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ORIGINAL PAPER

Identification of the zinc, copper and cadmium metalloproteome of the protozoon *Tetrahymena thermophila* by systematic bioinformatics

Christos T. Chasapis[1] · Claudia Andreini[2] · Athanasia K. Georgiopolou[3] · Maria E. Stefanidou[4] · Alexios Vlamis-Gardikas[5]

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Abstract *Tetrahymena thermophila* (*T. thermophila*) is a ciliated protozoon that can detect freshwater pollution by heavy metals ("whole-cell biosensor"). This work employed a systematic bioinformatics approach to predict and analyze the metalloproteome of *T. thermophila* for the essential Zn, Cu and the non-essential Cd. 3784 metal-binding proteins were identified compared to the 456 annotated so far in UniProt. The localization, functional classification, and the functionally enriched network of the newly identified metalloproteome are presented. Cd toxicity could be explained in terms of the metal replacing Cu and especially Zn in MAPKs, transporters and antioxidant enzymes. The predicted results for Cd toxicity and responses reflect those observed experimentally in different organisms after their exposure to Cd.

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✉ Christos T. Chasapis
cchasapis@iceht.forth.gr

[1] Institute of Chemical Engineering Sciences (ICE-HT), Foundation for Research and Technology, Hellas (FORTH), Stadiou St., Platani, 26504 Patras, Greece

[2] Magnetic Resonance Center (CERM) Department of Chemistry, University of Florence, 50019 Sesto Fiorentino, Italy

[3] Hellenic Open University, 26335 Patras, Greece

[4] Department of Forensic Medicine and Toxicology, School of Medicine University of Athens Goudi, 11527 Athens, Greece

[5] Department of Chemistry, University of Patras, Rion 26504, Greece

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Abbreviations

GO	Gene ontology
HMM	Hidden markov model
MAPK	Mitogen-activated protein kinases
MBP	Metal-binding pattern
MT	Metallothionein
RDGB	Retrieval of domains and genome browsing
SCO	Synthesis of cytochrome c oxidase
SOD	Superoxide dismutase
WCB	Whole-cell biosensor

Introduction

Metals in free or bound form are paramount to life (Frausto Da Silva and Williams 1991; Bertini et al. 2007; Festa and Thiele 2011). Almost 40% of all proteins with known structure bind to at least one metal ion (Shi and Chance 2008; Andreini et al. 2013). Iron (Fe) is the commonest metal followed by zinc (Zn), which is essential for two main reasons: it participates in the catalytic mechanism of enzymes and contributes to stabilization of protein structure (Andreini et al. 2006a; Murakami and Hirano 2008; Wilson et al. 2012). The content of Zn-binding proteins in the domain of Eukarya (8.8%) is significantly higher than that observed for Bacteria and Archaea (from 5 to 6%) (Andreini et al. 2006b). Copper (Cu) is also an essential metal, often employed by metal-binding enzymes to catalyze electron transfer reactions (Festa and Thiele 2012). Production of energy and antioxidant activity is two of the well-known metabolic functions utilizing Cu (Kim et al. 2008). The size of the Cu proteome is less than 1%

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in eukaryotes and prokaryotes (Andreini et al. 2008). Cadmium (Cd) is a non-essential metal with high biotoxicity even at very low concentrations. It can easily replace other bivalent metal ions (such as Zn^{2+}, Cu^{2+}, and Ca^{2+}) (Brzóska and Moniuszko-Jakoniuk 2001; Waalkes 2003; Remelli et al. 2016). The toxicity rank for eukaryotic microorganisms is Cd > Cu ≫ Zn (Gallego et al. 2007).

Protozoa have long been used as bioindicators of water quality and pollution (Nicolau et al. 2001; Jiang and Shen 2005). *Tetrahymena* sp. are ciliated protozoa that inhabit streams, lakes, and ponds. They are found almost everywhere, in a range of different climates. Based on their taxonomic classification (Fig. 1), *Tetrahymena* belong to *Hymenostomes* cilate protozoa with other members that are parasitic on fish or aquatic invertebrates. Among these is the protozoan *Ichthyopthirius multifiliis*, a common cause of death in aquaria and fish farms (Edward 2000). *Tetrahymena* sp. have been used extensively as eukaryote cell models in toxicology (Stefanidou 2013; Stefanidou et al. 2011; Loutsidou et al. 2012) and *T. thermophila* is mostly known for its use as a "whole-cell biosensor" (WCB) detecting pollution by heavy metals in aquatic or soil samples (Gutierrez et al. 2003, 2009; Shang et al. 2002; Amaro et al. 2011, 2014; Díaz et al. 2007). Due to their rapid and strong induction by metals and especially Cd, the promoters of the metallothionein (MT) genes *MTT1* or *MTT5* of the organism have been used to design metal biosensors

