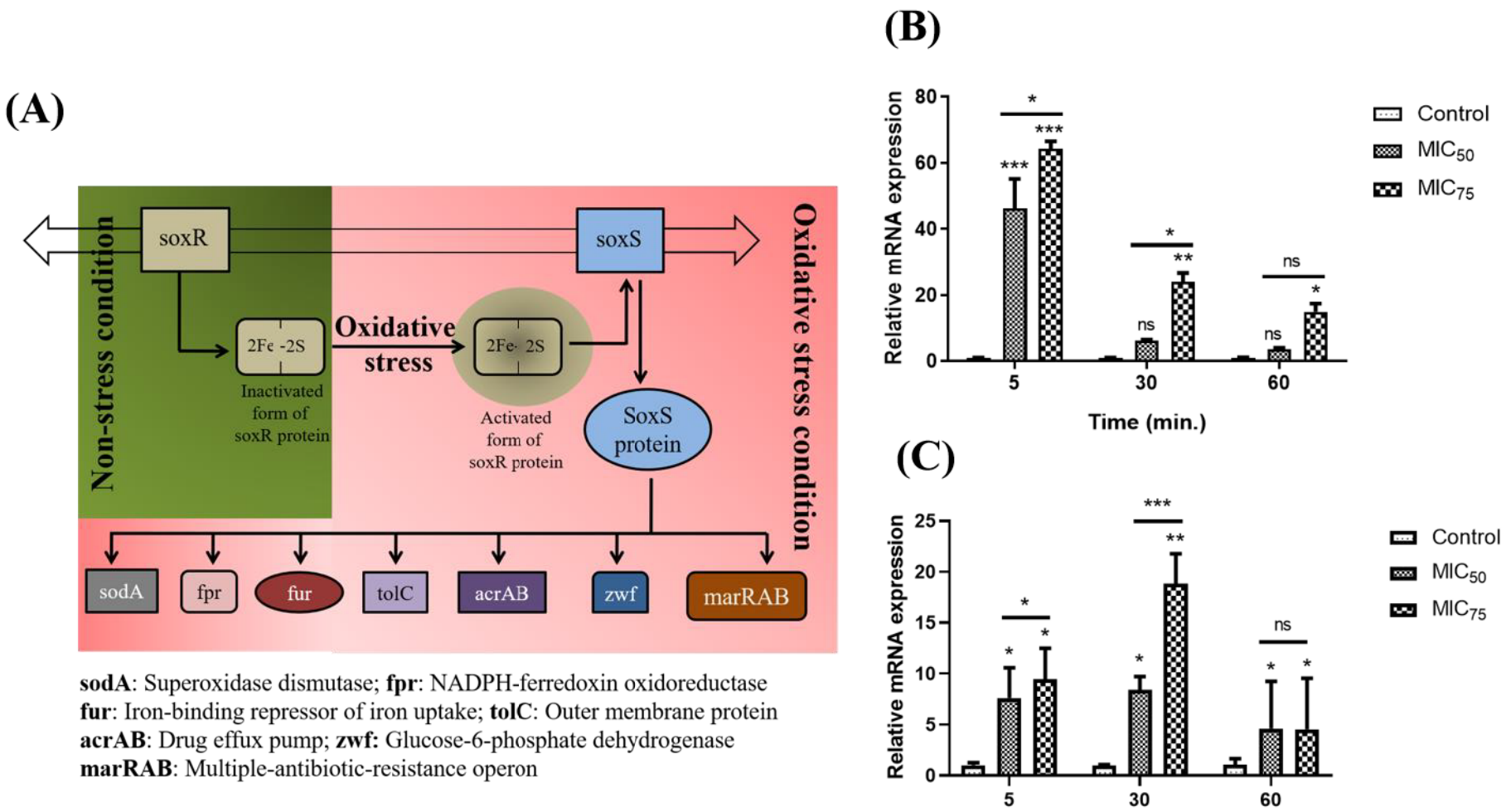
Once the silver ions enter inside the bacteria, what will happen next? It can be directly correlated with the toxicity level of silver. To find out the extent of silver's toxicity, we must know about the stress, faced by the bacterial cell. In general, the level of toxicity of any substance can be assessed by measuring the counterattack by the bacterial defense system. In *E. coli* K12 two regulatory defense (OxyR and SoxRS system) systems are present to overcome the oxidative stress (Seo et al., 2015). The *oxyR* regulon (set of genes) is triggered by H_2O_2, whereas the *soxRS* is triggered by the superoxide-generating (redox-cycling) agents (Nunoshiba et al., 1992). However, the transcriptional activity of OxyR has been reported to vary during normal cell growth in liquid cultures as it is adjusted to the endogenous H_2O_2 levels. Thus, in addition to the stress conditions, OxyR also function under normal physiological conditions (Pomposiello and Demple, 2001).

Conversely, the *soxRS* regulon respond to external agents that divert electrons from NADH or NADPH to molecular oxygen to generate a flux to superoxide (Fridovich, 1983). Under oxidative conditions, *E. coli* K12 induces production of 80 polypeptides. The synthesis of these polypeptides are governed by *soxRS* regulon at transcription level (Nunoshiba et al., 1992). The *soxRS* regulon is comprised of two main proteins namely SoxR and SoxS which are encoded by *soxR* and *soxS* genes, respectively, present on locus arranged head to head. SoxR protein is a homodimer (inactive form) which contains one [2Fe- 2S] cluster per subunit and constitutively expressed in the bacterial cells under non-stressed conditions.

During oxidative stress conditions, the SoxR get oxidized and in the oxidized form it stimulates the transcription of *soxS* gene (Imlay, 2013). The increased level of SoxS protein

then act as a secondary transcription factor and induces a chain of other regulons (Pomposiello et al., 2001), as depicted in Figure 4.9 A. Since *soxS* is a master regulator of defense mechanism, we have analyzed the differential gene expression of *soxS*. In case of silver ion treatment, a concentration-dependent increase in the expression of *soxS* gene was observed at the initial time point (5 min.) with ~ 64 fold up-regulation with the MIC_{75} treatment. However, at later time points (30 min. and 60 min.), decrease in the fold expression of *soxS* gene was observed suggesting the activation of the bacterial defense system by these time points (Figure 4.9 B). These results are in correlation to McQuillan et al. (2014), who showed expression of *soxS* gene in *E. coli* K12 treated with silver ions for 10 min. In case of L-Ag NPs treated bacterial cells, concentration and time-based increase in the fold expression of *soxS* gene was observed at 5 and 30 min. time points. Decrease in the expression of *soxS* gene at 60 min. time shows the activation of bacterial defense system (Figure 4.9 C). Careful analysis of obtained results shows ~ 64 fold up-regulation of *soxS* gene upon treatment with silver ions at the initial time points (5 min.), whereas only ~ 15 fold up-regulation was observed up to 30 min. time point upon treatment with L-Ag NPs. This confirms the controlled release of silver ions from L-Ag NPs. The increased expression of SoxS protein further enhances the ROS level in the bacterial cell by activating the expression of genes such as *sodA* (superoxide dismutase), *fpr* (NADPH-ferredoxin oxidoreductase), *fur* (iron binding suppressor of iron uptake), *tolC* (outer membrane protein), *acrAB* (drug efflux pump), *zwf* (glucose-6-phosphate dehydrogenase), *marRAB* (multiple antibiotic-resistance operon), etc. (Pomposiello et al., 2001).

