Molecular Analysis of the CusA Transporter Involved in Copper Resistance in Prokaryotes: Cloning, Phylogenetics, and Structural Modeling

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Abstract

Heavy metal toxicity is a well-known phenomenon. Same is the true even for essential metals when present above threshold levels. Various homeostatic and resistance mechanisms have aroused due to evolutionary pressures exerted by metal-rich environments. Copper is a dual faced metal that is essential as well as may be toxic for cellular processes. Efflux of excessive metal ions is one of the several resistance mechanisms in bacteria. CusA, an RND protein, plays a central role in a tripartite CusCBA efflux channel. In this study, the CusA from a copper-resistant Klebsiella pneumoniae strain was explored. The cusA gene were successfully amplified and cloned into a cloning vector and subjected to sequencing. The corresponding protein sequence was deduced and a comprehensive analysis was conducted, including its distribution and scope of homology in various bacterial taxa through multiple sequence alignment and phylogenetic tree construction. The findings revealed that CusA is widely distributed and conserved across a broad range of Proteobacteria, suggesting a crucial and widespread role in metal homeostasis and resistance. The protein was further characterized through structural modeling to predict its three-dimensional structure. The analysis identified key domains and functionally important conserved residues involved in binding and efflux of copper ions. The study, highlighting its evolutionary conservation, provides insights into its functional dynamics across diverse bacterial species. These results enhance the understanding of metal resistance mechanisms and may be beneficial for future research on bacterial adaptation to environmental stresses.

Keywords: *Klebsiella pneumoniae, CusA, RND protein, Copper resistance, Phylogenetics, Structural Modeling.*

Introduction

Essential metals play a fundamental role in various life processes. Among these, copper is the third most abundant metallic element in biological systems.

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Despite its essential role in sustaining life, it can be highly toxic, when its concentration surpasses a certain threshold, largely through generation of reactive oxygen species (ROS), interference with metal homeostasis, and improperly incorporation into proteins (Narendrula-Kotha et al.,2020). Given the delicate balance required for copper homeostasis, cells have evolved intricate regulatory mechanisms to maintain intracellular copper concentrations within narrow limits. Microorganisms, in particular, have developed resistance mechanisms to thrive in metal-polluted environments (Shafiq & Rehman, 2024). Molecular analyses have shown surprising similarities in copper resistance genes across diverse bacterial genera, suggesting a limited number of evolutionary pathways that have led to copper resistance.

Among bacteria, Gram-negative species must protect not only their cytoplasm but also their periplasmic compartments from copper-induced damage. One example of a copper resistance system found in Gram-negative bacteria is the Cus system. The Cus system includes a set of genes (Fig. 1) that encode proteins responsible for copper scavenging and efflux. These include CusRS, a two-component regulatory system that senses and activates the transcription of tripartite efflux channel. The CusA, CusB, and CusC proteins form a channel that spans the periplasm, allowing excess copper ions in cytoplasm to be expelled from the cell. A small periplasmic protein called CusF binds copper in periplasm and helps deliver it to the CusCBA system for efflux (Frank et al., 2003).

