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Molecular Characterization of a Copper Metallothionein Gene From a Ciliate *Tetrahymena farahensis*

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ABSTRACT

A new copper metallothionein (*TfCuMT*) gene has been identified from a locally isolated ciliate *Tetrahymena farahensis*. It contains 327 nucleotides encoding a peptide chain of 108 amino acids and belongs to class MTT2 and subfamily 7b. Amplification from both gDNA and mRNA confirmed the intronless nature of this gene. Like most of the metallohtioneins, cysteine residues contribute nearly 30% content with the specific CKC motifs. Structural repeats present in peptide sequence of *Tf*CuMT indicate internal duplication of gene at some stage of gene evolution. The predicted model of copper metallothionein protein showed that copper ions are mainly chelated by thiol sulfur of cysteine residues and are embedded in the folds of polypeptide chain. For in vivo expression of *TfCuMT* in *Escherichia coli* host cells the classical stop codons, which coded for glutamine in the ciliate were mutated to CAA and CAG through site directed mutagenesis. The mutated gene showed higher expression in pET28a expression vector compared with pET21a. Optimum expression was obtained after 6–8 h of 0.1 mM IPTG induction. Stability of His tagged *Tf*CuMT in 5% SDS was low, with half-life of about 104 min. Presence of 1.0 μM copper increased the expression level by 1.65-fold. Presence of 100 μM Cysteine in culture medium caused 2.4-fold increase in expression level. His tagged *Tf*CuMT was purified through affinity chromatography using NTN-His binding resin in the presence of 0.1 M imidazole and NaCl. The modeled structure of the TfCuMT showed a cleft for Cu binding with correct orientation of Cys residues in the motif CKC. J. Cell. Biochem. 117: 1843–1854, 2016.

KEY WORDS: *TETRAHYMENA FARAHENSIS*; COPPER METALLOTHIONEIN; SITE DIRECTED MUTAGENESIS; IPTG INDUCTION; *TfCuMT* GENE; COPPER; CYSTEINE; AFFINITY CHROMATOGRAPHY; MTT2 FAMILY OF METALLOTHIONEIN

Metallothioneins (MTs) are cysteine rich heterogeneous family of peptides involved in metal homeostasis and metal detoxification by forming metal-thiolate complex [Boldrin et al., 2002; Ramesh et al., 2009; Capdevila and Atrian, 2011]. They bind with metal ions, resulting in a metalloprotein complex which is accumulated into vacuole and later released as metallic complex [Diaz et al., 2006]. MTs are also known as antioxidants, protective against oxidative stress, DNA damage, angiogenesis and apoptosis [Minoru et al., 2009). MTs have also been found to rescue in Endoplasmic reticulum stress induced cardiac dysfunction [Yang et al., 2014]. On structural basis MT are broadly divided into two major (MT1 and MT2) and two minor (MT3 and MT4) isoforms. Most of the MT-1 members are cadmium induced, while MT-2 are copper induced MT. All MT-3 and MT-5 are cadmium MTs, while MT-4 is exclusively copper MTs [Diaz et al., 2007; Chaudhry and Shakoori, 2010].

Expression of MT1 and MT2 are mainly induced by reactive oxidative species (ROS), cytokines and metal ions including Cu, Cd, Hg, and Zn [Oliver and Eva, 2009]. Zn and Cu are regarded as the primary physiological inducers [Munger et al., 1985; Bremner, 1991]. The metal binding capacity of MTs varies for different metals and spcies of organisms [Siscar et al., 2014]. Although it has been

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established that the expression of MT is controlled at transcriptional level, but it is observed that sometimes there is lack of this relationship indicating that MT expression is also regulated at translational level [Boldrin et al., 2002].

MTs have a dynamic tertiary structure and there is a rapid exchange of ions in β domain as compared to α domain [Kagi and Kojima, 1987]. MTs are stable hydrophilic proteins [Blindauer, 2008] which indicate their presence in cytosol. These proteins are short of aromatic amino acids, which has facilitated the detailed optical spectroscopic studies to observe metal charge transfer transition using circular dichroism, electron absorption, and emission [Stillman, 1995; Vasak and Bogumil, 1997]. Histidine (His) residue is very infrequent in MT polypeptide chain, whereas positively charged amino acids Arg and Lys also have asymmetry in MTs [Gutierrez et al., 2011]. Interaction between metal ions and MT is complex one and it requires a series of reactions before reaching a thermodynamically stable stage. The folding mechanism of MT tells us about the rate of metal ions binding [Chan et al., 2002]. Coordination of Cu(I) with MT directs the polypeptide to wrap around the metal ion and form diagonal and trigonal geometery in mammals. Thiol group acts as a ligand for metal ions to form the clusters of metal thiolate which are involved in terminal coordination [Stillman, 1995; Fowle and Stillman, 1997; Henkel and Krebs, 2004].

MT genes are without any introns which are in consistent with the principle that genes with rapidly changing expression level have lower intron number. Presence of intron can delay regulatory responses [Jeffares et al., 2008]. Ciliate MTs are quite larger than vertebrate MTs so with higher molecular weight [Gutierrez et al., 2011]. Binz and Kagi [1999], on the basis of phylogenetic relationships, designated MTs as superfamily and divided it into 15 families representing different groups. Ciliates MTs belong to family 7 which are broadly divided into subfamilies 7a and 7b. Subfamily 7a contains cadmium/zinc binding ciliate MTs while subfamily 7b has copper binding MTs [Gutierrez et al., 2009]. Cysteine (Cys) residues are arranged in the form of clusters within primary structure of MT. In the case of CuMT, only typical MT clusters, that is, CXC are commonly observed [Gutierrez et al., 2011]. Subfamily 7b (CuMT) does not have clear motifs like subfamily 7a but have $CXCX_{2\text{-}5}CXC$ repeats [Amaro et al., 2008]. Moreover, lysine (Lys) residues are always adjacent to cysteine in subfamily 7b, while in subfamily 7a this association is scarce. Neighborhood of Lys reduces the reactivity of Cys residues and decreases the pKa value [Trinchella et al., 2008]. Up till now *Tetrahyemna* copper metallothionein (CuMT) has been reported from *T. thermophila* [Boldrin et al., 2002], *T. pigmentosa* [Boldrin et al., 2002], *T. pyriformis* [Fu and Miao, 2006; Santovito et al., 2007], *T. rostrata* [Amaro et al., 2008], and *T. tropicalis* [Chaudhry and Shakoori, 2010].

In this study a novel copper MT has been identified from recently reported ciliate *Tetrahymena farahensis* [Zahid et al., 2014] and has been successfully expressed in *Escherichia coli* expression system. The expressed protein has also been characterized and analyzed for folding and potential binding with copper ions.

MATERIALS AND METHODS

CLONING OF *TfCuMT* GENE FROM GENOMIC DNA

Genomic DNA was isolated from rapidly growing log phase *T. farahensis* cells. Log phase growing cells were starved for 18 h in 50 mM Tris buffer (pH 7.4). The cells were pelleted down and subjected to DNA isolation according to Chaudhry and Shakoori [2010]. Copper metallothionein gene of *T. farahensis* (*TfCuMT*) was amplified from genomic DNA using the following set of primers reported by Boldrin et al. [2008].

