Chapter 2

Metallothioneins, *Saccharomyces cerevisiae*, and Heavy Metals: A Biotechnology Triad?

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Additional information is available at the end of the chapter

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Abstract

Metal ions are the least sophisticated chemical species that interact or bind to biomolecules. The yeast Saccharomyces cerevisiae represents a versatile model organisms used in both basic and applicative research, and one of the main contributors to the understanding of the molecular mechanisms involved in the transport, accumulation, and homeostasis of heavy metals. With a negatively charged wall, the yeast cells are very good biosorbents for heavy metals. In addition to biosorption, the metabolically active cells take up heavy metals via the normal membrane transport systems. Once in the cell, the toxicity of the heavy metals is controlled by various mechanisms, including sequestration by metal-binding proteins, such as the metallothioneins. Metallothioneins are cysteine-rich proteins involved in the buffering of excess heavy metals, both essential (Cu and Zn) and nonessential (Cd, Ag, and Hg). S. cerevisiae has two innate metallothioneins, Cup1 and Crs5, intensively investigated. Additionally, S. cerevisiae served as a host for the heterologous expression of a variety of metallothioneins from different species. This review focuses on the technological implications of expressing metallothioneins in yeast and on the possibility to use these transgenic cells in heavy metal-related biotechnologies: bioremediation, recovery of rare metals, or obtaining clonable tags for protein imaging.

Keywords: metallothionein, *Saccharomyces cerevisiae*, heavy metal, bioremediation

1. Introduction

Biotechnology, which makes use of living organisms for technological purposes, is one of the applied fields that constantly benefited from the rapid advancements made in understanding life at molecular level. It is undoubtedly that the budding yeast *Saccharomyces cerevisiae* is one of the biotechnology's most versatile tools. Used since ancient times in bakery, brewery, and

wine making, the advent of molecular biology brought new glitter to this eukaryotic microorganism, and *S. cerevisiae* was practically reinvented. With an easy-to-manipulate genomics, with elegant and simple genetics, hosting molecules and biochemical processes well conserved along evolution, this microorganism served as a model organism for the discovery and understanding of numerous essential life mechanisms. Apart from playing an unique and undisputed role in basic research of life science, *S. cerevisiae* kept on adding value to its role in biotechnology, especially due to its capacity to heterologously express proteins, with various scopes: large-scale production of recombinant proteins of technological significance (enzymes, antibodies, and hormones); selection of strains with targeted characteristics and performances (metabolic engineering); environmental biotechnology (yeast surface display); and scientific reasons, such as elucidating the role of proteins from various organisms organism (yeast two-hybrid technique, expression of homologous counterparts from higher organisms in yeast, design yeast as model for human diseases, etc.) [1].

Metallothioneins (MTs) represent one of the numerous examples of proteins whose functions were investigated by heterologous expression in *S. cerevisiae* cells. MTs are low-molecular weight proteins that exist in most organisms from bacteria to humans, including yeasts [2]. MTs constitute an extremely heterogeneous family of cysteine-rich proteins (close to 30% of their amino acid content) that give rise to metal-thiolate complexes ensuing on metal ion coordination. MTs are considered to have many roles, being involved in protection against toxic metals, homeostasis/chaperoning of physiological metals, free-radical scavenging and antioxidative stress protection, control of oxidative state of the cell, antiapoptotic defense, etc. While some roles still remain obscure, it is widely accepted that all MTs have an undisputed capacity to buffer intracellular metal ions, especially Zn(II) and Cu(I) [3].

Heavy metals belong to a group of nondegradable chemicals naturally present in the environment. Numerous anthropogenic activities, especially the ones related to massive industrialization, intensive agriculture, or rapid urbanization led to important perturbations (accumulation, or in some cases, depletion) in heavy metal balance, with ecological, nutritional, and environmental impacts [4–10]. Some of the heavy metals (Co, Cu, Fe, Mn, Ni, and Zn) are essential for life in trace amounts, playing a pivotal role in the structure of enzymes and other proteins. Other heavy metals (Cd, Sb, Cr, Pb, As, Co, Ag, Se, and Hg) albeit not essential, interfere easily with the metabolism of essential heavy metals, competing for the various physiological transport systems as well as for the biomolecules they bind to. Essential or not, when present in high concentrations, heavy metals are strongly deleterious to living organisms due to nonspecific binding to proteins, often inducing oxidative stress, or disrupting biological membranes. Defense mechanisms against nonphysiological concentrations of heavy metal ions include excretion, compartmentalization in cell organelles, or increased synthesis of metal-buffering molecules, such as the MTs.

