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MINIREVIEW

Ciliate metallothioneins: unique microbial eukaryotic heavy-metal-binder molecules

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Abstract This article represents an updated review of ciliate metallothioneins (*Tetrahymena* species) including a comparative analysis with regard to well-known metallothioneins (MTs) from other organisms and discussion of their exclusive features. It opens with an introduction to ciliates, summarizing the main characteristics of these eukaryotic microorganisms and their use as cellular models to study metallothioneins and metal–eukaryotic cell interactions. It has been experimentally proved that at least three different metal resistance mechanisms exist in ciliates, of which bioaccumulation is the most studied. Structural comparative analysis reveals that *Tetrahymena* MTs have unique characteristics, such as longer length, a considerably higher cysteine content, different metal–MT stoichiometry values, the presence of new cysteine clusters, and a strictly conserved modular–submodular structure. Gene expression analysis reveals a multistress and differential response to diverse metals and other environmental stressors, which corroborates the classification of these MTs. An in silico analysis of the promoter sequences of some MT genes reveals the presence of conserved motifs that are probably involved in gene expression regulation. We also discuss the great advantages of the first ciliate whole-cell biosensors based on MT promoters from *Tetrahymena thermophila* to detect heavy metal ions in environmental samples.

Keywords Ciliated protozoa · Heavy metal bioaccumulation · Metallothioneins · Structural features · Gene expression

Abbreviations

cDNA	Complementary DNA
LC_{50}	Lethal concentration killing 50% of the cell population
MT	Metallothionein
qRT-PCR	Quantitative reverse transcription PCR
WCB	Whole-cell biosensor

Introduction (ciliates as microbial eukaryotic models)

Ciliated protozoa emerged about 10^9 years ago (Early Cambrian) before fungi and other eukaryotic models. Ciliates are one of three major evolutionary lineages that make up the alveolates, the other two lineages being dinoflagellates and parasitic apicomplexa. Ciliated protozoa are free-living eukaryotic microorganisms with a cosmopolitan distribution, adapted for living in both terrestrial and aquatic ecosystems. They are believed to be important grazers of bacteria and other microorganisms and this activity appears to stimulate the rates of carbon and nitrogen cycling in soils [1]. The absence of a cell wall in the vegetative stage of these eukaryotic microorganisms offers a higher sensitivity to environmental pollutants than other microorganisms, such as yeasts and bacteria, and therefore a faster cellular response [2–4]. A general feature

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of ciliates is nuclear dimorphism: two types of nuclei (micronucleus and macronucleus) are present in the same cell [5]. The ciliatology has given rise to two main ciliate models, *Tetrahymena thermophila* and *Paramecium tetraurelia*, which owe their importance to their "domestication" or adaptation to microbial laboratory work: they grow rapidly, giving a high cell density (approximately 10^6 cells/ml) in a variety of media and culture conditions, they are relatively large microorganisms (40–50 μm), which makes their manipulation easier, and they have a typical eukaryotic life cycle and sexual interaction (conjugation), which makes genetic analysis possible. Several good genetic analysis tools (e.g., nullisomic and knockout strains) and molecular genetics tools have been developed in both ciliate models, and the macronuclear genomes have already been sequenced in each model [6, 7]. Ciliates are eukaryotic cells with some metabolic traits that are more similar to those of human cells than yeasts or other eukaryotic microorganisms. After completion of the genome sequencing, both ciliate models show that they share a higher degree of functional conservation with human genes than do other microbial models. This can be seen in the better matching of relevant ciliate coding sequences to those in humans compared with nonciliate microbial models, e.g., humans and *T. thermophila* share more orthologs with each other (2,280) than are shared between humans and the yeast *Saccharomyces cerevisiae* (2,097) [6]. This is a good reason to use ciliates in ecotoxicology studies as models for humans or as eukaryotic models to analyze cell–metal interactions, representing an alternative to animal tests [2, 4]. Ciliates have contributed to fundamental biological discoveries such as lysosomes and peroxisomes, cilia components, the discovery of the first eukaryotic retrotranscriptase (telomerase), autocatalytic RNA capacity (ribozyme), and the function of histone acetylation in gene expression regulation. Finally, over the last 10 years, ciliates have increased our general knowledge of both metallothioneins (MTs) [8] and their different responses to metal stress [9–11].