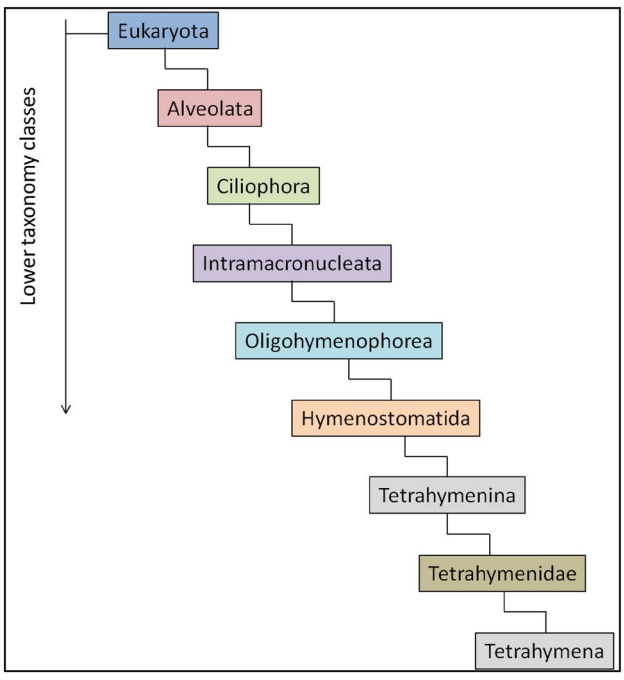

Fig. 1 Taxonomic classification of *Tetrahymena*. Hierarchical structure is based on Uniprot taxonomy data with taxon Id: 312017 for *T. thermophila*

(Shang et al. 2002; Amaro et al. 2014). The MTs of the ciliate are involved in metal resistance mechanisms, with metal bioaccumulation being the most known process. The formed metal–protein complexes are accumulated into vacuoles and finally released from the cell as non-toxic metallic complexes. The prioritation of bioaccumulation of metals in ciliates is Zn ≫ Cd > Cu. For *Tetrahymena* sp., Cd may displace Zn when both are present in the medium resulting in Zn deficiency (Gutiérrez et al. 2011; Chang 1996; Nilsson 1989).

The *T. thermophila* proteome consists of ~27,000 proteins, out of which only 11 have been manually annotated as metal-binding proteins in the protein database of UniProtKB/Swiss-Prot and 445 automatically annotated in UniProtKB/TrEMBL (Chasapis and Stefanidou 2016). The present work describes a combined approach of strategies based on structural data and annotation to predict the metal-binding proteins encoded by the genome of *T. thermophila* for Zn, Cu and Cd. The outcome was (1) the presentation of a novel probable metalloproteome of *Tetrahymena* ciliates for Zn, Cu and Cd and its involvement in the metabolism of the protozoon, and (2) the prediction of Zn and Cu homeostatic disturbances after poisoning by Cd.

Materials and methods

Identification of Zn-, Cu-, and Cd-binding proteins of *T. thermophila* (strain SB210)

We identified metalloproteins using the Retrieval of Domains and Genome Browsing (RDGB) strategy (Andreini et al. 2011). The RDGB protocol involves the usage of Pfam domains to identify putative homologs in any desired genome. Initially, we collected all 3D structures of Zn (3896), Cu (317) and Cd (2613)-binding proteins provided by the Protein Data Bank (PDB) and MetalPDB (Andreini et al. 2013). From these structures, we extracted each specific metal-binding pattern (MBP) involved in the interaction of specific proteins with the particulate metal. To remove proteins binding to Zn in a non-physiological manner, the experimental data (e.g., the crystallization conditions) for all PDB protein structures were examined in the publications describing the relevant structures. This step was important to remove proteins binding Zn in a non-physiological manner. For example, ~800 non-relevant structures were identified for Zn (data not shown), such as 1GUD where the coordination of the zinc ion was important in crystal packing but not in protein structure or function. To achieve maximal identification coverage for Zn-, Cu- and Cd binding, we selected for all Pfam domains whose description contained the words

