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Evaluation of Physiological Importance of Metallothionein Protein Expressed by *Tetrahymena cadmium metallothionein 1 (TMCd1)* Gene in *Escherichia coli*

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ABSTRACT

TMCd1 is a cadmium inducible metallothionein (MT) gene. In the present study the *TMCd1* gene of a ciliate protozoan has been expressed in *E. coli* and the function of the expressed TMCd1 protein as a metal-binding protein has been evaluated. The growth of *E. coli* cells expressing the GST fused TMCd1 proteins in the presence of cadmium metal clearly demonstrated the role of TMCd1 as a metal-binding protein. The metal accumulation experiments showed that the bacterial cells expressing the functional TMCd1 protein accumulated 19-fold more cadmium in contrast to control cells that lacked the TMCd1 protein expression. The results clearly demonstrate a physiological role of full length TMCd1 protein of a ciliate, expressed in *E. coli,* in cadmium metal sequestration and detoxification. J. Cell. Biochem. 113: 1616–1622, 2012.

KEY WORDS: METALLOTHIONEIN; HEAVY METALS; CADMIUM RESISTANCE GENE; *Tetrahymena*; CADMIUM; METAL UP-TAKE; SITE-DIRECTED MUTAGENESIS

Metallothionein (MT) is a low molecular weight and cystein-rich protein which is ubiquitously distributed in plants, animals, eukaryotic, and some prokaryotic microorganisms. It is a metal-binding protein having strong binding affinity for transition metal ions (Zn, Cd, or Cu) mainly of group 2a and 2b [Miles et al., 2000]. MTs are devoid of aromatic residues and on binding to metals they form metal thiolate clusters [Klaassen et al., 1999; Isart and Vasak, 2002]. MT genes can be induced upon metal binding such as copper, cadmium, mercury, zinc, and silver.

MTs, because of their metal-binding properties, are involved in storage and transportation of essential heavy metals (copper and Zinc) and also in detoxification of non-essential metals (for example, cadmium and mercury) [Coyle et al., 2002; Isart and Vasak, 2002]. Many MTs have been expressed in *E. coli* ranging from human MT-II [Odawara et al., 1995] to yeast MT [Sayers et al., 1993] to clarify their role in metal detoxification. However, to date, only one ciliate MT (TpyMT1) has been expressed and purified [Domenech et al., 2008]. *TMCd1* is a ciliate MT gene which was previously isolated from a ciliate *Tetrahymena tropicalis lahorensis* by Shuja and Shakoori [2007]. In 2009, Shuja and Shakoori isolated another cadmium-resistant gene—*PMCd1* in *Paramecium* sp. These MT genes were induced under cadmium metal stress which indicated that they play a role in cadmium detoxification [Shuja and Shakoori, 2009]. In order to determine the cadmium resistance and cadmium sequestration ability of TMCd1 protein, we expressed the full length TMCd1 protein in *E. coli* cells as a fusion to glutathione-S-transferase and determined the physiological importance of the expressed protein. This study is novel since it proves functional importance of full length TMCd1 protein, as evidenced by accumulation of cadmium by the cells expressing this protein.

MATERIALS AND METHODS

SITE DIRECTED MUTAGENESIS

The *TMCd1* cDNA (471 bp) cloned in pTZ57/RT vector was kindly donated by Dr. Rukhsana Shuja (School of Biological Sciences, University of the Punjab, Lahore). For subcloning purpose the *TMCd1* gene was PCR amplified to introduce the restriction sites for *Nco*I (Fermentas #ER0571) and *Hin*dIII (Fermentas #ER0501) at 5′ and 3′ ends of the gene, respectively. The PCR product was purified from 1% agarose gel and ligated to pTZ57R/T using T4 DNA ligase (Fermentas Ligation kit #K1214). In order to express the full length functional TMCd1 protein in *E. coli*, a point mutation was done to substitute the stop codon TAA (a universal codon for Gln in *Tetrahymena* gene) with the codon CAA at position 116 of the gene.