E. coli K12 is a peritrichous flagellar bacteria and possess 5-10 flagella all over its surface (Mears et al., 2014). Bacterial flagella have a complex structure and are involved in various functions such as motility of the bacteria, biofilm formation and adhesion to host surface (Liu and Ochman, 2007). The typical bacterial flagellum comprises of 6 working gears viz. a basal body, a motor, a switch, a hook, a filament, and an export apparatus (Macnab, 2003). The assembly and regulation of flagella is controlled by an array of genes (Liu and Ochman, 2007). Among all the flagellar genes, we selected *fliG* gene to study the effect of silver species on bacterial flagella as it is directly involved in the regulation of motor switch proteins, hence, any disturbance in the functioning of flagella in presence of silver species can be monitored. Differential expression analysis was performed for *fliG* gene at the previously mentioned conditions. Upon exposure of silver ions to bacterial cells, a small concentration-dependent change was observed at 5 min., which may be due to the initial interaction of silver ions with motor proteins at both the tested concentrations (Figure 4.10 A).



sodA: Superoxidase dismutase; **fpr**: NADPH-ferredoxin oxidoreductase
fur: Iron-binding repressor of iron uptake; **tolC**: Outer membrane protein
acrAB: Drug effux pump; **zwf:** Glucose-6-phosphate dehydrogenase
marRAB: Multiple-antibiotic-resistance operon

Figure 4.9: (A) Functioning of soxRS system; Differential expression of *soxS* gene upon the exposure of *E. coli* to (B) silver ion and (C) L-Ag NPs

However, at the later time points, the increased interaction of silver ions causes disturbance in the bacterial motor protein and leads to decrease the expression of *fliG* gene at 30 min. treatment. In general, biosynthesis of flagella is an energy consuming process. Hence, in adverse conditions when the growth potential of bacteria decreases, it down-regulates the biosynthesis of flagella in order to save energy to survive in the adverse conditions (Zhao et al., 2007). Likewise, decrease in the expression of *fliG* gene was observed under the exposure of silver ions as a strategy to survive under the oxidative stress conditions. At 60 min. of treatment, a small reduction in the down-regulation of *fliG* gene was observed, which might be due to the activation of bacterial defence system by this time point, which triggered the bacterial repair machinery. In case of L-Ag NPs, no significant change in the expression profile of *fliG* gene was observed at 5 and 30 min. of treatment. We believe that at initial time points (upto 30 min.) the concentration of silver ions released from the L-Ag NPs must not be in enough concentration to cause disturbance in the flagellar machinery. However, down regulation in the *fliG* gene expression was observed at 60 min. of treatment, which suggests the slow release of silver ions from L-Ag NPs by this time point (Figure 4.10 B).

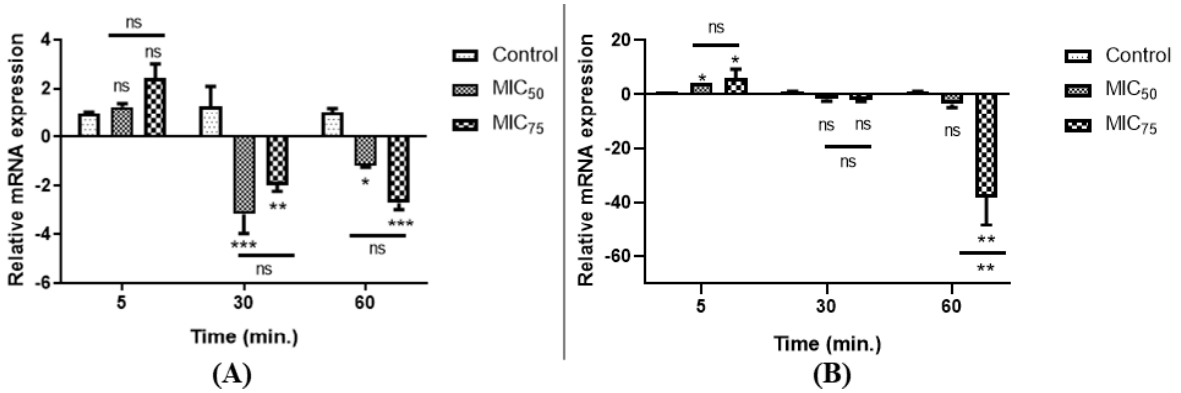


Figure 4.10: Differential expression of *fliG* gene upon exposure of *E. coli* to (A) silver ion and (B) L-Ag NPs.

Now a new question arises that whether the generated ROS levels are enough to affect the bacterial machinery like DNA/ protein processing or not? To figure out this question, we checked the extent of DNA damage upon silver species treatments. To do so, we have taken DNA damage repair machinery into consideration and analysed the differential regulation of SOS response.

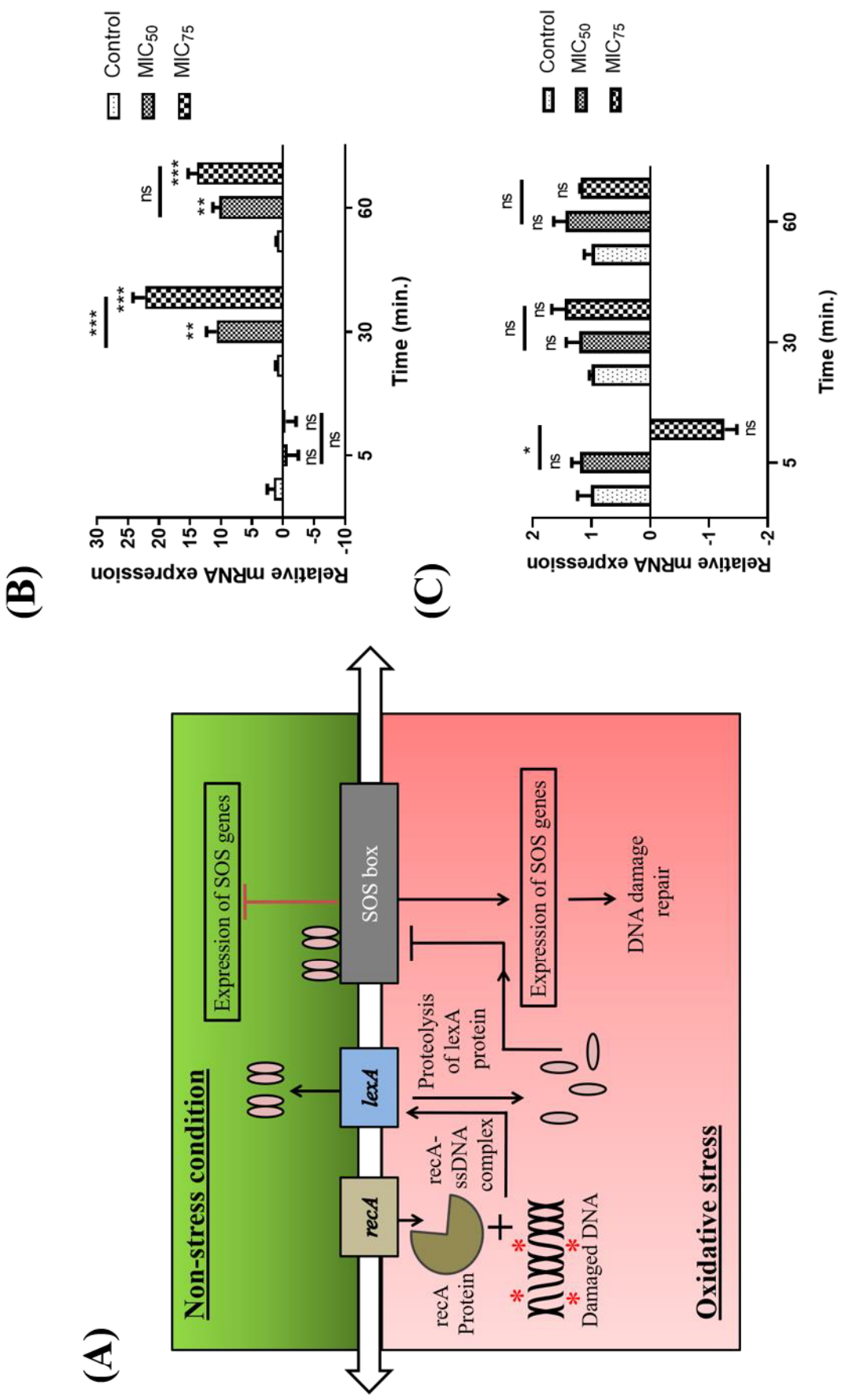


Figure 4.11: (A) SOS response system of bacteria; Differential expression of *recA* gene upon exposure of *E. coli* to (B) silver ion, and (C) L-Ag NPs

In *E. coli,* SOS response is a molecular programme that occur in response to DNA damage during stress conditions. The SOS response induce the activation of more than 50 unlinked genes which are often refffered as *din* (damage-inducible) genes. Under normal conditions, *recA* gene encode RecA protein which remain in inactive form. However, under stress conditions due to the action of any drug or agent, when DNA get damaged, the RecA protein binds to the developed ssDNA (due to damage) and form RecA/ssDNA nucleoprotein filament which activates the RecA (often reffered as RecA*). The activated RecA act as protease and cleave the LexA protein, which is a repressor of SOS regulon, resulting in the expression of SOS regulon. As the gene products of SOS regulon repair the DNA damage, the cell returns to the uninduced state and switch to reset (Figure 4.11 A) (Foster, 2005; Simmons et al., 2008; Kreuzer, 2013). In order to check the effect of silver species on the SOS response in *E. coli* K12, we analysed differential expression of *recA* gene.