Forward Primer 5′-ATGGAYACIYARACIYARACIAA-3′
Reverse Primer 5′-TCAGCATTTGCATTCAGCACA-3′

A 50 μl of PCR reaction mixture containing 1× Taq buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each forward and reverse primers, 2.5 U of Taq DNA polymerase and 400 ng of genomic DNA was incubated in an eppendorf tube in a thermocycler thermocycler with initial denaturation of 94°C for 5 min, followed by 35 cycles, each of denaturation at 94°C for 30 s, annealing at 52°C for 35 s, and elongation at 72°C for 30 s. Final extension was done for 7 min at 72°C. PCR amplified gene was analyzed using 1.5% agarose gel. Fermetas GeneJet™ gel extraction kit was used to purify the PCR amplified product.

Purified *TfCuMT* gene was ligated in pTZ57R/T using Fermentas InsTAclone™ PCR cloning kit (#K1214). The 25 μl of ligation mixture contained 2.5 μl of 10× ligation buffer, 165 ng of cloning vector (pTZ57R/T), 490 ng of amplified *TfCuMT* gene and 5 U of T4 DNA ligase. This mixture was incubated at 4°C for 12 h.

Freshly prepared *E. coli* DH5α competent cells (200 μl) were transformed with 7 μl of pTZ57R-*TfCuMT* recombinant plasmids. Recombinant colonies were confirmed through colony PCR and restriction of their isolated plasmids by using *EcoR1* and *HindIII* restriction enzymes in the presence of 1× Tango buffer.

Recombinant plasmids isolated by using Fermentas GeneGET™ Plasmid Miniprep kit were sent for sequencing to Center of Advance Molecular Biology (CAMB), Lahore. Sequence homology was checked through nucleotide BLAST at NCBI database.

CLONING OF *TfCuMT* FROM cDNA

Mid log phase *T. farahensis* culture was given copper stress (2 μg/ml). Isolation of total RNA and cDNA synthesis was performed according to Chaudhry and Shakoori [2010]. cDNA was used as a tempelate for in vitro amplification of *TfCuMT* gene. All the PCR condtions and cloning protocol were kept the same as described above. The sequence obtained from ABI Sequencer was compared with that of *TfCuMT* amplified from genomic DNA for the presence of any intron or variation of nucleotide sequences. Sequence alignment and phylogenetic relationships were found by using ClustalW2 and DNAStar softwares.

SEQUENCE BASED ANALYSIS OF TfCuMT PROTEIN

Nucleotide sequence of *TfCuMT* was translated into a peptide sequence using TRANSLATE tool of Expasy database. Homology of TfCuMT protein with other MTs was determined using protein BLAST at NCBI database.

Amino acid sequence of TfCuMT was analyzed to characterize the protein using different bioinformatics tools. The hydropathy analysis was performed to determine the hydrophobic and hydrophilic regions of the protein. ProtParam tool from Expasy database was used to determine molecular weight, isoelectric point (PI), half-life and nature of amino acids in protein. Sulfhydral group

of cysteine is involved in chelating metal ions. Metalmine software was used to check the cysteine residues involved in copper binding.

STRUCTURE MODELING AND COPPER COMPLEXING

Structure was modeled by utilizing LOMETS [Wu and Zhang, 2007], SEGMER [Wu and Zhang, 2010], MUSTER [Wu and Zhang, 2008], and Phyre2 [Kelley et al., 2015]. Different models were evaluated through Ramachandran plot utilizing PROCHECK [Morris et al., 1992].

Coordinates details were extracted from proteins complexed with Cu from PDB with their IDs; 1OT4, 2CQ9, 2HX7, AND 1KVJ. These extracted Cu coordinates were then added utilizing CHIMERA 1.1 [Pettersen et al., 2004] to all different structural models one by one and in combination.

PDBSum [Laskowski et al., 2005] was utilized to analyze the structural details of TfCuMT models with Cu one by one for metal binding residues, disulfide bonds and other secondary structure details.

MUTAGENESIS OF *TfCuMT*

Tetrahymena have slightly different genetic code than other organisms where universal stop codon TAA and TAG present at position 4, 6, and 97 encode glutamine instead of being a stop codon. Four stop codons were present in *TfCuMT* gene with respect to bacterial/yeast expression system. In order to express this MT gene in *E. coli*, these stop codons were mutated through site directed mutagenesis.

The two stop codons (TAG) present near N-terminus of *TfCuMT* gene were modified (CAG) by using mutagenic primer Xp1 (5′ CGCATATGGATA CGCAGACGCAGAC 3′). Restriction site for *Nde I* was also introduced at the 3′ end of gene to facilitate its cloning in expression vector. Similarly, TAA stop codon (codon for glutamine in *Tetrahymena*) present at +292 nt position was mutated to glutamine encoding CAA. For this mutagenic primer mut3 (5′ TCAGCATTTA-CATTCAGC ACAAGTGCAAGGGTTGCATTGGCAG 3′) was used. PCR amplification was performed by initial denaturation at 94°C for 5 min followed by 35 cycles, each of denaturation at 94°C for 45 s, annealing at 56°C for 30 s, and elongation at 72°C for 35 s. Reaction was completed with a final elongation for 8min at 72°C. PCR amplification of *TfCuMT* in the presence of mutagenic primers resulted in a product with required mutations. The mutated gene was cloned in pTZ57R/T and sequenced to confirm the induced mutations.

The fourth stop codon present in the center of gene at position +169 was mutated in two different PCR reactions. In 1st PCR reaction, 162 nucleotide fragment of *TfCuMT* (167–327 nt) gene was amplified using mut4 (5′ GTTGCCAATGCAATCCTTGT 3′) as forward primer, while previously used R3CUMT was used as reverse primer. PCR reaction was carried out with blunt end polymerase (KOD polymerase). The reaction mixture contained 1× KOD buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 2.5 U of KOD polymerase, 2 pmol of each forward and reverse primer and 200 ng of tempelate. The reaction mixture was incubated in thermocycler with initial denaturation of 5 min at 94°C, followed by 35 cycles, each of 50 s denaturation at 94°C, 30 s annealing at 56°C, and 25 s elongation at 72°C. A final extension was done at 72°C for 7 min. The PCR product was observed on 1.5% agarose gel. This 162 bp amplified product was named as megR.

In 2nd PCR reaction, *TfCuMT* gene with only inframe stop codon at +169 nt (cloned in pTZ57R/T plasmids) was used as template. The already used Xp1 primer was used as forward primer while megR was used as reverse primer. The reaction mixture ingredient had the same concentration as described above. Amplified product was subjected to T/A cloning and sequencing to confirm the mutation of all indigenous stop codons.

CLONING OF MUT-327 IN PET28A EXPRESSION VECTOR

Recombinant pTZ57R-327 was incubated at 37°C with *NdeI* and *EcoR1* in the presence of 2× Tango buffer to excise the mut-327 from the vector. pET28a was also restricted with both these enzymes to generate same sticky ends.

Mutated *TfCuMT* gene was cloned in pET28a. Ligation was performed by mixing 9 μl (80 ng) of mutated *TfCuMT* gene with 4 μl (28 ng) of restricted pET28a in the presence of 1× ligation buffer and 10 U of ligase enzyme. The recombinant plasmid was named as pET28a-mut327. *E. coli* DH5α competent cells were transformed with recombinant expression vector (pET28a-mut327). Cloning of mutated *TfCuMT* in pET28a was confirmed by restriction analysis. *E. coli* $BL28C^+$ cells were transformed with the recombinant pET28a-mut327 for the expression analysis.