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The site directed mutagenesis was done as described by Laible and Boonrod [2009] using the primers.

TMF (5′-AACCTGTTCGCTGGATGCTGTTG-3′) and TMR (5′-CAACAGCATAGCTTGAACAGGTT-3′) were complementary to each other and contained the desired mutation in the middle of their sequences. For thermocycling reaction, the whole construct was initially denatured at 94°C for 5 min. The recurring denaturation phase was set to 40 s at 94°C. The annealing temperature for the complementary mutagenic primers was set at 56°C for 25 s. For elongation, *pfu* polymerase (Fermentas #EP0501) was used and the elongation (25 cycles) was done at 72°C for 6 min. The reaction was stopped by heating the reaction mixture at 72°C for 15 min. The PCR product was then digested with *Dpn*I (Fermentas # ER1701) for 1 h at 37°C, and *E. coli* DH5α cells were transformed with this PCR product according to Sambrook and Russel [2001]. Plasmid was isolated from a single colony and the mutation was confirmed by DNA sequencing using ABI 3730 DNA genetic analyzer. The *TMCd1* gene was subcloned in pET-41a expression vector (Novagen #70559) using *Nco*I (Fermentas #ER0571) and *Hin*dIII (Fermentas #ER0501) for stable expression in *E. coli* BL21 DE3-RIL cells. The TMCd1 protein was expressed as a fusion protein with glutathione-S-transferase (GST).

PROTEIN EXPRESSION AND PURIFICATION

For expression analysis, the *E. coli* BL21 CodonPlus (DE3)-RIL cells were transformed with recombinant pET-41a plasmid. LB broth (20 ml) containing 30 μg/ml kanamycin in different flasks were inoculated with overnight grown culture of transformed cells. When the cells reached the mid logarithmic phase ($OD_{600} = 0.7$), the protein expression was induced by the addition of 0.5 mM lactose. After an hour of shaking incubation at 37°C, different concentrations of cadmium chloride (50–500 μM) were added to the cultures. Cultures were further grown for 11 h at 37°C. The cells were harvested by centrifugation at 7000*g* at 4°C and the expression pattern was analyzed on 12% SDS–PAGE.

For purification of recombinant protein, the cell pellet, obtained from 1 L of induced culture, was suspended in ice cold 1× GST binding/washing buffer (pH 7.5), and broken with brief pulses of sonication on ice in the presence of 2 mM PMSF, 0.4% V/V β-mercaptoethanol, and 0.5% V/V Triton X-100. The cell lysate was centrifuged at 6000*g* for 20 min. The filtered supernatant was then loaded onto 5 ml of GST-bound resin (Novagen #70541-3) that was pre-equilibrated with 1× GST binding/washing buffer. The unbound protein fractions were removed by washing the resin with 10 volumes of GST binding/washing buffer. The recombinant fusion protein (GST-TMCd1) was eluted with 10 ml of freshly prepared elution buffer (100 mg reduced glutathione in 50 mM Tris-Cl, pH 8). The protein fractions were analyzed on 12% SDS–PAGE and the fractions having high concentration of eluted fusion protein were collected.

COMPUTATIONAL ANALYSIS

The amino acid sequence of full length TMCd1 protein was deduced from the sequence of *TMCd1* gene by using Expasy Proteomic Translation Tool. Hydropathy plot was prepared by using Kyte and Doolittle [1982] hydropathy plot. Homology based three dimensional structure of the protein was predicted by I-Tasser Server [Zhang, 2008].