The obtained results showed that at initial time point (5 min.), silver ions could not induce the SOS regulon as the activation of this machinery requires the replication state of DNA which occur at ~20 min. in *E. coli* K12 (O'Donnell et al., 2013; Kreuzer, 2013). A concentration-dependent up-regulation in the expression of *recA* gene was observed from ~13 to ~22 fold with MIC_{50} and MIC_{75} of silver ions, respectively, after completion of replication cycle (30 min. time point). However, it decreased to ~15 fold up-regulation at 60 min. time point (for MIC_{75} treatment) which could be due to the completion of repair process for the damaged DNA as after which the bacteria no longer require the SOS response (Figure 4.11 B). Likewise, Merrikh et al. (2009) showed the RecA dependent DNA damage response in Δ*iraD E. coli* strain, where oxidative stress has been generated due to the loss of *iraD* gene, which is basically involved in the maintenance of sigma factor RpoS. Recently, it has been reported that oxidative stress generated due to the iron deficiency in *Caulobacter crescentus* has also activated the RecA protein dependent SOS response to repair the damaged DNA (Leaden et al., 2018). In case of L-Ag NPs treated bacteria, no significant change was observed in the expression pattern of *recA*, which confirms that bacterial DNA was not affected till 60 min. exposure of L-Ag NPs as they might have released very low concentration of silver ions by that time point (Figure 4.11 C).

Likewise, to check the damage in the intracellular proteins, protein chaperone was considered for the differential gene expression analysis. Protein chaperones play a significant role to prevent the misfolding and aggregation of cellular proteins. There are different families of chaperones present in bacterial cells and many of these express mostly under stress

conditions. The DnaK protein [homolog of Hsp70 (heat sensitive protein 70) of humans] is a most abundant and constitutively expressed chaperone in *E. coli* which is constitutively expressed under stress condition and is encoded by *dnaK* gene (Calloni et al., 2012). The DnaK protein consists of nucleotide binding domain & substrate binding domain. With the help of co-chaperone DnaJ and nucleotide exchange factor GrpE, the ATP-dependent reaction cycle of DnaK prevents the misfolding and initiates the refolding of cytoplasmic proteins under oxidative stress conditions (Figure 4.12 A) (Liberek et al., 1991; Calloni et al., 2012; Chiappori et al., 2015).

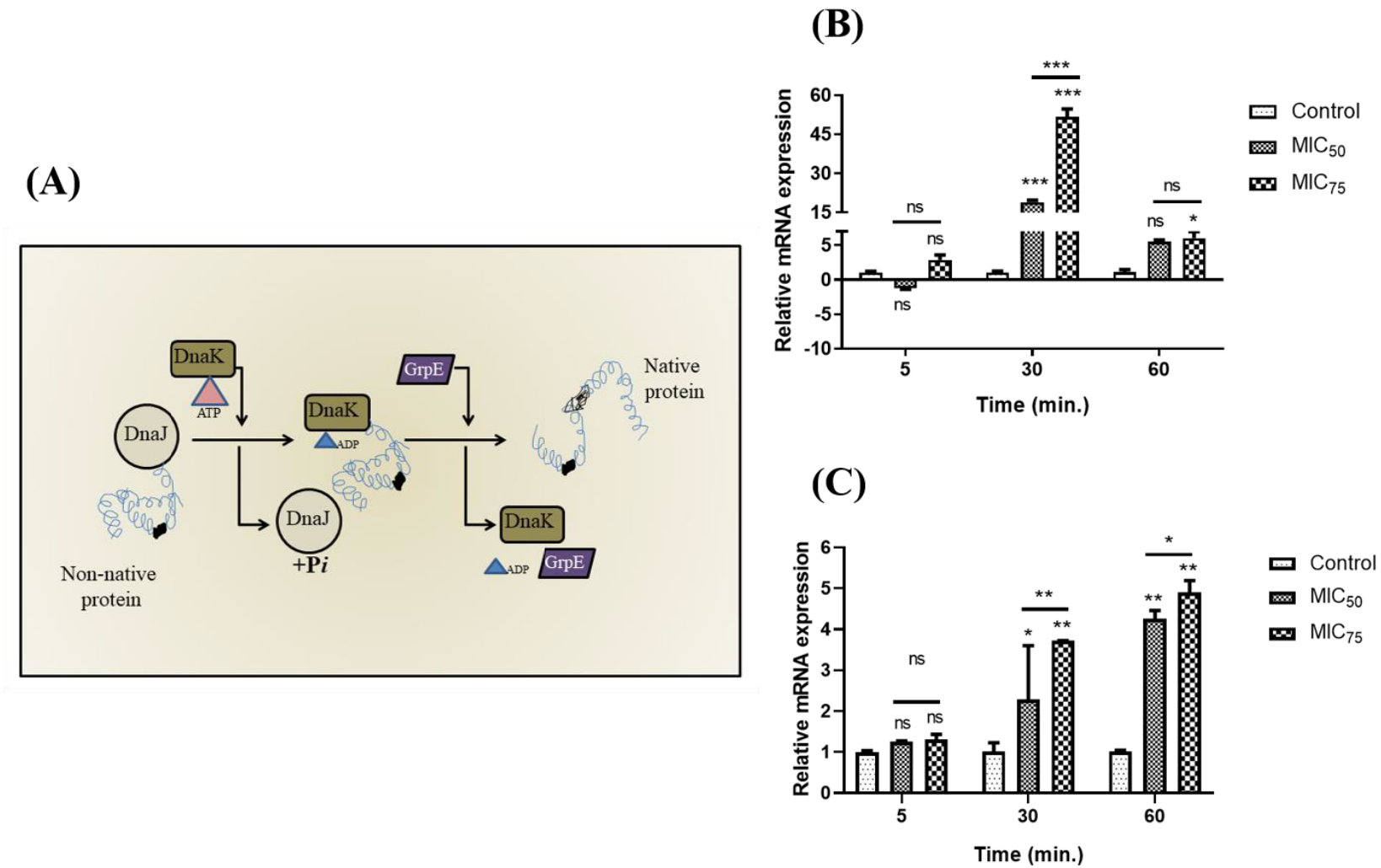


Figure 4.12: (A) Functioning of DnaK protein in bacteria; Differential expression of *dnaK* gene upon the exposure of *E. coli* to (B) silver ion and (C) L-Ag NPs

Differential expression pattern of *dnaK* gene upon exposure of *E. coli* to silver ions was found to be similar to that of *recA* expression pattern. Initially at 5 min. time point, no significant change was observed. But later on (at 30 min.) the expression reaches to maximum level to (~ 50 up-regulation). At 60 min. time point, the *dnaK* gene expression decreased to ~5 fold up-regulation which might be due the initiation of the protein repair process (Figure 4.12 B). Increase in the expression of *dnaK* can be correlated with activation of alternative sigma factor (σ^E), for the survival of bacteria (Kazmierczak et al., 2005). Activation of σ^E leads to the upregulation of molecular chaperone in order to prevent protein misfolding and aggregation (Rhodius et al., 2005; Rollauer et al., 2015). In case of L-Ag NPs treated bacteria, differential gene expression of *dnaK* was found to have the concentration and time-dependent up-regulation (Figure 4.12 C). It confirms the controlled release of silver ions from its L-Ag NPs as upto 60 min. time point the bacterial cells could not activate its protein defense system.

