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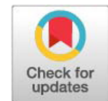
Copper Stress-Induced Transcriptional Regulatory Protein CusR also Regulates Silver Efflux in *Klebsiella pneumoniae* KW



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Key words
Heavy metal uptake, *cus* regulon, Metal efflux, CusCFBA system

ABSTRACT

Silver and silver containing compounds have had been in medicinal use since centuries. The antimicrobial properties of silver depend on its accumulation in bacterial cells. *Klebsiella pneumoniae* remains a major health concern around the globe due to its associated infections, such as, pneumonia, meningitis and bloodstream, surgical site and wound infections. The present study reports a locally isolated *K. pneumoniae* strain exhibiting high resistance (MIC=90mM) against Ag^+ and its accumulation potential. Transcription of Cus (Cu sensing) determinants is found to be upregulated in the presence of Ag^+. The transcriptional regulator CusR bound effectively with bidirectional *cus* promoter. STRING database analysis revealed that CusR possesses strong interactions and linkages with other Cus determinants and *via* ZraS with lead and zinc resistant genes. This regulator also possesses weak associations with Cue and Cut regulons and starvation sensing system.

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INTRODUCTION

Global mechanization and industrialization has led to continuous accumulation of toxic metals in the environment (Mosa *et al*., 2016). These metals not only pose threat to the environment but also to the living organisms, though metals are required for the functionality of several life processes such as being cofactors for enzyme functionality. These trace elements pose serious threat to the living organism if present beyond the tolerable limits (Franke *et al*., 2003; Kim *et al*., 2011). Silver ions are reported to be potent biocide. Several silver-binding membrane proteins are known to attract silver ions and by deriving energy from ATP hydrolysis, result in the uptake of silver ions inside the cells and initiate synthesis of silver nanoparticles (AgNPs) (McQuillan *et al*., 2012; Dakal *et al*., 2016). McQuillan *et al*. (2012) have suggested that the primary mechanism of action of silver nanoparticles is cell membrane dissolution. In addition, the dissolution of silver nanoparticles releases antimicrobial silver ions, which can interact with thiol-containing proteins in the cell wall and influence their functions. It is generally believed that easily ionized silver particles can affect the cell by the Trojan horse mechanism. Phagocytosis of AgNPs stimulates inflammatory signaling through the generation of reactive oxygen species (ROS) in macrophage cells, after which activated macrophage cells induce TNF-α secretion (Mikhailova, 2020). Silver nanoparticles (AgNPs) have been imposed as an excellent antimicrobial and antiviral agents

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Abbreviations
IPTG, Isopropyl thiogalactoside; MIC, minimum inhibitory concentration; MFP, membrane fusion proteins; OMF, outer membrane factor; RND, resistance-nodulation-division; ROS, reactive oxygen species. STRING, search tool for the retrieval of interacting genes.

being able to combat bacteria and viruses in vitro and in vivo causing infections (Salleh *et al*., 2020; Khina and Krutyakov, 2021). In combination with antibiotics, these ions have been used to kill multidrug resistant bacteria (Barras *et al*., 2018). They are also used in several medical devices such as wound dressings, implants, bone cement, shunts, catheters and endotracheal tubes (Sütterlin *et al*., 2017). Silver ions target the macromolecules of the cell such as DNA and protein, inhibiting cell transduction and growth. These ions also alter the respiration of *E. coli* as it has been considered a benign metal (Asiani *et al*., 2016). It is not known for the production of ROS, but it plays an important role in enhancing ROS production. Though, silver is less toxic to human cells (Mijnendonckx *et al*., 2013) its toxicity has been observed in the form of burns and argyria in some cases. It may also cause chest irritation and bronchitis (Prabhu and Poulose, 2012; Pelgrift and Friedman, 2013; Ayangbenro and Babalola, 2017).

The cells have developed different mechanisms to bypass the toxic effects of metals. The metal resistance genetic elements can be chromosomal or plasmid borne (Sotiriou and Pratsinis, 2011). Both play an important role in the accumulation and efflux of the metal out of the cell. A few prominent systems include members of RND (resistance-nodulation-division) superfamily transporters which catalyze active efflux of many heavy metals such as Cu, Ag, Cd resistant systems. These transporters become associated with two other classes of proteins – (1) the outer membrane channel proteins also known as outer membrane factors (OMF) such as AcrB of *E. coli* and MexB of *Pseudomonas aeruginosa* and (2) the periplasmic adapter proteins also known as membrane fusion proteins (MFP) such as AcrA of *E. coli* and MexA of *P. aeruginosa*.