EFFECT OF Cd^{2+} ON THE GROWTH OF *E. coli* EXPRESSING *TMCd1* GENE

The bacterial cultures, both with and without insert (*TMCd1* gene), were grown in LB medium containing 30 μg/ml kanamycin. At $OD_{600} = 0.7$, 0.5 mM lactose was added to the culture and after an hour $CdCl_2$ was added to various final concentrations (0–1000 μM). Cultures were further grown for 11 h, before the final cell densities were measured at OD_{600}. In another experiment the overnight grown cultures of control and test samples (as mentioned above) were used to inoculate 10 ml fresh LB medium supplemented with 250 μM $CdCl_2$. Cultures were induced with 0.5 mM lactose when the OD_{600} of the cultures reached a value of 0.6. The culture was further incubated at 37°C, and the cell densities measured at OD_{600} at various time intervals.

CADMIUM ACCUMULATION

The *E. coli* cells, harbouring pET-*TMCd1*, were grown overnight in LB medium containing 30 μg/ml kanamycin. Different flasks containing 20 ml LB broth were inoculated with this overnight culture. The cells were induced with 0.5 mM lactose and incubated at 37°C in a shaker till OD_{600} of 0.7. After an hour, different concentrations (50–500 μM) of cadmium chloride were added to cultures. Control cultures containing *E. coli* cells transformed with only pET-41a vector (without insert) were also treated in the same way as that of test cultures (pET-*TMCd1*). After 12 h of induction equivalent numbers of cells ($OD_{600} = 1$) were pelleted by centrifugation at 7000*g* for 5 min and the pellets were washed three times with 20 mM Tris-Cl (pH 7.5). To prepare the samples for atomic absorption spectroscopy, the pelleted cells were mineralized overnight for 24 h with 50 μl of 70% nitric acid at 50°C. Digested pellets were centrifuged in order to remove any insoluble product. Demineralized/deionized water (total volume 1 ml) was then added to mineralized product. Cadmium concentrations in the digested pellets were determined by atomic absorption spectrometer at a wavelength of 228.8 nm using air-acetylene flame. Standard curve was prepared by taking different known concentrations of cadmium chloride. The readings obtained were converted to amount of Cd^{2+} in ppm per 12×10^8 cells (1.5 ml) (on the basis of assumption that $OD_{600} = 1$ is equivalent to 8×10^8 cells). Student's *t*-test was applied using statistic software SPSS (version 13) to determine the significance of difference between control and test samples and the *P*-value from the *t*-test was calculated.

RESULTS

MUTATED *TMCd1* GENE

The mutated *TMCd 1* gene was cloned in *E. coli* DH5α using pTZ57R/T vector, and then sequenced with ABI DNA genetic analyzer, using MF-13 and MR-13 primers. The sequence alignment (Fig. 1) showed that a silent mutation TAA→CAA was successfully introduced in the gene.