Differential gene expression analysis of DNA and protein repair machineries showed the toxic effect of silver ions and L-Ag NPs, but this toxicity was found to be countered by the activation of a bacterial defense system at the later time points. Now a new question arises; does this toxicity affects the bacterial homeostasis. If yes, then how does bacteria maintain their metabolic state?

In general, the iron-sulfur cluster (ISC) maintain the bacterial homeostasis by functioning as an essential factor (prosthetic groups) in various chemical reactions where, inactive form of enzyme (Apoenzyme) is converted to the active form (Holoenzyme) by ISC system, which provides iron as a cofactor (Djaman et al., 2004; Roche et al., 2013). However, under oxidative stress conditions, ISC system doesn't function. Instead, the Suf system comes into the picture and becomes functional under iron deprived conditions via the activation of OxyR regulon (Figure 4.13) (Outten et al., 2003; Fontecave et al., 2005).

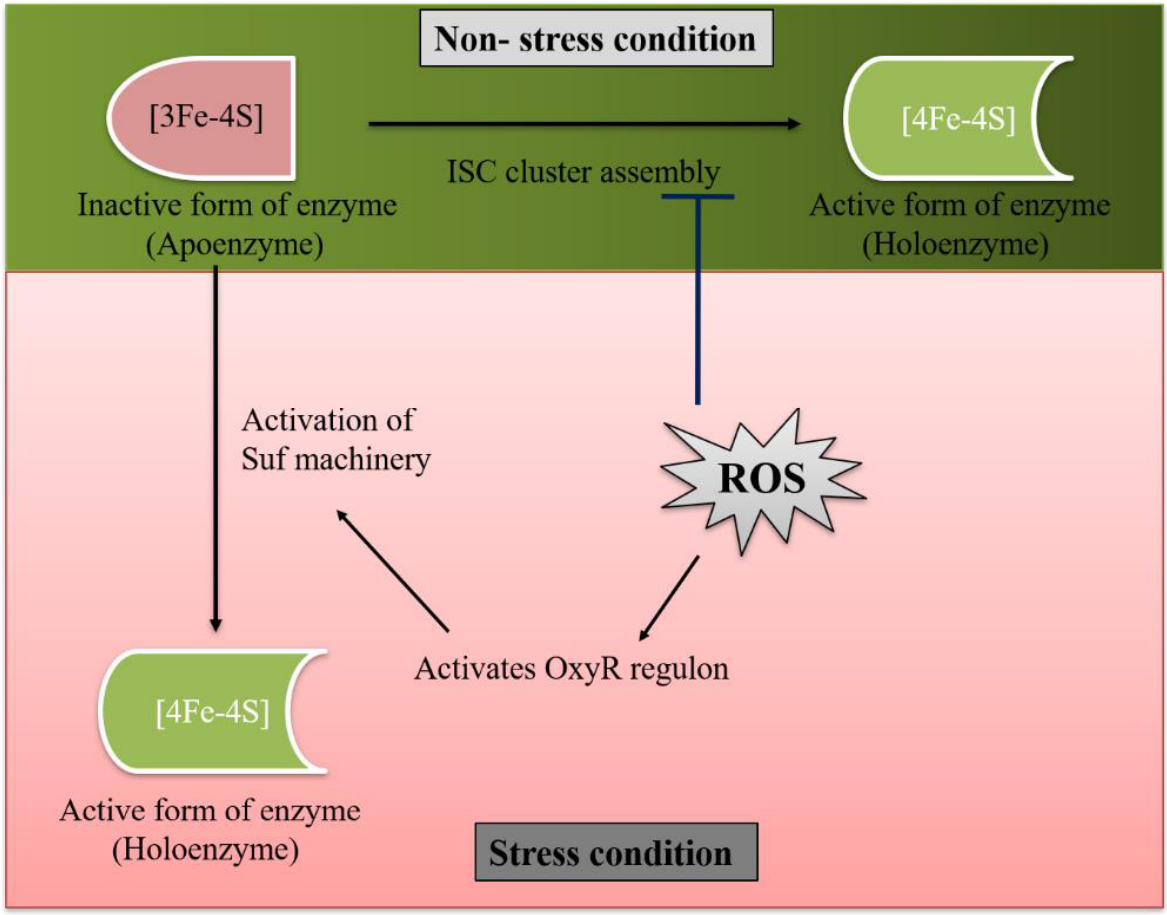


Figure 4.13: Correlation between the ISC system and Suf system in bacteria.

The functional form of Suf system is an assembly of six proteins i.e. SufABCDSE, which act together to form [Fe-S] clusters to maintain the bacterial homeostasis under oxidative stress (Fontecave et al., 2005). In this assembly, SufA functions as a scaffold protein whereas, SufS and SufE functions in the processing of sulfur. SufB along with SufC and SufD proteins play a final role in the formation of [Fe- S] clusters, which convinced us to select *sufB* gene for the differential expression studies upon exposure of *E. coli* K12 to the tested under the stress of silver species (Layer et al., 2007; Ayala-Castro et al., 2008; Dai and Outten, 2012) (Figure 4.14 A). The obtained results showed that at initial time point (5 min.), the exposure to silver ions lead to a concentration and time-dependent up-regulation of *sufB* gene, which reaches to its maximum level (~17 fold up-regulation) as one replication cycle get completed at 30 min. However, a subsequent decrease in the expression (~11 fold up-regulation) of *sufB* was observed at 60 min. time point due to the activation of the bacterial defense system, which leads to normalize the condition (Figure 4.14 B). Similar results were observed by Basak and Jiang (2012), who studied the response of the cAMP receptor protein (CRP) mutant strain of *E. coli* exposed to the hydrogen peroxide treatment. In case of L-Ag NPs, concentration and

time-based increase in the expression of *sufB* gene was observed upto 60 min. time point, which showed that in this case, the bacterial defense system was not fully activated as the maximum level of up-regulation was found to be only ~9 fold, which was ~17 in case of silver ions (Figure 4.14 C). This might be due to the controlled release of silver ions from L-Ag NPs, which at low concentrations does not allow the bacterial cells to activate its defense mechanism completely. These results suggest that the controlled release of ions from the L-Ag NPs could confuse the bacterial system by preventing the activation of defense system and reduce the chances of resistance development. However, in case of direct exposure of bacterial cells with silver ions, the sudden attack leads to activate the bacterial defense system, which allows to induce the required changes in the genome to counteract the situation, the so called resistance development (Panáček et al., 2018).

The obtained results showed that even after the entry of silver ions into the cell, the bacteria did not respond immediately as the bacterial cells are reported to tolerate micromolar concentrations of heavy metals including silver (Hobman and Crossman, 2015; Gurbanov et al., 2018). This property of metal tolerance resides in their genetic makeup upon transcriptional regulation of certain genes, which helps them to sustain under certain level of metal stress. As we have seen earlier in this chapter, bacterial defense system gets activated at the later time points of silver species treatment and enabled machineries such as DNA and protein repair machinery, activation of Suf system to maintain the homeostasis, etc.

Metals such as copper is required as a co-factor in several enzymatic reactions in *E. coli.* However, It can be extremely toxic at higher concentrations and require homeostasis which is mediated by sensors, transporters, chaperones and chelators (Arguello et al., 2013). In order to maintain the homeostasis and prevent toxic effects under metal stress conditions, the *E. coli* cells are known to possess the Cu and Ag efflux proteins located in the cell envelope encoded by *cusCFBA* operon (Lok et al., 2006; Franke, 2007) This operon has a homology with the silver resistance system (SilCFBA) of plasmid pMG101 in *Salmonella enterica* (Gupta et al., 2001; Pareek et al., 2018). Hence, *cusCFBA* was selected to study the efflux of silver ions from bacterial cells. The *cus* determinant machinery, consists of two operons, i.e. *cusRS* and *cusCFBA.* The *cusRS* act as a two-component pathway to sense the Cu^{+}/Ag^{+} (McQuillan et al., 2012) (Figure 4.15 A). *cusCFBA* is transcribed as a single mRNA starting from *cusC* gene which encode CusC protein (outer membrane factor which is homologous to TolC protein). CusF is a periplasmic protein which binds to Cu^{+}/Ag^{+} and interacts with the membrane protein efflux complex CusCBA, which spans the outer and cytoplasmic membrane (Outten et al.,

2001). Since *cusC* is the first to get transcribed among cusCFBA mRNA, it was selected to study the effect on efflux pump machinery upon exposure of *E. coli* to tested silver species. The silver ion treated bacterial cells showed a concentration and time-dependent up-regulation of *cusC* gene which reaches up to ~ 900 fold at 60 min. (Figure 4.15 B). This increase in the up-regulation can be correlated with the activation of bacterial defense machinery which got fully activated with time. However, L-Ag NPs treated bacteria showed very little and non-significant up-regulation of *cusC*. We believe that this may be due to the controlled release of silver ions from the surface of L-Ag NPs. Up-regulation of Cus operon in presence of silver has also been reported earlier (McQuillan et al., 2012).