The cytoplasmic borne *cus* regulon was selected for this study containing two operons one comprising four genes encoding structural proteins and second comprising of two genes, regulatory in nature. *cus* determinants consist of two operons, *cusRS* and *cusCFBA* transcribed in opposite direction. *cusRS* operon encodes a sensor histidine kinase CusS and a response regulator CusR. CusA, CusB and CusC interact to form an active channel spanning the periplasm and connecting the cytoplasm to the outer membrane. Each of these three component proteins is essential for metal efflux (Nikaido and Takatsuka, 2009). The construction of this tripartite complex suggests that the metals are exported directly into the external medium, rather than in the periplasm. Along with Cus regulon, Cue regulon also plays an important role in the metal efflux. Cue regulon comprises CopA which is involved in translocation of copper ions from cytoplasm to periplasm, CueO which oxidizes Cu^{+} to Cu^{++} in periplasm and CueR which is the transcription activator of the *cue* regulon (Outten *et al*., 2001).

Silver resistant bacteria were first isolated from the burn ward unit of the hospital in 1960s (Silver, 2003). Later these resistant strains were recovered from the environment especially in the remains of mines and waste water. Silver resistance has been best characterized in a plasmid pMG101 (Randall *et al*., 2015) which belongs to *Salmonella typhimurium*; it shows resistance to silver, mercury and tellurite (Mijnendonckx *et al*., 2013). Other systems that possess silver resistance include SilCBA, and CusCFBA systems, which are being controlled by SilP, SilF and CusR and CusS, respectively (Munson *et al*., 2000; Oshima *et al*., 2002; Silver, 2003; Mijnendonckx *et al*. 2013). PcoABCDRSE system is encoded by genes residing in a giant plasmid and its component PcoE has been reported to attach 48 silver ions thus making it unavailable for bacteria (Brown *et al*., 1995).

The present study deals with the structural and functional characterization of silver stress induced transcriptional regulatory protein encoded by *cusR* in a locally isolated KW strain of *Klebsiella pneumoniae* (Zulfiqar and Shakoori, 2012). Transcription of Cus (Cu sensing) determinants has been found to be upregulated in the presence of Ag^{+}. The transcriptional regulator CusR binds effectively with bidirectional *cus* promoter. STRING database analysis revealed that CusR possesses strong interactions and linkages with other Cus determinants and *via* ZraS with lead and zinc resistant genes. This regulator also possesses weak associations with Cue and Cut regulons and starvation sensing system.

MATERIALS AND METHODS

Klebsiella pneumoniae KW strain was first isolated by Zulfiqar and Shakoori (2012) from industrial effluents collected from Kot Lakhpat Industrial Area, Lahore. Stock of this strain was obtained from School of Biological Sciences, University of the Punjab, Lahore.

For administration of Ag^{+} stress, $AgNO_3$ salt was used throughout the study.

Determination of MIC of silver against K. pneumoniae *KW*

K. pneumoniae KW was allowed to grow in the LB agar medium supplemented with various concentrations of Ag^{+} (up to 120mM). The minimum concentration at which no growth was observed after 48 h incubation was considered as MIC of Ag^{+} against the bacterial strain.

Growth curve of K. pneumoniae *KW*

The bacterial growth was determined in the presence of various concentrations of Ag^{+} for which LB medium in

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sixteen Erlenmeyer flasks (200 ml capacity) was inoculated with 2 ml overnight cultures of KW and allowed to grow till OD_{600} 0.5±0.05. These log phase cultures were exposed to different concentrations of Ag^+ (20, 40, 60 and 80mM) along with control (No Ag^+). All these cultures were further grown at 37°C and 100 rpm. Cell density in these cultures could not be determined due to the formation of AgCl precipitates in LB broth that hindered the measurement of OD. However, cell density of each culture was determined by dropping 10µl of this culture on LB agar plates in triplicates at 0, 2, 4, 6 and 24 h of silver stress followed by incubation at 37°C. Any growth up to 48 h was checked. The experiment was repeated and 100 µl from each culture with 2h Ag^+ stress was spread on LB agar medium (spread plate method).

Effect of Ag^+ on growth of *K. pneumoniae* KW was also observed in the same way by using less concentrations of the metal (0.3, 0.5, 1, 2, 3, 4 and 5 mM) upto 8 h of the stress.

Amplification and cloning of cusR *gene*

Genomic DNA of *K. pneumoniae* KW was isolated as described by Rodriguez and Tait (1983). A 738bp DNA fragment containing *cusR* was amplified using specific forward and reverse primers with restriction sites for *Nde*I and *Hin*d III, respectively (Table I). A 50µl amplification reaction mixture was prepared as per manufacturer's guidelines (Fermentas Cat # EP0402). Amplification was carried out in Applied Biosystem 2720 thermal cycler.

For cloning, the amplified PCR product was gene cleaned and ligated in cloning vector pTZ57R by using Fermentas InsT/A clone PCR product cloning kit (Cat # K1214). *E. coli* DH5α competent cells were transformed with recombinant DNA. Cloning was confirmed by restriction analysis. The cloned *cusR* was subcloned in pET21a through *Nde*I and *Hin*dIII sites. BL21 codon plus competent cells were transformed with recombinant plasmid pET21a-cusR followed by confirmation through restriction digestion.