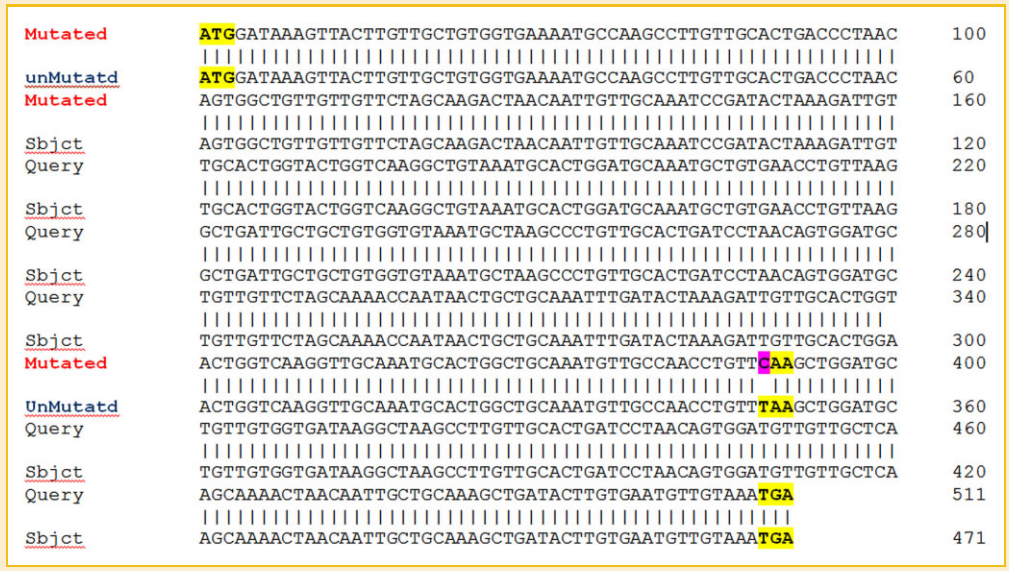

Fig. 1. Alignment of mutated sequence of *TMCD1* with the non-mutated sequence of the gene. The mutated region, initiation codon, and termination codons have been highlighted in yellow. Mutated base in the stop codon within the gene is shown in pink colour. Query, mutated *TMCd1* gene; sbjct, non-mutated *TMCd1* gene. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

PROTEIN EXPRESSION AND PURIFICATION

The colonies of *E. coli* BL21 CodonPlus (DE3)-RIL transformed with recombinant pET-41a containing full length mutated *TMCd1* gene, induced with different concentrations of lactose (0.1–0.7 mM) showed remarkable increase in protein expression (Fig. 2, which shows lactose concentration of 0.3–0.7 mM), compared with different concentrations of IPTG (0.1–0.7 mM) (Figure not given). In both these treatments 100 μM of $CdCl_2$ was added to maintain the stability of –SH groups. The maximum expression was observed at 0.5 mM lactose. Upon induction with lactose the GST fusion TMCd1 protein was seen as a 48 KDa band on coomassie brilliant blue stained 12% SDS gel. The molecular weight of the expressed protein was almost the same as that of theoretically calculated molecular weight of fusion protein. Densitometry of stained gels showed that the protein was expressed to a level of approximately 27% of the total cellular proteins.

Effect of cadmium metal on the expression of full length TMCd1 protein was analyzed by adding different concentrations of cadmium chloride (50 μM–700 μM). Increasing cadmium concentration increased the concentration of expressed protein due to the

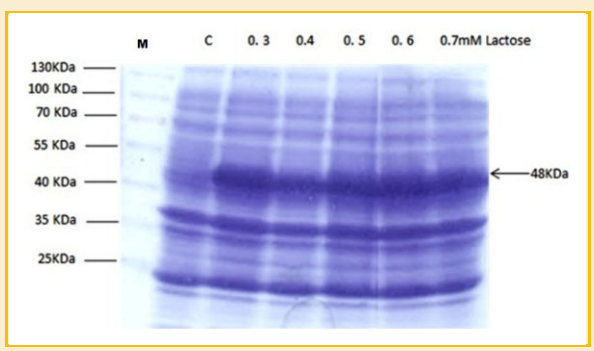

Fig. 2. Expression of GST-tagged TMCd1 protein after induction with 0.3, 0.4, 0.5, 0.6, and 0.7 mM lactose. One hundred micromolar of $CdCl_2$ was added to maintain the stability of –SH linkages. Lane C, un-induced control; Lane M, 170 kDa pre-stained molecular weight markers (Fermentas cat# SM0672). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