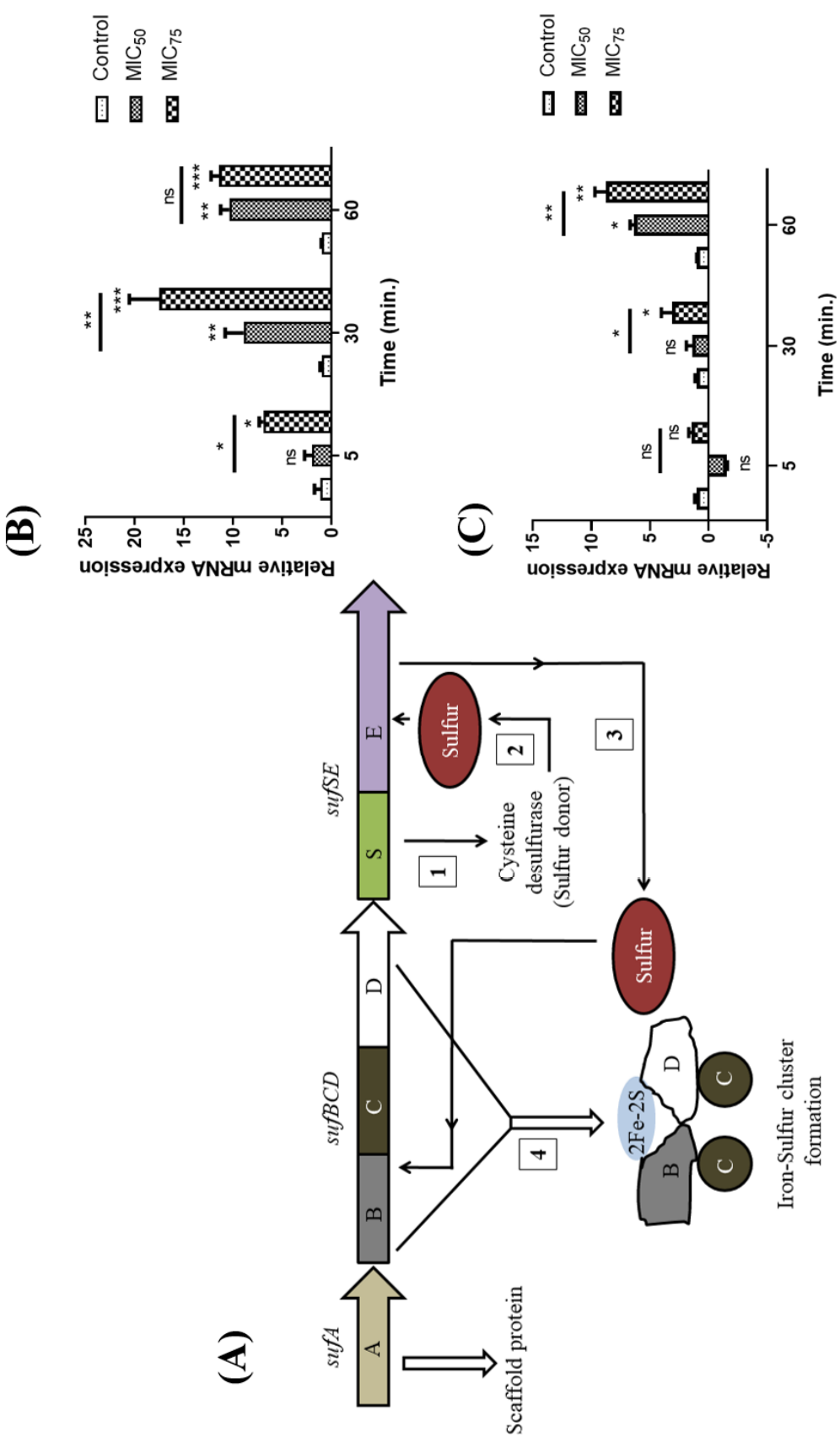


Figure 4.14: (A) Functioning of Suf system in bacteria; Differential expression of *sufB* gene upon exposure of *E. coli* to (B) silver ion and (C) L-Ag NPs

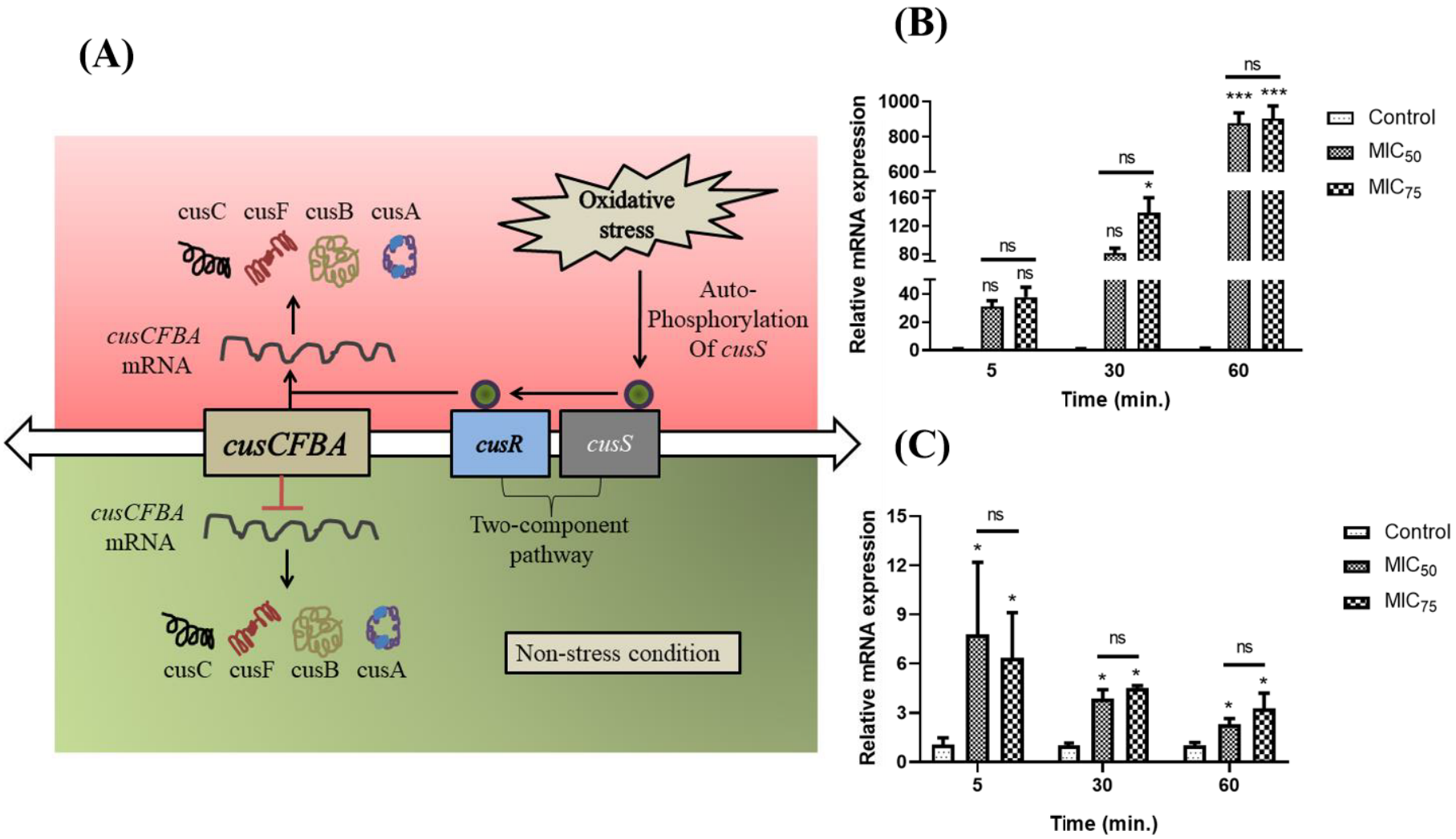


Figure 4.15: (A) Functioning of cusCFBA system in bacteria; Differential expression of *cusC* gene of *E. coli* exposed to (B) silver ions and (C) L-Ag NPs