Ciliate heavy metal bioaccumulation and metallothioneins

Among the six well-known metal resistance mechanisms present in prokaryotic and eukaryotic organisms, ciliates have at least two main mechanisms [3]: intracellular sequestration (bioaccumulation) of metal ions by binding to proteins (MTs) to prevent metal-sensitive cellular target interactions [12], and extracellular sequestration or biosorption, in which metal cations are passively adsorbed by extracellular polymers, cell walls, or capsules [13]. The latter resistance mechanism is present exclusively in ciliate resting cysts [3]. A rapid biotransformation of arsenate by biomethylation and biovolatilization has very recently been reported in *T. thermophila* [14]. Therefore, a third metal resistance mechanism (biotransformation) is also possible in ciliated protozoa. A fourth potential metal resistance mechanism in ciliates might be an active export, which is supported by (1) the large number (485) of putative genes encoding membrane transporters for inorganic cations in *T. thermophila* [6] and very probably in other ciliates, and (2) the analysis of several expression gene libraries from *T. thermophila* after metal treatments (unpublished data) revealed the presence of about 4.6% complementary DNAs (cDNAs) encoding membrane transporters, indicating the relative importance of these genes in metal detoxification processes. Among ciliates, metal bioaccumulation is the best known resistance mechanism. It involves intracellular sequestration by MTs, forming metal–protein complexes which are accumulated into vacuoles and finally released from the cell as nontoxic metallic complexes. From ciliate toxicological data, metal bioaccumulation seems to be a specific feature of each ciliate strain [9]. In general, the metal bioaccumulation ranking in ciliates is $Zn \gg Cd > Cu$. During ciliate metal bioaccumulation, Cd may displace Zn when both are present in the medium, so ciliates respond in a similar way to pluricellular organisms and mammalian tissues, in which the presence of Cd may cause a cellular Zn deficiency [15]. This competition between Cd and Zn has been detected in several ciliates, such as *Tetrahymena* sp. [16], *Uronema marinum* [17], *Drepanomonas revoluta*, *Euplotes* sp., and *Uronema marinum* [9]. Ciliate metal bioaccumulation has been detected by both transmission electron microscopy [18–20] and fluorescence microscopy [9, 11].

Although metal bioaccumulation has been detected in diverse phylogenetically distant ciliates, the presence of MTs has been reported in only two ciliate genera (*Tetrahymena* and *Paramecium*). With respect to the latter, no experimental data on its gene expression under metal stress have been given, so it should not be considered until expression experiments have been reported. Therefore, we have only considered as ciliate MTs those that are exclusively from different *Tetrahymena* species.

Ciliate MTs have been included in the MT superfamily classification system as family 7, which has been divided into two main subfamilies [8, 21, 22]: subfamily 7a, or CdMTs, and subfamily 7b, or CuMTs. Both subfamilies differ in metal induction patterns (Cd/Zn or Cu) and the pattern of Cys residue clustering [8, 22–26].

Most *Tetrahymena* MT genes were isolated as cDNA molecules and their alignment with the corresponding genomic DNA sequences revealed the absence of introns in all these MT genes. Genes with expression levels that change rapidly in response to environmental stress contain significantly lower intron numbers. The presence of introns can delay regulatory responses and they are selected

against genes with transcripts that require rapid adjustment to survive environmental changes [27]. In general, the gene expression of *Tetrahymena* MT genes is very fast, as reported in a CdMT gene (*MT-1*) from *T. pigmentosa*, in which an approximately tenfold increase in this transcript was detected after 30 min Cd treatment [28].

Structural comparative analysis of *Tetrahymena* metallothioneins

Twelve complete putative CdMT cDNA sequences from different *Tetrahymena* species have been reported and submitted to GenBank: two *T. pyriformis* sequences [abbreviated TpyrMT-1 (AJ005080) and TpyrMT-2 (AY765220)], three *T. thermophila* sequences [TtheMTT1 (DQ517937/EF195745/AY061892), TtheMTT3 (EF195744), and TtheMTT5 (DQ517936)], one *T. tropicalis* sequence (TtroMTT1; EF185997), one *T. rostrata* sequence (TrosMTT1; EU627174), one *T. pigmentosa* sequence (TpigMT-1; EU420056), one *T. vorax* sequence (TvorMT1; HQ166889), one *T. mobilis* sequence (TmobMT1; HQ166888), and two *T. hegewischi* sequences [ThegMT1 (HQ166890) and ThegMT2 (HQ166891)] [22–26, 28–30]. Four complete putative CuMT cDNA sequences from different *Tetrahymena* species have been already reported: two *T. thermophila* sequences [(TtheMTT2 (AY204351) and TtheMTT4 (AY660008)], one *T. pigmentosa* sequence (TpigMT-2; AF479586), and one *T. rostrata* sequence (TrosMTT2; EU627175) [22, 23, 28, 31].