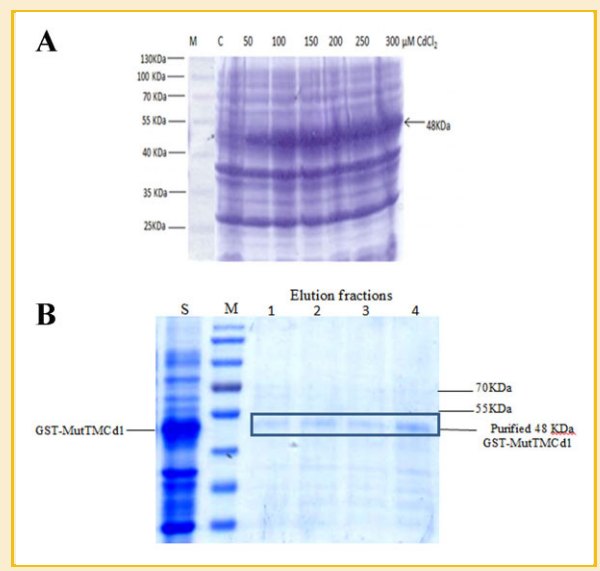

Fig. 3. A: Expression of MutTMCd1 protein in the presence of cadmium chloride. Lane 2, negative control (induced pET-41a without insert). Lanes 3–5, expression of TMCd1 protein in presence of different concentrations of cadmium chloride; M, 170 KDa pre-stained protein ladder (Fermentas # SM0672); (B) purified TMCd1 protein. Lane S, supernatant obtained after sonication. Lane M, prestained protein ladder. Lane P, 48 KDa GST-TMCd1 protein eluted by 100 mM glutathione. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

stabilization of expressed protein upon binding to metal ions. Maximum expression of protein was observed when $CdCl_2$ in the range of 100–300 μM was added to the induced cultures (Fig. 3A). The expression of the TMCd1 protein was reduced when the final concentration of cadmium salt was increased beyond 300 μM.

Solubility analysis of TMCd1 protein revealed that more than 70% of the protein was in soluble form. The soluble protein obtained after sonication of pellet was purified, as mentioned in Materials and Methods section, using GST bound resin. Analysis of coomassie brilliant blue stained SDS gel indicated that TMCd1 protein was 18% of the total protein in crude extract, whereas the purified protein was approximately 60% pure and major impurities or other proteins expressed in bacteria were removed during the washing of resin (Fig. 3B).

TMCd1 PROTEIN

Translated sequence of full length TMCd1 protein indicates that it is composed of 156 amino acids and is rich in cystein content. It contains 47 cystein residues which make 30.13% of the total residues of the protein (Fig. 4).

Hydropathy plot [Kyte and Doolittle, 1982] of the GST-TMCd1 showed that the protein contains more amino acids in hydrophilic region than the hydrophobic region (Fig. 4C).

The homology based three-dimensional (3D) structure of TMCd1 protein was predicted by ITasser protein prediction software [Zhang, 2008] and modulated on PyMOL protein modeling software (Fig. 5). Theoretically considering that all Cys residues in MTs are involved in metal binding, the stoichiometry for CD-MTs is Cd7(Cys)20 [Klaassen et al., 1999]. Assuming that this condition exists in *Tetrahymena* TMCd1 protein containing 47 cysteins is predicted to bind 16 Cd.

RESISTANCE TO CADMIUM METAL IONS

E. coli BL21 DE3-RIL cells with and without *TMCd1* were grown in LB medium containing various concentrations of $CdCl_2$. The OD_{600} values of the *E. coli* cells transformed with pET-*TMCd1* were quite significantly high at *P*-value 0.05 as compared with *E. coli* cells without *TMCd1* gene. pET-*TMCd1* transformed *E. coli* cells were able to tolerate $CdCl_2$ to a concentration of 900 μM. In contrast, the *E. coli* cells without *TMCd1* (control) were unable to tolerate $CdCl_2$ above 300 μM and the OD_{600} values significantly fell after 200 μM (Fig. 6A). Likewise the OD_{600} values at various time intervals for the *E. coli* pET-*TMCd1* cultures grown in LB medium supplemented with 250 μM $CdCl_2$ was almost double compared to the control cells without *TMCD1* gene (significance at P 0.05) (Fig. 6B).