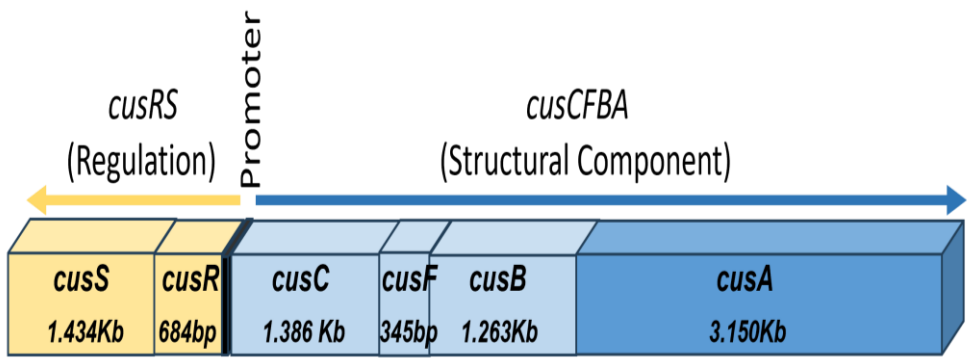

Fig. 1. Schematic diagram showing arrangement of genes in *cus* determinants of *K. pneumoniae* KW. *cusCFBA* operon (blue colour) codes for efflux channel proteins (CusC, CusB and CusA) and a periplasmic chaperon (CusF) while *cusRS* operon (orange colour) codes for a transcription regulator (CusR) and a copper sensor (CusS). The promoter in this case is bidirectional in nature.

CusA is central pump protein belonging to the resistance nodulation cell division family (RND). RND proteins are secondary transporters probably energized by proton-substrate antiport. It functions as a copper-specific pump and works in concert with CusB, a membrane fusion protein, and CusC, an outer membrane channel, to form a tripartite complex that spans the periplasmic space. CusA actively transports copper ions out of the cell, thereby protecting vital cellular components from copper toxicity (Klenotic & Yu, 2024).

This study focused on investigating the structural characteristics of CusA, a key protein involved in copper resistance, within a specific copper-resistant bacterial isolate. Using advanced computational modeling and bioinformatics approaches, the research aimed to analyze the protein's architecture in detail. By examining the distribution of CusA across different bacterial taxa and its evolutionary relationships, the study hopes to uncover significant insights into the mechanisms of bacterial copper resistance. Understanding these structural and evolutionary aspects could provide valuable information for enhancing knowledge in microbial resistance systems and may offer potential applications in biotechnology and environmental microbiology.

Material and Methods

Isolation of genomic DNA

A Cu(II) resistant locally isolated bacterial strain *K. pneumoniae* KW (Acc. No. AB642256) reported by Zulfiqar and Shakoori (2012) was grown in 1mM Cu(II) supplemented LB medium . Genomic DNA of the isolate was extracted as described by Rodriguez and Tait (1983). The quality and quantity of DNA was asessed by taking spectrum between 200_{nm} and 280_{nm} in a UV spectrophotometer (Bio Spec-1604 Shimadzu) as well as resolving on 0.8% agarose gel.

Amplification of cusA

Primers (cusA-F1 and cusA-R1) were designed from the flanking region of gene *cusA* (Table I). These primers were designed from the sequence of *K. pneumoniae* subsp. *pneumoniae* MGH (ACC # CP000647) (McClelland et al., 2006) using online available Primer3 version 0.4.0 program (Rozen and Skaletsky, 2000). The 50µl mixture of amplification reaction contained 1.5mM $MgCl_2$, 250µM dNTPs, 2µM each primer, 1x Taq Polymerase buffer, 5 units of Taq polymerase and 100ng genomic DNA of *K. pneumoniae* KW. The PCR cycle comprised of an initial denaturation for 5min at 94 °C followed by 30 cycles each of denaturation at 94°C for

1min, annealing at 58°C for 90sec and extension at 72°C for 2min with final extension at 72°C for 10min.

The PCR product was run on 0.8% agarose gel using GeneRuler^{TM} DNA ladder mix (Fermentas Cat # SM0331) as a reference. The required amplicon was excised from gel and extracted through Qiagen gel extraction kit (Cat # 28704) as described by manufacturers.

Cloning of cusA

The purified DNA of *cusA* was ligated to a TA cloning vector pTZ57 R/T by using Fermentas InsT/A clone PCR Product Cloning Kit (Cat # K1214) according to manufacturers' instructions.

Competent cells of *E. coli* strain DH5α were prepared by ice cold $CaCl_2$ method (Sambrook and Russell, 2001). These competent cells were transformed with the ligation mixture (recombinant vector) and the tranformants were spread on LB agar plates containing 100 µl of ampicillin (100 mg/ml), 133 µl of 0.1 M IPTG (Fermentas Cat # R0393) and 133 µl of 20 mg/ml X-gal (Fermentas Cat # R0402). The transformants were allowed to grow at 37 °C for overnight. Blue and white colonies appeared. Positive colonies (White in color) with recombinant plasmids were selected for further processing.