In Table 1, we compare several features of *Tetraymena* MTs with regard to MTs present in most organisms. They are considerably longer than vertebrate MTs. Their lengths fall in the top 1% of the more than 500 nonredundant Cys-rich (more than 25% Cys residues) proteins in GenBank annotated as MT-like, so, likewise, they have molecular masses greater than those of vertebrate MTs (Table 1). They are classified as stable proteins with an estimated half-life of about 30 h. Their average hydropathicity values are −0.54 (CdMTs) and −0.44 (CuMTs) (indicating their hydrophilic character and a higher protein flexibility), which are lower than those for vertebrate MTs (−0.15 as an average value) [32]. A general feature of MTs is the relative scarcity of aromatic amino acids. All *Tetrahymena* CuMTs and some CdMTs (TpyrMT-1, TpyrMT-2, TrosMTT1, TmobMT1, and TvorMT1) lack aromatic amino acids, but other *Tetrahymena* CdMTs are less typical because they contain aromatic residues (Phe > Tyr > Trp). Another infrequent residue in the amino acid sequences of MTs is the His residue. It is generally absent in MTs, as in most *Tetrahymena* MTs. However, as recently reported, His residues, the most frequent Zn ligands in enzymatic sites, are also present (one to four residues) in MTs from a variety of species (bacteria, fungi, plants, and animals), thereby enhancing the relative affinity for Zn in comparison with Cd [33]. Only one *Tetrahymena* CdMT (TtheMTT3) and two CuMTs (TtheMTT2 and TtheMTT4) contain His residues.

Another general feature of MTs (including those from *Tetrahymena*) (Table 1) is the extreme asymmetry in the ratio of positively charged amino acids Lys and Arg. Other peculiar amino acid asymmetry ratios of *Tetrahymena* MTs with regard to vertebrate MTs are the Ile to Leu and the Ser to Thr ratios (Table 1). We do not know the meaning (if any) of these differences.

Cys residues are the main elements in MT composition because they are the chelating points for heavy metals, forming metal–thiolate clusters [34, 35]. Although the average Cys percentage in *Tetrahymena* MTs is within the average Cys percentage of vertebrate MTs, the total amount of *Tetrahymena* MT Cys residues is considerably higher (up to 54 residues) (Table 2) than that in vertebrate MTs (seven to 21 residues). These Cys residues are arranged in clusters, such as XCCX, CXC, and XXCXX (unclustered Cys residues), where X is any amino acid. These are typical clusters in MTs, but possible additional clusters (CCC, CXCC, or CXCXC) have been found in *Tetrahymena* CdMTs (Table 2). CuMTs from this ciliate only have typical MT clusters (Table 2). In *Tetrahymena* CdMTs, about 32% of the total Cys residues are clustered in the CCC motif, which is occasionally found in MTs from other organisms, such as a CdMT from the annelid *Eisenia foetida*, a putative MT from the yeast *Yarrowia* (*Candida*) *lipolytica* (P41928), a CuMT from the arthropod *Callinectes sapidus*, and an MT isoform from the mollusk *Crassostrea virginica* [36–38]. XCCX motifs are also abundant in *Tetrahymena* CdMTs (about 33.8% of the total Cys residues), whereas in CuMTs, Cys residues are mostly

Table 1 Comparison of the main features of *Tetrahymena* metallothionein (MTs) with regard to other (mainly vertebrate) MTs

Feature	Other MTs	*Tetrahymena* CdMTs	*Tetrahymena* CuMTs
Length (amino acids)	25–82	99–191	78–108
Molecular mass (kDa)	2.5–8	10.5–20	8.2–11.2
Cys (%)	16–32	24.2–30.1	28.2–29.6
Aromatic/His	0–2[a]/0–4[a]	0–4/0–2[b]	0/0–1[c]
Lys/Arg	Lys ≫ Arg	Lys ≫ Arg	Lys ≫ Arg
Ile/Leu	Ile ≤ Leu	Ile ≥ Leu	Ile ≤ Leu
Ser/Thr	Ser > Thr	Ser ≤ Thr	Ser < Thr
Metal binding capacity[d]	$7Cd^{2+}$/ $12Cu^{+}$	8–19 Cd^{2+}	13–19 Cu^{+}

[a] Few MTs

[b] Only TtheMTT3 (two residues)

[c] Only two isoforms

[d] These are theoretical values for *Tetrahymena* MTs (see the text)

Table 2 Distribution of Cys cluster types among *Tetrahymena* MTs

Species	CCC	CXCC	CXCXC	XCCX	CXC	XXCXX	Total Cys residues[a]
CdMTs (subfamily 7a)							
TpyrMT-1	4	2	0	5	1	1	31
TpyrMT-2	6	5	0	6	4	1	54
TtheMTT1	6	3	0	8	2	1	48
TtheMTT3	2	2	1	9	3	3	42
TtheMTT5	1	1	0	5	1	6	24
TtroMTT1	6	3	0	8	2	0	47
TrosMTT1	4	2	0	6	2	0	34
TpigMT-1	4	2	0	6	2	0	34
TvorMT1	6	3	0	8	2	1	48
TmobMT1	6	3	0	9	3	0	51
ThegMT1	4	2	0	6	2	0	34
ThegMT2	4	1	0	8	7	4	49
Cys (%)[a]	32	17.5	0.6	33.8	12.5	3.4	
CuMTs (subfamily 7b)							
TtheMTT2	0	0	0	0	15	2	32
TtheMTT4	0	0	0	0	15	2	32
TpigMT-2	0	0	0	1	12	2	28
TrosMTT2	0	0	0	1	9	2	22
Cys (%)[a]	0	0	0	3.5	89.4	7	

