

# Molecular Analysis of the Two-Component CusS/CusR Regulon for Copper Resistance: Cloning, Phylogenetics, and Structural Modeling

Soumble Zulfiqar\* and Abdul Rauf Shakoori

School of Biological Sciences, University of the Punjab, Quaid-I-Azam Campus, Lahore 54590, Pakistan

## ABSTRACT

Copper resistance in bacteria is mediated by regulatory systems that sense and respond to metal stress. In this study, the CusS and CusR from a copper-resistant *Klebsiella pneumoniae* strain were explored, which form a two-component regulatory system crucial for copper detoxification. The genes were successfully amplified and cloned into a vector. Their sequences were obtained through commercial services. The corresponding protein sequences were deduced and a comprehensive analysis was conducted, including multiple sequence alignments and phylogenetic tree construction. The findings reveal that CusS and CusR are conserved across a broad range of Proteobacteria, suggesting a widespread role in metal resistance. The proteins were further characterized through structural modeling to predict their three-dimensional conformations. The analysis identified key domains involved in the regulatory mechanisms of copper resistance. The study highlights the evolutionary conservation of this two-component system and provides insights into its functional dynamics across diverse bacterial species. These results enhance our understanding of metal resistance mechanisms and could inform future research on bacterial adaptation to environmental stresses.

## Article Information

The article was presented in 42nd Pakistan Congress of Zoology (International) held on 23-25th April 2024, organized by University of Azad Jammu & Kashmir, Muzaffarabad, Pakistan.

## Authors' Contribution

SZ performed wet and dry lab work and wrote the manuscript. ARS supervised the whole work.

## Key words

*Klebsiella pneumoniae*, CusS/CusR regulon, Copper resistance, Phylogenetics, Structural modeling

## INTRODUCTION

Copper is an essential micronutrient required in several biological processes, but it becomes toxic when present in excessive amounts. Elevated copper levels, exacerbated by rapid industrialization, have led to widespread contamination of food chains, resulting in significant health issues for both humans and wildlife. Effective management of copper contamination is crucial for filtering it out of industrial waste to prevent its entry into food chains. While traditional chemical and physical methods for cleaning industrial waste can be expensive and environmentally damaging, the advent of biotechnology has introduced microbes with simple genomes and

structures as invaluable tools that offer a more sustainable and cost-effective solution. By leveraging microbes that can tolerate and detoxify high concentrations of copper, environmental contamination can be mitigated. Understanding the mechanisms bacteria use to handle toxic metals is fundamental for optimizing these biotechnological strategies and enhancing our ability to address metal pollution more effectively.

Since the early 1970s extensive research has been carried out in the field of metal resistance systems. Metal resistance systems may have developed shortly after prokaryotic life started and are present in nearly all bacterial types (Ji and Silver, 1995). Selected genes arose to deal with toxicity of metals at the same time as mechanisms for sugar and carbon sources (Bhagirath *et al.*, 2019; Ji and Silver, 1995). Resistance can be transferred among organisms through conjugation or transduction (Harnett and Gyles, 1984). Investigations of copper-resistant bacteria have revealed several mechanisms. However, recent molecular analyses suggest surprising similarities between copper resistance genes from diverse bacterial genera that were described as having different resistance mechanisms. The number of evolutionary paths that have led to copper resistance in bacteria may therefore be more limited than it would seem from the number of

\* Corresponding author: [soumbles.sbs@pu.edu.pk](mailto:soumbles.sbs@pu.edu.pk), [soumbles@gmail.com](mailto:soumbles@gmail.com)  
1013-3461/2024/0065 \$ 9.00/0



Copyright 2024 by the authors. Licensee Zoological Society of Pakistan.

This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

proposed mechanisms. The finding of chromosomal genes with similarity to plasmid-borne copper resistance genes in pseudomonads and the identification of chromosomal genes which function in copper uptake and management in *Escherichia coli* have provided insight into copper resistance genetic determinants evolution in several bacterial species (Cooksey, 1993).

The emerging similarities between copper resistance systems from different bacterial taxa and from distinct geographical locations could be explained by plasmid transfer between bacteria, probably assisted by long-distance transport of resistant bacterial strains by human activities. However, considering that chromosomal copper transport and management systems could be common in different bacterial taxa, then independent evolutionary modifications of related ancestral transport genes could also have led to related copper resistance gene systems in different taxa. If parallel evolution of resistance from related but somewhat diverged ancestral genes occurred, then it would not be surprising to find some differences in specific functional and regulatory mechanisms, as is observed for the related copper resistance systems in *Pseudomonas syringae* from the United States and in *E. coli* from Australia. The characterization of chromosomal genes involved in copper transport and management may help to clarify their evolutionary relationship to the plasmid-borne resistance systems (Cooksey, 1993). Suggested copper resistance mechanisms in bacteria include reduced uptake by permeability barrier, active efflux of the metal, intracellular compartmentalization/sequestration, extracellular sequestration, enzymatic detoxification of a metal to a less toxic form, and reduction in metal sensitivity of cellular targets (Bhagirath *et al.*, 2019; Bruins *et al.*, 2000).

In contrast to Gram-positive bacteria, a Gram-negative bacterium not only needs to safeguard the cytoplasm but also the vital periplasmic compartments from metal-induced damage. A family of related transport systems found in Gram-negative bacteria is that of the proton-driven CBA-transport systems. These are involved in export of metal ions, xenobiotics, and drugs (Tseng *et al.*, 1999; Poole, 2001) and work under both anaerobic and aerobic conditions (Munson *et al.*, 2000).

