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Molecular Characterization of Znt Regulon for Multi-Metal Resistance in *Klebsiella pneumoniae* KW

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ABSTRACT

Heavy metals such as zinc, lead and cadmium, at high concentrations, are toxic to microorganisms. A chromosomally encoded *znt* operon plays an important role in multi-metal resistance in some members of Enterobacteriaceae. However, its role has not yet been explored in *K. pneumoniae*. This study focused on molecular characterization of *ZntR*, a transcriptional regulator of *znt* operon. The minimum inhibitory concentration of zinc, lead and cadmium were 10, 10 and 4 mM, respectively against *K. pneumoniae* KW. The strain also exhibited metal uptake and storage ability. Having amplified and cloned *zntR* gene, expressed and purified ZntR protein showed binding with *zntA* promoter. Increased transcripts of *zntR* in the presence of metal ions revealed its metal inducible nature. The molecular dynamics simulation and protein-metal ion docking studies of the *K. pneumoniae ZntR* protein are being reported for the first time.

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Authors' Contribution
ARS and SZ designed the project. HS performed the experiments. AT-AK performed transcriptional profiling and in silico analysis. SZ helped in analysis of results and experiments. HS prepared the first draft which was later improved by SZ and ARS. ARS provide laboratory facilities and supervised the whole study.

Key words
Cadmium, Heavy metals, *K. pneumoniae*, Multi-metal Resistance, Profiling of ZntA, Zinc

INTRODUCTION

Metals play an important part in the metabolic processes of microorganisms. Some of them are essential nutrients which include calcium, cobalt, chromium, copper, iron, potassium, magnesium, manganese, sodium, nickel and zinc while others like gold, lead, cadmium and mercury are non-essential metals. Essential metals are involved in different biological reactions in addition to stabilizing protein structure and synthesizing bacterial cell wall (Gadd, 1992; Gadd and White, 1993) and thus are important for various metabolic processes. For example magnesium, involved in DNA/ RNA synthesis, is required for muscles contraction, transmission of neurological signals, insulin metabolism and cardiac excitability (Gröber *et al*., 2015), calcium is needed for muscle contraction, neurological signal transmission, blood clotting and formation and strengthening of bones and teeth (Piste *et al*., 2012) and potassium is important for the functioning of cells, tissues and organs especially heart (Clausen and Poulsen, 2013). However, both essential and non-essential metals, in high concentrations, pose toxic effects by displacing other essential metal ions from their native binding sites or interacting with various ligands (Gadd, 1992; Gadd and White, 1993). Excess of these metals usually ends up with metabolic disorders. Zinc, in higher concentrations, may have some adverse effects such as muscular pain and stiffness, loss of appetite, nausea and irritability. Cadmium may lead to renal failure, disturb the endocrine function and cause the prostate cancer (Plum *et al*., 2010). Excess of toxic metal ions interact with cellular proteins and cell components and result in reactive oxygen species production (Noroozi *et al*., 2017; Andreini *et al*., 2008).

Zinc homeostasis involves tissue specific expression and localization of 2 zinc transporter (ZnT) families: The 10 members of the ZnT/SLC30 family move zinc from the cytosol into the secretary pathway or out of the cell. Cellular zinc homeostasis is achieved by multiple processes, including zinc influx into the cells, zinc efflux out of the cells and compartmentalization and storage within the cells (Kambe *et al*., 2004, 2021). These processes are accomplished by a diverse family of proteins including zinc transporters and zinc binding proteins. Zinc transporters distribution on the plasma membrane and organelle mem-

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brane. Two distinct but interacting classes of zinc transport proteins that contain 24 members have been identified so far, including the zinc importers (SLC39/ZIPs) (Kambe *et al*., 2004) and zinc exporters (SLC30/ZnTs) (Kambe *et al*., 2004). The ZIP family has 14 members (ZIP1-ZIP14) and moves zinc from extracellular spaces or intracellular organelles into the cytosol. The ZnT family contains 10 members (ZnT1-ZnT10) and is responsible for exporting zinc from cytosol to extracellular space or intracellular organelle (Jeong and Eide, 2013). Generally cytosolic zinc levels are regulated positively by ZIPs and negatively by ZnTs, whereas organelle zinc levels are regulated positively by ZnTs and negatively by ZIPs. There is virtually no free zinc in the cells (Bafaro *et al*., 2017). Metallothionein and glutathione function as reservoirs of intracellular zinc.

Bacteria are considered to be an important tool for the detoxification/removal of heavy metals from the environment because they have small size and are easy to cultivate. Several bioremediation methods have been established based on bacteria like *Micrococcus* and *Bacillus* spp. Bacteria through chromosomal rearrangements, transposon and plasmid-mediated resistance systems may have adapted metal resistance. The efficient bioremediation strategies are based upon the resistance mechanisms of microorganisms. These mechanisms include extracellular sequestration, intracellular sequestration, active transport of heavy metal ions and enzymatic detoxification. Understanding of resistance mechanism in metal tolerant bacteria helps to devise the bioremediation strategy.

Klebsiella pneumoniae is a pathogenic bacterium. An important characteristic of this specie is its bioremediation ability for metals like mercury, cadmium and some important organic compounds nevertheless the detailed mechanism of resistance against these metals has not been explored in this bacterium in detail. In this study, an already reported copper resistant *K. pneumoniae* strain exhibited multi-metal resistance and metal uptake abilities revealing its bioremediation potential. Findings of this study including upregulated expression of ZntR (a transcriptional regulator) in the presence of zinc, lead and cadmium and its binding with the promoter of Znt A (a type of ATPase involved in metal translocation) will help in understanding the activation of resistance mechanism in *K. pneumoniae*. Bioinformatic analyses showing the binding of metal ions (Zn^{+2}, Pb^{+2} and Cd^{+2}) with homology based predicted 3D structure of Cue R further support the results.