Confirmation of insert through restriction analysis

Recombinant Plasmid was isolated through alkaline lysis method as described by Birnboim and Doly (1979) from 1.5ml overnight culture of positive transformants grown in ampicillin supplemented LB broth and subjected to RNase treatment using Ribonuclease A (Fermentas Cat # EN0531).

Presence of the insert (*cusA*) in the isolated recombinant vector was confirmed through restriction analysis. The recombinant plasmid (200 ng) was subjected to single restriction with 10 units of either *Hind* III (Fermentas Cat # ER0501) in the presence of 1x Red buffer or *Eco* RI (Fermentas Cat # ER0271) in the presence of 2x Y tango buffer as well as double restriction with both enzymes in the presence of 2x Y tango buffer. In each case, the volume was made up to 25µl with nuclease free water and each reaction mixture was incubated at 37°C for 2-3hours. The digested products were run along with DNA marker on 0.8% agarose gel .

Sequencing of *cusA*

For sequencing, the recombinant plasmid was isolated and purified through QIAprep® Spin Miniprep Kit (Cat # 12125). Sequencing was first performed using vector specific M13F and M13R primers. To get sequences of internal region of the construct, more primers were designed from already sequenced regions. Details of these primers used for bidirectional sequencing is given in Table I. Sequencing was carried out on ABI PRISM 310 Automated DNA sequencer (Applied Biosystems) through a core facility of sequencing available in the School of Biological Sciences, University of the Punjab.

Table 1. ***Primers Used for Amplification of Cusa and Sequencing of Both Strands of the Amplicon*.**

Primer ID	Primer sequence 5'⟶ 3'	Position
cusA-F1	CCACGCGCACTAAGGAACGGAAC	5190395 *
cusA-R1	CCAGAAGAATGGCGCCACTATAGCC	5187167 *
cusA-F2	GCTCCCTACAGGACTGGTTC	494
cusA-F3	CTCAGCTACAAGCTGCTGGAAG	1036
cusA-F4	TCCTGATGGGCTTCTGGATCC	1520
cusA-F5	CTGGATAAAACCGTGCGTCTGC	1969
cusA-F6	GGCATCAGTGTGTCCTACTCC	2581
cusA-R2	GCCAGATATAGCAGCACGAA	2693
cusA-R3	TCTCGCGCTGAATATCAATG	2218
cusA-R4	CAATCAGCAGGGTAGTCTTCG	1651
cusA-R5	TCATGATAAAGGCGAAGCAC	1165
cusA-R6	GGGTCGACGACAATCTGATAC	602

***** 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

Multiple sequence alignment

The nucleotide sequences obtained were joined and *K. pneumoniae* KW *cusA* gene sequence was submitted to DNA Data bank of Japan (DDBJ) with the accession number AB641116.

Protein sequence of CusA was deduced from the nucleotide sequence of the gene. This sequence was subjected to protein BLAST analysis available online on NCBI site to calculate the identity of CusA. Proteins homologous to CusA were also identified and short listed.

Determination of phylogenetic relationship

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

Membrane topology of each protein was predicted through hydropathy profile of each of six proteins of cus determinants through online available software Protter (Omasits *et al.*, 2014).

Determination of three-dimensional structure

To determine the tertiary structure of CusA protein, homology modeling was carried out by online available SWISS model server (Arnold *et al.*, 2006). Template structure, for this, was first identified on the basis of maximum similarity. The deduced protein sequence of CusA along with this template was submitted for automated modeling. These models were viewed by DeepView - Swiss-PdbViewer version 4.0 (Guex and Peitsch, 1997). Literature was surveyed for determining if any of the proteins under study functionally exist as oligo/multimeric form. Tertiary structures of these oligo/multimers were modeled using Symmdoc server (Schneidman-Duhovny *et al.*, 2005).