Table 1 Number of metalloproteins predicted in *T. thermophila* genome

Organism	Total proteome	Zn proteins	Cu proteins	Cd proteins
T. thermophila	26,976	2431 (9%)	19 (0.07%)	2295 (8.5%)

"zinc"/"Zn", "copper"/"Cu", "cadmium"/"Cd" and those related in the literature to Zn^{+2}, Cu^{+2} and Cd^{+2} binding, respectively. Then, we curated these domains manually to create a set of Pfam Zn-, Cu-, and Cd-binding domains, most of which have associated MBPs (Table S2). This set served as the starting point for search in the *T. thermophila* proteome.

The proteome of *T. thermophila* (strain SB210) was downloaded from the protein resource UniProt (http://www.uniprot.org/proteomes/UP000009168). Each protein was scanned for the occurrence of Pfam domains as defined by the previous step, using the HMM search function of HMMER 3.1b2 and a threshold of 0.05 for the *E* value (as calculated by Pfam). False positives were filtered out by searching MBPs in the retrieved protein sequences, and rejecting those lacking the MBPs. The MBP filter was applied assuming that the predicted proteins contained all the ligands of the MBP with spacing within each sequence maintained within ±20% of total number of amino acids (or ±1 amino acid for short spacing).

Functional classification, localization and construction of functionally grouped GO annotation network of the predicted Zn-, Cu-, and Cd-binding proteins

A functional classification of the putative metalloproteins of *T. thermophila* was obtained using the FunCat2 tool of the Pedant database (http://pedant.gsf.de/) (Walter et al. 2009). Prediction of the subcellular localization of these proteins was based on the integration of data from Pedant and UniProt web sources. In addition, all predicted metalloproteins were analyzed in terms of their gene ontology (GO) (Ashburner et al. 2000) functional annotation. For the construction, visualization and analysis of the GO enriched network, gene clusters encoding the Zn-, Cu- and Cd- metalloproteins were loaded to the ClueGO v.2.0.0 (Bindea et al. 2009) plug-in of Cytoscape v.3.0.0 (Shannon et al. 2003). In this program, each node represents a biological process (BP), while the edges reflect the relationships between the GO terms based on the overlap of their associated genes. The enrichment significance of the GO terms is reflected by the size of the nodes. The functional grouping of the GO terms is based on the kappa score calculated by ClueGO (Huang et al. 2007).

Results

The *T. thermophila* genome was analyzed for the occurrence of Zn-, Cu-, and Cd-binding proteins using the RDGB approach (Andreini et al. 2011). Following this protocol (1) all the Zn-, Cu-, and Cd-binding patterns from the PDB, and (2) all the Zn-, Cu- and Cd-binding domains from the Pfam database were retrieved to browse the target proteome. A total of 3784 potential unique metalloproteins (taking account the overlap between the three predicted metalloproteomes), comprising 2431 putative Zn-binding (724 Pfam domains), 19 Cu-binding (63 Pfam domains) and 2295 Cd-binding (204 Pfam domains) proteins were detected (Tables S1–S2). Altogether, these proteins represent 17.5% of the whole proteome of the protozoon (Table 1).

Zn-binding proteins (Zn proteome)

The present bioinformatics analysis suggested that the Zn proteome might constitute up to 9% of the entire proteome of *T. thermophila*. Most of the functions involving the Zn proteome were related to protein fate and modification (27.4%), followed by metabolism (26.4%), transcription (16.4%), cell cycle and DNA processing (13.5%) (Fig. 2). Zn-binding proteins related to protein fate and modification included Zn-dependent peptidases, proteases and proteins related to ubiquitination. Many transcription factors containing Zn-finger domains and several metal transporters apart from the exclusive Zn-specific transporters were identified. Some of the identified Zn-binding proteins are involved in nucleic acid-related processes and metabolism (Table S1). Accordingly, members of the Zn proteome were