Expression analysis of CusR

For expression analysis, 100 ml LB broth supplemented with ampicillin (100µg/ml) was inoculated with 1ml overnight culture of $BL21C^+$ cells containing pET21a-cusR and allowed to grow at 37ºC till OD_{600} 0.6. IPTG was added (0.15mM final conc.) and culture was placed at 17ºC with shaking (120rpm). In control, no IPTG was added. The culture was centrifuged and the cell pellet was resuspended in 10ml of 20mM Tris-Cl (pH 8.0). It was sonicated (10 sec pulse with 15 sec rest) for 15 min at 60hz till the sample became transparent. Samples were checked by running on 12% SDS-PAGE. Maximum expression was observed in insoluble fraction (Fig. 4A). In order to proceed further with soluble form of protein, transformed cells were initially grown at 37°C and when OD reached 0.5-0.8, the temperature was raised to 45°C for 30 min. and then cooled at 20ºC. The culture was later induced with 0.1mM IPTG, and allowed to grow overnight at 17°C. This resulted in partial solublization of CusR as shown in Figure 4B in which a thick 25 kDA band of CusR is visible in total cell lysate (T) as well as in both supernatant (S) and pellet (P) fractions.

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Table I. Primers used in this study.

Primers	Primer sequences (5'--- 3')	Target	Amplicon size	Used for
cusR-F	CCTCATATGAAGATTTTGATTGTC	*cusR*	738 bp	Amplification for cloning
cusR-R	GCGAAGCTTATAAAGAAGGTCAG			
GyrA-F	TACGCGGTATACGACACCAT	*gyrA*	91 bp	Realtime PCR for relative quantification of mRNAs in response to Ag^+
GyrA-R	CGATGGAACCAAAGTTACCC			
$CFBA_{RT}$-F	CGCAGTGCATATCCTGTTG	*cusCFBA*	124 bp	
$CFBA_{RT}$ -R	AACGAAGGCGTAAGACTGCT			
RS_{RT}-F	CCTCAACGGCTATCACCTG	*cusRS*	90 bp	
RS_{RT}-R	ACGATATCCCAACCGTTCAC			
Cpq-F	GTCAAACGCGTGAAAGAGAG	*copA*	80 bp	
Cpq-R	GTCACATGGGCTTCAGTGAG			
Coq-F	CCCTGAATGCCACTACCTG	*cueO*	106 bp	
Coq-R	TCCGCCAGCTGGTTAGTAAT			
Crq-F	GGGTTTAACCTGGAAGAGTGC	*cueR*	111 bp	
Crq-R	GTTCTCGATATCCGCCACTT			
pRS-F	GGTACCTAGCTGTATTGAGC	*CusRS* promoter	298 bp	Promoter activity in response to Ag^+
pRS-R	TCTAGATCTTCACGGCAGGC			

The underlined nucleotides in cusR-F and cusR-R show restriction sites for *Nde*1 and *Hin*d III, respectively.

Isolation and purification of protein

The lysed cells as described above were subjected to fractional ammonium sulphate precipitation with 20%, 30%, 40%, 50%, 60%, 70% and 80% of ammonium sulphate. Each time, required amount of ammonium sulphate was slowly added and the sample was allowed to stand on ice for 2h for maximum precipitation. Fractions were centrifuged at 6000 x g. Supernatants and pellets were stored at each step of precipitation. After analysis on SDS-PAGE, two fractions obtained after 20 and 30% precipitation were pooled and dialyzed against 50mM Tris-Cl buffer (pH 8.0) with continuous shaking at 4°C. Buffer was changed at 4h intervals.

Proteins obtained after ammonium sulphate precipitation were fractionated by anion exchange chromatography using HiTrap QFF-5ml column on FPLC system AKTA purifier (GE healthcare). Protein (15mg) was loaded on the column. Fractions were collected, pooled and later run on 12% SDS PAGE. Partially purified CusR protein was quantified using Bradford reagent.

Gel shift assay

CusR protein (15µg) was incubated with *cusRS* promoter amplified by using pRS-F and pRS-R primers (Zahid *et al*., 2012, Table I) at 37°C for half an hour. The mixture was run on 4% polyacrylamide gel (prepared in 1X TAE buffer). Promoter, promoter + protein, BSA and protein marker were run at 80 V. Gel was stained with EtBr solution and any shift in mobility of *cusRS* promoter was observed.