MATERIALS AND METHODS

Bacterial isolate

In this study, a copper resistant bacterial strain of *K. pneumoniae* KW (AB641121) was obtained from School of Biological Sciences, University of the Punjab. This strain was isolated from toxic metals laden waste water sample, collected from Kot Lakhpat Industrial Estate, Lahore (Zulfiqar and Shakoori, 2012).

Metal used in the study

Stock solutions of metals, used in this study, included 1 M zinc chloride (13.63 g of $ZnCl_2$ in 100 ml of de-ionized water), 1 M cadmium chloride (22.84 g of $CdCl_2.2.5H_2O$ in 100 ml of de-ionized water) and 1 M lead acetate (32.52 g of $Pb(C_2H_3O_2)_2$ in 100 ml of de-ionized water).

Determination of resistance level and growth curves

Resistance of *K. pneumoniae* KW strain was checked against three metals i.e. zinc, cadmium and lead. Minimum inhibitory concentration (MIC) against KW was determined by allowing it to grow at 37°C on LB agar plates supplemented with different concentrations of each metal under study. The lowest concentration which showed no growth after 48 h was considered as MIC.

To study the effect of metals on growth of *K. pneumoniae*, 1 ml of overnight bacterial culture was inoculated in ten flasks each containing 100 ml LB broth, followed by incubation at 37°C and 150 rpm. In 2h old culture (early log phase), three of these flasks were supplemented with 0.5, 1 and 2.5 mM Cd^{+2}, three with 3, 5 and 8 mM Zn^{+2}, and three with 3, 5 and 8 mM Pb^{+2}. In one flask (control culture), no metal ions were added. Growth was determined at 1h regular intervals up to 9 h. Where required, an aliquot was spread on LB plate.

Determination of metal uptake ability

K. pneumoniae KW culture was grown at 37°C at 120 rpm. In 2 h old culture, metal stress was given that included 3mM Zn^{+2}, 3mM Pb^{+2} and 0.5mM Cd^{+2}. At regular intervals of time, an aliquot from each culture was centrifuged and cell pellet was digested with 50 µl Conc. HNO_3 and volume was made 1ml by the addition of de-ionized water. Metal content in each cell pellet was measured through atomic absorption spectrometry (AAS) (Thermo Unicam-SOLAAR) using air-acetylene flame. The ability to uptake the respective metal by the strain was calculated as µg metal/mg cell dry weight. For determination of cell dry weight of each cell pellet, relation of OD to cell dry weight of the strain under study was used as reported by Imran *et al*. (2021).

DNA isolation and amplifications of zntR *gene and* pzntA *promoter*

Genomic DNA of bacterial specie was extracted according to the method described by Wilson (2001) from the overnight culture with a few modifications. Primers were designed through Primer3 software (Table I) to amplify the *zntR*, and *pzntA* via polymerase chain

reaction.

Table I. Sequences of the primers used to amplify *zntR* gene and *pzntA* promoter.

	Primers for	Sequences 5`→3`
1.	amplification of *zntR* KpZntR	F GGAACGTCATATCCTCGATG
		R AACTCGGCGCGAGTGTAG
2.	amplification of promoter *pzntA*	F GGCCACTTCCTGATCGTG
		R CGTCTGGAGTCGACATGG
3.	introduction of *Nde*I site in *zntR* KpZntRe F	GGAGTACATATGTATCGTATTGGTG
4.	realtime PCR for *gyrase* subunit A (housekeeping gene) *Gyr A*	F TACGCGGTATACGACACCAT
		R CGATGGAACCAAAGTTACCC
5.	realtime PCR for *zntR*	F AGTCGAAAGGGATCGTTCAG
		R AACAGTAAACGCTGCTGTGG

The thermal cycle conditions comprised initial denaturation at 95°C for 5 min, followed by 30 cycles, each of denaturation at 95°C for 45 sec, annealing at 54°C for 45 sec and extension at 72°C for 60 sec. The final extension was done at 72°C for 10 min.

The thermal cycle conditions for amplification of *pzntA* comprised initial denaturation at 95°C for 5 min, followed by 30 cycles, each of denaturation at 95°C for 15 sec, annealing at 54°C for 20 sec, and extension at 72°C for 30 sec. The final extension was done at 72°C for 10 min. The amplified products were analyzed on 1% agarose gel. Amplicons were extracted from agarose gel by using Thermo Scientific Gene JET PCR Purification Kit (Cat# K0701) and sequenced.

zntR was cloned in cloning vector pTZ57 by using Fermentas InsT/A clone PCR product cloning kit (Cat # K1214) according to manufacturer's instructions in DH5α. Cloning of the desired fragment was confirmed through *Sac*I and *Hin*dIII double restriction analysis. *Nde*I site was introduced at 5' end of the gene through amplification by using primers KpZntRe-F (with *Nde*I site) and KpZntR-R. The gene was subcloned in pET-21a(+) (Novagen) through *NdeI* and *BamHI* sites while BL21C^{+} cells acted as host.