The copper translocating Cus system is encoded on the chromosome as a determinant comprising of two operons transcribed in opposite directions (Li *et al.*, 2023). One operon encodes the regulators CusRS that form a two-component regulatory system. CusS is a histidine kinase located at the cytoplasmic membrane probably sensing copper ions in the periplasm. CusR acts as a response regulator. The CusRS, a sensor/regulator pair, activates the adjacent but divergently transcribed genes *cusCFBA*

(Bhagirath *et al.*, 2019; Munson *et al.*, 2000; Oshima *et al.*, 2002). CusA, CusB and CusC interact to form an active channel spanning the periplasm and connecting the cytoplasm to the outer membrane. Thus, CusCBA plays a role in efflux of excess cellular copper. A unique feature of the Cus system is the presence of a small periplasmic metallochaperone protein, CusF transporting copper to the CusCBA efflux complex and thus facilitating copper detoxification of the periplasm.

The present study aimed to explore the structural features of the CusRS two-component system in a copper-resistant bacterial isolate, utilizing computational and bioinformatics techniques. Study of its distribution and evolutionary relationship in bacterial taxa is likely to provide valuable information to the field of bacterial copper resistance.

## MATERIALS AND METHODS

### Isolation of genomic DNA

A Cu<sup>++</sup> resistant bacterial isolate *Klebsiella pneumoniae* KW (Acc. No. AB642256) reported by Zulfiqar and Shakoori (2012) was grown in LB medium supplemented with 1mM Cu<sup>++</sup>. Genomic DNA of the strain was isolated as described by Rodriguez and Tait (1983) and subjected to RNase treatment. The quantity and quality of DNA was observed by taking spectrum between 200<sub>nm</sub> and 280<sub>nm</sub> in spectrophotometer (Bio Spec-1604 Shimadzu) as well as running on 0.8% agarose gel.

### Amplification and cloning of *cusRS*

The regulatory operon comprising of *cusR* and *cusS* were amplified from the isolated genomic DNA. The primers used in amplification reaction included forward primer *cus RS-F1* and reverse primer *cus RS-R1* (Table I). These primers were designed from the sequence of *Klebsiella pneumoniae* subsp. *pneumoniae* MGH (ACC # CP000647) (McClelland *et al.*, 2006) using online available Primer3 version 0.4.0 program (Rozen and Skaletsky, 2000).

The PCR reaction mixture (50 µl) contained 250 µM dNTPs, 1.5 mM MgCl<sub>2</sub>, 1x Taq Polymerase buffer, 2 µM each primer, 5 units Taq polymerase and 100-200 ng genomic DNA. The PCR cycle consisted of initial denaturation of 5 min at 94 °C followed by 30 cycles each of denaturation at 94 °C for 1 min, annealing at 58 °C for 90 sec and extension at 72 °C for 2 min with final extension at 72 °C for 10 min.

The PCR product was resolved on 0.8 % agarose gel using DNA ladder mix (Fermentas Cat # SM0331) as reference. The band, observed at right position, was excised from the gel through Qiagen gel extraction kit (Cat

# 28704) and ligated in pTZ57R/T vector using Fermentas InsT/A clone PCR Product Cloning Kit (Cat # K1214) according to manufacturers' instructions. *E. coli* DH5 $\alpha$  cells made competent by ice cold CaCl<sub>2</sub> method were transformed with the recombinant plasmid pTZ57/cusRS (Sambrook and Russell, 2001). Positive transformants, selected on the basis of blue white assortment, were grown in the presence of ampicillin and the recombinant plasmid was purified through method reported by Birnboim and Doly (1979).

**Table I. Primers used for amplification and sequencing of *cusR* and *cusS* of *Klebsiella pneumoniae* KW.**

| Primer ID | Primer sequence (5' ? 3') | Position            |
|-----------|---------------------------|---------------------|
| cus RS-F1 | GGCGTGACGGGAAAATGACAAAAC  | 5193500 – 5193524 * |
| cus RS-R1 | TTATCTCCGGCCTGGTGCAAACTTC | 5195815 – 5195791 * |
| RS-F2     | GTAAAACCATTCGCCTTTGC      | 408-427             |
| RS-F3     | CATGATCCATTCGGTGAAGG      | 876-895             |
| RS-F4     | CATCGTGCTGTTGTGGTCT       | 1374-1393           |
| RS-R2     | CACTTCATCGGCCAGATCC       | 1800-1782           |
| RS-R3     | ATGGATAAGGCCATCAGCAG      | 1292-1273           |
| RS-R4     | GGTGGCGAGGCTAATAAAGA      | 846-827             |

\* The positions of primers are according to *K. pneumoniae* subsp. *pneumoniae* MGH (ACC # CP000647). For rest of the primers, the position indicates number of nucleotides away from 5' end of the amplicon

The presence of insert was confirmed through restriction analysis. The recombinant plasmid was subjected to single digestion with either of *Pst*I and *Xba*I and double digestion with both of these endonucleases in the presence of 1x Y tango buffer. The digested products along with DNA marker were resolved on 0.8 % agarose gel.

For sequencing, the recombinant plasmid was isolated and purified through QIAprep® Spin Miniprep Kit (Cat # 12125). Sequencing was first performed using M13 F and M13 R primers. To get sequence of internal region, primers were designed from already sequenced regions (Table I). Sequencing was carried out on ABI PRISM 310 Automated DNA sequencer (Applied Biosystems) through a core facility of sequencing available in School of Biological Sciences, University of the Punjab.

#### Sequence alignment of *CusS* and *CusR* proteins and analysis

The nucleotide sequences of the two regulatory genes *cusR* and *cusS* were used to deduce respective protein

sequences. These sequences were subjected to protein BLAST analysis available online on NCBI site (<http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) to calculate the identity of the *cus* determinants. The predicted protein sequences were also analysed for conserved domains through NCBI's Conserved Domain Database (CDD) (Marchler-Bauer *et al.*, 2009).

The multiple alignments of the deduced protein sequences were performed through online clustal W program available on EMBL-EBI site (<http://www.ebi.ac.uk/Tools/clustalw2/>) along with homologous proteins of some other strains of *Klebsiella pneumoniae*, *Klebsiella variicola*, *Klebsiella oxytoca*, other members of family Enterobacteriaceae (*Escherichia coli*, *Citrobacter koseri*, *Cronobacter sakazakii*, *Edwardsiella ictaluri*, *Salmonella enterica*, *Serratia marcescens* and *Shigella sonnei*) and organisms representing at inter-order (*Shewanella putrefaciens*, *Pseudomonas aeruginosa* and *Acinetobacter junii*) and at inter-class level (*Caulobacter segnis*, *Ralstonia pickettii*, *Herminiimonas arsenicoxydans* and *Thiobacillus denitrificans*).