Functional analysis of cus regulon against silver

Ag^+ with final concentration of 1, 2, 3, 4 and 5mM was added in 20 ml of log phase culture (2h old) of *K. pneumoniae* KW in five flasks. In control, no Ag^+ was added. After 15 min of Ag^+ stress, RNA isolation, cDNA synthesis and qRT-PCR of Cus genetic determinants (*cusCFBA* and *cusRS*) along with Cue genetic determinants (*copA, cueO* and *cueR*) was carried out as described by Zulfiqar and Shakoori (2012). The internal control was gyraseA. Results were recorded as described by Pfaffl (2001). The primers used for qRT-PCR are shown in Table I.

In silico *analysis of CusR and pcusR*

The 3D structure of CusR was predicted via homology modelling through Protein Homology/Analogy Recognition Engine V 2.0 (Phyre2) (Kelley *et al*., 2015). STRING software (https://string-db.org) was used to study CusR interaction possibilities with other cellular proteins.

DNA sequence of a region encompassing cusR promoter (pcusR) was subjected to various online tools such as BPROM (Solovyev and Salamov, 2011) and virtual footprint (Münch *et al*., 2005) to predict regulatory sites including -10 and -35 regions, cusR binding box and transcription start site.

Statistical analysis

Microsoft Excel was used to calculate the mean values of the data, while the Chi-square test was used to calculate the level of significance using JMP Statistical Package Software (Version 5.0.1.a, SAS Institute Inc., Cary, NC). Confidence level at 95% was determined and $P<0.05$ was used as significance level in all statistical analyses.

RESULTS

MIC

K. pneumoniae KW was able to grow in the presence of Ag^+ up to 90 mM. Therefore, MIC of Ag^+ for this strain was determined as 120 mM.

Effect of Ag^+ on the growth of K. pneumoniae *KW*

Effect of Ag+ on the growth of *K. pneumoniae* KW is shown in Figure 1. Addition of the metal in low concentrations (≤ 5mM) resulted in immediate death of a significant portion of the population and reduced the cell density in each culture. At 2h stress, there appeared a few or no colony, however, it looked the cells that somehow survived started growing and significant growth was there at 4 and further hours (Fig. 1C).

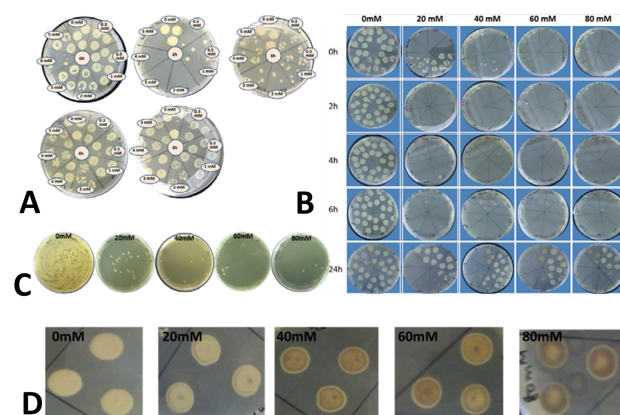

Fig. 1. Effect of Ag^+ on growth of *Klebsiella pneumoniae* KW and metal uptake. (A) Ag (0.3-5mM) was added in log phase cells and 5µl was spotted with 2h time intervals. Addition of the metal appeared to be highly toxic and strength of colonies decreased with increasing concentration at time 0 and no visible growth at 2hs. Growth reinitiated at 4 h that increased afterwards. (B) Addition of higher concentrations caused further delay and it took 6 h for cells to adapt and growth reinitiation. (C) Spreading of 100µl at 2h post metal addition showed the presence of small number of alive cells. (D) Brown coloration of colonies

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show silver accumulation in the cells.

Addition of higher concentrations of the metal (>20mM) resulted in immediate death of almost whole population. At 2 and 4 hours stress, either a few or no colony was observed (Fig. 1A). However, spreading of 100µl at two hours stress resulted in the appearance of a few colonies (Fig. 1B) that were not observed when only 5 µl was spotted. At 6h of stress a significant increase in number of colonies was observed and after 24 h stress heavy growth was there though it was still significantly less than the control.

Ag^+ uptake ability

After 24 h of silver stress, a brown rim appeared around periphery of each colony. The color and area of this rim gradually increased with the increase in Ag^+ concentration showing the uptake of Ag^+ by *Klebsiella* cells (Fig. 1D).

CusR expression and purification

CusR expression was taken in BL21 cells under IPTG induction. Figure 2A shows the three fractions; total cell protein (T), soluble (supernatant, S) and insoluble (pellet, P) proteins of the recombinant cells grown at 37 °C. Induced band of CusR roughly around 25 kDa in the pellet fraction showed that the protein was expressed in insoluble form. Figure 2B shows partial soluble form of CusR when cells were given heat shock followed by growth at 20 °C

Figure 2C shows the dialyzed sample and the fractions obtained from anion exchange chromatography. CusR containing fractions obtained through FPLC were later on pooled to get partially purified CusR.