Determination of domains architecture

The predicted protein sequence was also analyzed for conserved domains through InterPro Scan (https://www.ebi.ac.uk/interpro/).

Results

Amplification and Cloning of *cusA*

Successful amplification and cloning of whole gene of *cusA* was carried out as depicted through gels at each step in the figure 2.

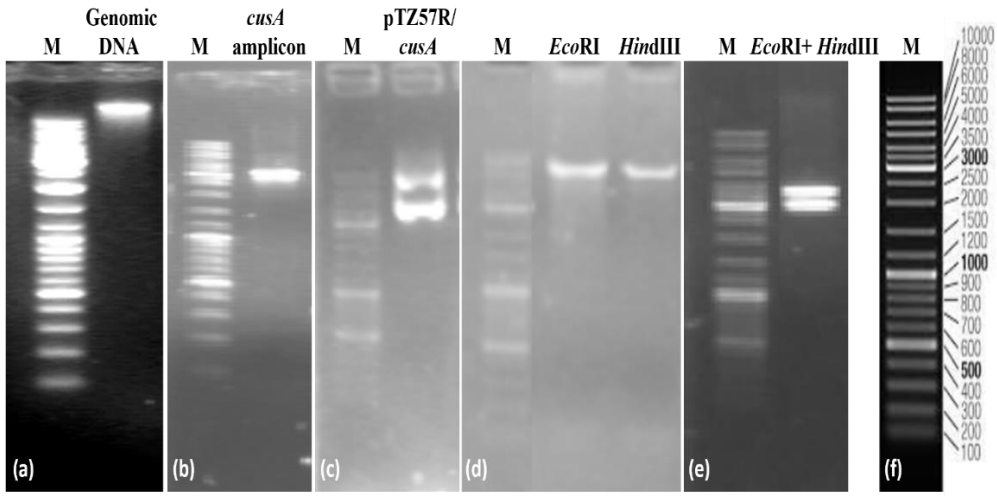

Fig. 2. Amplification and cloning of *cusA.* (a) genomic DNA of *K. pneumoniae* KW, (b) amplified product of *cusA* (3.229 kb), (c) Recombinant plasmid, (d) digested products of recombinant plasmid with single endonuclease (e) and with two endonucleases, (f) M represents DNA marker (Fermentas cat # SM0331).

Multiple alignment of homologous CusA proteins

BLAST analysis of the deduced protein sequence of CusA revealed that CusA is widely present in prokaryotes. The detail of homologous proteins selected in this study for multiple alignment is given in Table II. These included homologous proteins reported in some strains of *K. pneumoniae*; other species of the *Klebsiella* genus including *K. variicola* and *K. oxytoca*; some members of family Enterobacteriaceae other than *Klebsiella* including *Escherichia coli*, *Cronobacter sakazakii*, *Citrobacter koseri*, *Salmonella enterica*, *Edwardsiella ictaluri*, *Shigella sonnei* and *Serratia marcescens*; species representing at inter-order level including *Pseudomonas aeruginosa*, *Shewanella putrefaciens* and *Acinetobacter junii* as well as some species at inter-class level including *Caulobacter segnis, Thiobacillus denitrificans, Herminiimonas arsenicoxydans* and *Ralstonia pickettii*,). Scope of similarity was revealed through multiple alignment of the deduced *K. pneumoniae* CusA protein sequence along with homologous protein sequences in other bacterial species.