Expression and purification of ZntR

ZntR protein expression under IPTG induction was obtained by culturing transformants (with recombinant pET vector) in LB broth having ampicillin (100 µg/ml) at 37 °C with shaking at 120 rpm. Same procedure was opted for control using cells transformed with vector only. Conditions for maximum protein expression including inducer (IPTG) concentration and time of induction were optimized

For ZntR purification, cells expressing ZntR were lysed through sonication and expressed protein mixture was subjected to ammonium sulphate precipitation at 4°C. Fractions with ZntR protein were pooled and dialyzed to eliminate small molecular weight proteins and salts. Protein concentration of dialyzed sample was estimated by coomassie dye-binding assay (Bradford, 1976) by using rotiR-quant bradford protein assay kit (Carl-Roth Company). Protein obtained after dialysis was filtered through a membrane filter of pore size 0.45 µm and fractionated by anion exchange chromatography using HiTrap QFF-5 mL column on fast protein liquid chromatography (FPLC) system, ÄKTA purifier (GE Healthcare) (Sambrook and Russel, 2001). Purified recombinant protein along with molecular mass markers (BenchMarkTM Protein Ladder) was analyzed on SDS-PAGE.

Molecular characterization of ZntR

Activity assay for recombinant ZntR

Activity assay for ZntR protein was done according to Rowe and O'Gara (2016) by incubating the protein (15 µl) with promoter *pzntA* (5µl). Incubation was given for 30 min on ice. The mobility shift of *pzntA* in the presence of *ZntR* was checked on PAGE designed for DNA (6% Acrylamide, 1x TAE, 50 µl 10 % APS, 4 µl TEMED, dis. H2O up to 5ml).

Effect of metals on expression of ZntR

Effect of zinc, cadmium and lead on expression of *zntR* at transcriptional level was analyzed. Overnight culture (1ml) was inoculated in four flasks each containing 20 ml LB broth followed by incubation at 37°C and 150 rpm. At OD_{600} 0.4-0.6, one of these flasks was supplemented with 1mM Cd^{+2}, one with 3mM Zn^{+2}, and one with 3mM Pb^{+2}. In control culture, no metal ions were added. After 15 min of metal addition, total RNA from each culture was isolated using TRIzol (Chomczynski, and Sacchi, 1987), subjected to DNase treatment (Fermentas Cat # EN0521) and cDNA was synthesized from 4 µg total RNA using RevertAid™ M-MuLV reverse transcriptase (Fermentas Cat # EP0442).

Each cDNA was used to amplify a target sequence of *zntR* as well as *gyrA*, an internal control. The reaction (25 µl) contained 1X Maxima™ SYBR Green qPCR Master Mix (Fermentas Cat # K0221), 0.3 uM each of respective forward and reverse primers, 2.5 µl of 100x diluted cDNA. All reactions were carried out in triplicates. Thermal cycling was performed using a two-step cycling protocol. It consisted of initial denaturation at 95 ºC for 10 min, 40 cycles each of 95 ºC for 15 sec, 60 ºC for 1.0 min and fluoresce detection. To verify the specificity and identity of the PCR product, melt curve analysis was performed

between 60 ºC and 90 ºC with reading after every 0.5 sec.

N fold change in mRNA level of *zntR* in response to metal ions was calculated against control with no metal through Pfaffl method (Pfaffl, 2001).

Molecular dynamics simulation and docking of ZntR with metal ions

As the crystal structure of ZntR in *K. pneumoniae* is not reported yet, its 3D structure was predicted via homology modelling through Protein Homology/analogY Recognition Engine V 2.0 (Phyre2) (Kelley *et al*., 2015). Molecular dynamics simulation was carried out to computationally determine the stability of the predicted 3D structure of the protein under physiological conditions. The 3D structure of the protein ZntR, obtained via homology modelling, was simulated for 10 ns time frame using gromacs simulation software and tool (Abraham *et al*., 2015). To further see the overall stability of the protein molecules during the period of 10 ns MD run, radius of gyration (Rg) was analyzed along with a calculation of root mean square deviation (rmsd) along the run.

The molecular docking of ZntR with zinc, cadmium and lead ions was performed using autodock tools (ADT) (Morris *et al*., 2009). The results were based on minimum energy score having the most favorable docking conformation. Moreover, the best pose was selected and analyzed using UCSF chimera software (Pettersen *et al*., 2004).

RESULTS

Effect of metals on growth

Minimum inhibitory concentrations (MICs) of Zn^{+2}, Pb^{+2} and Cd^{+2} against *K. pneumoniae* KW strain were found to be 10 mM, 10 mM and 4 mM, respectively (Fig. 1).