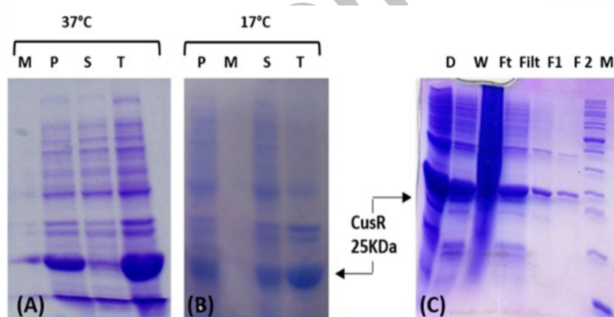

Fig. 2. Expression and purification of CusR. A, total cell lysate (T), supernatant (S) and pellet (P) of un-induced and 0.1mM IPTG induced transformants grown at 37 °C. B, total cell lysate (T), supernatant (S) and pellet (P) of induced transformants, first given heat shock and then grown at 20 °C. C, dialyzed sample and FPLC fractions of CusR; dialysed sample (D), washout (W), Flow through (F), filtered sample (Filt). The subsequent lanes show FPLC fractions. Lane M: Bench mark protein unstained ladder.

cusRS *promoter and its binding with* CusR

Figure 3 shows the binding of *cusRS* promoter (298bp) with partially purified CusR. *cusRS* promoter alone appeared at 298bp position. However, *cusRS* promoter + protein CusR mixture appeared slightly above indicating slow movement of *cusRS* promoter due to its binding with CusR protein and hence increased mass. *cusRS* promoter + BSA (negative control) appeared at the level of *cusRS* promoter alone (298bp) indicating no interaction.

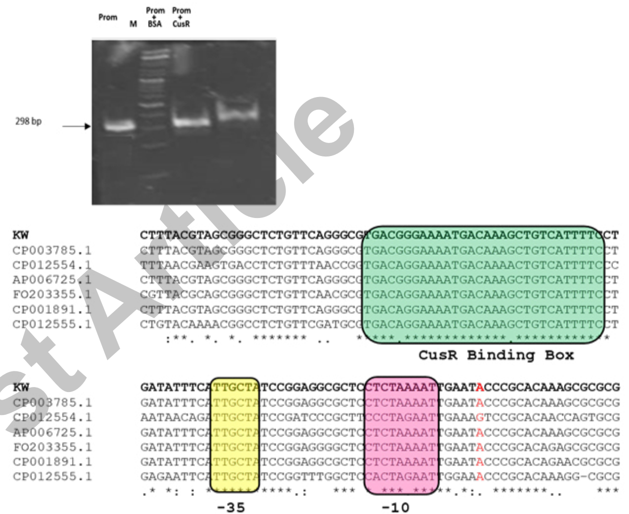

Fig. 3. 4%-PAGE to show DNA-Protein interaction (Gel shift assay). Lane 1: the amplified bidirectional cusRS promoter (Prom), Lane 2: DNA Ladder Mix (SM006), Lane 3: BSA + cus promoter, Lane 4: promoter bound to CusR. Below it is shown multiple alignment of promoter cusRS sequence from *K. pneumoniae* KW, *K. pneumoniae* HS11286 (CP003785.1), *K. pneumoniae* NTUH-K2044 (AP006725.1), *K. aerogenes* KCTC 2190 (FO203355.1), *K. variicola* At-22 (CP001891.1), *Raoultella ornithinolytica* strain 18 (CP012555.1), and *Citrobacter freundii* strain P10159 (CP012554.1). Figure shows palindromic sequence of CusR binding box (Green color box), -10 box (yellow color box), -35 box (pink color box) conserved among different species of family Enterobacteriaceae. A/G in red font represents predicted transcription start site.

Promoter cusRS *sequence analysis*

CusR regulates bidirectional *cusRS* promoter. The regulatory elements present in this promoter include a CusR binding box, -35 box, -10 box and transcriptional start site from 'A' nucleotide (Fig. 3). For multiple alignment, CLUSTALW was used to check sequence homology with already reported *cusRS* promoter sequences from *K. pneumoniae* and some other species of

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family Enterobacteriaceae.

Effect of Ag^+ on expression of copper responsive cus *genetic determinants*

mRNA levels of both *cusRS* and *cusCFBA* increased in response to all concentrations of Ag^+ added in the medium. However, this increase was not consistent with increase in Ag^+ in the medium. In the presence of 1mM Ag^+, *cusRS* and *cusCFBA* mRNA levels increased 8 and 70 times, respectively as compared to the control (Fig. 4A). However, lesser fold increase of both polycistronic mRNAs was observed in the presence of 2mM Ag^+. A very profound increase was observed with further increase in the metal concentration (3mM). At this level, maximum expression was found (25 and 210 fold increase of *cusRS* and *cusCFBA*, respectively than those in the absence of Ag^+). When Ag^+ concentration was further increased, both the RNA levels were correspondingly upregulated, though this increase was much less as compared to those in the presence of 3mM Ag^+.