Table 2. *CusA Sequences Used To Draw Phylogenetic Relationship*

Sr. No.	Organism	CusA accession #
1	*K. pneumoniae* subsp. *pneumoniae* MGH 78578	ABR80086.1
2	*K. pneumoniae* subsp. *pneumoniae* NTUH-K2044	BAH61534.1
3	*K. pneumoniae* subsp. *rhinoscleromatis* ATCC 13884	EEW38763.1
4	*K. pneumoniae* 342	ACI07181.1
5	*K.* sp. 1_1_55	EFD83113.1
6	*K. variicola* At-22	ADC60414.1
7	*Escherichia coli* SMS-3-5	ACB18346.1
8	*Citrobacter koseri* ATCC BAA-895	ABV13720.1
9	*Cronobacter sakazakii* ATCC BAA-894	ABU79422.1
10	*Edwardsiella ictaluri* 93-146	ACR69292.1
11	*Salmonella enterica* subsp. enterica serovar Tennessee	ZP_04654235.1
12	*Serratia marcescens*	CAE51673.1
13	*Shigella sonnei* Ss046	AAZ87295.1
14	*Shewanella putrefaciens* CN-32	ABP73997.1
15	*Ralstonia pickettii* 12J	ACD26784.1
16	*Acinetobacter junii* SH205	EEY91706.1
17	*Thiobacillus denitrificans* ATCC 25259	AAZ97286.1
18	*Herminiimonas arsenicoxydans*	CAL60743.2
19	*Caulobacter segnis* ATCC 21756	ADG10982.1
20	*Pseudomonas aeruginosa* PA7	ABR80764.1

Phylogenetic analysis

Phylogenetic analysis of the selected CusA protein sequences was carried out through construction of neighbor joining tree (Fig. 3). It was found that CusA was widely present and highly homologous in all the bacterial species under consideration. CusA of *K. pneumoniae* was 97-99 % homologous to other *Klebsiella* spp. The homology at family level was between 83% and 90%. Species of order alteromonadales and those of order pseudomonadales exhibited 85 % and 66-67 % similarity, respectively. Species of beta protobacteria and alpha protobacteria (different classes) showed 64-67 % and 66 % homology in terms of CusA protein sequence.