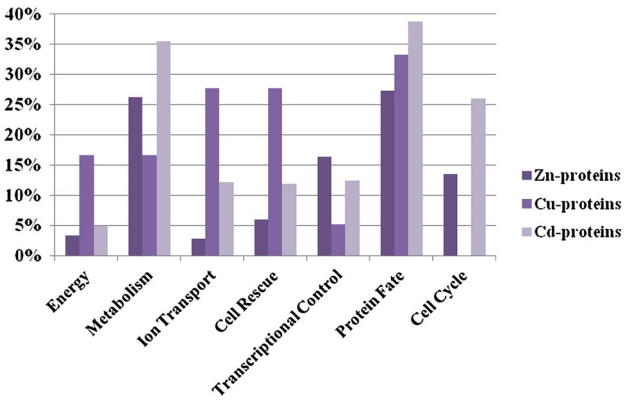

Fig. 2 Functional classification of Cu-, Fe-, and Zn-binding proteins encoded by the *T. thermophila* genome. Metalloproteins were classified according to the FunCat2 scheme

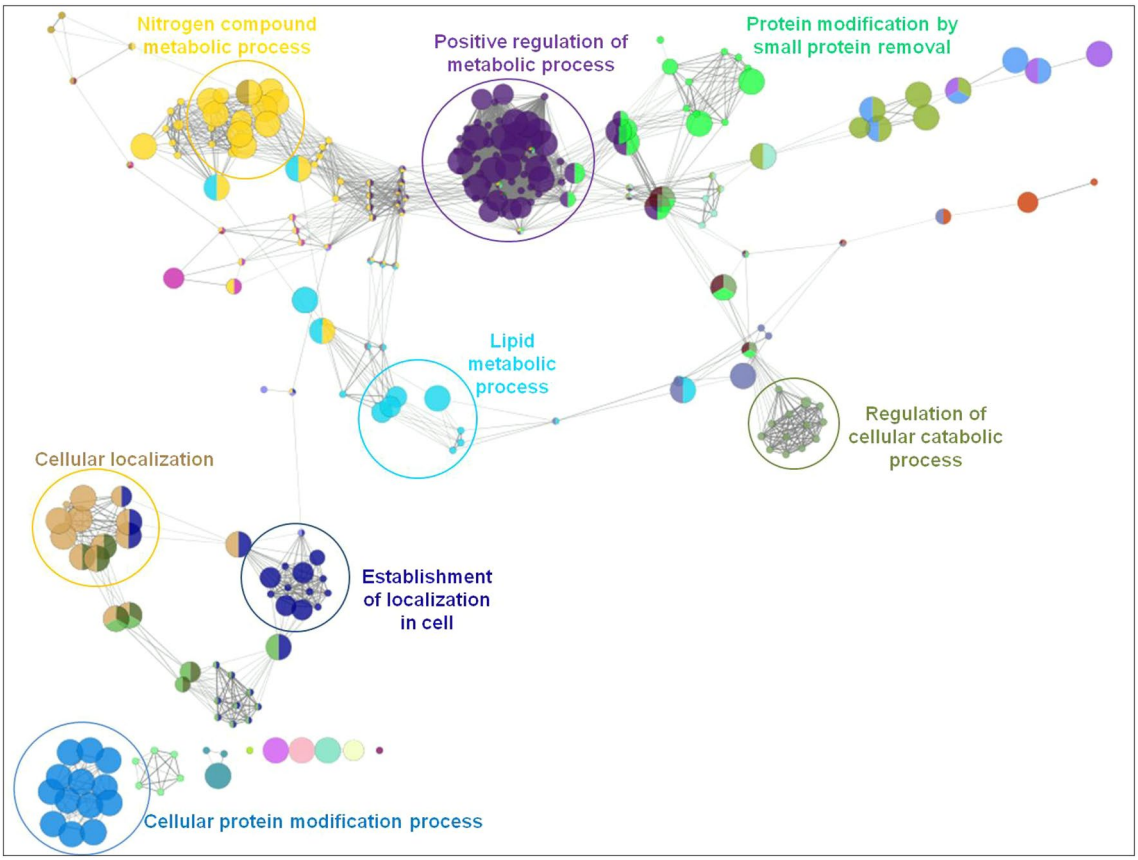

Fig. 3 Functionally enriched network of the predicted Zn proteins in *T. thermophila* calculated by ClueGO (kappa score ≥0.3) and only the most significant groups are shown. *Node color* represents the class that they belong. *Mixed coloring* means that the specific node belongs to multiple classes. Ungrouped terms are not shown