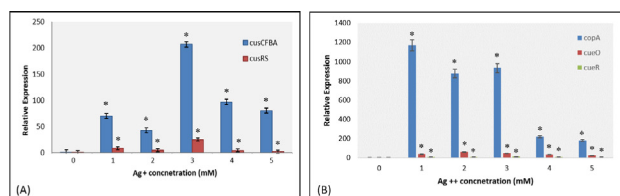

Fig. 4. Effect of Ag^+ (1-5 mM) on transcription of *cus* and *cue* genes. (A) Expression of *cusCFBA* and *cusRS* was upregulated in the presence of 1mM Ag^+ that increased with higher concentrations of Ag^+ upto 3mM. However, further increase in the metal resulted in lesser upregulation. (B) Transcription of *cue* regulon genes (*copA*, *cueO* and *cueR*) also upregulated in the presence of Ag^+. In this case, maximum upregulation was observed at 1mM Ag. *cue* regulon was found to be 6 times more upregulated as compared to *cus* regulon in response to the metal.

Effect of Ag+ on expression of cue *genetic determinants*

Change in expression level of Cus regulon (CusCFBA and CusRS) at transcriptional level was also compared to another regulon viz; Cue regulon (CopA, cueO and CueR). *copA* showed maximum upregulation (1168 times) in the presence of 1mM Ag^+ in the medium. This decreased slightly (up to 932 times than control) with increase in Ag^+ up to 3mM. However, with further increase in the metal concentration (4 -5mM), upregulation greatly decreased (remained upto 180 times compared to control). *cueO* and *cue*R exhibited upregulation up to 59.7 and 10.3 times, respectively in response to Ag^+ and followed the same trend in expression in response to varying concentrations of Ag+ as exhibited by other genes under study (Fig. 4B).

Structural analysis of CusR

Amino acid sequence was used to find some physical properties using the web tool protparam (https://web.expasy.org/protparam). Total amino acids were 227, isoelectric point was 5.49 and molecular weight predicted was 25 kDa.

CusR associations and interactions with other proteins

Protein-protein interactions at sub-cellular level were calculated through STRING database (Fig. 5). This analysis yielded a multiple node interaction networks map. The interaction network analysis contained a total number of nodes to be 11 and 37 edges with 6.73 average node degree. The clustering coefficient was 0.915, and the PPI enrichment value was 9.47e-11. It was found that CusR interacts with factors involved in copper homeostasis such as Cus, Cue and Cut. This analysis also showed strong relation of CusR with ZraS that affects regulation of zinc and lead homeostasis. A weak association between CusR and RspR, a regulator of starvation sensing system, was also found.

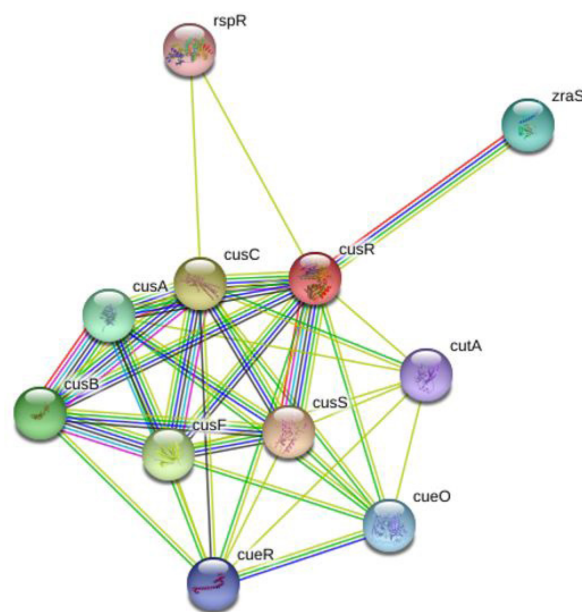

Fig. 5. CusR protein-protein interaction analysis through STRING server shows its major role in copper homeostasis (via Cus, Cue and Cut factors) in addition to regulation and sensing of some other heavy metals (*via* zraS) as well as starvation (*via* rspR).