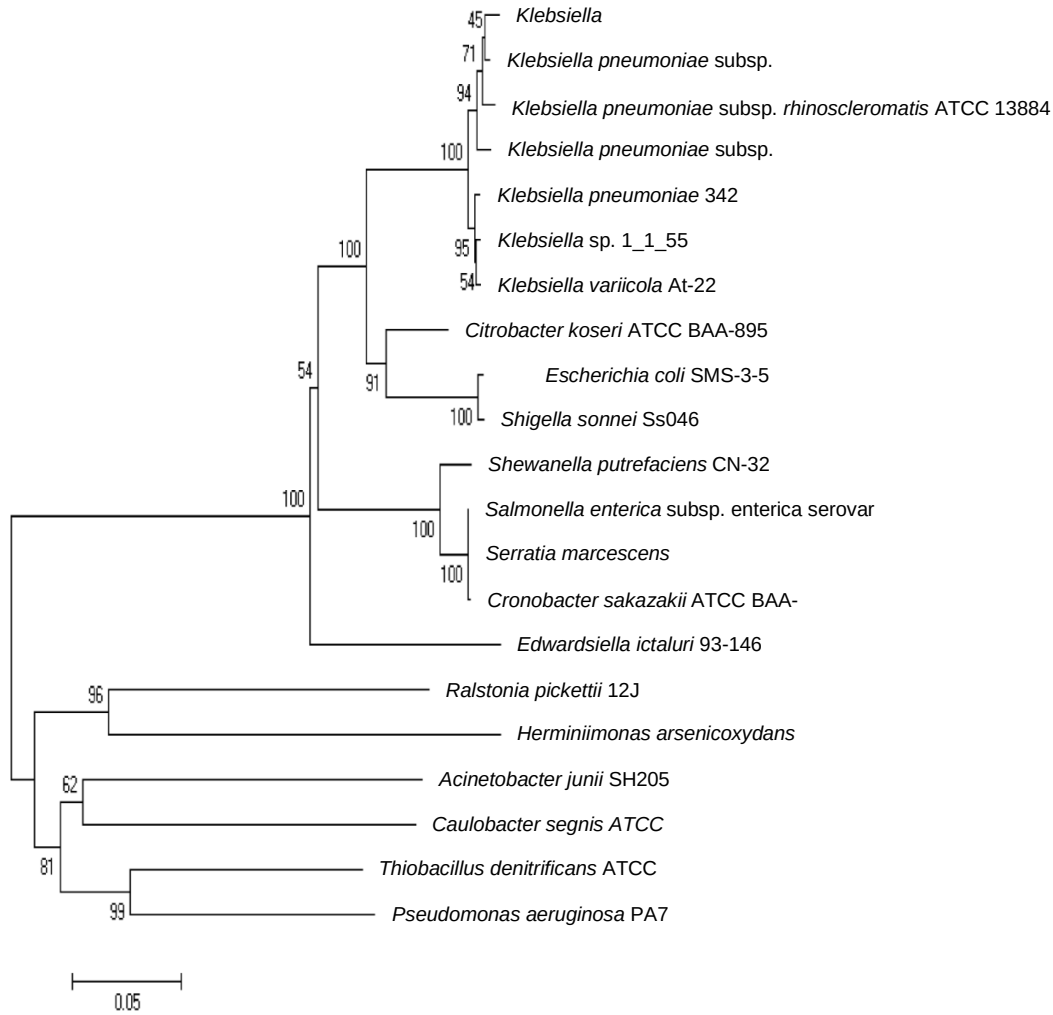

Fig. 3. Neighbor joining tree showing phylogenetic relationship of CusA protein sequence of *K. pneumoniae* KW with those of other bacterial species. Bootstrap values shown at branch points express percentage of 1000 replications. Bar of 0.05 represents substitutions per residue.

Structural analysis of CusA

CusA, a large inner membrane protein, was composed of 1049 residues. Three-dimensional model of this protein revealed that it was composed of two segments (Fig. 4). One of these segments was formed of only α helices. Hydropathic

analysis showed these helices formed twelve trans-membrane (TM) regions (Table III) spanning plasma membrane.

Table 3. ***Transmembrane Regions in CusA Protein***

No.	N terminal	Transmembrane region	C terminal	length
1	14	FLVMMAALFLSIWGTWTII	32	19
2	338	LSYKLLEEFIVVALVCALFL	357	20
3	364	LVAIISLPLGLCFAFIMM	381	18
4	390	IVSLGGIAIAVGAMVDAAIV	409	20
5	449	ALFISLLIITLSFIPIFTL	467	19
6	486	MAGAALLAIVAIPILMGFWI	505	20
7	538	TTLLIALLSILTVVWPLN	555	18
8	874	LMVPMTLMIIFVLLYLAF	981	18
9	898	LLIITSVPFALVGGIWFL	915	18
10	924	VATGTGFIALAGVAAEFGVV	943	20
11	987	TVAVIIAGLLPILWGTGA	1004	18
12	1013	AAPMIGGMITAPLLSLFIIP	1032	20

In CusA, two large loops were predicted; first loop between TM 1 and TM 2, and second loop between TM 7 and TM 8 (Fig. 4). These two loops formed the other segment present in the periplasm. This contains a pore domain and a docking domain. The pore domain can be divided in PN1 and PN2 subdomains present in the first periplasmic loop and PC1 and PC2 present in the second periplasmic loop. Similarly, docking domain forming upper edge of CusA also contains two subdomains: DN and DC present in the first and second periplasmic loops of the protein, respectively. Two small cytoplasmic loops between TM4 & TM5 and TM10 & TM11 were also predicted in CusA.