Phylogenetic relationship among these sequences was inferred through neighbor-joining method (Saitou and Nei, 1987) using MEGA 4 software (Tamura *et al.*, 2007). The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004). Tree topology was evaluated by the bootstrap re-sampling method of Felsenstein (1985) based on 1000 replications.

#### Determination of membrane topology of regulatory proteins

Membrane topology of *CusR* and *CusS* proteins was predicted through hydropathy profile of each protein through online available software SOSUI (Mitaku *et al.*, 2002).

#### Determination of three-dimensional structure of regulatory proteins

Homology modeling for determining the tertiary structure of protein was carried out by using SWISS model server (Arnold *et al.*, 2006). Template structure was identified on the basis of maximum identity. This template structure along with the deduced protein sequence was submitted for automated modeling. The model obtained was viewed by deep view- Swiss-PdbViewer version 4.0 (Guex and Peitsch, 1997). Literature was surveyed for determining if any of the proteins, under study, functionally exists as oligo/multimeric form. Tertiary structures of these oligo/multimers were modeled using Symmdoc server (Schneidman-Duhovny *et al.*, 2005).

## RESULTS

### Characteristics of *cusRS*

Figure 1 shows an intact band of the isolated genomic DNA appeared on gel indicating its integrity (Fig. 1a).  $OD_{260/280}$  was found to be 1.89 representing high quality of the isolated DNA.

A single band of *cusRS* amplicon (2.316 kb) was observed in the agarose gel with no nonspecific amplification (Fig. 1b). In no-template control reaction (NTC), no band was observed. The recombinant plasmid purified appeared in the form of three bands representing nicked, coiled and supercoiled forms of the molecule (Fig. 1c). Presence of a single ~ 5.1 Kb band in result of single restriction and two bands approximately at 2.8 and 2.4 Kb in double digestion confirmed the successful cloning of *cusRS* in the cloning vector (Fig. 1d).

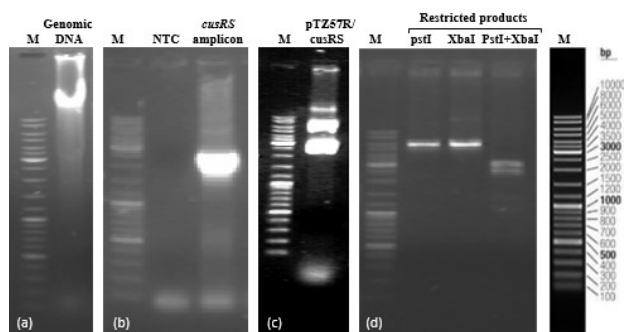


Fig. 1. Cloning of *cusRS*. (a) Genomic DNA of *K. pneumoniae* KW (b) *cusRS* amplicon (2.316 kb) (c) recombinant plasmid pTZ57R/*cusRS* (d) Restriction products of single and double digestion of the recombinant plasmid. Lanes M represent DNA marker (Fermentas cat # SM0331).

The DNA sequences of *cusS* and *cusR* obtained were submitted to DNA Data Bank of Japan (DDBJ) with the accession numbers AB640883 and AB641120, respectively. NCBI BLAST analysis revealed an 11 bp overlapping region (5' ATGCCCGCTAA) in the coding sequence of the two genes where ATG acts as start codon for *cusS* and TAA serves as stop codon for *cusR*.

In *K. pneumoniae* KW, the predicted CusS and CusR proteins were 477 and 227 amino acids in length, respectively. Blast analysis revealed that CusS and CusR proteins are widely and exclusively present in proteobacteria. Multiple alignment of each deduced protein sequence along with homologous protein sequences in other bacterial systems revealed its scope of similarity.

### Phylogenetic analysis of regulatory proteins

Phylogenetic analysis of each deduced protein

sequence revealed its relationship at various levels, ranging from intra species up to inter class level.

### *CusS*

Phylogenetic relationship of CusS was revealed through construction of neighbor joining tree (Fig. 2). CusS protein sequence of *K. pneumoniae* KW showed 96–98 % similarity with homologous proteins in the same genus. Among other members of family enterobacteriaceae, *Citrobacter* was the most homologous (69 %) followed by *Escherichia* (65 %) and *Shigella* (64 %). *Salmonella*, *Cronobacter* and *Serratia* were next, all showing 57 % homology. *Edwardsiella* was observed to be the least homologous (53 %) within the family Enterobacteriaceae. CusS in *Shewanella* was found slightly more homologous (56%) than *Edwardsiella*. Pseudomonadales (*Pseudomonas* and *Acinetobacter*) of same gamma class and members of beta proteobacteria (*Ralstonia*, *Herminiimonas* and *Thiobacillus*) possessed similarity between 32–40 %. No remarkable homologue of CusS was found in *Caulobacter*, a member of alpha proteobacteria.

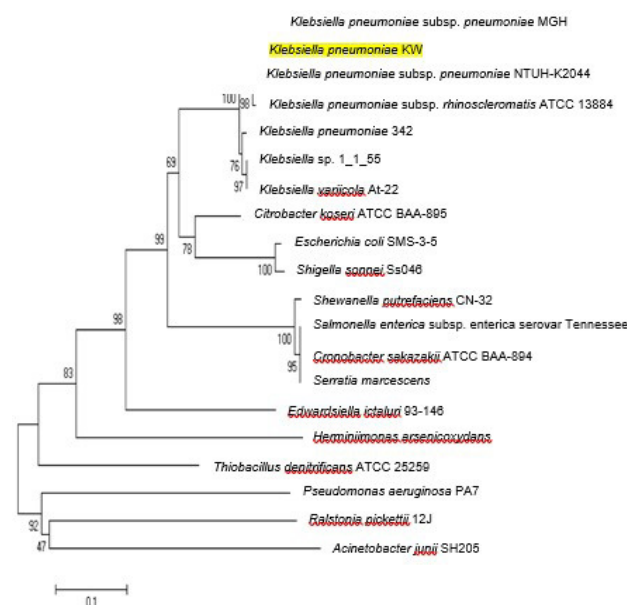


Fig. 2. Neighbor joining trees showing relationship of CusS protein of *K. pneumoniae* KW among various bacterial strains. Bootstrap values expressed as a percentage of 1000 replications are given at branching points. In each tree, bar represents substitutions per residue position.