A cluster is defined as any group of contiguous residues in which any two Cys residues are separated from one another by, at most, one non-Cys residue (X); every Cys belongs to a single cluster. The numbers refer to Cys cluster counts, except where indicated otherwise

XXCXX unclustered Cys

[a] Numbers refer to Cys residue counts

clustered in the CXC motif (89.4% of the total Cys residues) (Table 2).

Considering that in vertebrate MTs all Cys residues are involved in heavy metal binding and the stoichiometry is therefore $Cd_7(Cys)_{20}$ for CdMTs or $Cu_{12}(Cys)_{20}$ for CuMTs, and assuming that this is also applicable to *Tetrahymena* MTs, we can calculate the theoretical binding capacity of these MTs (Table 1). For instance, TtheMTT1, TvorMT1, and ThegMT2 might bind approximately 17 Cd atoms, TtheMTT3 15 Cd atoms, TtheMTT5 eight Cd atoms, TrosMTT1, TpigMT-1, and ThegMT1 approximately 12 Cd atoms, TtroMTT1 16 Cd atoms, TmobMT1 18 Cd atoms, TpyrMT-2 19 Cd atoms, and TpyrMT-1 11 Cd atoms. Some of these data have been experimentally corroborated: 12 Cd^{2+} per mole of protein or 11 Cd^{2+} per polypeptide for TpyrMT-1 [39, 40]. A stable $Cd_{16}(Cys)_{48}$ complex in vitro has recently been suggested [41] for TtheMTT1 (a value similar to the theoretical value assigned to this CdMT) and $Cd_{11}(Cys)_{32}$ has been suggested for TtheMTT2, with the metal-to-Cys ratio being about 1:3 for both MTs. It was found that Cu^{2+} cannot replace Cd from the Cd_{16}–TtheMTT1 complex, but Cu^{2+} can replace Cd^{2+} from the Cd_{11}–TtheMTT2 complex. These data confirm the classification of TtheMTT1 and TtheMTT2 as CdMT and CuMT, respectively. With regard to *Tetrahymena* CuMTs, TtheMTT2 and TtheMTT4 (which are identical proteins, approximately 99% identity) [23] might bind approximately 19 Cu^{+}, TpigMT-2 16 Cu^{+}, and TrosMTT2 13 Cu^{+}. Although a more intensive analysis of the real metal binding capacity of these ciliate MTs is necessary, we can conclude that the number of metal ions per molecule that these MTs might bind is considerably higher than that of vertebrate MTs (Table 1).

Decimal numbers of Cys residue percentages from all *Tetrahymena* MTs are pure or semipure periodic numbers, indicating the existence of a periodic pattern of these amino acid residues in the protein sequence [8], as has also been detected in most MTs from other organisms. A remarkably regular and hierarchical modular organization has been reported in *Tetrahymena* CdMTs (Fig. 1) [8, 22, 23]. All members of *Tetrahymena* subfamily 7a (CdMTs) are readily and unambiguously divided into segments initially defined by the criterion that every segment carries a CXCCK motif at its C-terminus (with several exceptions) (Fig. 1), denominated as "modules." These range in length from 27 to 56 amino acids and are separated by two to nine amino acid "linkers." From this point of view, TpyrMT-1, TtheMTT5, TrosMTT1, TpigMT-1, and ThegMT1 present