Overnight culture of transformed $BL21C^+$ in the presence of ampicillin (0.1 μg/ml) was used as inoculum (1%) for LB medium. Inoculated medium was kept in shaking incubator at 37°C until OD_{580} reached 0.4 to 0.8. Induction of IPTG (0.2 mM) was given for 6 h for the expression of cloned *TfCuMT*.

SAMPLE PREPARATION FOR SDS-PAGE

Protein expression was analyzed on SDS PAGE. For this, 10 ml of 6 h cell culture was pelleted down by centrifugation at 9,800*g* for 10 min at 4°C. The pellet was washed with 50 mM Tris buffer (pH 7.5). Cells were resuspended in 50 mM Tris buffer with a final OD of 15 at 600 nm. About 500 μl of this suspension was subjected to sonication for 1 min (pulse of 20 s and cooling of 2 min on ice) using ultrasonication machine (Sonic Vibro). Sonicated sample (20 μl) was mixed with 5 μl 5× SDS loading buffer (50% glycerol, 10% SDS, 0.5 M dithiothreitol, 0.25% bromophenol blue, 0.25 M Tris-Cl pH6.8) and denatured in boiling water for 5 min. Pattern of expressed protein was observed using SDS-PAGE.

OPTIMIZATION OF PROTEIN EXPRESSION

The expression level of protein was optimized by varying concentration of IPTG, and induction time. Effect of cysteine and copper ions on expression was also determined. For each parameter, overnight culture of transformed *E. coli* $BL21C^+$ was inoculated in LB medium. IPTG effect on expression was determined by using 0.05, 0.1, 0.25, 0.5, 0.75, and 1.0 mM concentrations. Optimum time of expression was found by measuring expression after every 2 h, till 12 h. Effect of copper ions on expression was determined by inducing culture in the presence of 1, 5, 10, 20, and 50 μM copper ions. In another experiment medium was supplemented with 50 and 100 μM cysteine.

EXPRESSION AS SOLUBLE OR INSOLUBLE FRACTION

IPTG induced culture of transformed $BL21C^+$ was pelleted down and resuspended in 50 mM Tris-Cl of pH 7.5. Samples (500 μl) were taken

in sterile eppendorf tubes and sonicated for 5 min at the pulse rate of 60 for 20 s with a cooling gap of 2 min. After sonication sample was centrifuged at 12,000*g* for 10 min to separate the sobluble fraction (supernatant) and inclusion bodies (pellet). Inclusion bodies were resuspended in equal volume of 50 mM Tris-Cl, pH 7.5. Both fractions were loaded on gel for SDS-PAGE to determine expression of protein as soluble or insoluble fraction.

PREPARATION OF INCLUSION BODIES

Inclusion bodies were prepared by disrupting the cells through sonication using Ultrasonic Processor (Sonics). Pellet of 5 L culture was resuspended in 95 ml Tris-Cl buffer (pH7.5) and sonicated for five pulses of 1 min each with 5 min gap in between at 60 amplitude using 12 mm probe. Supernatant was removed by centrifugation at 5,800*g* value for 10 min and pellet was resuspended in the same volume of Tris-Cl pH 7.5. Same process of sonication was repeated for 5–6 times, each time an aliquot was saved for analysis on SDS-PAGE. On completion of sonication 200 μl aliquot was taken for protein estimation and remaining sample was centrifuged at 6,800*g*. Pellet was washed 2–3 times with Tris-Cl buffer (pH 7.5) and saved at −80°C till further use.

SOLUBILIZATION OF INCULSION BODIES

For solubilizing inclusion bodies, guanidine hydrochloride (GnHCl) buffer of pH10 (6 M GnHCl, 100 mM Tris-Cl and 1 mM EDTA) was used. A maximum of 6 mg of proteins was solubilized in 1 ml of buffer. Sample was centrifuged at 5,800*g* for 10 min at 4°C to separate cell debris from the soluble proteins. Supernatant was mixed with 2 mM dithiothreitol (DTT) and incubated at 37°C for 30 min for complete reduction of thiol groups (SH) of cysteine residues present in TfCuMT protein.

PURIFICATION OF RECOMBINANT TfCuMT

pET28a has a sequence of six His residues. This His-tag is attached to the C-terminal of expressed protein. Recombinant *TfCuMT* was purified using affinity chromatography according to Cui et al. (2012).

RESULTS

COPPER METALLOTHIONEIN GENE

Copper metallothionein gene (*TfCuMT*) was amplified through PCR using degenerate primers. A 327 bp product was obtained (Fig. 1). The sequencing results showed 83% homology of amplified *TfCuMT* with already reported *T. thermophila* copper metallothionein (*MTT2*).

Amplified product was T/A cloned in pTZ57R/T and *E. coli* DH5α cells were transformed with pTZ57R recombinant plasmid containing 327 bp *TfCuMT*. The plasmid isolated from recombinant colonies was linearized after restriction with *HindIII* and gave two bands viz. 2.8 kb and ~400 bp after restriction with *EcoR1* and *HindIII* (Fig. 1C).

CHARACTERISTICS OF *TfCuMT*

Translated sequence shows that 327 nucleotide sequence of *TfCuMT* encodes a 108 amino acids polypeptide chain with ATG as start codon and TGA as stop codon. TAG and TAA which are stop codons for bacteria and other eukaryotes encode glutamine in ciliates and are present within the coding region of the gene. TAG codon is present at positions 4 and 6 while TAA is present at 56 and 96 positions. The gene is intronless as evident by the cDNA sequence. The protein has cysteine as the most abundant amino acid residue making 30.6% of the total amino acids, while lysine and threonine account for 10.2% each. Calculated molecular weight of the protein is 11.36 kDa and theoretical PI is 8.0. Its instability index is −40.47 showing that this protein is unstable. Figure 1D shows the *TfCuMT* nucleotide sequences and the resulting amino acids sequence.

Copper MT polypeptide showed conserved cysteine residues which are in agreement with their function. The polypeptide chain consists of one CxxC, one CxCC, and fourteen CxC motifs. These motifs of cysteine amino acids are arranged in consistent repeats, that is, CxCxxCxCxxxCxC, CxCxxCxxCxxCxCxxCxC, CxCxxCxCxxCxCC, CxCxxCxCxxCxC, and CxCxxCxCxxCxC. Multiple sequence alignment of *TfCuMT* with already reported *Tetrahyema* copper MT (Fig. 2A) shows that CxC motif are highly conserved.

PHYLOGENETIC RELATIONSHIP OF TfCuMT WITH OTHER CILIATES

Phylogenetic relationships of *TfCuMT* with other members of subfamily 7b of MTs using CdMT (subfamily 7a) as outgroup is shown in Figure 2B. The phylogenetic tree shows relatively close homology of *TfCuMT* with the copper MTs of *T. thermophila* and *T. tropicalis*.

CONSERVE RESIDUES ANALYSIS

Amino acid sequence alignment was performed using T-Coffee database. Multiple sequence alignment of TfCuMT shows it has a regular organization with a number of conserved residues like MTs of other species of *Tetrahymena*. Fig. 2C shows that peptide positions 13, 15, 24, 32, 34, 42, 44, 47, 53, 61, 63, 69, and 71 are highly conserved for cysteine residues while peptide position 1, 10, 26, 36, 48, 51, and 65 are moderately conserved for methionein, threonine, cysteine, proline, lysine, cysteine, and glycine, respectively.