A

Residue name	Count	% age in protein
Ala A	6	3.8%
Arg R	0	0%
Asn N	11	7%
Asp D	11	7%
Cys C	47	30.13%
Glu E	3	1.92%
Gln Q	4	2.6%
Gly G	15	9.6%
His H	0	0%
Ile I	0	0%
Leu L	0	0%
Lys K	19	12.2%
Met M	1	0.64%
Phe F	1	0.64%
Pro P	8	5.1%
Ser S	10	6.4%
Thr T	16	10.25%
Trp W	0	0%
Tyr Y	0	0%
Val V	4	2.6%

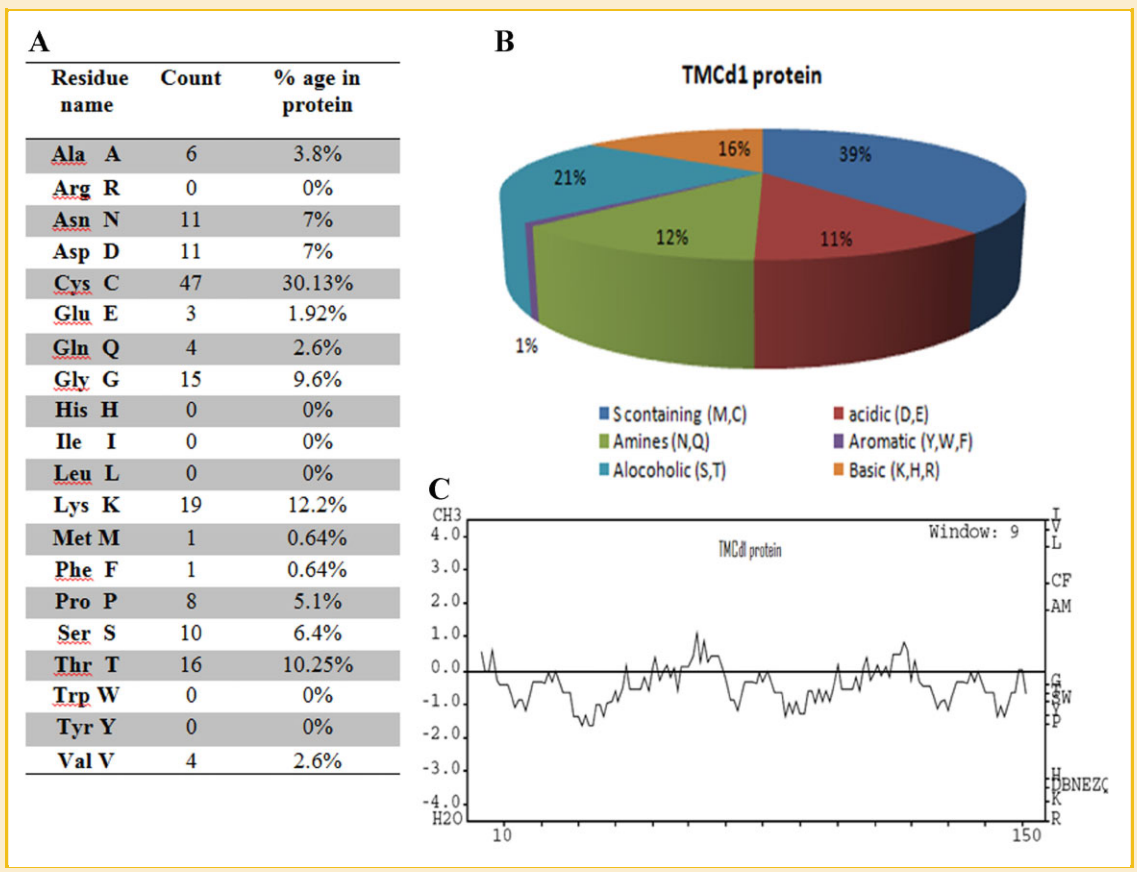

Fig. 4. A: Amino acid composition of full length TMCd1 protein. B: 3D Pie chart showing contribution of each group of amino acids in the TMCd1 protein. C: Hydropathy plot (window size 9) of TMCd1 protein. Y-axis represents hydrophobicity score and X-axis represents the position of amino acids in protein sequence. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Fig. 5. Homology-based three-dimensional predicted model of full length TMCd1 protein (grey colour) displaying the distribution and position of cysteine residues