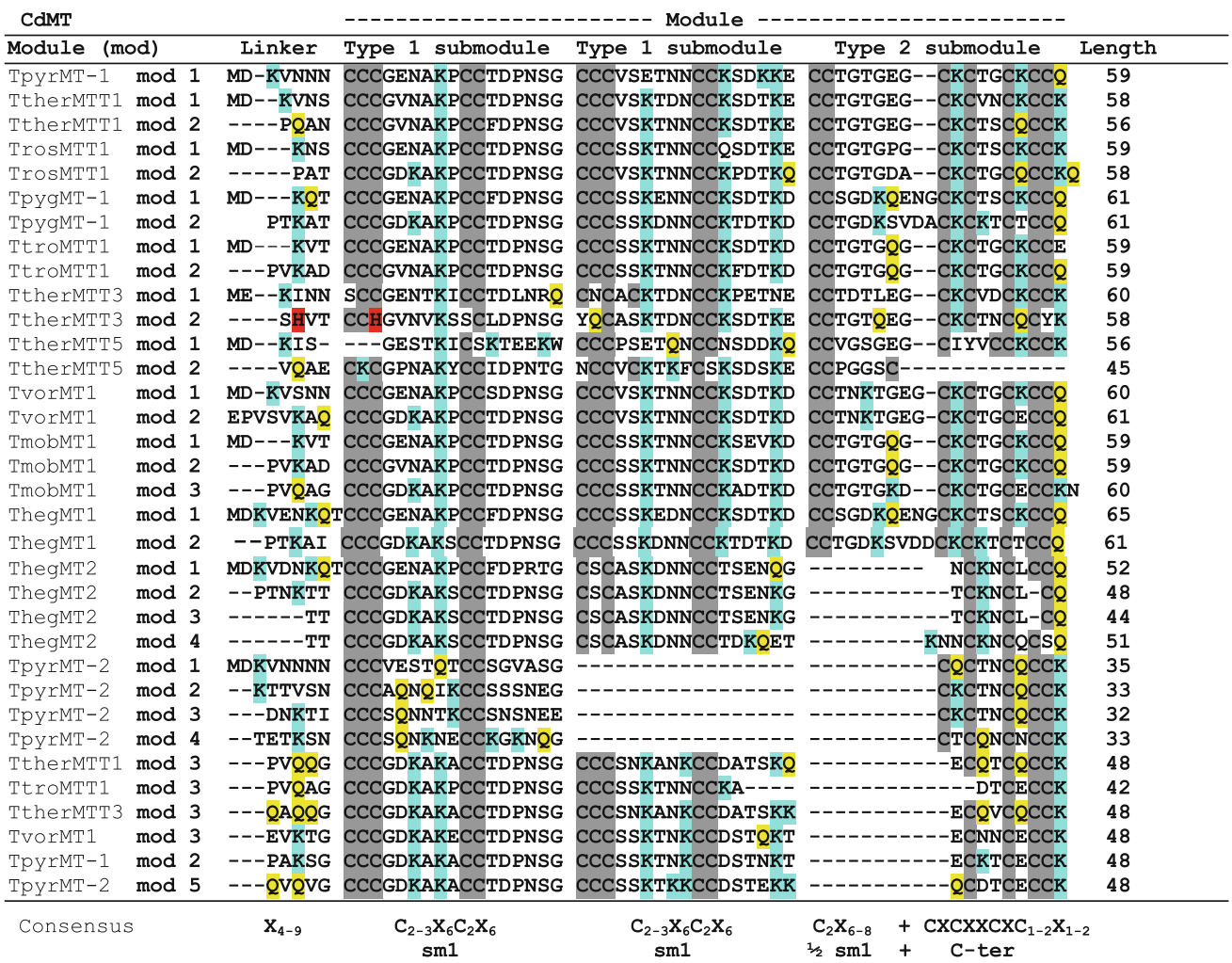

Fig. 1 Modular structure of *Tetrahymena* subfamily 7a (Cd metallothioneins, CdMT). Multiple alignments were made using the T-coffee program, followed by visual inspection and manual adjustment. See the text for further explanation. Areas shaded in *gray* show conserved Cys residues in submodules. Areas shaded in *blue* and *yellow* show Lys (K) or Gln (Q) residues, respectively. His (H) residues are shown by *red shading*. *sm1* type 1 submodule, *C-ter* C-terminus

a bimodular structure, TtheMTT1, TtheMTT3, TtroMTT1, TvorMT1, and TmobMT1 have three modules, ThegMT2 has four modules, and TpyrMT-2 (the longest MT) has five modules (Fig. 1). These modules appear to consist of two types of submodule: type 1 submodules have the consensus sequence $C_{2–3}X_6C_2X_6$ (with few exceptions), whereas the complete type 2 submodules might be represented as $C_2X_{6–8}$ + $CXCXXCXC_{1–2}X_{1–2}$ (approximately half of a type 1 submodule plus the C-terminus) (where the last "X" is K in approximately 48%, Q in 45%, or E/N in 3%). In most cases, these modules are formed by two type 1 submodules and one type 2 submodule (Fig. 1), although several exceptions are observed, such as TpyrMT-2 (which has four modules formed by only one type 1 submodule and half of a type 2 submodule). The second or third module of several CdMTs has two type 1 submodules and half of a type 2 submodule (Fig. 1).

Tetrahymena CuMTs (subfamily 7b) do not have such clear modular structures as CdMTs, but from their alignment a structural organization based on $CKCX_{2–5}CXC$ repeats (where in a few cases Lys may be substituted by other amino acids) might be considered [23].