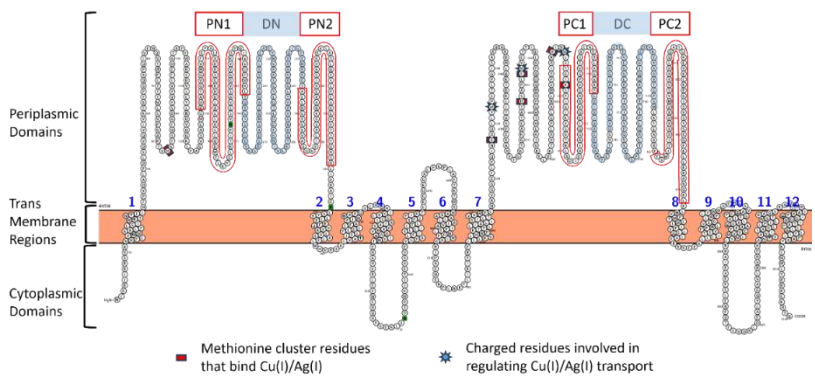

Fig. 4. Structural insights in CusA of *K. pneumoniae*. CusA is a transmembrane protein with twelve membrane spanning helices (TM1-TM12). Two large periplasmic

loops between 1st and 2nd TMs and 7th and 8th TMs form functional domains; the pore domain consisting of PN1 (137-181), PN2 (276-329), PC1 (821-872) and PC2 (673-723) and the docking domain consisting of DN (182-275) and DC (724-812). Two small loops between 4th and 5th TMs and 10th and 11th TMs are present in the cytoplasm.

As the functional form of CusA is a homotrimer that is stabilized through its interaction with homehexameric CusB, the three-dimensional structure of both the monomeric and trimeric forms of CusA were computationally designed through Symmdoc server as shown in figure 5. The pore/channel through which efflux of excessive Cu (I)/Ag(I) ions takes place is visible in the trimeric form top or bottom plane.

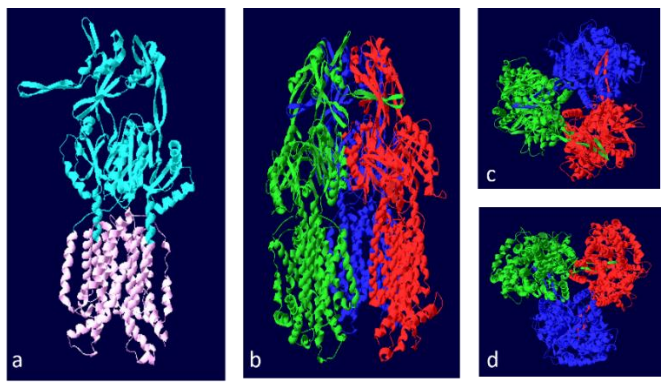

Fig. 5. Three dimentional structure of CusA. (a) Ribbon diagram of CusA monomer. The periplasmic and transmembrane segments are shown in blue and pink colors, respectively. (b) Ribbon diagram of CusA homotrimer in membrane plane. Each monomer of CusA is represented with a different color. (c) CusA homotrimer viewed from top and (d) from bottom.

Functionally Conserved residues in CusA

Structural insight and multiple alignment of CusA sequence over a broad range of prokaryotes revealed some functionally important amino acids that remained conserved during evolutionary pathway. These can be classified as;

1. **Methionine cluster and pairs**
   Several methionine residues are found conserved in CusA of the bacterial domain of life. Amongst these methionine residues at positions 573, 623 and 672 form a methionine cluster that coordinates and binds with Cu(I) and Ag(I) ions.

Other than these, there are several other methionine residues in periplasmic as well as TM domains that are found conserved. These include methionine at positions 230, 381, 403, 410, 501, 640, 812, 944, 988, 1011, 1018 and 1022. Some of these are thought to form Methionine pairs that facilitates ion shuttling from cytoplasm or periplasm to exterior of the cell. Additionally, methionine residues at positions 271, 391 and 486 are also thought to form pairs though these are found conserved in this study with one exception where these are replaced by either valine or leucine, both non-polar amino acids like methionine itself.

2. **Charged amino acids**

Some charged amino acids including acidic amino acids (aspartic acid at 617 and glutamic acid at 567 and 625) and basic amino acids (arginine at 83 and 669 and lysine at 678) are also found conserved. These are thought to be involved in stabilizing methionine and Cu(I)/Ag(I) ion coordination and influencing conformational changes required for channel opening in CusA.