predicted having nucleolar localization. Cytoplasmic, mitochondrial and plasma membrane (perhaps metal transporters) localizations were also predicted for the Zn-binding proteins (Table S1). The enriched GO network of the predicted Zn proteome consisted of 253 BP nodes and 1230 edges and was structured on 30 kappa-score groups. Figure 3 shows eight significant groups of GO terms (cellular protein modification processes, cellular localization, establishment of localization in cell, lipid metabolic processes, nitrogen compound metabolic processes, positive regulation of metabolic processes, protein modification by small protein removal and regulation of catabolic processes) and the connections of BP nodes based on the overlap of their associated genes To estimate the importance of Zn protein-encoding genes in mediating biological responses, topological analysis of the functional enriched network was applied to evaluate the number of interactions in which every single node was involved (Table S4). By this analysis, BP nodes related to positive regulation of metabolic process (GO:0009893) and protein metabolic processes (GO:0032270) were the most connected GO terms with 55 links. Furthermore, some of the BP nodes (20% of the total nodes, Table S4) were connected with more than two functional groups, indicating that their associated genes (~30% of the Zn proteome encoding genes with available GO ontology) could regulate multiple cellular processes.

Cu-binding proteins (Cu proteome)

The 19 Cu-binding proteins predicted for *T. thermophila* constituted only 0.07% of the entire proteome. Such proteins were the high-affinity Cu transporters and Cu-containing enzymes (e.g., cytochrome *c* oxidase and Cu, Zn superoxide dismutase, both essential for mitochondrial function and related to the response to Cu stress in yeasts (Banci et al. 2011). Protein fate (folding and stabilization, 33.3%), ion transport (27.7%), and cell rescue (27.7%) were the most enriched functional categories (Fig. 2). The prediction of cellular localization for Cu-binding proteins was in agreement with their functional analysis and annotation (Table S1). A group of the Cu-containing proteins (15% of the predicted Cu proteome) were enzymes related to general metabolism and localized in the cytoplasm.

Some Cu-binding proteins (C oxidase copper chaperone and cytochrome C oxidase biogenesis Cmc1-like protein) were predicted cytosolic because of their participation in the respiratory electron transfer chain. The ClueGO functionally annotated network for the predicted Cu proteome had 20 BP nodes and 77 edges. The functional enriched network was structured on two kappa-score groups; the first consisted of BP nodes involved in general transport of ions and Cu^{2+}. The second of BP nodes related to detoxification and response to inorganic and toxic stress. Network analysis suggested that the BP nodes of the second group were related to responses to reactive oxygen species (GO:0000302), oxidative stress (GO:0006979), and toxic or inorganic substances detoxification (GO:0009636). These nodes provided the most connected GO terms with 17 links (Table S4).

Cd-binding proteins (Cd proteome)

The predicted *T. thermophila* Cd proteome constituted up to 8.5% of the entire proteome, with 2295 Cd-binding proteins identified. The list included the well-known proteins induced by Cd in *Saccharomyces cerevisiae* (enzymes with antioxidant properties (thioredoxins), heat shock proteins and proteases) (Vido et al. 2001), enzymes associated with glutathione biosynthesis and homeostasis (e.g., glutathione S-transferase, glutathione peroxidase) and other metabolic enzymes (even with Zn-binding motifs) (Table S1). Furthermore, ciliate MTs that fall into the 7a subfamily (Díaz et al. 2007) belonged to the predicted Cd proteome. This result was in agreement with the central assumption that MTs in *Tetrahymena* provide cellular protection from heavy metals (Gutiérrez et al. 2011). Protein fate and modification constituted the most enriched functional category (38.8% of Cd proteome) followed by general cell metabolism (35.5%), cell cycle and DNA processing (26.1%), ion transport (12.2%), and cell rescue and defense (11.9%) (Fig. 2). Cytoplasmic and plasma membrane localizations were the most prevalent for the Cd metalloproteome. The ClueGO functionally grouped annotation network (structured on 23 kappa-score groups and consisting of 253 BP nodes and 1011 edges, Table S4) revealed seven significant groups of GO terms (ion transmembrane transport, signal transduction by protein phopshorylation, mitotic cell cycle and cell death, responses to oxidative stress, mitogen compound metabolic processes, and cellular component organization, Table 2) that were activated by or were sensitive to Cd.