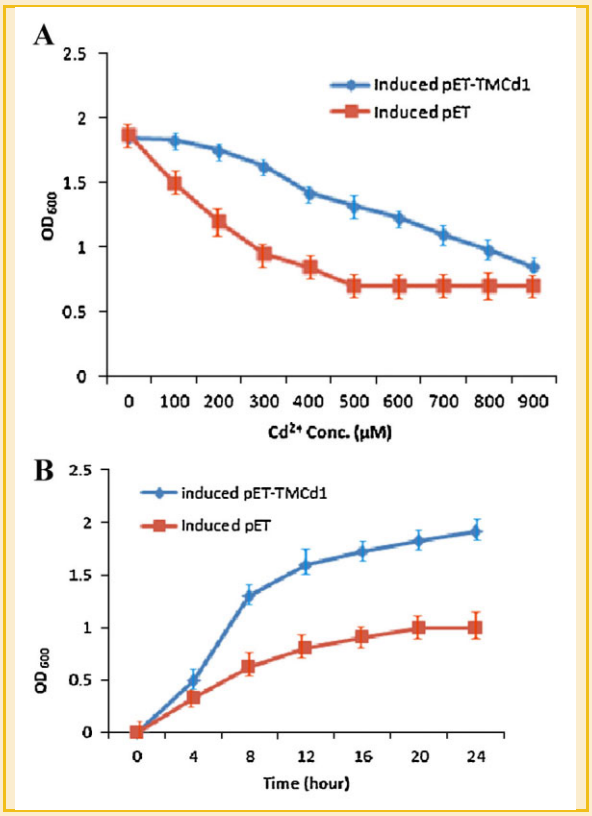

Fig. 6. A: Effect of increasing Cd^{2+} concentrations on the growth of *E. coli* cultures supplemented with various concentrations of $CdCl_2$. All values are an average of three independent experiments. B: Growth of *E. coli* test (induced pET-*TMCd1*) and control (induced pET) cultures in the presence of 250 μM Cd^{2+}. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

ACCUMULATION OF CADMIUM BY THE CELL

In order to determine the bioaccumulation of Cd^{2+} ions by *E. coli* BL21 DE3-RIL cells containing pET-41a-*TMCd1* plasmid, different concentrations of metals were added to the test and control media. The *E. coli* BL21 DE3-RIL cells expressing TMCd1 protein accumulated Cd^{2+} ions effectively at different Cd^{2+} concentrations (50–300 μM). The control cells that lack the TMCd1 protein did not show any significant accumulation of metal (Table I). Furthermore, the cells grown under high cadmium concentrations (above 300 μM) had no significant bioaccumulation of Cd^{2+} (data not shown).

The highest bioaccumulation level was 5.37ppm/12×10^8 cells or 7.9 μM/mg of cell wet weight (Table I). The comparison of test and control samples showed that the test cultures accumulated maximum of 19 fold more Cd^{2+} in contrast to control cells. The results were significant at *P*-value 0.01.

DISCUSSION

Metallothioneins (MT) are cystein-rich proteins and their role in metal homeostasis and sequestration is well documented by many scientists [Coyle et al., 2002]. In present study *TMCd1* encoded full length MT protein was expressed successfully in *E. coli* and role of this MT in conferring resistance to transformed *E. coli* cells to cadmium stress was found.

As a first step of this study the *TMCd1* gene was mutated by site-directed mutagenesis at position 116 in order to get expression of full length protein. The sequencing and BLAST results of mutated *TMCd1* gene confirmed the mutagenesis. There are many complications concerning the expression of MTs intracellularly. The high cysteine content of MTs interferes with the redox pathways

TABLE I. Cadmium Accumulation by Transformed *E. coli* Cells.