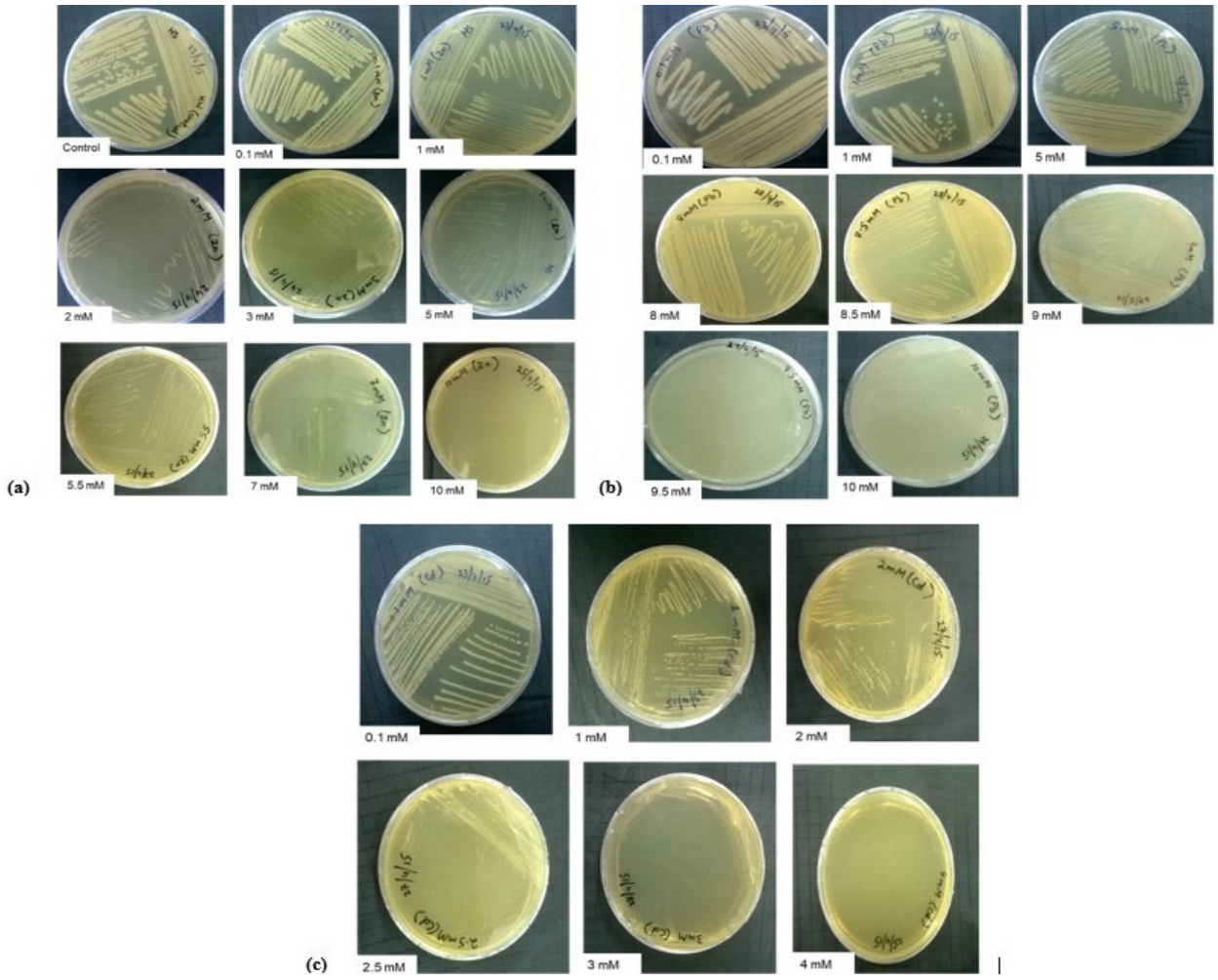

Fig. 1. Minimum inhibitory concentrations of heavy metals. Growth of KW was analyzed in the presence of various concentrations of (a) Zn^{+2} ($ZnCl_2$), (b) Pb^{+2} ($Pb(C_2H_3O_2)_2$) and (c) Cd^{+2} ($CdCl_2.2.5H_2O$).

Figure 2 shows growth curves of *K. pneumoniae* KW in the presence of different concentrations of metals under study. Increasing concentrations of metal in the medium retarded the growth of bacteria and resulted in prolongation of lag phase.

Specific growth rate/min of the strain was $2.85x10^{-1}$ in the control culture that reduced to $2.44x10^{-1}$ in the presence of 3mM Zn^{+2}, $1.74x10^{-1}$ in the presence of 3mM Pb^{+2} and $1.78x10^{-1}$ in the presence of 0.5mM Cd^{+2} representing insignificant effect. However, addition of metal in higher concentrations (5 and 8mM Zn^{+2}, 1 and 2.5mM Cd^{+2} and 5 and 8mM Pb^{+2}) in early log phase cultures resulted in halted growth till the end of experiment (9 h). An aliquot of each culture, streaked in LB agar medium, showed that cells were alive but no multiplication was taking place during this period.

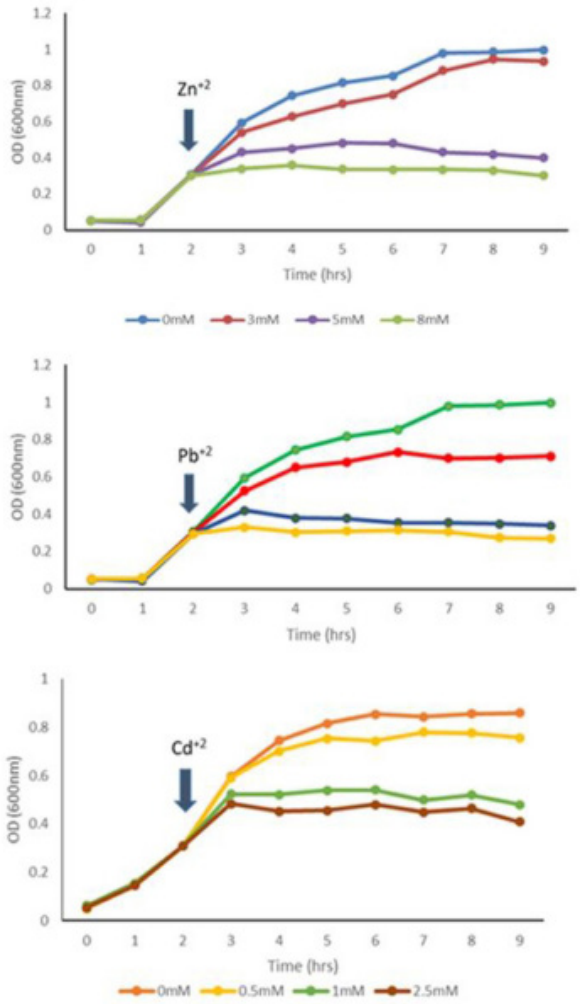

Fig. 2. Effect of Zn^{+2}, Pb^{+2} and Cd^{+2} on growth of multi-metal resistant *K. pneumoniae* KW.