Cd/Zn- and Cd/Cu-binding proteins

Although Cd^{2+} is a non-essential metal ion, it can easily replace Zn^{2+} or Cu^{2+} in metalloproteins (Friedman 2014).

Table 2 The most significant functional groups of the predicted Cd-binding proteins in *T. thermophila* calculated by ClueGO (kappa score ≥0.3)

	Functional groups of GO terms for the predicted Cd-binding proteins
1	Ion transmembrane transport
2	Signal transduction by protein phopshorylation
3	Mitotic cell cycle and cell death
4	Responses to oxidative stress
5	Nitrogen compound metabolic processes
6	Cellular component organization
7	Cellular nitrogen compound metabolic processes

In the PDB (Fig. 4, top), the transition metal ions most often substituted by Cd are Zn and Cu (2.6 and 2.2% of substituted sites, respectively). In almost 50% of cases, the substitution may affect the first coordination sphere by changing the coordination number or/and geometry with respect to the native metal ion (Fig. 4, bottom), leading to inactivation of the enzymes. Based on the present analysis, 959 (39.5%) and five proteins (26.3%) of Zn and Cu proteome, respectively, were identified in the Cd proteome (Fig. 5a, Table S3). Since these proteins might constitute the ensemble of possible Zn^{+2} and Cu^{+2} substitutions by Cd^{+2}, we grouped them according to their functions (Fig. 5b). Most of the 959 Zinc-binding proteins belonged to the mitogen activated protein kinases (MAPK) cascade (such as: serine/threonine kinase domain proteins). The rest of them were critical for organelle organization, responses against superoxide (such as Cu, Zn SOD), the mitotic cell cycle, intracellular signal transduction, the regulation of protein localization and the transport of Zn^{+2} (such as Zip proteins, a family of metal transporters first identified in plants (Kambe et al. 2014 and references therein). Enzymes were also some of the Cu-binding proteins that were identified as potential Cd targets (such as Cu, Zn SOD protein, SCO1/Senc family of proteins) (Table S3).

Discussion

Metal-binding proteins constitute a significant proportion of the total proteome in diverse organisms from bacteria to man (Christopher et al. 2010). This work describes an in silico prediction of the Zn, Cu and Cd metalloproteome for *T. thermophila,* an organism used for toxicological studies of heavy metals, especially in environmental studies. The advantage of the presented computational approach (the RDGB strategy) is that the metal-binding domains retrieved from the target proteome were based not based on the Pfam library but also on the