Initial cone (μM) of Cd^{2-} in the medium	Cd^{2-} (ppm) accumulated/6 mg (cell wet weight) by culture (12×10^8 cells)	Cd^{2-} (ppm) accumulated/6 mg (cell wet weight) by treated culture (12×10^8 cells)	Fold increase with reference to control cells
0	0	0	0
50	0.23 ± 0.035	$2 \pm 0.88^*$	8.7-fold
100	0.28 ± 0.021	3.25 ± 0.71	13-fold
150	0.25 ± 0.106	4.26 ± 0.81	17-fold
200	0.35 ± 0.098	5.37 ± 0.31	15.3-fold
250	0.26 ± 0.042	4.95 ± 0.53	19-fold
300	0.25 ± 0.043	4.2 ± 0.53	16.8-fold

*$\pm$Standard deviation of mean values.

inside the cytosol of the cell [Raina and Missiakas, 1997; Mejare and Bulow, 2001]. In order to circumvent the problem of instability of expressed protein, TMCd1 protein was expressed with GST tag at its N-terminal end. As a result, the expression of protein was greatly enhanced and the high level of protein expression was observed. Experimental analysis showed that about 70% of the TMCd1 protein was expressed in soluble form. MTs are more stable and less prone to proteolytic cleavage in metal bound state as compared to unbound state. Therefore, in order to stabilize the three-dimensional structure of protein various concentrations of cadmium metal were used. The most stable expression of TMCd1 protein was achieved when the cadmium in a range of 100–300 μM was added to the cell cultures. When the metal concentration was increased above 300 μM the expression of protein was decreased because too much metal content becomes toxic for cell and might interfere with protein expression. The purified TMCd1 protein was of the same size (48 KDa) as theoretically calculated size of full length TMCd1 protein.

The high metal-binding property of MTs has widely been exploited in order to enhance the tolerance and accumulation or sequestration of metals by bacteria. Chen and Wilson [1997] overexpressed a pea MT in *E. coli* cells and bioaccumulation experiments on the transformed bacterial cells revealed that the cells were able to tolerate and accumulate low concentrations of mercury metal. When *E. coli* BL21 (DE3)-RIL cells transformed with pET41a-*TMCd1* were tested for the ability to tolerate cadmium metal, a remarkable difference was noted between the pET-41a-*TMCd1* transformed cells expressing TMCd1 protein and the cells transformed with only pET-41a vector, as depicted by OD_{600} values. The *E. coli* cells that lack the protein were unable to grow under high metal stress. The results clearly indicated that expression of TMCd1 MT protein confers resistance to Cd^{2+} in transformed *E. coli* cells.

The bioaccumulation experiments revealed that the *E. coli* cells expressing TMCd1 protein accumulated the cadmium to a great extent. The cells expressing TMCd1 protein accumulated Cd^{2+} as high as 5.37ppm/12×10^8 cells or 7.9 μM/mg of cell wet weight. The induced *TMCd1* transformed *E. coli* cells accumulated Cd^{2+} 19-fold higher than the control cells that lack *TMCd1* gene. The results were significant as depicted by the *P*-value (0.01) obtained by Student's *t*-test. The results suggest that the expressed TMCd1 protein is in functional form and is able to sequester Cd^{2+} metal inside the *E. coli* cells by formation of metal thiolate clusters. The results were also in agreement with previous findings that the induced *TMCd1* gene was involved in Cd^{2+} metal detoxification [Shuja and Shakoori, 2007].

In this study the MT protein was expressed to prove its functional importance as depicted by Cd accumulation by cells expressing the TMCd1 protein. Without adding metal the protein could not be stably expressed although the high metal content became toxic for cells and interfered with expression. The expression and cadmium accumulation experiments provide the proof for physiological significance of expressed TMCd1 protein.

CONCLUSIONS

In conclusion, TMCd1 MT protein was expressed efficiently and stably in *E. coli* cells. The growth and accumulation experiments performed under Cd^{2+} pressure clearly implied a role for TMCd1 protein in protecting and conferring the resistance to transformed *E. coli* cells to higher concentrations of external cadmium metal ions. The expression and cadmium accumulation experiments provide the proof for physiological significance of expressed TMCd1 protein.

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