Metal uptake ability

Metal uptake in terms of µg metal per mg cell dry weight was determined. Results are shown in Figure 3. With passage of time, a gradual increase in metal amount in cells was observed. However, the rate of uptake per h was not constant. In the figure, area in see green color represents higher uptake rate as compared to white area. Maximum metal accumulation in cells was found to be 32 µg Zn^{+2}, 56.86 µg Pb^{+2} and 20.26 µg Cd^{+2}/mg cell dry wt.

Amplifications of zntR *and* pzntA

Figure 4 shows genomic DNA of *K. pneumoniae* KW and PCR products, run on 1% agarose gel. Appearance of bands at 810 bp and 130 bp represented amplicons of *zntR* and promoter region of *zntA* designated in this study as *pzntA*, respectively.

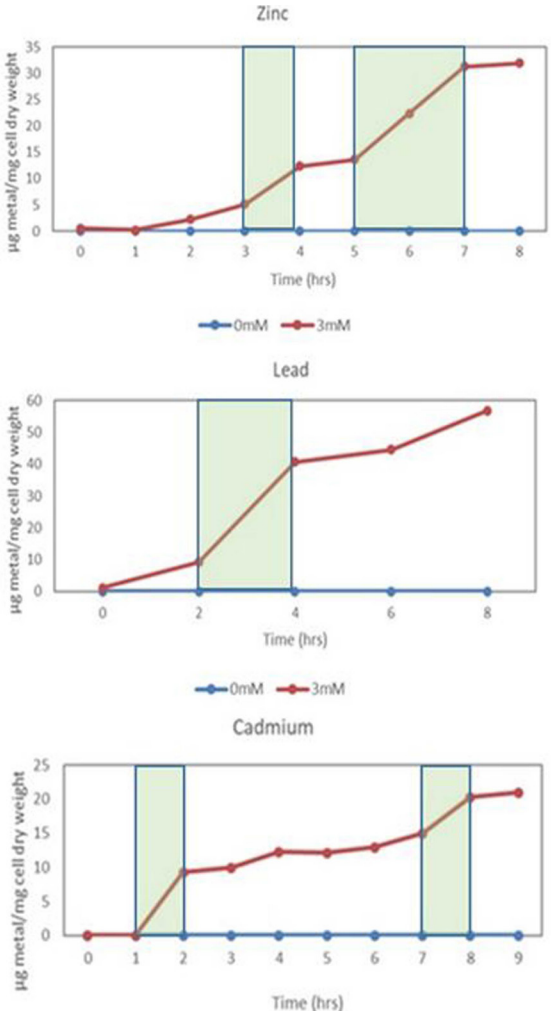

Fig. 3. Metal uptake by *K. pneumoniae* in the presence of 3mM Zn^{+2}, 3mM Pb^{+2} and 1mM Cd^{+2}. See green shaded area represents rapid uptake rate.

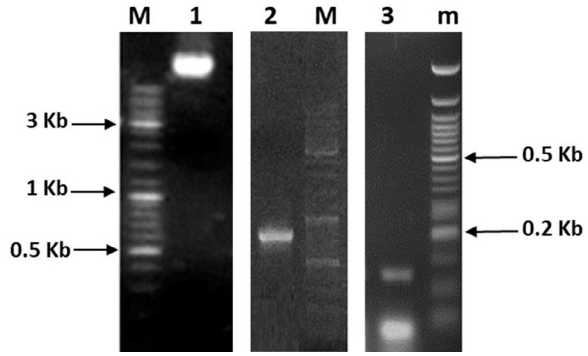

Fig. 4. 1% agarose gel showing genomic DNA isolated from *Klebsiella pneumoniae* KW (Lane 1), amplified products of ZntR (810 bp) (Lane 2) and promoter *pzntA* (Lane 3). DNA ladders used were Fermentas Cat # SM0331 (Lane M) and Fermentas Cat # SM0613 (Lane m).

Expression of ZntR

Considerably high expression of ZntR under IPTG induction was observed in cell lysate (representing all expressed proteins) (Fig. 5A). ZntR expressed partly in soluble form (supernatant fraction) and partly in insoluble form (pellet fraction). Samples from control (cells transformed with vector only) did not show any band at position comparable with that of ZntR in IPTG induced ones.

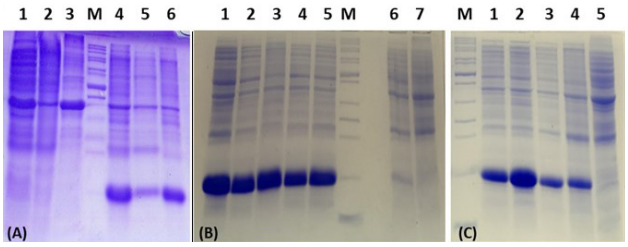

Fig. 5. The expression of recombinant protein ZntR after IPTG induction (A) Lanes 1, 2, and 3: proteins from cell lysate, supernatant and pellet from control culture, respectively; Lanes 4, 5, and 6: proteins from cell lysate, supernatant, and pellet from culture expressing ZntR, respectively. Bands at 15kDa in lanes 4-6 represent ZntR protein. (B) Optimization of IPTG concentrations for ZntR expression. Lanes 1, 2, 3, 4 and 5: IPTG induction at concentrations of 0.01, 0.05, 0.1, 0.5 and 1 mM, respectively; Lane 6: No IPTG and lane 7: pET21 vector without ZntR insert. (C) Optimization of IPTG induction time. Lanes 1, 2, 3, and 4: IPTG induction for a time of 2, 4, 6, and 8 hr, respectively; Lane 5: No IPTG. Lane M: Protein ladder (Benchmark cat.no. 10747-012).

Expression of ZntR in the presence of different concentrations of IPTG and a range of induction time revealed that maximum expression of ZntR was obtained in the presence of 0.1 mM IPTG with 4 h of induction period (Fig. 5B, C).