### *CusR*

Phylogenetic analysis of CusR of *K. pneumoniae* KW revealed that this protein was more homologous upto wider level as compared to other regulatory protein, CusS. Figure 3 depicts the phylogenetic status of this protein

among bacterial strains under consideration. CusR was found 98–99 % homologous in all *Klebsiella* spp., 93 % in each of *Escherichia* and *Shigella*, 92 % in *Citrobacter*, 87 % in each of *Salmonella*, *Cronobacter* and *Serratia* and 84 % in *Edwardsiella*. Thus, the same trend was observed as for CusS. *Shewanella* harboured 85 % homologue of CusR of *K. pneumoniae* KW. Members of beta proteobacteria (*Ralstonia*, *Herminiimonas* and *Thiobacillus*) were found more closely related (65–69 %) as compared to pseudomonadales (*Pseudomonas* and *Acinetobacter*) of the alpha proteobacteria (62 %). Gamma proteobacteria (*Caulobacter*) were observed as the least homologous (44 %).

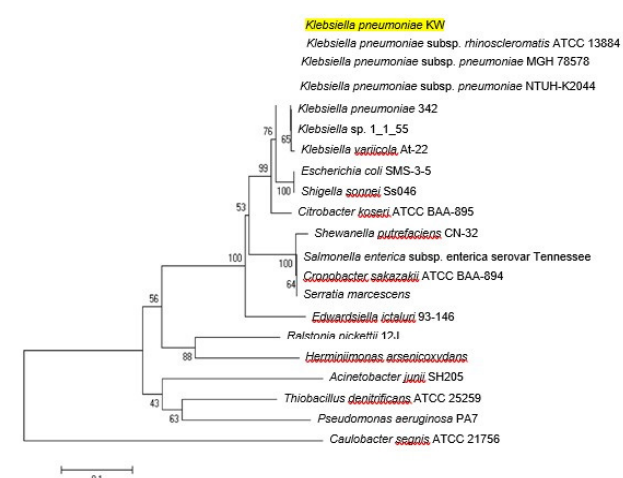


Fig. 3. Neighbor joining trees showing relationship of CusR protein of *K. pneumoniae* KW among various bacterial strains. Bootstrap values expressed as a percentage of 1000 replications are given at branching points. In each tree, bar represents substitutions per residue position.

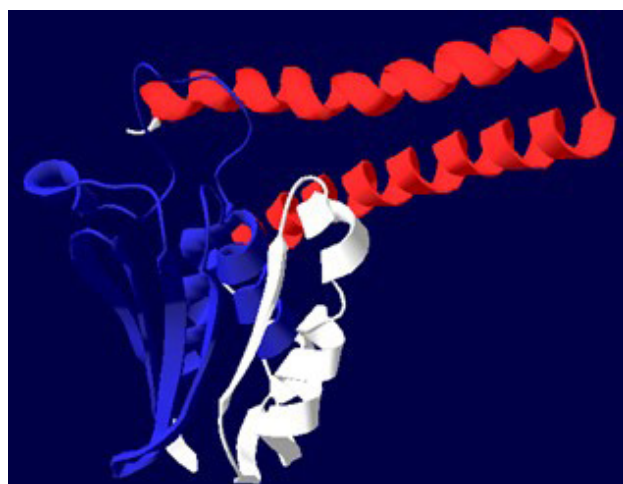


Fig. 4. Ribbon diagram of CusS. His KA domain is shown in red colour and HATPase\_C domain is blue.

### Structural analysis of CusS

Structural analysis of CusS and CusR included determination of three-dimensional structures on basis of homology modeling and topological analysis.

CusS, a sensor protein comprised 477 residues. Three-dimensional structure of CusS (Fig. 4) comprised of residues 255 to 477, as the template was available for only this part of protein. No appropriate template with HAMP domain along with N-terminal side was found in the database.

Conserved Data Domain (CDD) showed that this protein constituted three domains; HAMP, His KA and HATPase\_C domains (Fig. 5). The structural and sequence analysis of this protein revealed many features of the three domains.

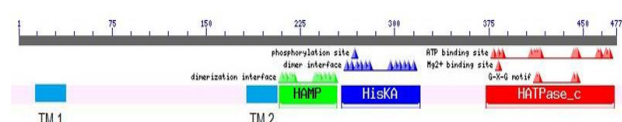


Fig. 5. Structural organization of CusS. TM 1 and TM 2 are trans-membrane regions followed by HAMP, His KA and HATPase-C domains.

HAMP domain (Histidine kinase, Adenylyl cyclase, Methyl-accepting protein, and Phosphatase) was formed of 206–254 residues. This domain contained two alpha helices connected by an extended linker. A dimerization interface was found in this domain involved in the formation of a parallel four-helix bundle. Figure 6 shows its multiple alignment with related domains found in CDD.

His KA domain (Histidine Kinase A) was composed of 256–320 residues. Dimerization and phosphoacceptor sites were observed in this domain. A histidine residue (position 269) involved in trans-autophosphorylation was found conserved in phosphoacceptor site. Phosphoacceptor site and dimerization interface are shown in the Figure 6 which harbors multiple alignment of related protein sequences available in CDD.

HATPase\_c domain (Histidine kinase-like ATPase) playing its role in ATP binding, comprised residues 373 – 475. Two specific GXG motifs were found conserved in this domain.