DISCUSSION

Metal resistance

K. pneumoniae, known to cause ventilator associated

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pneumonia, has been a major component of soil microflora. It has been known to be resistant to metals such as Ag^{+}, Hg^{++} and Cd^{++} much more than other species - *Pseudomonas fluorescens, Pseudomonas aeruginosa, Proteus mirabilis* and *Staphylococcus* sp. (Filali *et al*., 2000; Wei *et al*., 2009; Vardhan *et al*., 2019). Silver has been used in medical field due to its less toxic effects on humans however its uncontrolled use has caused resistance in microorganisms posing a threat. Bacterial isolates from burn ward units possess high level of resistance against Ag^{+} (Norton and Finley, 2021). Panacek *et al*. (2018) reported *E. coli* grown in liquid medium containing silver nanoparticles. Our results have shown that this bacterium is able to resist high levels of silver that is 90mM. MIC of silver against two *Klebsiella* spp. and an *Enterobacter* sp. has been previously reported to be about 512mg/l, 256mg/l and 5mM, respectively (Finley *et al*., 2015; Randall *et al*., 2015; Sütterlin *et al*., 2017). Bacterium under study has also shown resistance against 5mM Cu^{++} (Zulfiqar and Shakoori, 2012), 1mM Au^{+}. It also has shown resistance against zinc, mercury, lead and cadmium (Imran *et al*., 2021).

Growth of K. pneumoniae *and metal uptake*

Addition of silver in the growing cultures resulted in immediate death of the cell population and a very few cells could survive. In the presence of low concentrations of the metal, cells appeared to adapt and a significant number of colonies were visible after four hours that increased further at later time points. However, in the presence of these concentrations, the growth rate appeared to be dose dependent. In the presence of higher concentrations (≥20mM), cells took longer time to adapt and reinitiate growth of the metal but the growth rate was independent of the metal concentration.

Appearance of brown rim around colonies with silver stress showed metal uptake ability of *K. pneumoniae* KW cells. Increase in the intensity of the brown ring was also observed with increase in the metal concentration in the medium and depicting the dose dependent uptake. There are several studies which show accumulation of heavy metals in various microorganisms. Shakibaie *et al*. (2008) have reported accumulation of 0.35% Cu^{++} and 0.3% Zn^{++} per mg dried biomass. Mohamed and Abo-Amer (2012) reported that *Gemella* and *Micrococcus* could reduce 55% and 36% Pb and Cd, respectively from the environment. Although the resistance mechanism in these bacteria was plasmid borne, the reduction of metals was very high in contrast to the present study where the Ag^{+} uptake by the bacterium was 3.74%, 6.60% and 4.92% for 20, 30 and 60 mM Ag^{+}, respectively in the medium.

The biphasic growth curve of *K. pneumoniae* in Figure 6 shows multiplication of bacterial cells in the presence of Ag^{+}. The model suggests, prior to metal addition, a normal growth pattern is shown. Addition of the meal proves to be lethal and results in the immediate death of the cell population with survival of a very few cells. The time required for the restoration of growth is found to be concentration dependent. During this, the bacterial cells turn on their metal efflux system, bringing metal concentration to the subtoxic level that restores the bacterial growth.

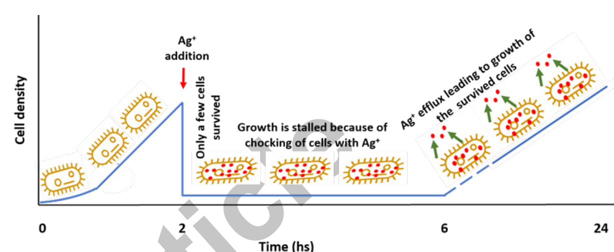

Fig. 6. Proposed growth trend of *K. pneumoniae* KW against increasing concentration of silver.

Effect of Ag^{+} *on expression of* cus *genetic determinants*

Expression of *cus* determinants increased significantly in the presence of different concentrations of Ag^{+} that indicated their possible role in sensing and resistance against Ag^{+}. He *et al*. (2021) measured *cusR, S, C, F, B* and *A* transcripts levels individually and reported almost equal upregulation in response to Cu^{++} however, fold increase in *cusCFBA* transcripts, observed in this study, in response to Ag^{+} is significantly high as compared to *cusRS* transcripts. As the concentration increased till 3mM, the more fold increase in the expression was observed. This difference in fold increase is according to the requirement of end products to make cell survive in higher concentration of metal. However, further increase in concentration of the metal ions resulted in lesser fold increase in both transcripts level. Most probably cells had shifted to some other SOS like mechanism.