Figure 6 shows various steps of ZntR purification process. Ammonium sulphate precipitation was done as an initial step towards purified ZntR. Of the different salt concentrations, 40 % showed maximal protein precipitation and hence considered to be optimal for further downstream experiments (Fig. 6A). Selected fraction was dialyzed to remove unwanted proteins and salts and subjected to FPLC. Figure 6B and 6C show fractions and chromatogram elution profile, respectively. The peaks in chromatogram depicted where highest amount of recombinant ZntR protein was eluted. Total 8 fractions were collected and analyzed on SDS-PAGE. These fractions were eluted between 26-100 % of NaCl gradient. Fractions 5-8 showed maximum protein elution. Figure 6D shows partially purified ZntR protein obtained after dialysis of pooled FPLC fractions.

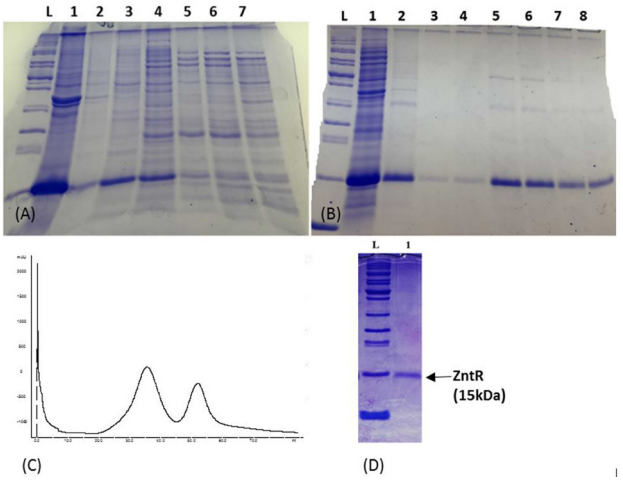

Fig. 6. Purification of ZntR (A) Fractionation by ammonium sulphate precipitation. Lanes 1, 2, 3, 4, 5, 6, and 7: Fractions obtained at 20%, 30%, 40%, 50%, 60%, 70%, and 80% saturation levels of ammonium sulphate, respectively. (B) Fractionation by anion exchange chromatography. Lane 1: Sample before FPLC; Lanes 2-8: Fractions obtained from anion exchange column HiTrap QFF. (C) Chromatogram showing elution profile of recombinant ZntR [X-axis, volume of buffer (mL), Y-axis $OD280_{nm}$ mAbs unit]. The peak represents maximum protein elution between 30-40% NaCl gradient. (D) SDS-PAGE showing protein after dialysis. Lane 1: Partially purified ZntR protein. Lane L: Protein ladder (Benchmark cat.no. 10747-012).

Activity assay for recombinant ZntR

The gel mobility shift assay was performed to check

the interaction of recombinant ZntR protein with *pzntA*. In the gel, in lane 1 where purified *pzntA* alone was loaded, band appeared at 130 bp, in accordance with the size of amplicon whereas in lane 2 where a mixture of *pzntA* and ZntR after an incubation was loaded, band of *pzntA* appeared slightly at upper position (Fig. 7). Retarded movement of the promoter in the gel was due to greater density because of its binding with ZntR protein.

Fig. 7. Activity assay for recombinant ZntR. Lane 1: promoter *pzntA* alone, Lane 2: promoter *pzntA* and ZntR appeared at comparatively higher molecular weight position.

Effect of metals on expression of ZntR

Figure 8 shows the effect of Zn^{+2}, Cd^{+2} and Pb^{+2} transcripts level of *zntR* on revealing its multi-metal inducible nature. Highest expression was found in the presence of lead (3.98 folds) followed by zinc (2.71 folds) and cadmium (1.64 folds).

Molecular dynamics simulation studies of ZntR

Molecular dynamics simulation studies performed to compute the stabilities of the 3D predicted structure of ZntR showed that the protein ehhibited a dynamic behavior under simulation conditions, as can be seen from its RMSD fluctuations (Fig. 9A). Also, the radii of gyration of ZntR showed fluctuating behavior again indicating its dynamic behavior during 10 ns simulation period (Fig. 9B). Moreover, the radii of gyration of ZntR along y- and z- axes showed fluctuations till 2.5 ns after which it behaved rather stably overall. However, the radius of gyration along z-axis showed instability throughout the simulation period.

Docking of ZntR with metal ions

The structure of the protein is represented in rainbow

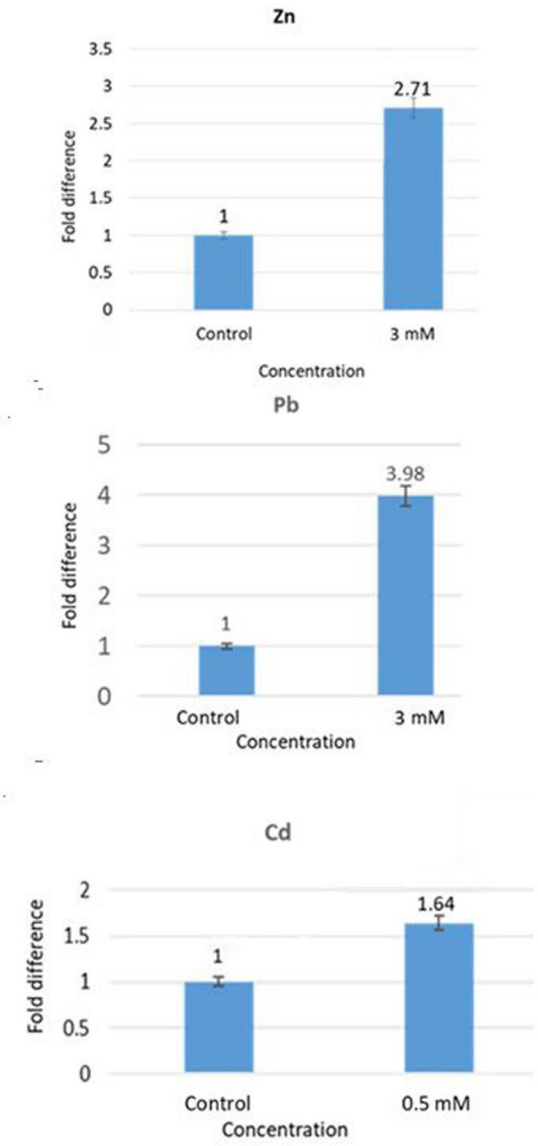

Fig. 8. Effect of Zn, Pb and Cd on the transcription of *zntR*. Upregulated expression of *zntR* indicates its induction by the metals.