Another system for the efflux of Cu^{+}/Ag^{+} in *E. coli* is Cue system which is regulated by CueR regulon (Figure 4.16 A). The CueR regulon concurrently controls *cueO* and *copA* where, CueO is a putative multicopper oxidase and CopA is Cu(I)-translocating P-type ATPase which is localized in the inner cell membrane (Grass and Rensing, 2001; Outten et al., 2001; McQuillan et al., 2012).

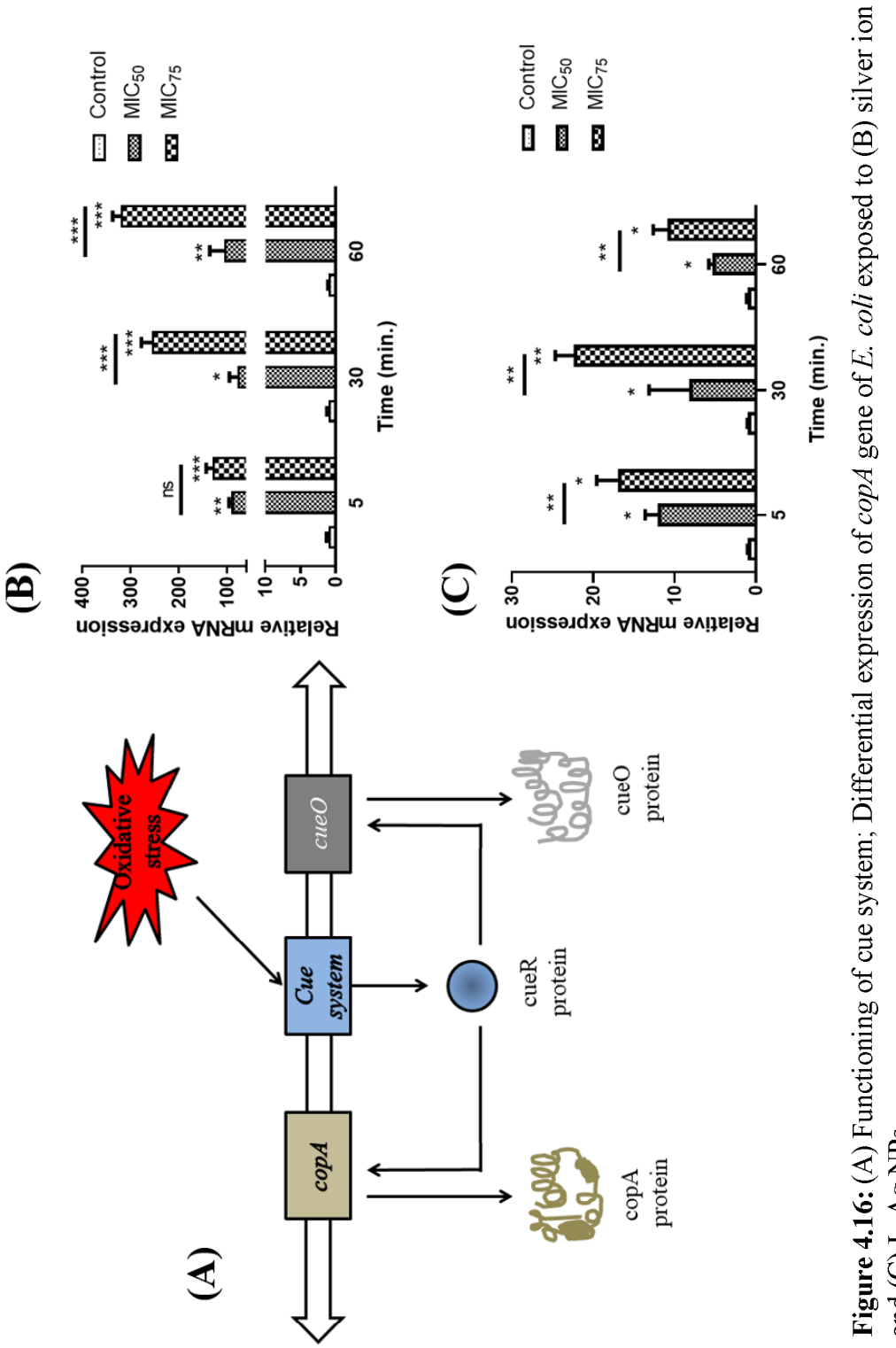


Figure 4.16: (A) Functioning of cue system; Differential expression of *copA* gene of *E. coli* exposed to (B) silver ion and (C) L-Ag NPs

At elevated cytosolic Cu^{+}/Ag^{+} concentrations, *cueR* gene products (CueR protein) up-regulate the expression of *copA* and *cueO* genes. The resulting CopA and CueO proteins translocates silver and copper cation from the cytoplasm to periplasm, where they are transferred to CusF of CusCFBA system in periplasm along with ATP hydrolysis. There are chances of oxidation of Cu in periplasm which can affect the periplasmic proteins. However, their oxidation was prevented by the CueO protein as it donates electrons from its Cu atom to molecular oxygen and thus, reverse the effect of Cu oxidation. However, as silver cannot be easily oxidised to Ag^{+2}, it cannot damage the periplasmic components and get effluxed by CusCFBA system from the periplasmic to outside the cell (Grass and Rensing, 2001; Rensing et al., 2000; Outten et al., 2001; Nies, 2003). That's why *copA* was selected for the differential gene expression studies. Similar to *cusC*, the expression pattern of *copA* was found to be time and concentration-dependent in case of silver ions treated bacteria as both these genes play a pivotal role in the efflux of silver ions (Figure 4.16 B). However, the expression of *cusC* gene was much highly upregulated in comparison to *copA* gene. It is because CopA is an inner membrane protein and explicitly involved in transporting silver ions from cytoplasm to the periplasm. Other than CopA, many other transporters also transport the silver ions into the periplasmic space. Hence, due to the higher concentration of silver ions in the periplasmic space, more amount of CusC protein (an outer membrane efflux protein) is required to transport it outside cell. Hence, higher expression of *cusC* gene was observed in comparison to *copA* gene. Bacteria treated with L-Ag NPs also showed the time and concentration-dependent up-regulation of *copA* at 5 and 30 min. time points. However, at later time point (60 min.), the expression of *copA* got reduced which may be due to the removal of most of the intracellular silver ions from the bacterial cell (Figure 4.16 C). This shows that L-Ag NPs released a limited amount of silver ions because of its potential of controlled release in comparison to the direct exposure of silver ions where a lot of silver ions attacked simultaneously on bacteria, which resulted in much higher expression of efflux pump. Regulation of *cus* and *cueO* regulons was also analysed by McQuillan et al., (2012 and 2014) also reported the up-regulation in the expression of *cusCFBA* and *cueO* regulon under the stress treatment of silver ions/ Ag NPs for 10 min. Increase in the up-regulation of these regulon's could be related to the generation of resistance against silver as *cusC* and *copA* showed maximum up-regulation among all the genes during present study. Among these two regulons, *cus* regulon is mainly recognized to confer resistance against low level of silver treatment (Munson et al., 2000; Hobman and Crossman, 2015).

The above results showed that the complete activation of homeostasis machinery/defense system takes at least 20 min. in case of *E. coli* cells exposed to silver ions. Whereas, L-Ag NPs exposed cells do not show activation of defense system fully due to the controlled release of ions from the nano form.