EXPRESSION OF MUTATED *TfCuMT* IN BL21C^{+}

Universal Stop codons TAA and TGA present at 4, 6 and 97 positions were mutated using mutagenic forward and reverse primers. Recombinant plasmid pTZ57R-327 was used as template (the cloned template is advantageous as the PCR product appearing at 327 bp size was mutated). The amplified PCR product of 327 bp was subjected to T/A cloning. Fourth and last stop codon present at 57 position was mutated by using a mega primer of 162 nucleotides as reverse primer. Mega primer was opted as an alternative to whole plasmid synthesis method for removal of stop codon from the center of the *T. farahensis CuMT*. Mutated *TfCuMT* was cloned in cloned in pET28a to get an optimum expression.

E. coli BL21C^{+} were transformed with recombinant pET28a. Expression of *TfCuMT* using pET28a expression vector was also helpful in its purification during downstream processes.

EFFECT OF IPTG, TIME, COPPER, AND CYSTEINE ON THE EXPRESSION OF *TfCuMT*

Figure 3a shows the effect of different concentrations of IPTG on expression of *TfCuMT*. Expression increased to 7.3-, 7.9-, 7.5-,

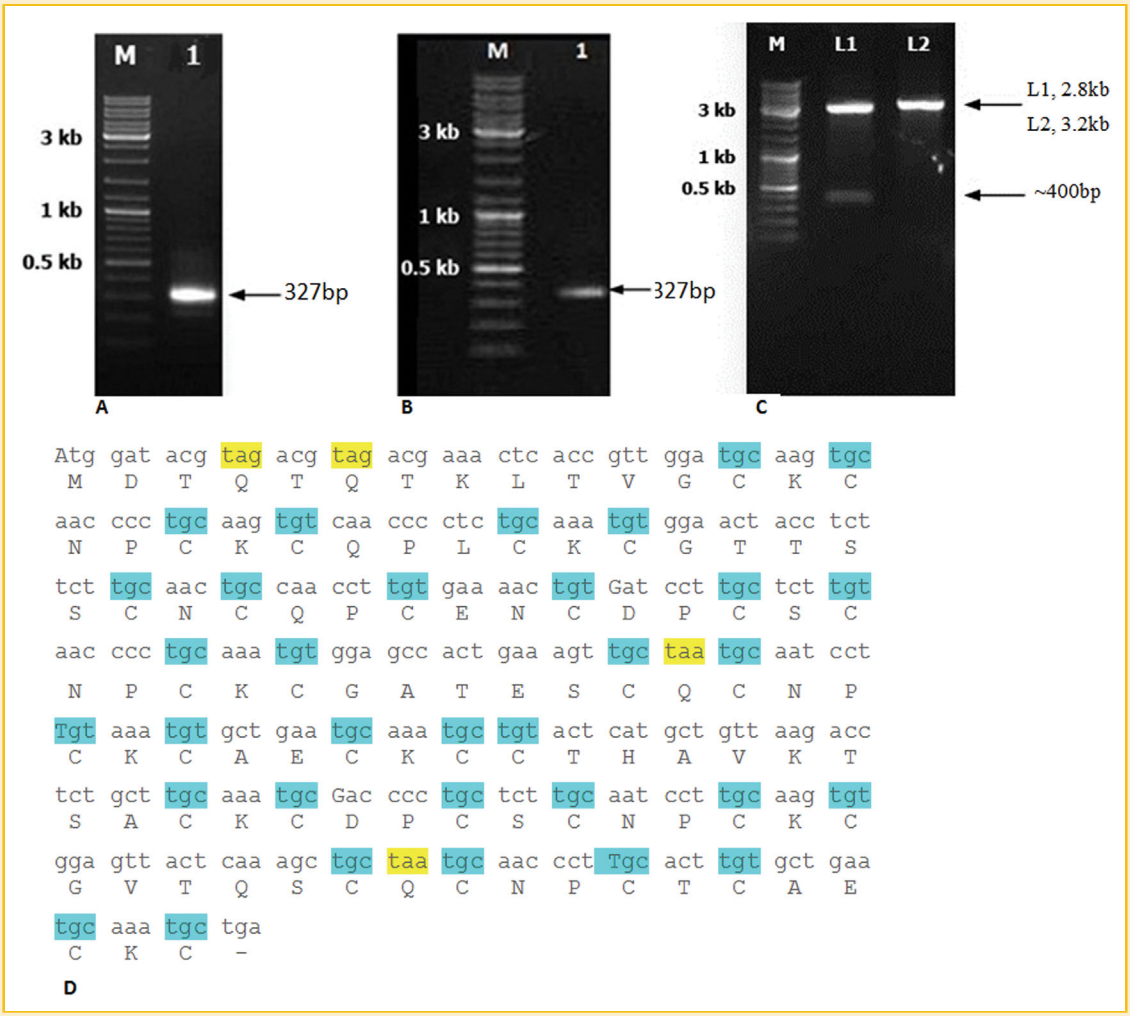

Fig. 1. Copper metallothionein gene– *TfCuMT*. A: PCR product (327 bp) of *TfCuMT*. B: gene clean of amplified *TfCuMT*. C: Restriction analysis of plasmids isolated from *TfCuMT* containing recombinant pTZ57R-327. L1 shows double restriction of recombinant pTZ57R-327 with *EcoR1* and *HindIII* while L2 shows single restriction of recombinant pTZ57R-327 with *HindIII;* M, DNA marker. D: Nucleotide sequence of *TfCuMT* and deduced amino acid sequence of polypepetide chain. Cysteine codons are highlighted in blue while stop codon within the peptide sequence are highlighted in yellow color.

7.4-, 6.9-, and 5.0-folds at 0.05, 0.1, 0.25, 0.5, 0.75, and 1.0 mM IPTG concentrations, respectively. Quantification results showed 0.1 mM IPTG as the optimum concentration for *TfCuMT* expression. Expression of *TfCuMT* increases with the passage of time up to 8 h after induction. An optimized expression is observed after 6–8 h of IPTG induction. At later stages expression level is slightly decreased. Expression increased about 19-, 14.5-, 15.5-, 15.6-, 15.0-, and 13.4-folds after 2, 4, 6, 8, 10, and 12 h, respectively (Fig. 3b).

MTs are metal chelating proteins. In order to check the stability of MT in the presence of copper, *TfCuMT* was expressed under different concentration of copper. Figure 3c shows that low copper concentration (1 μM) was optimum for MT expression, while higher concentration suppressed the expression of copper concentrations. Addition of cysteine in the medium at the time of induction resulted in 1.8-fold increase at 50 μM cysteine concentration, while 2.4-fold increase in expression was observed in the presence of 100 μM cysteine concentration (Fig. 3d).

STABILITY OF TfCuMT

Stability of TfCuMT as apometallothionein was determined at 37°C. For this purpose protein inclusion bodies were suspended in 5% SDS and incubated at 37°C. SDS PAGE showed protein degraded about 12.5% within 20 min. This shows that the expressed protein had a half-life of about 1 h and 43 min.