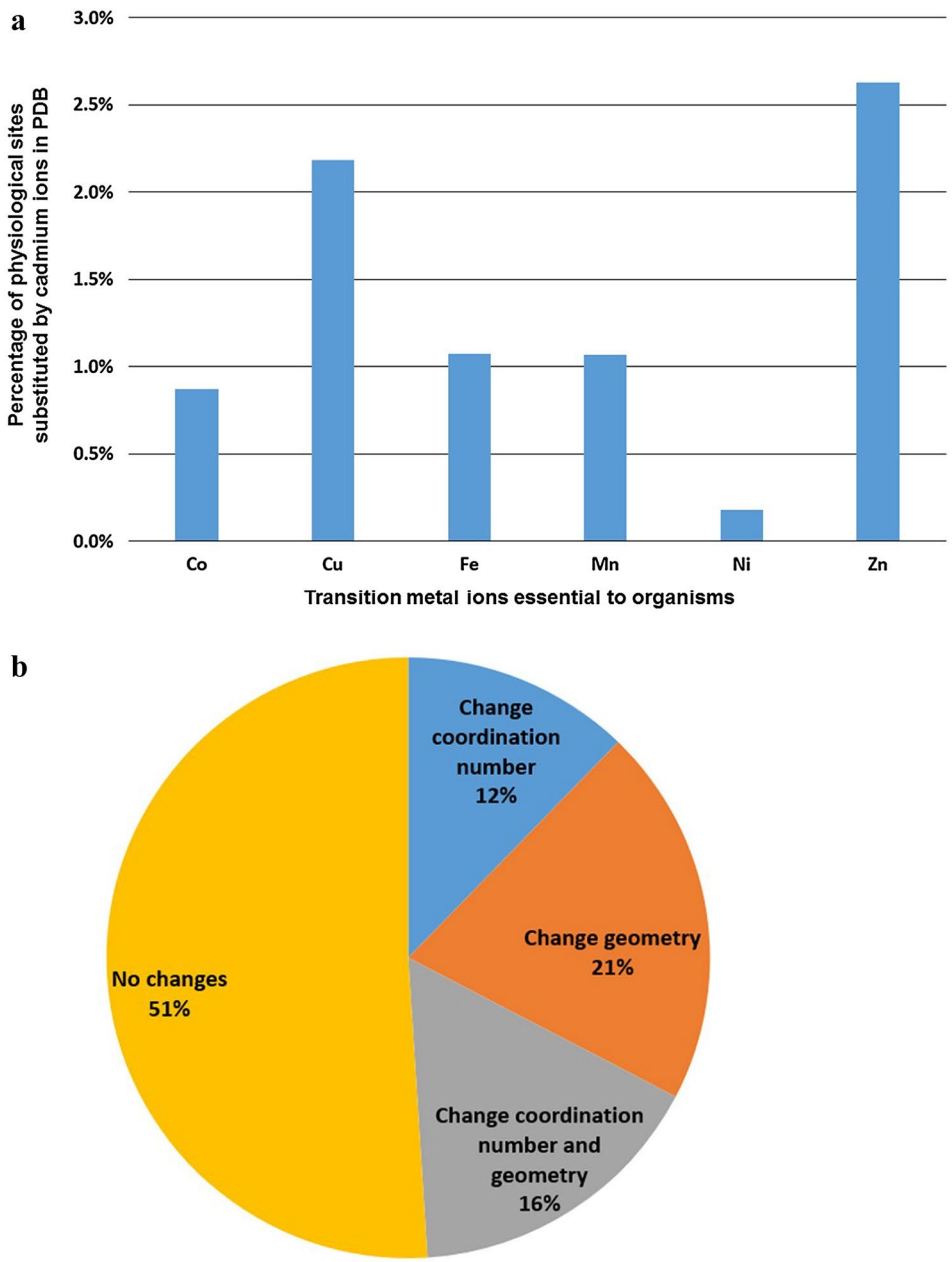

Fig. 4 **a** Fraction of physiological metal sites substituted by Cd^{2+} in the PDB for each essential transition metal ion. **b** Effects of the coordination of cadmium on the first coordination sphere of the site (coordination number, coordination geometry) with respect to the physiological metal ion

metal-binding patterns extracted from metalloproteins from other organisms with known 3D structures available from PDB. The 3784 altogether identified possible metalloproteins (14% of the proteome) included 2431 Zn- (9% of the proteome), 19 Cu- (0.07%) and 2295 Cd-binding proteins (8.5%). These results are in agreement with data from other organisms: in Eukarya, Zn-binding proteins constitute up to 9% of the proteome and in Bacteria and Archaea 5–6% (Andreini et al. 2006b). In eukaryotes and prokaryotes, the size of the Cu proteome was below 1% in (Andreini et al. 2008).

The higher frequency for the Zn-binding proteins was somehow expected because Zn^{2+} is one of the most abundant metal ions in living organisms. Zn-binding proteins were predominantly involved in metabolic processes and protein fate, while 27.6% of the Zn metalloproteome could regulate multiple cellular pathways. Cu-binding proteins were mainly involved in ion transport and response to inorganic substance, and Cd-binding proteins in the general cell metabolism, cellular defense against heavy metals, the regulation of cell cycle and death.

Among the predicted Cd-binding proteins were essential enzymes with typical Zn- or Cu-binding motifs. This observation is in agreement with the ability of Cd^{+2} to replace Zn^{+2} and Cu^{+2}, a well-known process known