Along with the Cus regulon, Cue regulon also plays an important role in the metal efflux. Therefore, the expression of genes constituting Cue regulon was also assessed at the transcriptional level and compared with that of Cus regulon. Cus regulon comprises CopA, a P-type ATPase that is known for the translocation of excess Cu^{+} from cytoplasm to periplasm (Rensing and Grass, 2003); CueO, a multi copper oxidase that oxidises Cu^{+} to less toxic form Cu^{++} in the periplasm and CueR, the transcription activator of *cue* regulon (Outten *et al*., 2001). These genes were also upregulated in response to Ag^{+} though fold increase of *copA* was very much high (upto

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1168 fold) that remained high in the presence of 1-3mM Ag^+. However, further increase in metal concentration in the medium resulted in lesser upregulation that remained ~ 200 times more as compared to the control. *cueO* and *cueR* transcripts were increased upto ~60 and ~10 times in the presence of 1-3 mM Ag^+ and like *copA* these two genes also exhibited lesser upregulation in the presence of further increased concentrations of Ag^+ in the medium. Zulfiqar *et al*. (2019) also find similar expression pattern of *cueO* in response to copper.

cusRS *promoter and its binding with* CusR

The exact mechanism behind the protein binding affinity of CusR with *cusRS* promoter is not known. Urano *et al*. (2017) used labeled purified protein CusR and observed its binding affinity with the *cusRS* promoter and another *hiuH* promoter containing a sequence highly similar to CusR box. Results showed that CusR had four-fold increase affinity to bind with *cusRS* promoter in comparison to *hiuH* promoter. We have also observed CusR binding with its respective promoter.

CusR interactions

CusR interactions with other proteins were analyzed using STRING data base. Strongest interaction is found between CusR and CusS. This data base also shows the biological and molecular roles of these proteins; involvement of CusR in copper and silver ion homeostasis, copper ion transmembrane transporter activity and efflux pump complex formation.

Mechanistic analysis of Cus regulon

cus regulon consists of regulatory (*cusRS*) and structural genes (*cusCFBA*). It is suggested, once CusS (sensor histidine kinase) senses metal ions, it gets phosphorylated, and transphosphorylates a transcriptional regulator CusR, which binds with the bidirectional promoter (Munson *et al*., 2000). The effect of a small increase in these two proteins multiplies when the promoter is activated. Figure 7 shows production of CusC, CusB and CusA proteins which are the structural components of transmembranous channel for efflux of Ag^+. The tripartite complex consisting of an outer membrane protein (CusC), inner membrane protein (CusA) and a membrane fusion protein (CusB) forms an efflux channel (Kim *et al*., 2011). Whereas, a metal chaperon (CusF) is present in periplasm where it binds the metal ions and shift these to the efflux pump, for their export out of the cell (Loftin *et al*., 2005). Therefore, more number of these proteins is required for more metal to be exported.

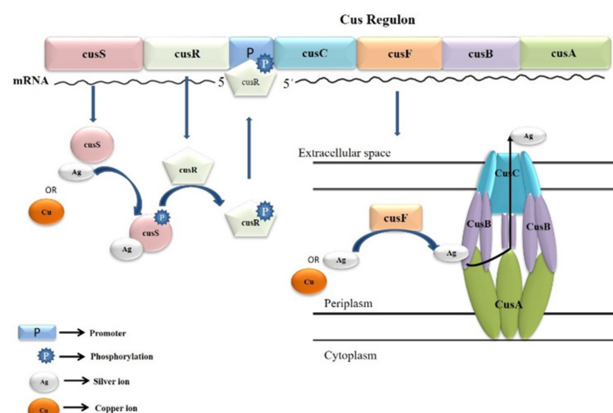

Fig. 7. Suggested functional model of Cus Regulon. The transmembrane efflux channel CusCBA consists of CusA, inner membrane protein; CusC, outer membrane protein and CusB, membrane fusion protein. CusF, a metallochaperone in periplasm, binds Ag^+ and brings it to the efflux channel. The regulatory component of cus regulon CusRS which is a two-component regulatory system consists of CusS, sensor kinase and CusR, response regulator. The Cus regulon is activated in the presence of metal ions (Ag^+, Cu^{++}, Au^+), for which CusS is auto-phosphorylated in the presence of metal ion (in this case Ag^+) and trans-phosphorylates CusR, which binds with bidirectional *cus* promoter to activate RNA polymerase and transcription factor complex (adapted from Kim *et al*., 2011).

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CONCLUSIONS AND RECOMMENDATIONS

Enterobacteriacae has been explored a lot in terms of metal resistance mechanisms. Each of both metals, silver and copper, acts as an inducer of two component system pathway. This study presents different behavior of *K. pneumoniae* KW in response to Ag^+ in contrast to Cu^{++} (Zahid *et al*., 2012; Zulfiqar and Shakoori, 2012) and Au^+ (data not published yet).

The occurrence of silver resistance mechanisms in gram negative pathogenic bacteria can prove to be important tool of manipulation for surveillance on the spread and emergence of silver resistant strains and the utility of silver compounds for medicinal purposes.

DECLARATIONS

Data availability statement

All data generated or analyzed during this study are included in this published article.

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Ethical approval and consent to participate

This study was approved by the Ethical Committee of the School of Biological Sciences. No consent was required for this study.

Declaration of competing interests

The authors have not declared any financial and personal and personal relationship with the people or organization that could inappropriately influence this research work.

Statement of conflict of interest

The authors have declared no conflict of interest.

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