PURIFICATION OF RECOMBINANT TfCuMT

His-tagged TfCuMT was purified using nickel affinity chromatography column. Crude protein was loaded on nickel column and unbound protein was removed using wash buffer. At 0.1 M imidazole and NaCl, TfCuMT was eluted as pure product (single band). Figure 4 shows different fractions of proteins obtained during nickel affinity chromatography.

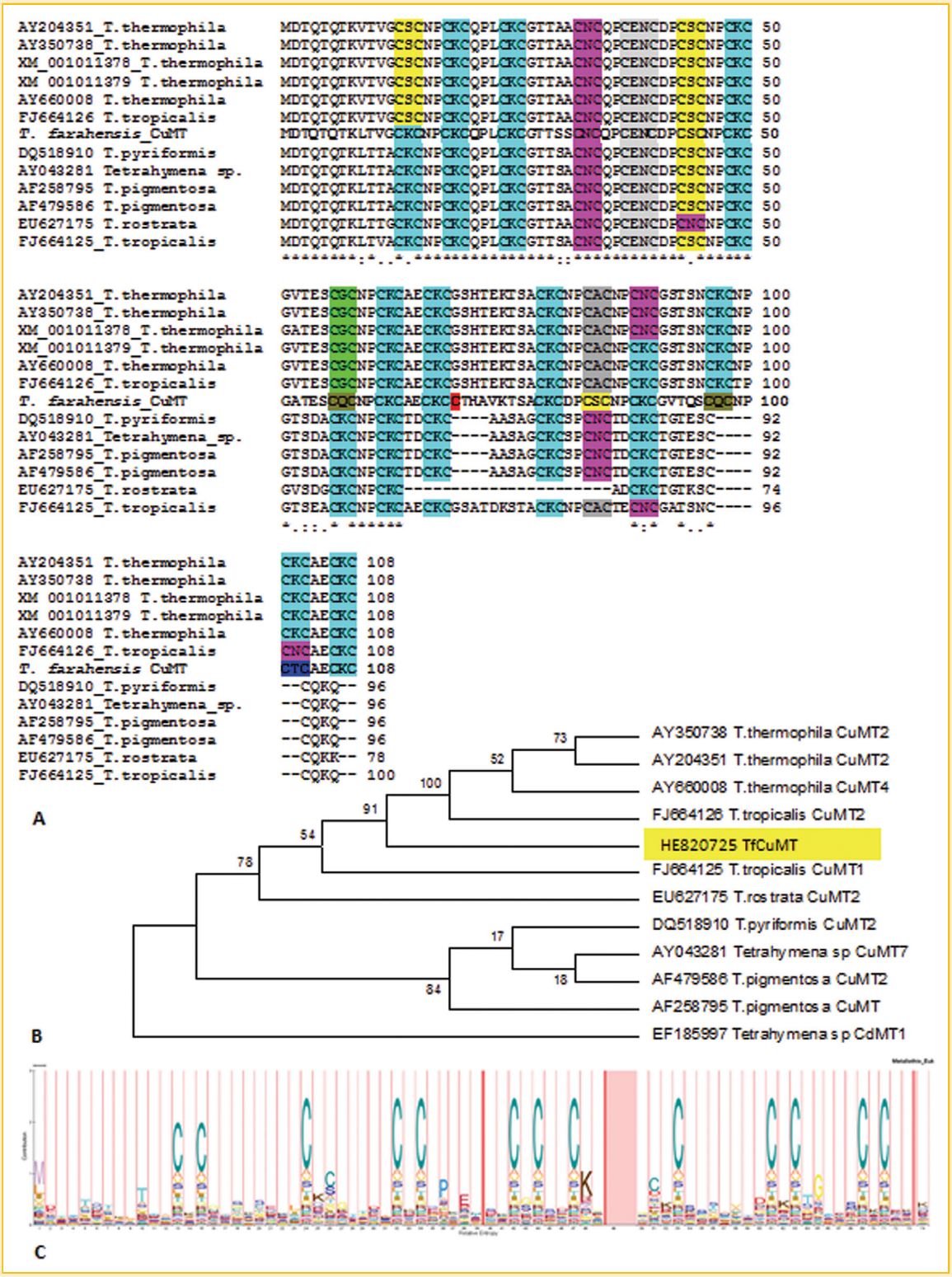

Fig. 2. A: Multiple sequence alignment of TfCuMT protein with other *Tetrahymena* copper metallothioneins (MTT2) of different species of genus *Tetrahymena*. MTT2 specific CXC motifs are highlighted. Cadmium metallothionein (MTT1) specific CCC motifs are virtually absent in Copper metallothioneins. B: Phylogenetic tree of *TfCuMT* on the basis of Neighbor Joining method. Mega5.2 software was used to construct the tree at 1000 bootstrap value. C: Graphical representation of conserved amino acids in TfCuMT. The large size "C" indicate highly conserved sequences. Medium size indicates moderately conserved sequences while small size means variable regions. It appears that CxC blocks are quite conserved.