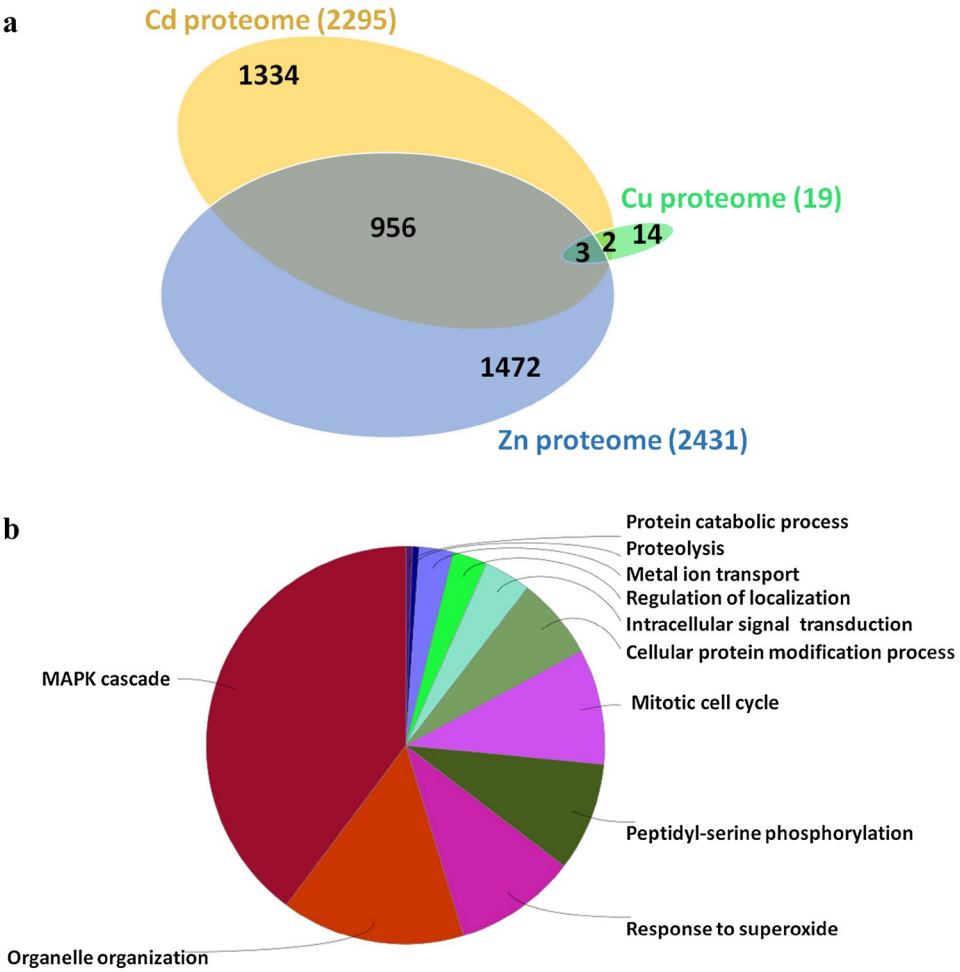

Fig. 5 **a** Venn diagram of the predicted Zn, Cu and Cd proteome; **b** Functional grouping of Zn-binding proteins that are potential targets for binding to Cd

as molecular mimicry (Chmielowska-Bąk et al. 2013). In this study, 39.5% of the Zn and 31% of the Cu proteome could apparently bind to Cd. The binding motifs that could replace Zn^{+2} by Cd^{+2} belonged mostly to protein members of the MAPK cascade suggesting that the latter may be affected by increases in environmental Cd. In plants, MAPK cascades were indeed activated by the presence of Cd (Chmielowska-Bak et al. 2014 and references therein). Zip transporters that are normally responsible for Zn cellular uptake were also potential targets for Cd-binding (Table S3). In fact, increased Cd concentrations induced the expression of the Zip family of proteins in different organisms (Chauchene et al. 2011; Dalton et al. 2005; Lee and An 2009). Cation channel proteins were identified as possible members of the predicted Cd proteome suggesting that together with the Zip transporters they could constitute the main candidates for the Cd uptake in *T. thermophila.* On the other hand, no Cu transporters were predicted to bind to Cd suggesting a selectivity of the Zn transporters for Cd uptake. Noteworthy is the significant number of protein members of the Cd metalloproteome (similar to that for Zn-binding proteins), possibly reflecting the evolution of organisms in relatively Cd-free environments; if Cd was a common element, its deleterious character should have driven evolution to more selective binding.

Conclusions

The computational results predicted the metalloproteome for Zn, Cu and Cd for *T. thermophila* and confirmed the toxicity of Cd^{2+} in view of its ability to substitute Zn^{2+} and Cu^{2+} in some critical enzymes of the organism. This work could be useful for studies related to individual Zn, Cu, and Cd-binding proteins of *Tetrahymena* or their homologues and could offer insights into the effects of Cd poisoning of many other aquatic organisms. Systematic examination of the upstream sequences of the genes identified as metalloproteins for *T. thermophila* could provide novel promoters for the detection and monitoring of environmental heavy metal pollution by WBCs.

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