We then intended to understand that whether the exposure to silver species cause metabolic inactivation of bacteria? To answer this, we have taken the toxin-antitoxin (TA) system of bacteria into the consideration. In *E. coli*, around 36 TA systems are present, out of which the MazE- MazF is one of the most studied system (Amitai et al., 2004; Tan et al., 2011; Hall et al., 2017). The MazEF system belongs to the type II toxin-antitoxin system, where toxin and anti-toxin protein interact together to form a complex which protect the cell from the action of toxin. Among MazE and MazF proteins, MazE (labile protein) is the antitoxin and MazF is the toxin (stable protein). Under stress conditions, MazE protein get degraded by a serine protease ClpPA rendering synthesis of MazF (Amitai et al., 2004; Engelberg-Kulka et al., 2006) (Figure 4.17 A). The MazF toxin protein then inhibit the synthesis of cell survival proteins, while causing the expression of specific genes responsible for the cell death (Zhang et al., 2003). Considering the role of MazF in programmed cell death, we chosen *mazF* for differential gene regulation study. We found that at initial time point (5 min.), bacteria treated with silver ions showed down-regulation of *mazF* gene which might be due to the unusual changes in the structure of mazEF promotor sequence, termed as alternating pailndrome (Marianovsky et al., 2001). However, after completion of one replication cycle, activation of DNA damage machinery leads to the up-regulation of *mazF* to inactivate the metabolic function in the damaged cells at 30 min. Later on, at 60 min. time point, bacteria started recovering from the silver ion stress as the expression of *mazF* reduced in comparison to 30 min. time point (Figure 4.17 B). Hazan et al. (2004) also studied the MazEF mediated cell death in *E. coli* exposed to various stresses like DNA damage, high temperature (50°C), & oxidative stress and observed the mazEF mediated cell death in all the stress conditions. However, L-Ag NPs treated bacterial cells showed unusual and non-significant regulation of *mazF* gene, which might be due to the controlled release of silver ions in lesser concentration from L-Ag NPs (Figure 4.17 C).

Table 4.3 summarizes the relative fold expression of all the 10 selected genes upon exposure of *E. coli* to silver ions and L-Ag NPs. Figure 4.18 represents the heat map of all the differentially expressed genes in *E. coli* upon exposure to silver ions and L-Ag NPs in

comparison to untreated control. for 5, 30 and, 60 min, which suggests the comparative expression analysis between all the genes at different time points.

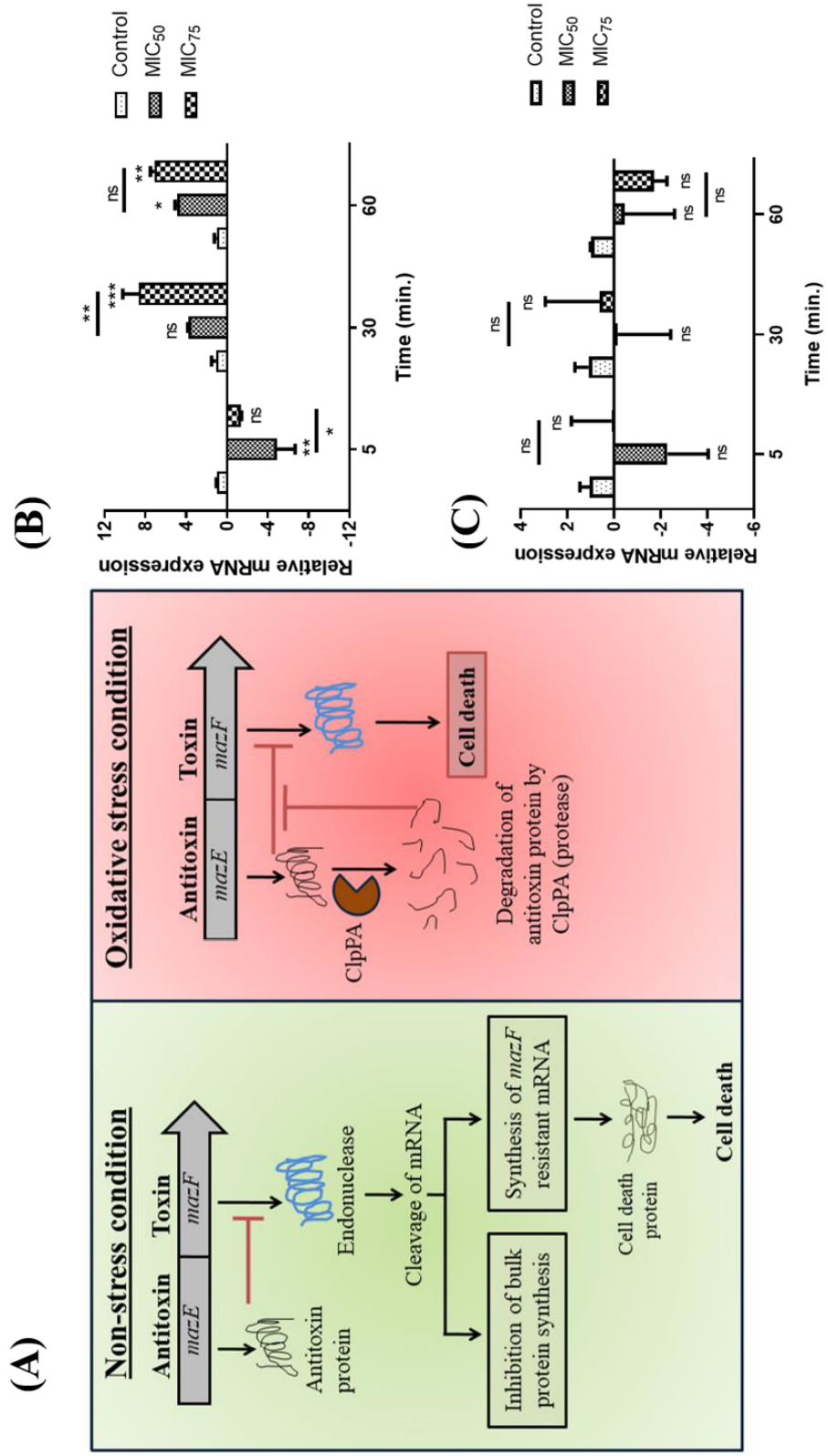


Figure 4.17: (A) Functioning of mazEF system in bacteria; Differential expression of *mazF* gene upon exposure of *E. coli* to (B) silver ion and (C) L-Ag NPs

Table 4.3: Relative fold expression profiling of the 10 selected genes upon exposure of *E. coli* to silver species.

Genes		Silver Ions			L-Ag NPs		
		5 min.	**30 min.**	**60 min.**	**5 min.**	**30 min.**	**60 min.**
FliG	**MIC_{50}**	1.24	-3.17	-1.20	4.42	-1.66	-3.62
	MIC_{75}	2.44	-1.98	-2.71	5.89	-2.20	-38.15
WbbH	**MIC_{50}**	-2.37	-6.15	-2.65	0.03	2.19	-1.45
	MIC_{75}	-1.88	-24.25	-6.98	-1.38	2.11	-2.75
AqpZ	**MIC_{50}**	1.95	-1.28	2.01	6.07	-.20	-.19
	MIC_{75}	2.14	-1.12	-1.26	10.48	-2.96	-2.2
soxS	**MIC_{50}**	46.26	6.15	3.67	7.62	8.37	4.55
	MIC_{75}	64.20	24.03	14.93	9.43	18.83	4.62
recA	**MIC_{50}**	-0.71	10.70	10.26	1.19	1.20	1.44
	MIC_{75}	-0.42	22.28	13.85	-1.26	1.45	1.19
dnaK	**MIC_{50}**	-1.29	18.90	5.47	1.25	2.28	4.26
	MIC_{75}	2.88	51.63	6.04	1.30	3.72	4.90
sufB	**MIC_{50}**	1.99	8.94	10.40	-1.54	1.39	6.38
	MIC_{75}	6.95	17.48	11.43	1.46	3.09	8.77
cusC	**MIC_{50}**	30.91	81.30	877.8	7.80	3.85	2.32
	MIC_{75}	37.76	138.53	904.22	6.35	4.51	3.27
copA	**MIC_{50}**	90.90	78.40	105.90	12.06	8.17	5.35
	MIC_{75}	130.08	255.85	320.38	16.96	22.41	10.89
mazF	**MIC_{50}**	-4.33	3.77	4.87	-2.28	-0.15	-0.45
	MIC_{75}	-1.33	8.65	7.14	0.10	0.61	-1.71