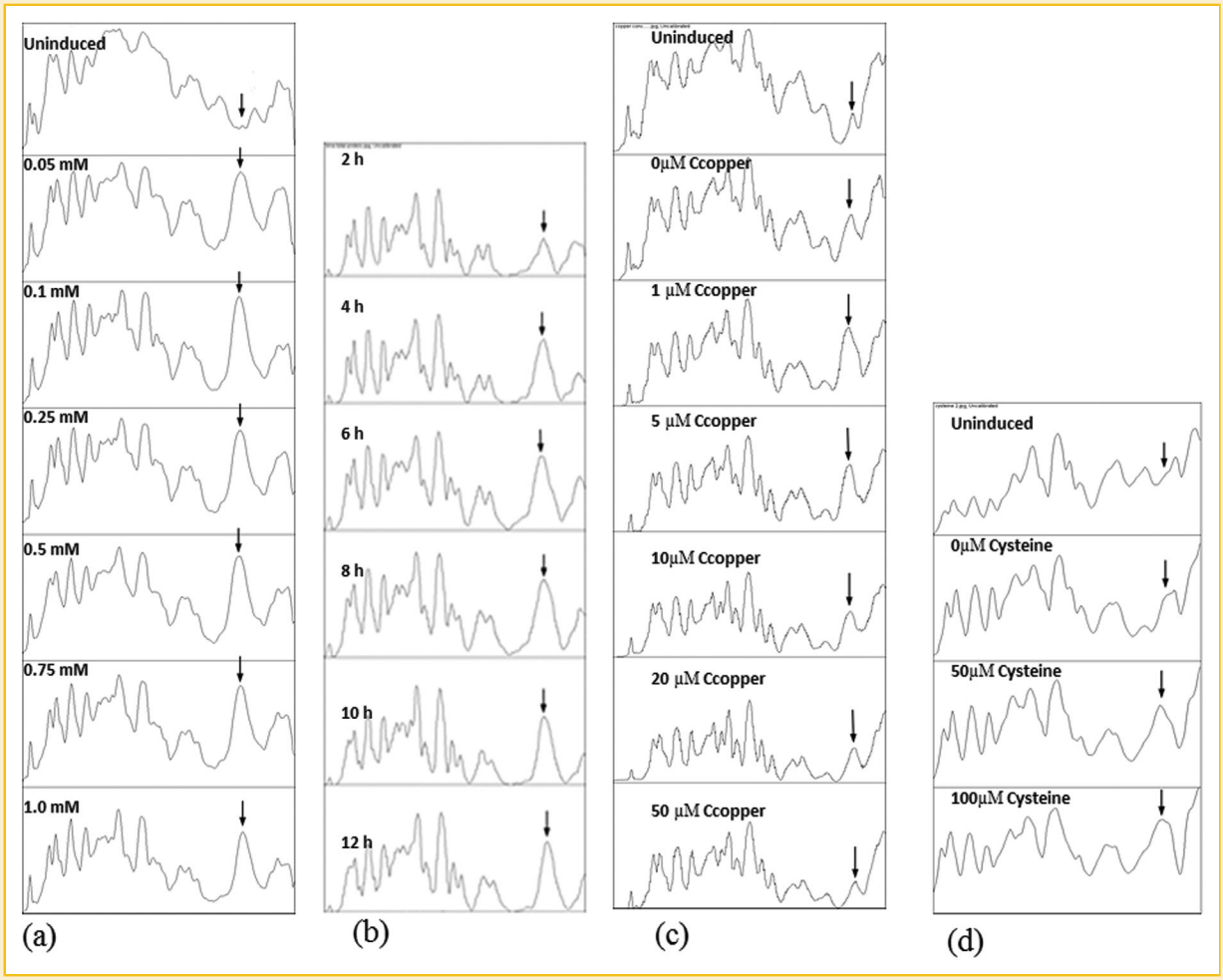

Fig. 3. Expression of His tagged *TfCuMT* in *E.coli* BL21C^{+}. a: expression of *TfCuMT* at different concentrations of IPTG after 6 h of induction. 0.1 mM appears optimum for the expression of His tagged *TfCuMT*. b: Expression of *TfCuMT* at different intervals of time. Expression level increases with increase in time upto 8 h after which there was a slight decrease in the expression of *TfCuMT*. c: Effect of different concentrations of copper on the expression of *TfCuMT*. 1 μM copper caused an increase in expression while further increase in copper concentration caused a decrease in expression level. d: Effect of cysteine presence on the expression of *TfCuMT*. Expression of TfCuMT is directly propotional to amount of cysteine added.

3D STRUCTURE OF *TfCuMT*

Amongst the different structures modeled by different methods one modelled by Kelley et al. [2015] was found to be the most accurate as depicted by Ramachandran plot results (Fig. 5a). Additionally, this structural model was the only structure with the most compact shape and with higher order of secondary structural elements (Fig. 5b, top).

After addition of Cu to the modelled structure by Phyre2 was found to contain the cleft for Cu binding with correct orientation of cysteine residues located in the motif "CKC" (Figs. 5 and 6). The PDBSum results also supported the fact that Cu ion correctly fitted in the cleft involving binding of Cys 48 and 63 with this ion (Fig. 6). Moreover, both these Cys residues were located in the CKC motifs. Additionally, painting the molecular surfaces also depict the fact that Cu becomes embedded in the protein fold (Figs. 5b, bottom) helping the protein to fold correctly in a relatively compact structure compared to other extended structures modelled by other methods. Furthermore, the CuMt complex was also checked for its potential binding details utilizing CheckMyMetal web server [Zheng et al., 2014]. The results further confirmed the potential disulfide linkage between Cys 48 and Cys 63 (Fig. 6), one ionic interaction of each sulfur, of the two Cys residues, with Cu and three potential co-ordinations with alpha and beta carbon of Cys 48 and with beta carbon of Cys 63 (Fig. 7). All these analyses augment the fact that the predicted structure of CuMt is the nearest to the native structure of the protein.

DISCUSSION

Ciliate MT belong to family 7 of MTs, which is subdivided into subfamily 7a and subfamily 7b, mainly on the basis of the structural arrangement of cysteine residues, their lysine

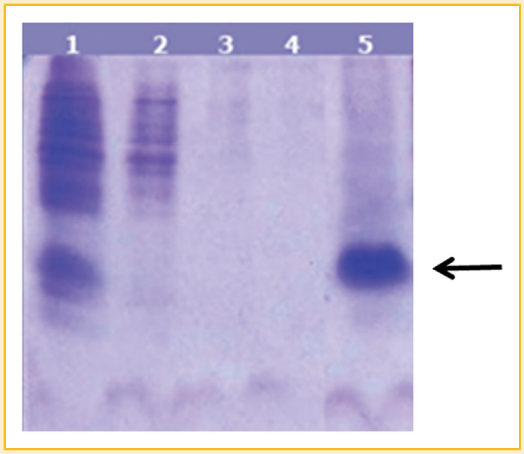

Fig. 4. Purification of TfCuMT using nickel affinity chromatography. 1, total cell proteins; 2, flow through during protein loading; 3, wash buffer flow through; 4, elusion at 0.05 M imidazole and NaCl; 5, elusion at 0.1 M imidazole and NaCl.

neighborhood, phylogenetic analysis and expression with respect to specific metal [Diaz et al., 2007]. In the present study, a new copper MT gene has been amplified from genomic DNA and cDNA of a locally isolated copper resistant *T. farahensis*. This local isolate has been described as new species on the basis of SSr RNA and cytochrome c oxidase subunit 1 [Zahid et al., 2014]. Nucleotide sequence of *TfCu*MT from both sources confirms the absence of introns. This is in accordance with all previously reported MTs from different species of *Tetrahymena* [Piccinni et al., 1999; Santovito et al., 2001; Boldrin et al., 2003; Fu and Miao, 2006; Shuja and Shakoori, 2007; Boldrin et al., 2008]. This characteristics of MT is helpful in rapid response against the sudden stress of metal ions [Leignel et al., 2005; Santovito et al., 2007]. BLAST results and multiple sequence alignment of *TfCu*MT showed that it is a member of subfamily 7b due to its structural similarity and lysine arrangement.

The amplified *TfCuMT* nucleotide sequence showed 83% homology with already reported copper MT gene of *T. thermophila*. The amplified gene contains 327 nt and it encodes 108 amino acid peptide sequence with TGA as universal and only stop codon for ciliates. The theoretical molecular weight of this protein is 11.6 kDa. This confers the finding of Gutierrez et al. [2009] that *Tetrahymena* MTs are quite larger than vertebrate MTs. Although *Tetrahyemena* MTs are larger enough, they have cysteine residues within the average range (16–32%) of other MTs [Gutierrez et al., 2009].

Sequence analysis showed the presence of 30.6% cysteine residues within peptide sequence of TfCuMT. Santovito et al. [2001] also purified copper binding MT from copper induced sample of *T. pigmentosa*. cDNA sequence analysis showed that it consisted of 29% cysteine residues arranged in characteristic motifs of metal binding proteins [Santovito et al., 2001].