Another independent structural feature that also differentiates both *Tetrahymena* MT subfamilies is the physical relationship of Lys to Cys residues along the polypeptide backbone. The Lys residues of CuMTs have a strong tendency to be juxtaposed with Cys (CKC), as occurs in mammalian MTs. On the other hand, in subfamily 7a (CdMTs), Cys-juxtaposed Lys residues are more scarce and are concentrated in type 2 submodules. It has been reported that having an adjacent basic residue considerably decreases the p*K* value and consequently the reactivity of Cys residues [42]. Therefore, it is possible that *Tetrahymena* CuMTs may have a metal buffering

capacity that differs from the one theoretically assigned to them.

All these differential structural features of *Tetrahymena* MTs have led to them being divided into two well-characterized subfamilies (7a, or CdMTs, and 7b, or CuMTs) [22]. The proposed phylogenetic tree obtained from the alignments of amino acid sequences [23] corroborates this classification.

Gene duplication is one of the main steps in the generation and evolution of new genes and seems to be the main mechanism involved in MT evolution, as noted by several authors [43–45]. This assumption is easily deduced from the existence of conserved Cys pattern repeats throughout the MT polypeptide molecule. A similar hypothesis has been reported to explain *Tetrahymena* MT evolution [22, 23, 30, 46], and an updated model to explain the possible evolutionary history of *Tetrahymena* CdMTs has recently been reported [8].

Gene expression and multistress response

MTs can be induced by an extensive range of different stimuli or environmental stressors, such as metals, oxidative stress, heat or cold shocks, hormones, cytokines, pH changes, starvation, and a large variety of chemicals or drugs. For this reason, these proteins are considered as multistress proteins. MTs do not seem to be essential proteins, but rather provide survival advantages during times of stress [47, 48].

Likewise, the *Tetrahymena* MT family presents a multistress response because its gene expression can be induced by very different stressors [22–24, 30, 49]. Comparisons of quantitative real-time PCR values obtained for *Tetrahymena* MT genes by various authors is not easy, owing to differences in experimental conditions and/or quantitative reverse transcription PCR (qRT-PCR) protocols. However, general qualitative considerations may be inferred from already published qRT-PCR data (Table 3). In general, the induction values of *Tetrahymena* CdMT genes caused by Cd are higher than those caused by Cu (Cd > Cu), whereas in the only CuMT gene analyzed by qRT-PCR (*TrosMTT2*) to date the average induction value caused by Cu is higher than that caused by Cd (Cu > Cd) (Table 3). Secondly, the ranking of relative fold-induction values can change depending on the duration of metal treatment. When arsenate (As^{5+}) is used, it occupies the second or third place of the fold-induction ranking for *Tetrahymena* CdMTs (Table 3); however, the CuMT gene (*TrosMTT2*) is downregulated after 1 or 24 h treatment. Thirdly, Pb induces the expression of *Tetrahymena* MT genes and occupies first or third place in the relative fold-induction ranking. Several *Tetrahymena* CdMT genes achieve their highest expression levels under Pb treatment, such as *TtheMTT5* and *TrosMTT1* [22, 23]. MTs are induced by Pb in rats, humans, and fishes [50–52]. Pb is second to Cd in its ability to displace Zn from hepatic ZnMT and can displace Cd from the CdMT complex [53, 54]. In plants, transcriptome analysis reveals that many genes respond similarly to Pb and Cd [55]. It has recently [56] been reported that La^{3+} induces the expression of both *TtheMTT1* and *TtheMTT2* genes, whereas fluorescence analysis indicates that La^{3+} binds to both *T. thermophila* MTs.

Oxidative stress caused by H_2O_2 or paraquat induces MT gene expression, including both *Tetrahymena* CdMT and CuMT genes [22–24, 30, 49]. It has also been suggested that TtheMTT1 and TtheMTT2 have antioxidant ability, after the appearance of disulfide bonds from the CdMT complexes was detected when they reacted with NO

Table 3 Comparison of relative fold-induction rank of *Tetrahymena* MT genes under different heavy metal stress, obtained by quantitative reverse transcription PCR analysis

MT gene	Relative fold-induction value rank	Length of treatment	References
TpyrMT-1	Cd > Cu > Zn > Hg	1 h	[24]
TpyrMT-2	Cd > Cu > Zn > Hg	1 h	
TtheMTT1	Cd > Hg > Cu > Zn	30 min	[49]
	Cd > Zn > Pb > Cu > Ni	1 h	[22]
	Cd > Pb > As > Cu > Zn > Ni	24 h	
TtheMTT3	Zn > Cd > Pb > Ni > Cu	1 h	
	Cd > Zn > As > Ni > Cu > Pb	24 h	
TtheMTT5	Pb > Cd > Zn > Cu > Ni	1 h	
	Pb > As > Cd > Cu > Zn > Ni	24 h	
TrosMTT1	Cd > Pb > As > Cu > Zn	1 h	[23]
	Pb > Cd > As > Zn > Cu > Ni	24 h	
TrosMTT2	Cu > Pb > Cd > Zn > As	1 h	
	Cu > Zn > Cd > Pb > Ni	24 h	
TpigMT-1	Hg > Pb > Cd > Cu > Zn	1 h	[30]