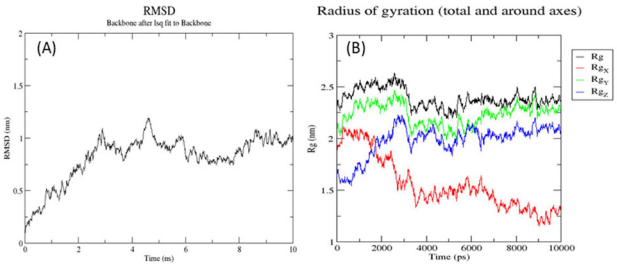

Fig. 9. Root man square deviation (RMSD) (A) and radii of gyration (B) of 3D predicted structure of ZntR protein.

colors with N terminus in blue and C-terminus in red (Fig. 10). The docking results showed that binding sites for cadmium and lead were at a pocket near the N terminus of the protein while zinc binding site was near the C-terminus of the receptor protein molecule.

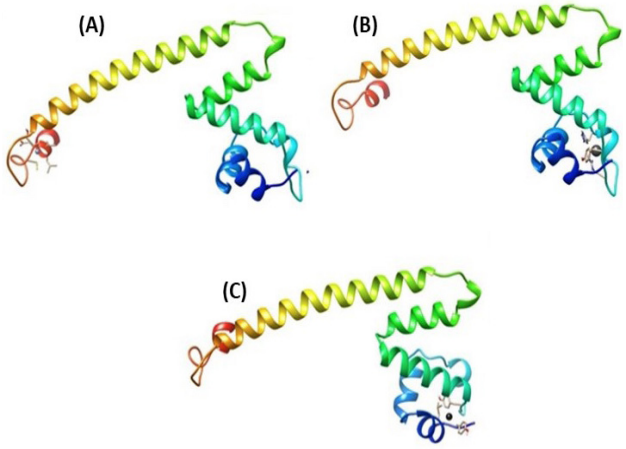

Fig. 10. Binding of metal ions with ZntR. Full protein view. (A) Zinc; (B) Lead; and (C) Cadmium.

The interacting residues of ZntR with Zn^{+2}, Pb^{+2} and Cd^{+2} are depicted in A1, B1 and C1, respectively, of Figure 11. Cadmium has shown interaction with TYR-40, LEU-7 and TYR-2; lead with HIS-29, ARG-38 and TYR-40 mainly; and the amino acids interacting with zinc ion were CYS-124, SER-125, ILE-126, LEU-127, GLU-128 and ALA-129.

The hydrophobicity analysis of the binding pockets of the protein with respect to the three metal ions revealed that cadmium binding site was in relatively hydrophilic pockets, while the binding pockets of lead and zinc appeared to be relatively hydrophilic and neutral, respectively. Moreover, an analysis of the hydrophobicity being provided by the interacting amino acid residues only, revealed similar patterns (Fig. 11B1, B2, B3).

When distances between the closest approaching atoms of the interacting amino acids of ZntR and the metal ions were calculated, the cadmium ion showed a distance of 3.0 Å. 2.8 Å, and 3.1 Å with the C3 atoms of TYR-2, TYR-40 and LEU-7, respectively. Besides, the distances between cadmium and O2 of TYR-2 was 2.5 Å and with the H of LEU-7 was 3.2 Å. The distances of lead ion with the C3 of ARG-32 and HIS-29 were 3.6 Å and 3.2 Å, respectively, while that with H of ARG-38 was 3.1 Å. Similarly, an analysis of zinc ion revealed it to be at a distance of 2.8 Å and 3.6 Å from C3 atoms of CYS-124 and GLU-126, respectively (Fig. 12).

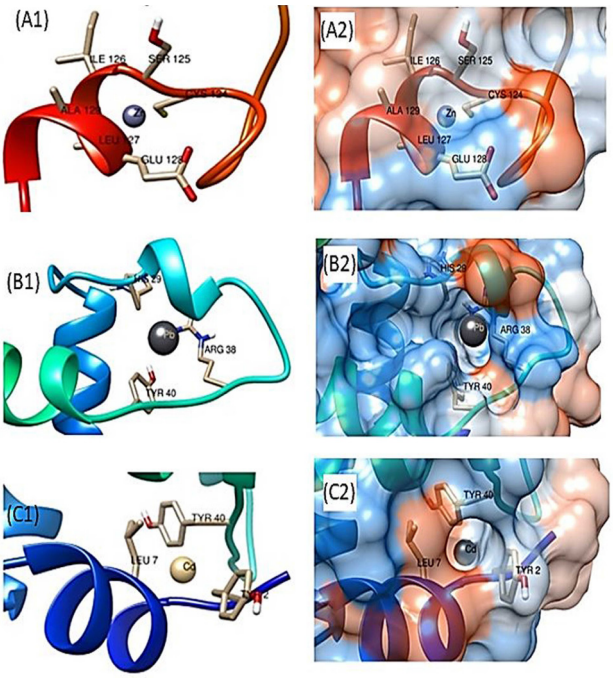

Fig. 11. Zoom in metal binding pockets and hydrophobicity view of ZntR for zinc (A1and 2), lead (B1and 2) and cadmium ions (C1and 2), respectively.