Most of the cysteine residues of *TfCu*MT are arranged as "CxC" motifs forming major repeats. Here "x" in most cases is lysine while serine, glutamine, threonine, and asparagine are also present. Lysine to arginine ratio is not observed due to total absence of arginine in the primary structure of *TfCu*MT. Most of the amino acids are uncharged, while positively or negatively charged amino acids contribute up to 18%. Boldrin et al. [2008] reported that numerous cysteine residues arranged in different motifs is a typical characteristic of all MTs. The CxC motifs are distributed throughout peptide length of subfamily 7b (copper metallothioneins) while in case of 7a (cadmium metallothionein) these CxC motifs are mainly restricted near to C-terminal [Diaz et al., 2007]. Diaz et al. [2007] has also reported presence of lysine in CxC motifs and absence of CCC cluster in subfamily 7b. Both of these characteristics are also present in the structure of TfCuMT. However, there is one CC motifs which has been observed very rare in copper metallothionein (7b). Coyle et al. [2002] has reported the absence of aromatic amino acids in all typical MT, later on aromatic amino acid was reported in MTT1 from *T. tropicalis* and *T. thermophila* [Diaz et al., 2007; Shuja and Shakoori, 2007]. Presence of only one histidine residue in *Tf*CuMT is also in close agreement with Gutierrez et al. [2009] findings that histidine is very rare in most of the reported MTs. Arginine is reported to be absent in all *Tetrahymena* CuMT [Diaz et al., 2007; Gutierrez et al., 2009]. Lysine is exclusively used as positive amino acid in presently reported TfCuMT like most of the other MTs [Amaro et al., 2008]. TfCuMT is devoid of modular structures however, structural repeats are present with replacement of K at some point by N, T, or Q. Amaro et al. [2008] has also reported the presence of $CXCX_{2-5}CXC$ structural repeats in copper MTs. The structural repeats in *Tetrahymena* MTs are probably the result of duplication of some ancestral fragment [Boldrin et al., 2003] and further diversification of duplicated genes gave rise to the subfamilies of *Tetrahymena* MT [Diaz et al., 2007; Santovito et al., 2007]. Multiple sequencing alignment of TfCuMT using T-coffee database shows that most of the conserved cysteine residues are present in first 70 amino acids peptide length. This is because most of the reported MTs have amino acids residues below 80.

The hydropathy plot shows that all the domains of *Tf*CuMT have hydropathicity value quite lower than 1.8 indicating that the protein is cytoplasmic in nature. pKa curve showed isoelectric point to be pH8.4 which is consistent with the general trend of PI of already reported MTs. The isoelectric point of most of the MTs is either neutral or slightly basic [Gutierrez et al., 2009].

MUTAGENESIS OF *TfCuMT*

Santovito et al. [2001] has reported the sequence analysis of CuMT of *T. pigmentosa* confirming TGA as only stop codon like other members of genus *Tetrahymena* and other ciliates. The universal stop codon TAA and TAG encodes glutamine in *Tetrahymena* [Horowitz and Gorovsky, 1985; Harper and Jahn, 1989; Shuja and Shakoori, 2007]. Presence of stop codon results a truncated expression of ciliate MT in bacterial expression system as reported by Shuja et al. [2013]. Mutagenesis resulted in expression of a full length protein which is useful to characterize the protein and analyze its metal binding capacity. Suleman and Shakoori [2012] have also reported the functional analysis of a full length expressed mutated cadmium MT.

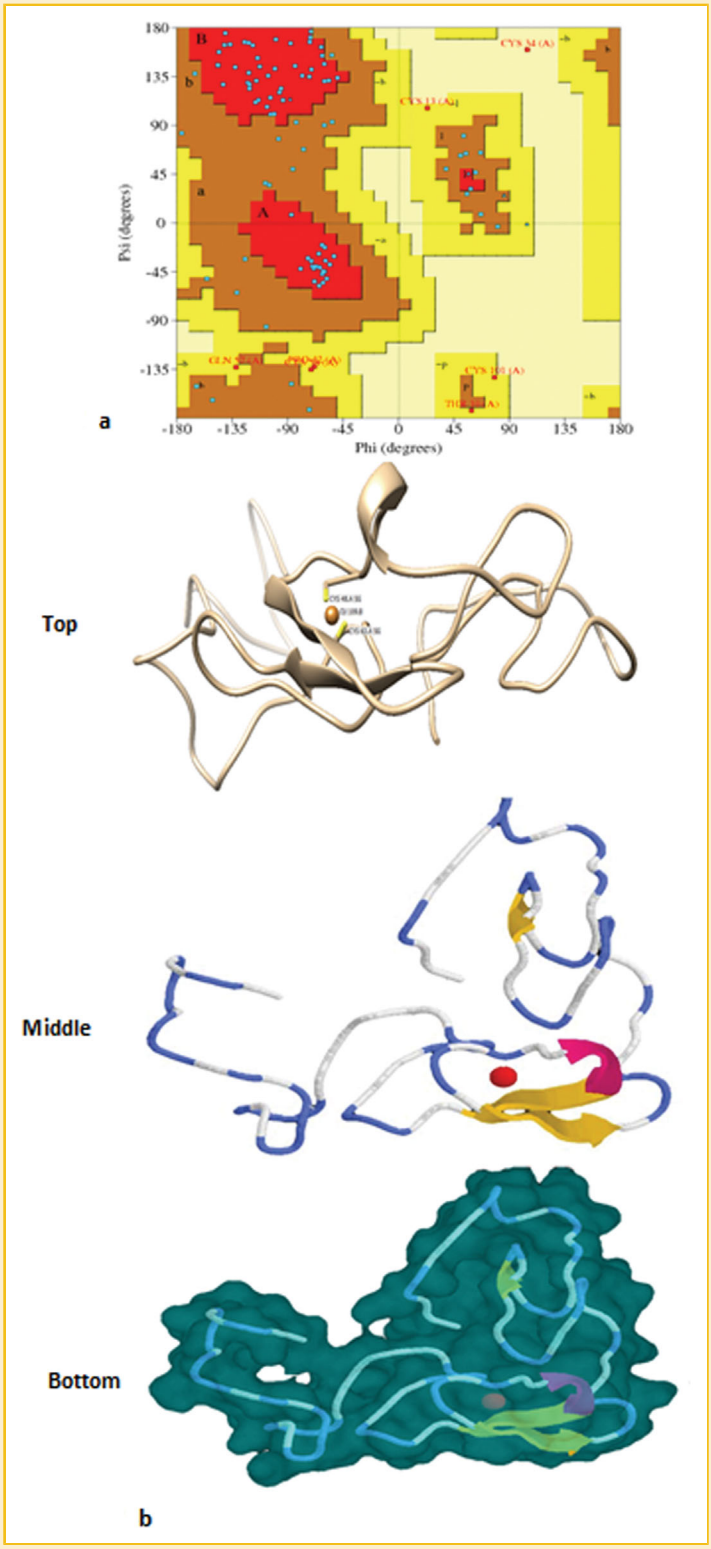

Fig. 5. A: Ramachandran plot of most favourable structure of TfCuMT showing that most residues are present the allowed regions. B: Three dimensional structure of TfCuMT showing highest number of strucutural elements. Addition of copper showing its chelation with Cystein residues present at 48 and 63 peptide postion (top, centre). painting of polypeptide backbone shows the copper ions are embaded into proein folds (bottom).