### Topological analysis of CusS

Topological analysis of CusS revealed that this was a membrane protein, comprising two trans-membrane regions TM1 (TRLTFFISLATVIAFFAFTWIMI) and TM2 (AELISAASIISLLIIAIVLFVVY) each of which was 23 residues in length. Figure 7 reveals that both of these two regions were present towards N-terminal side of the three domains. TM1 was present near the N-terminal end spanning residues 11–33 while TM2 (182–204) was

present just before the HAMP domain.

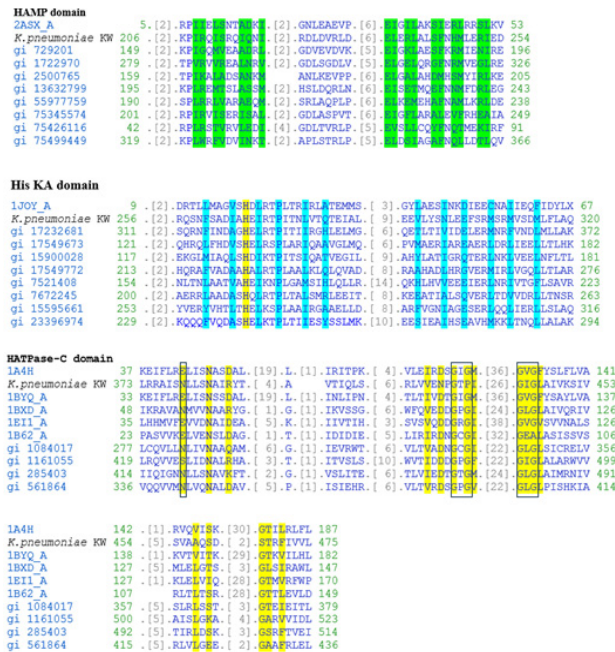


Fig. 6. Multiple alignment of the three domains in CusS of *K. pneumoniae* KW along with related domain sequences found in CDD. In HAMP domain, residues involved in dimerization interface are highlighted with green colour. In His KA domain, phosphoacceptor site and residues involved in dimerization are highlighted with yellow and blue colours, respectively. In HATPase-C domain, residues involved in ATP binding are highlighted with yellow colour while Mg binding site and GXG motives are boxed with blue and black boundaries, respectively.

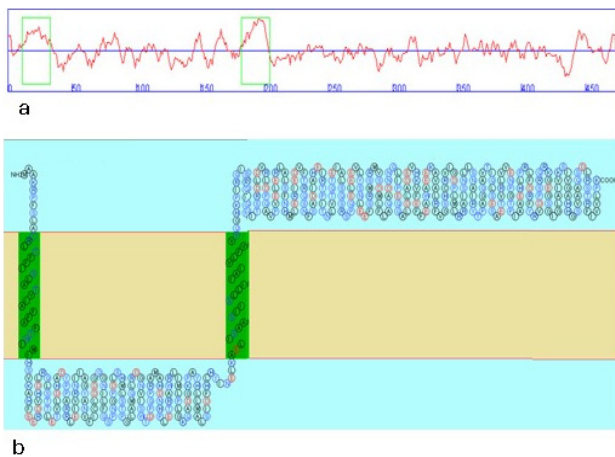


Fig. 7. Topology of CusS. Hydropathic graph (a) reveals two trans membrane regions. Topological model (b) shows amino acid residues constituting the two trans membrane regions (shown in green boxes).

### Structural analysis of CusR

CusR protein, the response regulator, consisted of 227 amino acids. Figure 8 reveals ribbon diagram of its three-dimensional structure designed by homology modeling while Figure 9 shows its structural organization.

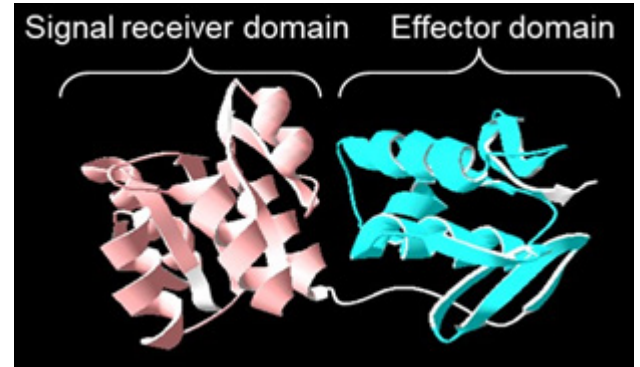


Fig. 8. Ribbon diagram of response regulator CusR. CusR is composed of two domains. Signal receiver domain is shown in pink colour and effector domain is shown in blue colour.

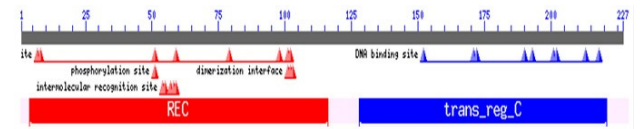


Fig. 9. Structural organization of CusR. This protein is composed of Signal receiver domain (REC) followed by effector domain (trans-reg-C).

Three-dimensional structure revealed that CusR protein possessed two domains: An N-terminal REC domain followed by trans\_reg\_C domain. REC (Signal receiver domain) belonging to REC superfamily, was 113 amino acids in length spanning amino acid 4–116. It consisted of five  $\beta$  strands and five  $\alpha$  helices. Sequence analysis of this domain through CDD (Fig. 10) revealed that it contained active site, phosphorylation site, intermolecular recognition site and dimerization interface. A conserved aspartate residue (at position 51) was found present in phosphorylation site. The intermolecular recognition site was present in loop 5, is in close proximity to the active site. The dimerization interface was formed of residues KPF in  $\alpha$  4– $\beta$  5– $\alpha$  5 interdomain interface surface.