3. **Amino acids involved in CusA and CusB Interaction**

Homotrimer of CusA is surrounded with homohexamer of CusB. The residues involved in formation of salt bridges between the two oligomers are considered to be glutamic acid at 155, glutamine at 584 and arginine at 771 and 777 positions. Amongst these both arginine residues are conserved. While, neither of lysine at 594, arginine at 147 and glutamine at 198 that are involved in hydrogen bond formation between CusA and CusB is conserved.

Discussion

Where copper being its requirement in electron transport chain and other metabolic processes is an essential heavy metal, at the same time it becomes potent toxic when present in surplus amounts. Therefore, a very fine balance in cellular copper concentration is a prerequisite for smooth metabolism. Extensive effort has been and still is being carried out to understand the mechanism/s that lie behind this fine balance. In this regard, a sufficient number of research reports have been published that have reported and discussed the proteins that carryout or influence somehow copper homeostasis and resistance. One of these resistance mechanisms is to expel the excessive copper out of cell. A tripartite protein complex comprising CusCBA along with a small periplasmic chaperon CusF is a widely distributed efflux pump in bacterial species. CusA of this channel belongs to RND family specifically binds toxic metal ions, copper (Cu(I)) and silver (Ag(I)), and through exporting them

maintains cellular homeostasis (Routh et al., 2011). This study focuses on comparative analysis of CusA sequence in various bacterial species that cover upto class level.

The phylogenetic relationship of CusA protein sequences of selected bacterial species depicted in form of neighbor joining tree revealed a general trend that alteromonadales were closer to enterobacteriales in terms of CusA homology as compared to that with pseudomonadales. Alteromonadales were even more homologous than *Edwardsiella* that belongs to family enterobacteriaceae. Whereas, at interclass level, beta proteobacteria were found to be more closely related in terms of CusA sequence to gamma proteobacteria than alpha proteobacteria.

CusA is a transmembrane protein pump that has a large periplasmic segment comprising of pore domain that form an efflux channel and a docking domain involved in Cu(I)/Ag(I) ion binding and their export . Several amino acid residues in the CusA protein have been identified as functionally critical for metal ion binding and transport, playing key roles in the pump's efficiency and specificity. Each monomer of CusA comprises of 1049 amino acids with forty-four methionine residues. Though, CusA does not have the highest methionine content in absolute percentage terms compared to all proteins, the functional concentration of methionine residues-especially within critical regions- makes it fair to describe it as functionally methionine-rich. The clustering of methionine residues at strategic points highlights the importance of this amino acid for CusA's unique role in metal resistance (Long et al., 2010).

Out of total fort four methionine residues, nineteen were found to be highly conserved in the three classes of bacteria, underscoring their essential role in metal ion transport and homeostasis. Amongst these, the three methionine residues (M573, M623, and M672) involved in copper ion binding are highly conserved across all prokaryotic species without exception. The methionine residues that form pairs and facilitate ion shuttling through the channel are also highly conserved across selected prokaryotic species (Su et al., 2010). However, in rare cases, one of the three residues is replaced by valine or leucine in only a single species each; for example, Val at position 271 or 391 and Leu at position 486. In all other species, methionine is consistently found at these positions. Similarly, the charged amino acids involved in interaction between conserved methionine and Cu(I)/Ag(I) are also highly conserved without exception (Su et al., 2012). The amino acids that interact CusB, either through salt bridges or hydrogen bonding, are found to be less conserved.

Conclusion

In summary, the mechanisms that bacteria use to manage copper homeostasis provide insight into the evolutionary pressures exerted by metal-rich environments and may offer valuable information for future studies on microbial resistance to heavy metals. Moreover, CusA can be regarded as a methionine-rich protein in a functional sense, as methionine residues are overrepresented in key functional regions and play essential roles in metal ion binding and export. This makes methionine a defining feature of CusA's structure and function within the CusCBA efflux pump.

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