in vitro [40]. However, other *Tetrahymena* CdMT genes (*TpyrMT-2* and *TrosMTT1*) are not significantly induced by H_2O_2 or paraquat [23, 24]. Starvation (the most common environmental stress), mainly after 24 h exposure, induces the gene expression of several *Tetrahymena* MT genes [22, 23]. The *TtheMTT5* gene is expressed during ciliate conjugation (a sexual process induced by starvation), as revealed by the existence of several expressed sequence tags encoding this gene, isolated from conjugating cells [22].

Although the multistress character of *Tetrahymena* MTs is evident, a differential gene expression level exists under the same stressor among different MT isoforms from the same subfamily and the same *Tetrahymena* species, and among different species, as seen in different mammalian MT isoforms [57, 58]. The three CdMT isoform genes from *T. thermophila* present differential expression patterns. TtheMTT5 and TtheMTT1 seem to be general stress and metal detoxification MTs. Transformed strains of *T. thermophila* (GFPMTT5) with the plasmid construct P_{MTT1}*::GFP::MTT5* (which includes the MTT1 promoter, green fluorescent protein as the reporter gene, and the MTT5 open reading frame) have shown themselves to be about 10 times more resistant to Cd with regard to the wild-type strain (unpublished data), indicating that an increase in the CdMT (*TtheMTT5*) gene dose affects the Cd lethal concentration killing 50% of the cell population (LC_{50}) value of this ciliate. It also indicates that the main function of this CdMT (TtheMTT5) is probably heavy metal detoxification. The TtheMTT3 isoform (a His-containing CdMT that can enhance the relative affinity for Zn, in comparison with Cd) could be involved in the intracellular homeostasis of Zn.

An interspecific stress response diversity is shown among MT genes of *T. thermophila* and *T. rostrata* with regard to heat-shock stress. Both *TrosMTT1* and *TrosMTT2* genes are considerably overexpressed at 42 °C (more than 100-fold induction), whereas *T. thermophila* MTs either show a low expression or are not expressed at all at this temperature [22, 23, 49]. These differences might be explained by the consideration that *T. rostrata* is a facultative parasite in various vertebrates and is probably more sensitive to temperature changes than other *Tetrahymena* species [59].

The induction of transcription of several MT genes by a variety of stress conditions suggests that cell exposure to one type of stress may lead to the acquisition of tolerance against another stress type. This cross-protection, detected in some organisms, suggests that there is at least partial overlap between genome responses to different types of stress [60]. This overlapping genome expression comprises general stress-responsive genes, such as heat-shock or MT genes.

MT genes of many organisms are mainly regulated at the transcriptional level [58]. Very few *cis*-acting regulatory elements have been identified experimentally in *Tetrahymena*. An MT conserved motif (MTCM1) was identified by in silico analysis in the putative promoter region of the three *T. thermophila* CdMT isoforms and the promoter region of the *TpyrMT-1* gene [22]. Most of the MTCM1 copies include a sequence (TGANTCA, where "N" means any nucleotide) that is similar to the sequence (TGA[G/C]TCA) binding the eukaryotic AP-1 transcription factor [61]. In *S. cerevisiae* an AP-1 transcription factor (YAP-1) is involved in response to oxidative stress and metal resistance [62]. Likewise, transcription factors similar to AP-1 have been detected in the MT promoters of insects (*Drosophila melanogaster*) and mollusks (*C. virginica*) [37]. AP-1, also known as c-jun, is a member of the bZIP superfamily of eukaryotic DNA binding transcription factors.

This MTCM1 motif is present six times in *TtheMTT1*, twice in *TtheMTT3*, and 13 times in *TtheMTT5* [22]. The promoter region of *TtheMTT5* has a 416-bp tandem duplication (96% identical to one another); five copies of MTCM1 are present within each duplication and another three copies are outside the duplications and nearer the start codon. The quantitative expression analysis of the three *T. thermophila* CdMT genes showed that *TtheMTT5* is the most strongly induced, whereas *TtheMTT3* is the least induced by diverse environmental stressors (*TtheMTT5* $\gg$ *TtheMTT1* $>$ *TtheMTT3*) [22]. The presence of the duplicated promoter region in *TtheMTT5* might be related to the high expression level of this CdMT gene when compared with *TtheMTT1* and *TtheMTT3* genes. Likewise, the number of real polyA sites, detected from different cDNA libraries (seven in *TtheMTT5*, two in *TtheMTT1*, and one in *TtheMTT3*), might show different messenger RNA formation capacity levels.