Trans-reg-C (effector domain) was 94 amino acids in length spanning from residues 128 to 221. There were seven  $\beta$  strands and three  $\alpha$  helices. Sequence analysis through CDD (Fig. 10) revealed that the effector domain contained DNA and RNA polymerase binding sites

required for its interaction with a specific site (cusR box) present in the promoter region of *cus* determinants and the RNA polymerase leading to enhanced transcription of four structural genes *i.e.*, *cusC*, *cusF*, *cusB*, *cusA*.

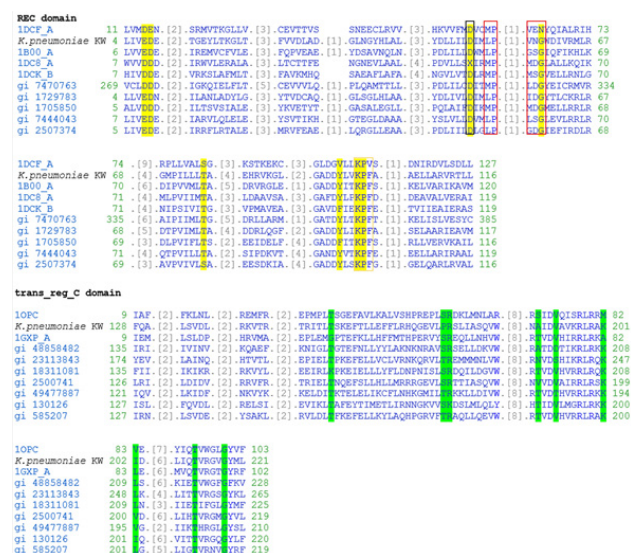


Fig. 10. Mustiple alignment trans\_reg\_C domain in CusR of *K. pneumoniae* KW along with related protein sequences found in CDD. In REC domain, residues contributing active site are highlighted with yellow colour while phosphorylation site, dimerization interface and intermolecular recognition site are boxed with black, orange and red boundaries, respectively. In trans\_reg\_C domain, residues involved in DNA binding are highlighted with green colour.

In topological analysis of CusR no membrane associated region was observed and CusR was found as a soluble protein.

## DISCUSSION

Copper is an essential transition metal. Owing to its redox property, it plays very important role in many biological processes. The same metal exerts lethal effects when present at higher concentration. Organisms have evolved some homeostatic and resistance mechanisms to cope this problem. These mechanisms include extra and intra cellular sequestration, compartmentalization, reduction of metal into less toxic form and active and passive efflux. Many bacteria have been reported that harbor one or more of these resistance mechanisms (Li *et al.*, 2014).

In this study, a bacterial strain *K. pneumoniae* KW was used. This strain exhibits high resistance to copper with minimum inhibitory concentration of 6.0 mM. It

uptakes a significant amount of  $\text{Cu}^{++}$  (~ 24  $\mu\text{g}/\text{mg}$  dry cell weight within 5 h in the presence of 3 mM  $\text{Cu}^{++}$  in the medium) and effluxes almost all the accumulated copper within 24 h (Zulfiqar and Shakoory, 2012).

The gram-negative bacteria deal with high amounts of copper mainly through export of excess copper. Passive efflux of copper is reported in *E. coli* and other bacteria through *cus* determinants (Li *et al.*, 2023). *cus* determinants include six genes grouped in form of two operons; *cusRS* and *cusCFBA*. These operons are present divergently and are regulated in opposite directions by a same promoter (Frank *et al.*, 2001, 2003). *cusRS* is regulatory in nature and is formed of two genes including *cusS* and *cusR* (Munson *et al.*, 2000). CusS and CusR constitute two components regulatory system in which CusS senses copper and CusR which is a response regulator binds DNA and enhances transcription of *cus* determinants (Fu *et al.*, 2020). *cusCFBA* constitutes four structural genes including *cusC*, *cusF*, *cusB* and *cusA* (Bhagirath *et al.*, 2019).

Considering the efflux ability of *K. pneumoniae* KW, an attempt was made to explore its *cusRS* system. For this, *cusS* and *cusR* genes were amplified from genomic DNA of the strain and cloned in a cloning vector. The nucleotide sequences were used to deduce the respective protein sequences that were later analyzed through multiple alignments with homologous proteins of protobacteria, construction of phylogenetic trees, three-dimensional modeling, and topological analysis.

Multiple alignments and phylogenetic analysis of deduced protein sequences of both *Cus* determinants through construction of neighbor joining trees revealed many features. It was found that *Cus* system exists widely in proteobacteria. In case of each *Cus* determinant, alteromonadales were found to be more homologous to enterobacteriales as compared to pseudomonadales. Surprisingly, alteromonadales were homologous even more than *Edwardsiella*, a member of family enterobacteriaceae. At interclass level, beta proteobacteria were found to be more homologous to gamma proteobacteria as compared to alpha proteobacteria in terms of *Cus* determinants homology. Moreover, it was found that CusS was highly homologous even at wider range, while homology of CusR was considerably low.

Analysis of deduced protein sequence of CusS through CDD and three-dimensional model revealed that this protein contains three domains; HAMP domain, His KA domain and HATPase\_c domain. All these domains are present towards C terminal portion of the protein. HAMP domain contains two alpha helices connected by an extended linker (Aravind and Ponting, 1999; Swain and Falke, 2007). The dimerization interface present in this domain contains polypeptide binding site through which

two HAMP domains form a parallel four-helix bundle (Dunin-Horkawicz and Lupas, 2010). His KA domain contains dimerization and phosphoacceptor sites. Histidine Kinase A homo dimers are formed through parallel association of two domains creating 4-helix bundles. Then trans-autophosphorylation occurs at a conserved Histidine residue in phosphoacceptor sites by the catalytic domain of the histidine kinase (West and Stock, 2001). This phosphoryl group is subsequently transferred to the Asp acceptor residue of a response regulator protein (Stock *et al.*, 2000). HATPase\_c domain plays its role in ATP binding. Two specific GXG motives are found conserved in this domain (Dutta and Inouye, 2000).