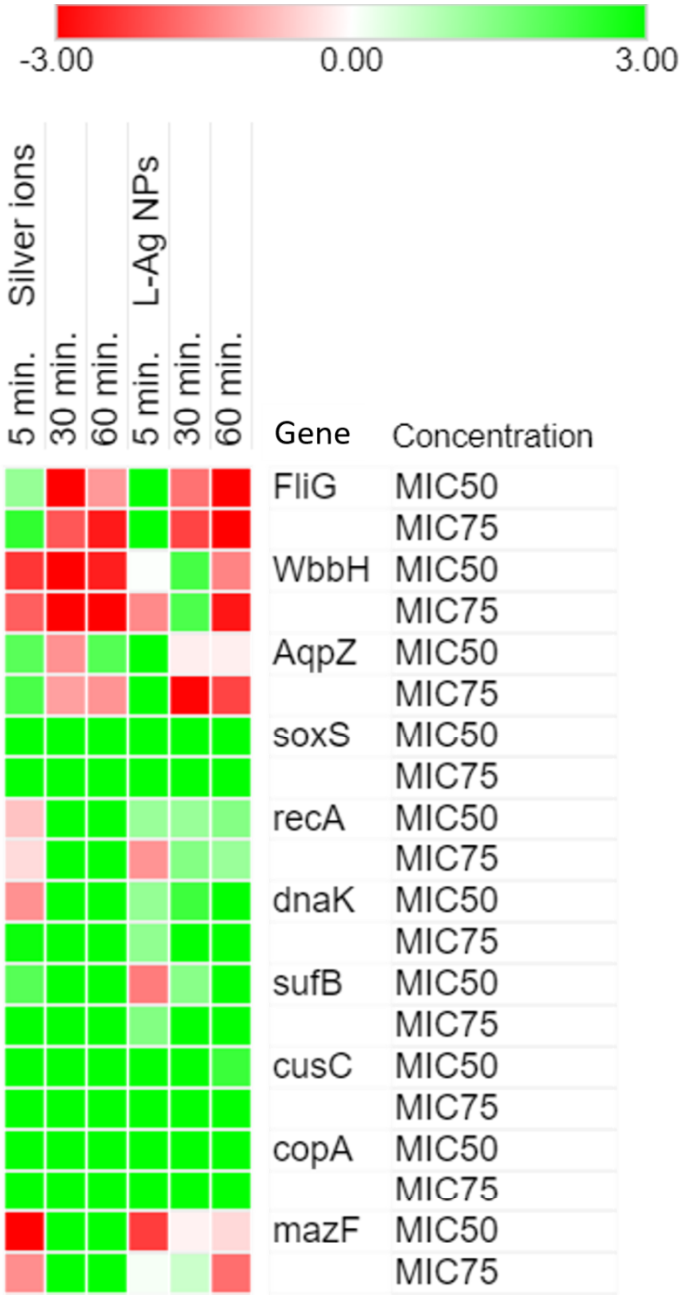


Figure 4.18: Heat map representing the expression pattern of studied genes in *E. coli* cells exposed to silver ions and L-Ag NPs (Green and red colour denote the up-regulated and down-regulated genes, respectively, in comparison to control).

4.3.3 Kinetics of cellular uptake of silver

To find out the concentration of silver ions which entered inside the *E. coli* K12, the cells treated with the MIC_{75} of silver ions and L-Ag NPs for 5, 30 and 60 min. were separately subjected to acid digestion. It resulted in release of silver ions from the bacterial cytoplasm, which were measured by the ICP-OES analysis (McQuillan et al., 2012; Jain et al., 2015). The obtained results showed that bacterial cells treated with $AgNO_3$ (silver ions) had more

intracellular silver ion concentration in comparison to the cells treated with L-Ag NPs (Figure 4.19). Uptake of less silver ions in case of bacterial cells exposed to L-Ag NPs showed the controlled and sustained release of silver ions, which makes it better antibacterial agent in comparison to silver ions ($AgNO_3$).

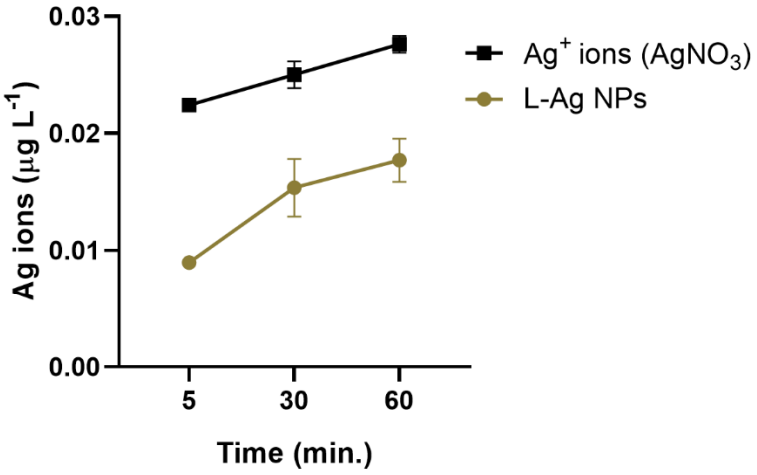


Figure 4.19: Concentration of silver ions in *E. coli* cells exposed to MIC_{75} of silver species. The data are expressed as mean ± standard error of two independent experiments.

4.4 Conclusion

This chapter presents the studies to divulge the mechanistic aspects behind the antibacterial action of silver ions and L-Ag NPs. To do so, initially, biochemical assays were performed to get the primary picture of antibacterial potential of silver species. Based on literature survey, ROS generation and membrane damage assays were selected for the biochemical analysis of antibacterial action. *E. coli* K12 was exposed to different concentrations (MIC_{25}, MIC_{50}, MIC_{75}, and MIC_{100}) of silver ions and L-Ag NPs. A concentration-dependent increase in ROS and membrane was observed with both the tested silver species. Since silver ions attack directly on the bacterial cell and L-Ag NPs functioned as a reservoir and slowly released silver ions, the extent of damage/leakage in the bacterial cell was more in case of silver ions. Apart from providing glimpse about the antibacterial potential of silver ions and L-Ag NPs, these assays helped us to decide their treatment concentration for differential gene expression studies. As MIC_{25} did not induce much changes in the bacterial machinery and treatment with MIC_{100} affects the metabolic growth of bacteria, MIC_{50} and MIC_{75} were selected for the differential gene analysis.

Based on the review of literature, ten diverse genes from various physio-biochemical pathways of bacteria were selected. The basis of selection was their probable role under the action of silver species. For the differential gene expression studies, bacteria cells were exposed to MIC_{50} and MIC_{75} of silver ions and L-Ag NPs, separately for 5, 30, and 60 min. Time of treatment was selected based on the bacterial replication time (~ 20 min.). Differential expression of *soxS, recA, dnaK, sufB, cusC, copA* and *mazF* confirmed that L-Ag NPs acted as a reservoir and allowed controlled and constant release of silver ions. Expression profile of *wbbH* and *aqpZ* showed that direct exposure of silver ions leads to the membrane damage, allowing the entry of silver into the bacterial cell.

Controlled release of ions from the surface of L-Ag NPs allowed the bacterial cells to function normally till the accumulation of threshold amount of silver ions which triggered the action of defence system, thus, reducing the chances of resistance development in bacteria. However, in case of direct exposure of silver ions, their sudden attack leads to activate the bacterial defence system quickly. In long term, such treatment may force the bacterial machinery to induce changes in their genome to counteract the situation and develop resistance against silver ions, similar to the well-known antibiotic resistance problem. The obtained results advocate that L-Ag NPs can be used as effective antibacterial agent.