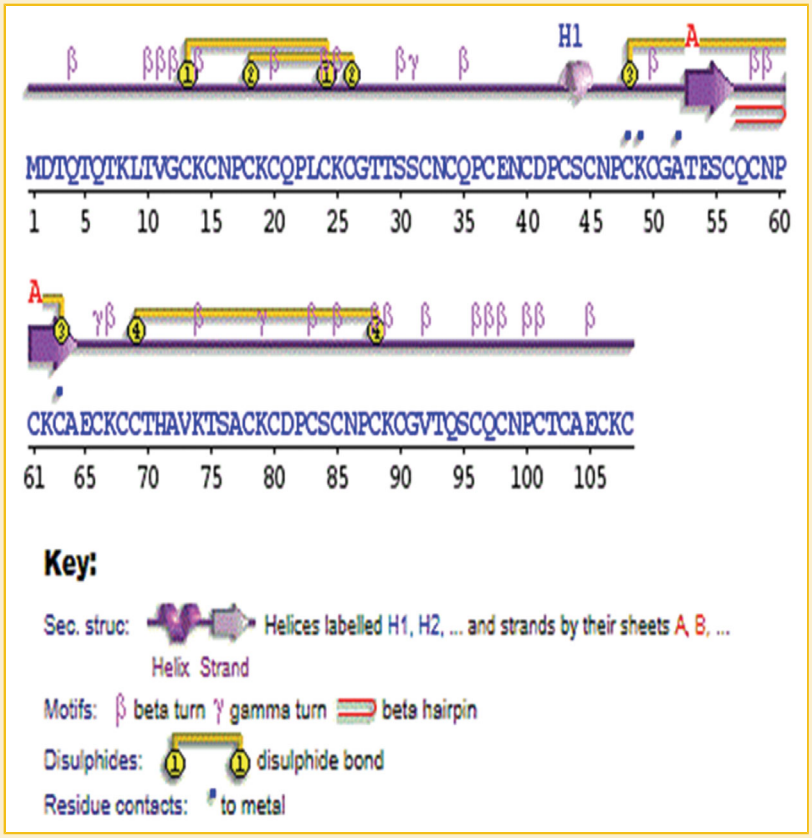

Fig. 6. Structural analysis of of TfCuMT using PDBsum. β turns appear to be the most frequent secondry structural elements. Four disulphide bonds are also present between Cys_{13}, Cys_{24}; Cys_{18}, Cys_{26}; Cys_{48}, Cys_{66}; and Cys_{69}, Cys_{88}, respectively.

EXPRESSION AND PURIFICATION OF TfCuMT

E. coli BL21C^{+} host cells were transformed with mutated gene cloned in pET21a expression vector for expression purpose. Very low expression of *Tf*CuMT was observed. Shuja et al. [2013] have reported no detectable expression of CdMT of *T. tropicalis lahorensis* using pET21a expression system. The lower level of expression may be due to instability of apoproteins and susceptibility to metal ions [Durnam and Palmiter, 1981]. *Tf*CuMT was cloned in pET28a to facilitate the purification of protein. Expressioon in pET28a also resulted twofold increase in expression as compared to pET21a expression vector. Shuja et al. [2013] have reported no detectable expression of CdMT of *T. tropicalis lahorensis* using pET21a expression system. The lower level of expression may be due to instability of apoproteins and susceptibility to metal ions [Durnam and Palmiter, 1981]. Probably protein expressed in pET28a was stabilized due to N-terminal His tag. These results are better in comparison with diffused expression of CdMT in pET28a by Shuja et al. [2013]. The yield of proteins is increased when these are expressed as fusion proteins to reduce the proteolytic attack by proteases [Mbikay et al., 1981].

The availability of cysteine residue for synthesis of MT is another limiting factor for the expression of MT. *Tf*CuMT is a cysteine rich protein, while cysteine is a low abundance amino acid in *E. coli*. A gradual increase in the expression level was observed by increasing an input of cysteine concentration in the medium. This shows that cysteine acts as a limiting factor for expression of *Tetrahyemena* copper MT in *E. coli* BL21C^{+}.

*Tf*CuMT showed optimum expression after 6–8 hr of IPTG induction. Optimum expression was obtained at 0.1 mM IPTG induction further increase in IPTG conc. did not show any considerable increase in expression level.

pET28a does not contain any copper inducible promoter; however, an increased expression level was observed in the presence of 1 μM copper. This increase in expression is probably due to chelation of copper ions with *Tetrahymena* copper MT, resulting a reduced degradation of expressed protein. Shuja et al. [2013] have also reported stabilized expression of tagged fusion protein in the presence of 0.4 mM Cd^{2+}.

N-terminal His tag of recombinant *Tf*CuMT is also a primary requirement for single step purification using Ni affinity column chromatography. NTN resin buffered with 6 M GnHCl resulted in elusion of purified His tagged *Tf*CuMT using 0.1 M imidazole and 0.1 M NaCl. His_6-MTT2 of *T. thermophila* was successfully expressed in *E. coli* and purified using Ni-NTA [Wang et al., 2011]. Fusion proteins have also been reported to be purified through affinity chromatography using intein tag or by Q column chromatography [Hong et al., 2001; Yu et al., 2002]. *Tetrahymena* cadmium MTs of *T. pyriformis* and *T. tropicalis lahorensis* have been successfully

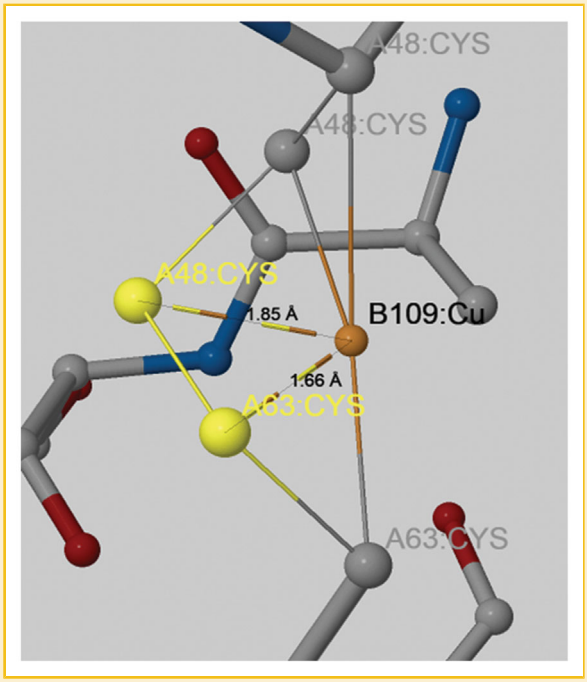

Fig. 7. *Tetrahymena farahensis* CuMt complex confirm the potential disulfide linkage between Cys 48 and Cys 63 using CheckMyMetal web server. Copper ion making bonds with sulfur as well as carbon atoms of both Cysteine residues [Zheng et al., 2014].

expressed and purified as fusion proteins [Domenech et al., 2008; Shuja et al., 2013].

CONCLUSION

A new copper metallothionein (*TfCuMT*) gene isolated from a locally isolated ciliate *Tetrahymena farahensis* contains 327 nucleotides encoding a peptide chain of 108 amino acids. It is intronless and contains about 30% cysteine residues with the specific CKC motifs. Structural repeats present in peptide sequence of *Tf*CuMT indicate internal duplication of gene at some stage of gene evolution. The predicted model of copper metallothionein protein showed that copper ions are mainly chelated by thiol sulfur of cysteine residues and are embedded in the folds of polypeptide chain. For in vivo expression of *TfCuMT* in *E. coli* host the classical stop codons were replaced with Gln, which showed higher expression in pET28a than in pET21a. Optimum expression was obtained after 6–8 h of 0.1 mM IPTG induction. The modeled structure of the TfCuMT showed a cleft for Cu binding with correct orientation of Cys residues in the motif CKC.

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