Biotechnological use of ciliate metallothioneins

Bioassays and biosensors are good tools for assessing metal pollution because they have the ability to react with or detect only the available fraction of metal ions, whereas common analytical methods are unable to distinguish whether fractions of metals are available or unavailable to biological systems [63]. *Tetrahymena* MTs might be good tools (biological sensing elements) for developing classic biosensors because these proteins are larger than other MTs and have a higher metal binding capacity. In environmental biomonitoring, global parameters such as bioavailability, toxicity, and genotoxicity cannot be tested using molecular recognition or chemical analysis, and can only be assayed using whole cells. Over the last 10 years or so, the concept of the whole-cell biosensor (WCB) has been introduced by

several authors as a very useful alternative to classical biosensors [64]. A WCB uses the whole prokaryotic or eukaryotic cell as a single reporter, incorporating both bioreceptor and traducer elements. In general, living systems used as WCBs are cells that are experimentally modified to incorporate the transducer capacity. Two types of bioassays using WCBs may be considered: "turn off" and "turn on" assays. In "turn on" assays, a quantifiable molecular reporter is fused to a specific gene promoter known to be activated by the target environmental pollutant (such as heavy metals). Some of the best gene promoter candidates to make a WCB are *Tetrahymena* MT gene promoters (such as those from *TtheMTT1* and *TtheMTT5* genes), which respond very quickly (less than 1 h) and strongly to heavy metal exposure. Indeed, both TtheMTT1 and TtheMTT2 promoters have already been used for overexpression of homolog and heterolog genes in *T. thermophila*, and are therefore important biotechnological tools for expressing eukaryotic proteins, some of which are difficult to express in prokaryotic cells [26, 31, 65, 66]. Recently, we have reported the first two ciliate WCBs to detect heavy metals [67]. These WCBs (MTT1Luc and MTT5Luc) are based on MT promoters from *TtheMTT1* or *TtheMTT5* genes of *T. thermophila* linked with the eukaryotic luciferase gene as the reporter gene (*MTT1::LucFF* and *MTT5::LucFF*). These transformed strains were used to design "turn on" bioassays to detect bioavailable heavy metals in polluted soil and aquatic samples in about 2 h. Validation of these WCBs was conducted using both artificial and natural samples, including methods for detecting false positives and negatives. The main features of these WCBs can be summarized in the following points:

1. Luciferase activity in MTT1Luc and MTT5Luc can be measured as efficiently in intact viable cells as in permeabilized cells, and a similar induction is detected with these in vivo and in vitro approaches [67]. This indicates that the luciferin uptake in *Tetrahymena* is more efficient than in other eukaryotic model systems that have been used as WCBs (e.g., WCBs based on *S. cerevisiae* or *Caenorhabditis elegans* require that cells first be permeabilized) [68, 69].
2. In both WCBs, all metals tested induce luciferase expression at concentrations lower than their respective LC_{50} values. Although both strains are effective WCBs, important differences between MTT1Luc and MTT5Luc exist: MTT5Luc responds more strongly to diverse heavy metals than MTT1Luc and a differential response for each type of metal has likewise been reported [67]. In general, these results are consistent with qRT-PCR analysis of the *T. thermophila* CdMT genes after similar metal treatments [22].
3. Experiments with previous EDTA exposures show that these WCBs only respond to bioavailable metals.
4. A comparison of the *Tetrahymena* MTT1Luc and MTT5Luc sensitivity levels with the sensitivity levels of eukaryotic or prokaryotic WCBs already published [67] shows that both *Tetrahymena* WCBs are often the most sensitive microbial eukaryotic metal biosensors, sometimes exceeding available bacterial WCBs as well.
5. Although a large number of metal WCBs have been developed, most have not been evaluated using natural samples [70]. Both MTT1Luc and MTT5Luc can detect bioavailable heavy metals in natural soil or aquatic samples, including samples with metal concentrations lower than the maximum values established by a European Union directive [67].

This first ciliate WCB might be the starting point for a more extensive use of these eukaryotic microbial models to design classic biosensors or WCBs for detecting diverse organic or inorganic environmental pollutants or biotechnologically relevant substances.

Concluding remarks

Ciliate MTs have unique features compared with MTs from other organisms. The most important of these are (1) an unusually high molecular mass and length with regard to most known MTs; (2) the presence of new Cys clusters, mainly CCC clusters; (3) a considerable number of Cys residues, allowing a greater total number of metal ions with which these MTs can bind in comparison with other MTs; (4) a highly conserved Cys periodic pattern, which divides amino acid sequences into modules and submodules; (5) two well-characterized and separate subfamilies with well-differentiated features; and (6) a fast and strong induction level of the gene expression, which constitutes a good molecular tool for designing biosensors of interest for environmental or biotechnological applications.

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