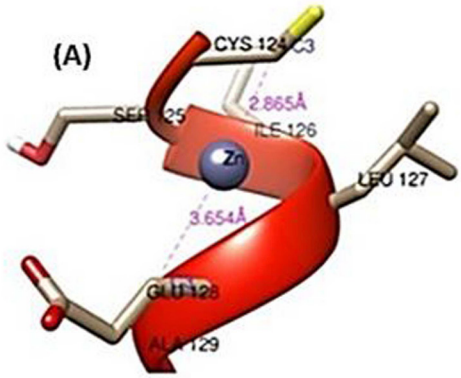

Fig. 12. Zoomed in view of interacting residues only. Distances are shown in magenta while names of interacting atoms are in medium blue. (A) Zinc; (B) Lead; and (C) Cadmium.

DISCUSSION

The main aim of the study was to explore the multi-metal resistance potential of a copper resistant *Klebsiella* sp. and to determine the molecular basis of resistance. The strain exhibited resistance against the three metals among

which high tolerance level was for zinc and lead (MIC = 10 for both the metals) while cadmium was proved to be more toxic for cells (MIC = 4).

This strain was isolated from the industrial effluents of Kot Lakhpat area where an industrial zone comprising > 450 industrial units is present. These effluents carry toxic metal compounds in significant proportion (Azeem, 2009). The occurrence of metal resistant bacteria in such sites has been supported by various studies focusing on bacterial isolations from different water sources contaminated with heavy metal ions of various types. Consequently, the acquired resistance in *K. pneumoniae* KW is most commonly attributed to the heavy metal ions' load in effluents from which the isolation was performed. In a case study, resistant strains of *E. coli* were found in metal contaminated water of Yamuna, a river near Delhi, India, and a positive correlation was found between minimum inhibitory concentrations of the strains and heavy metal content of the river water (Bhardwaj *et al*., 2018). Some other studies including susceptibility studies of biofilms towards metal toxicity in the environment (Harrison *et al*., 2004); studies focusing on the resistance gained by microbiota of aquatic animal species (Hacioglu and Tosunoglu, 2014); identification of *E. coli* metal resistant strains isolated from wastewater sites in Assiut city, Egypt (Abskharon *et al*., 2008); and occurrence of the resistant strains of *E. coli* in hospital waste waters in India (Alam and Imran, 2014) also supported the development of resistance in microbes present in contaminated environments.

While studying the metal uptake ability of *K. pneumoniae* KW, although metal uptake was observed for the three metals i.e., zinc, lead and cadmium, differences with respect to uptake rate were there with greater accumulation per mg cell dry weight was observed for lead (56.86 µg) followed by zinc (32 µg) and cadmium (20.26 µg). Cells exhibited 2.8 and 1.58 times more uptake of lead and zinc, respectively as compared to cadmium. Similar to this work, cadmium resistance and accumulation has also been reported in *K. pneumoniae* CBL-1 isolated from industrial wastewater (Rehman, 2012) and *K. michiganensis* MCC3089 isolated from a rice field in vicinity of a steel plant (Mitra *et al*., 2018). *Cupriavidus necator* and *Pseudomonas aeruginosa* exhibiting enhanced zinc and cadmium uptake and bioremediation abilities have also been reported (Ferreira *et al*., 2018; Chellaiah, 2018).

To study the binding of ZntR protein with the promoter region of *zntA* gene, gel shift assay was done. For that matter, the prerequisites included cloning and expression of ZntR protein and amplification of *zntA* promoter region and their purification. The gel shift assay clearly indicated a physical interaction between the two molecules as after incubation with the protein, the mobility of promoter DNA in gel became slower as compared to that with no protein. Previous studies have also validated the interaction of ZntR protein with the promoter of *zntA*, albeit in a different species *S. aureus* and *E. coli* and found this regulatory protein to have dual regulatory role, that is, both its own regulation and *zntA* regulation (Singh *et al*., 1999; Outten *et al*., 1999). Besides ZntR, there is another regulator of zinc homeostasis known as zinc uptake regulator (Zur) also shown to be functioning via regulation of its relevant genes including *znuA*, *znuCB* and *ykgM-RpmJ2* in *Yersinia pestis* (Li *et al*., 2009). Similarly, there is a protein Zap1 in yeast which is also a DNA binding protein responsive to zinc ions (Frey *et al*., 2011).

Comparative quantification of *zntR* mRNA levels through realtime PCR revealed that the gene expression was upregulated in the presence of the three metals revealing its multi-metal responsive nature. The highest upregulation of the said gene's mRNA was observed under the influence of lead, followed by second highest by zinc and then lastly by cadmium. Up to best of our knowledge, this is the first study that reports the transcriptional regulation of ZntR under the respective heavy metal ions stress. The correlation of intracellular zinc, lead and cadmium content of microbial cells with the increased levels of ZntR mRNA further corroborates the results.

The molecular dynamics simulation and protein-metal ion docking studies of the *K. pneumoniae* KW ZntR protein are being reported, up to best of our knowledge, for the first time. An important point to emphasize is the dynamic behavior of the ZntR being exhibited under simulation conditions. This is quite in line with this protein's inherent nature of folding and unfolding which is required for it to bind with its respective promoter regions of the genes, it has regulatory effects on (Gomes and Stafshede, 2010; Brocklehurst *et al*., 1999).

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Statement of conflict of interest

The authors have declared no conflict of interest.

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