CDD and three-dimensional model of CusR revealed that this response regulator was composed of two domains; REC domain and trans-reg-C domain (Pao and Saier, 1995). REC domain is also known as signal receiver domain. It contains active site, phosphorylation site, intermolecular recognition site and dimerization interface. Upon receiving signal from CusS, this domain is transphosphorylated at a conserved aspartate residue present in phosphorylation site. Active site possessing an acidic pocket coordinates  $Mg^{++}$  required for event. Phosphorylation leads to a conformational change creating an exposed hydrophobic surface. This event triggers the formation of homodimers through the dimerization interface. CusR in homodimeric form binds to DNA and RNA polymerase through trans\_reg\_C domain also known as effector domain (Martinez-Hackert and Stock, 2001; Kenney, 2002). The effector domain is activated upon phosphorylation of REC domain which induces the cellular response. The effector domain contains DNA and RNA polymerase binding sites through which it interacts with a specific site (cusR box) present in the promoter region of *cus* determinants and the RNA polymerase. The binding of response regulator to RNA polymerase and DNA enhances the promoter activity in both directions leading to increased levels of transcription of four structural genes *i.e.*, *cusC*, *cusF*, *cusB* and *cusA* in one direction and two regulatory genes *i.e.*, *cusR* and *cusS* in the other direction.

Our findings emphasize the importance of the CusRS system in the broader context of bacterial copper resistance, highlighting its evolutionary significance across diverse bacterial taxa. These results may be helpful for future studies to explore the potential of targeting these systems in efforts to control toxic metals-based pollution.

## CONCLUSION

Findings of this study not only deepen the understanding of the structural and functional aspects of the CusRS system in *Klebsiella pneumoniae* but also

underscore its critical role in bacterial copper resistance. These insights contribute to the broader field of environmental microbiology and suggest new avenues for exploring targeted strategies to mitigate copper toxicity in both natural and industrial settings.

## DECLARATIONS

### Statement of conflict of interest

There is no competing interest.

## REFERENCES

- Aravind, L. and Ponting, C.P., 1999. The cytoplasmic helical linker domain of receptor histidine kinase and methyl-accepting proteins is common to many prokaryotic signaling proteins. *FEMS Microbiol. Lett.*, **176**: 111-116. <https://doi.org/10.1111/j.1574-6968.1999.tb13650.x>
- Arnold, K., Bordoli, L., Kopp, J. and Schwede, T., 2006. The Swiss-model workspace: A web-based environment for protein structure homology modelling. *Bioinformatics*, **22**: 195-201. <https://doi.org/10.1093/bioinformatics/bti770>
- Bhagirath, A.Y., Li, Y., Patidar, R., Yerex, K., Ma, X., Kumar, A. and Duan, K., 2019. Two component regulatory systems and antibiotic resistance in Gram-negative pathogens. *Int. J. mol. Sci.*, **20**: 1781. <https://doi.org/10.3390/ijms20071781>
- Birnboim, H.C. and Doly, J., 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.*, **7**: 1513-1523. <https://doi.org/10.1093/nar/7.6.1513>
- Bruins, M.R., Kapil, S. and Oehme, W.F., 2000. Microbial resistance to metals in the environment. *Ecotoxicol. environ. Saf.*, **45**: 198-207. <https://doi.org/10.1006/eesa.1999.1860>
- Cooksey, D.A., 1993. Copper uptake and resistance in bacteria. *Mol. Microbiol.*, **7**: 1-5. <https://doi.org/10.1111/j.1365-2958.1993.tb01091.x>
- Dunin-Horkawicz, S. and Lupas, A.N., 2010. Comprehensive analysis of HAMP domains: Implications for transmembrane signal transduction. *J. mol. Biol.*, **397**: 1156-1174. <https://doi.org/10.1016/j.jmb.2010.02.031>
- Dutta, R. and Inouye, M., 2000. GHKL, an emergent ATPase/kinase superfamily. *Trends Biochem. Sci.*, **25**: 24-28. [https://doi.org/10.1016/S0968-0004\(99\)01503-0](https://doi.org/10.1016/S0968-0004(99)01503-0)
- Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**: 783-791. <https://doi.org/10.1111/j.1558-5646.1985.>

- tb00420.x
- Franke, S., Grass, G. and Nies, D.H., 2001. The product of the *ybdE* gene of the *Escherichia coli* chromosome is involved in detoxification of silver ions. *Microbiology*, **147**: 965-972. <https://doi.org/10.1099/00221287-147-4-965>
- Franke, S., Grass, G., Rensing, C. and Nies, D.H., 2003. Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. *J. Bact.*, **185**: 3804-3812. <https://doi.org/10.1128/JB.185.13.3804-3812.2003>
- Fu, B., Sengupta, K., Genova, L.A., Santiago, A.G., Jung, W., Krzemiński, Ł., Chakraborty, U.K., Zhang, W. and Chen, P., 2020. Metal-induced sensor mobilization turns on affinity to activate regulator for metal detoxification in live bacteria. *Proc. natl. Acad. Sci. USA*, **117**: 13248-13255. <https://doi.org/10.1073/pnas.1919816117>
- Guex, N. and Peitsch, M.C., 1997. Swiss-Model and the Swiss-Pdb viewer: An environment for comparative protein modeling. *Electrophoresis*, **18**: 2714-2723. <https://doi.org/10.1002/elps.1150181505>
- Harnett, N.M. and Gyles, C.L., 1984. Resistance to drugs and heavy metals, colicin production, and biochemical characteristics of selected bovine and porcine *Escherichia coli* strains. *Appl. environ. Microbiol.*, **48**: 930-945. <https://doi.org/10.1128/aem.48.5.930-935.1984>
- Ji, G. and Silver, S., 1995. Bacterial resistance mechanism for heavy metals of environmental concern. *J. indust. Microbiol.*, **14**: 61-75. <https://doi.org/10.1007/BF01569887>
- Kenney, L.J., 2002. Structure/function relationships in OmpR and other winged-helix transcription factors. *Curr. Opin. Microbiol.*, **5**: 135-141. [https://doi.org/10.1016/S1369-5274\(02\)00310-7](https://doi.org/10.1016/S1369-5274(02)00310-7)
- Li, L.G., Cai, L., Zhang, X.X. and Zhang, T., 2014. Potentially novel copper resistance genes in copper-enriched activated sludge revealed by metagenomic analysis. *Appl. Microbiol. Biotechnol.*, **98**: 10255-10266. <https://doi.org/10.1007/s00253-014-5939-5>
- Li, L., Ma, J., Cheng, P., Li, M., Yu, Z., Song, X., Yu, Z., Sun, H., Zhang, W. and Wang, Z., 2023. Roles of two-component regulatory systems in *Klebsiella pneumoniae*: Regulation of virulence, antibiotic resistance, and stress responses. *Microbiol. Res.*, **272**: 127374. <https://doi.org/10.1016/j.micres.2023.127374>
- Marchler-Bauer, A., Anderson, J.B., Chitsaz, F., Derbyshire, M.K., DeWeese-Scott, C., Fong, J.H., Geer, L.Y., Geer, R.C., Gonzales, N.R., Gwadz, M., He, S., Hurwitz, D.I., Jackson, J.D., Ke, Z., Lanczycki, C.J., Liebert, C.A., Liu, C., Lu, F., Lu, S., Marchler, G.H., Mullokandov, M., Song, J.S., Tasneem, A., Thanki, N., Yamashita, R.A., Zhang, D., Zhang, N. and Bryant, S.H., 2009. CDD: Specific functional annotation with the conserved domain database. *Nucl. Acids Res.*, **37**: D205-D210. <https://doi.org/10.1093/nar/gkn845>
- Martinez-Hackert, E. and Stock, A.M., 2001. The DNA-binding domain of OmpR: Crystal structures of a winged helix transcription factor. *FEMS Microbiol. Lett.*, **203**: 11-21.
- McClelland, M., Sanderson, E.K., Spieth, J., Clifton, W.S., Latreille, P., Sabo, A., Pepin, K., Bhonagiri, V., Porwollik, S., Ali, J. and Wilson, R.K., 2006. *The Klebsiella pneumoniae genome sequencing project*. Genetics, genome sequencing center, 4444 Forest Park Parkway, St. Louis, MO 63108, USA.
- Mitaku, S., Hirokawa, T. and Tsuji, T., 2002. Amphiphilicity index of polar amino acids as an aid in the characterization of amino acid preference at membrane-water interfaces. *Bioinformatics*, **18**: 608-616. <https://doi.org/10.1093/bioinformatics/18.4.608>
- Munson, G.P., Lam, D.L., Outten, F.W. and O'Halloran, T.V., 2000. Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. *J. Bact.*, **182**: 5864-5871. <https://doi.org/10.1128/JB.182.20.5864-5871.2000>
- Oshima, T., Aiba, H., Masuda, Y., Kanaya, S., Sugiura, M., Wanner, B.L., Mori, H. and Mizuno, T., 2002. Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. *Mol. Microbiol.*, **46**: 281-291. <https://doi.org/10.1046/j.1365-2958.2002.03170.x>
- Pao, G.M. and Saier, M.H. Jr., 1995. Response regulators of bacterial signal transduction systems: Selective domain shuffling during evolution. *J. mol. Evol.*, **40**: 136-154. <https://doi.org/10.1007/BF00167109>
- Poole, K., 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *J. mol. Microbiol. Biotechnol.*, **3**: 255-264.
- Rodriguez, R.L. and Tait, R.C., 1983. *Recombinant DNA techniques: An introduction*. Addison-Wesley Publishing Co. London, pp. 162-163.
- Rozen, S. and Skaletsky, H.J., 2000. Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics methods and protocols: Methods in molecular biology* (eds. S. Krawetz and S. Misener), Humana Press, Totowa, NJ. pp. 365-386. <https://doi.org/10.1385/1-59259-192-2:365>

- Saitou, N. and Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**: 406-425.
- Sambrook, J. and Russel, W.D., 2001. *Molecular cloning: A laboratory manual*, 3<sup>rd</sup> ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schneidman-Duhovny, D., Inbar, Y., Nussinov, R. and Wolfson, H.J., 2005. Geometry based flexible and symmetric protein docking. *Proteins*, **60**: 224-231. <https://doi.org/10.1002/prot.20562>
- Stock, A.M., Robinson, V.L. and Goudreau, P.N., 2000. Two-component signal transduction. *Annu. Rev. Biochem.*, **69**: 183-215. <https://doi.org/10.1146/annurev.biochem.69.1.183>
- Swain, K.E. and Falke, J.J., 2007. Structure of the conserved HAMP domain in an intact, membrane-bound chemoreceptor: A disulfide mapping study. *Biochemistry*, **46**: 13684-13695. <https://doi.org/10.1021/bi701832b>
- Tamura, K., Dudley, J., Nei, M. and Kumar, S., 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, **24**: 1596-1599. <https://doi.org/10.1093/molbev/msm092>
- Tamura, K., Nei, M. and Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. natl. Acad. Sci. (USA)*, **101**: 11030-11035. <https://doi.org/10.1073/pnas.0404206101>
- Tseng, T.T., Gratwick, K.S., Kollman, J., Park, D., Nies, D.H., Goffeau, A. and Saier, M.H., 1999. The RND permease superfamily: An ancient, ubiquitous and diverse family that includes human disease and development proteins. *J. mol. Microbiol. Biotechnol.*, **1**: 107-125.
- West, A.N. and Stock, A.M., 2001. Histidine kinases and response regulator proteins in two-component signaling systems. *Trends biochem. Sci.*, **26**: 369-376. [https://doi.org/10.1016/S0968-0004\(01\)01852-7](https://doi.org/10.1016/S0968-0004(01)01852-7)
- Zulfiqar, S. and Shakoori, A.R., 2012. Molecular characterization, metal uptake and copper induced transcriptional activation of efflux determinants in copper resistant isolates of *Klebsiella pneumoniae*. *Gene*, **510**: 32-38. <https://doi.org/10.1016/j.gene.2012.08.035>