S. cerevisiae has been thoroughly investigated, and many mechanism involved in heavy metal transport and homeostasis have been elucidated in this organism [11–16], preparing the grounds for development of techniques used to engineer *S. cerevisiae* cells for increased

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heavy metal accumulation and improved tolerance. The present review focuses on the studies that relate heterologous expression of MTs in *S. cerevisiae* to the metal-binding characteristics observed and to the possibility to use them for biotechnological purposes.

2. Innate and heterologous expression of MTs in *S. cerevisiae*

Apart from being classified on the basis of their structural homology or on taxonomic criteria, MTs are also classified on molecular functionality grounds, starting from their innate metal-binding abilities, into Cu(I)- and Zn(II)-thioneins, with the representative nonessential models Ag(I) and Cd(II), respectively [3, 17]. This is based on the formation of homometallic MT species when they are produced in metal-enriched media; this classification is not regarded as absolute, since cross-affinity is often noticed for Zn(II)-thioneins binding Cu(I) and vice versa [3].

S. cerevisiae has two structurally different MTs, Cup1, and Crs5. Cup1 has been classified as the strictest Cu(I)-isoform (genuine Cu(I)-thionein) [3]. Cup1 biosynthesis is copper-activated at transcriptional level via the copper-binding transcription factor Ace1/Cup2 [18–21] providing the principal method of cellular removal and sequestering the extremely toxic Cu(I) [21–23]. Although substantially divergent from vertebrate and plant MTs, the arrangement of 12 cysteine residues, which is a hallmark of metal-binding proteins, is partially conserved. In contrast to the MTs from higher eukaryotes, Cup1 is responsible only for Cu(I) and Cd(II) ion tolerance *in vivo* [24, 25], albeit capable of binding other metal ions *in vitro* [26]. This is in contrast to the MTs found in higher eukaryotes, which are typically capable of detoxifying an array of metal ions.

Considered a secondary copper-resistant agent in *S. cerevisiae*, Crs5 is nonhomologous to the paradigmatic Cup1, determining survival under Zn(II) overload in a *CUP1*-null background. Its overexpression prevents the deleterious effects exhibited on the *cup1Δ crs5Δ* double knock-out mutant by exposure to combined Zn(II)/Cu(II), similarly to mouse MT1 Zn-thionein, but not to Cup1. Numerous similar observations denoted that Crs5 has a dual metal-binding behavior, being significantly closer to Zn(II)-thioneins than to Cu(I)-thioneins [23, 27, 28].

Following the discovery and characterization of Cup1, many newly discovered MTs were characterized by heterologous expression in *S. cerevisiae* (**Table 1**).

In plants, the first evidence for the role of MTs in Cu(II) and Cd(II) tolerance was provided by expressing two *Arabidopsis thaliana* MT genes in MT-deficient yeast cells. For example, when expressed in *cup1Δ* knock-out mutant, both *At*MT1 and *At*MT2 complemented the *cup1Δ* mutation by providing a high level of resistance to $CuSO_4$ and moderate resistance to $CdSO_4$ [29]. Later, all four types of plant MTs were checked as metal chelators by expressing *A. thaliana* MT cDNAs (*At*MT1a, *At*MT2a, *At*MT2b, *At*MT3, *At*MT4a, and *At*MT4b) in the Cu(II) and Zn(II)-sensitive yeast mutants, *cup1Δ* and *zrc1Δ cot1Δ*, respectively. All four types

MT expressed	Source organism	Behavior in *S. cerevisiae*	Reference
*At*MT1a, *At*MT2a, *At*MT2b, *At*MT3, *At*MT4a, and *At*MT4b	*Arabidopsis thaliana* Model plant organisms, metal nonaccumulator	Complement Cu(II) and Cd(II) sensitivity of a *cup1Δ* mutant; *At*MT4a, and *At*MT4b conferred greater Zn(II) tolerance and higher accumulation of Zn(II) than other MTs to the *zrc1Δ cot1Δ* mutant	[29, 30]
*Jc*MT2a	*Jatropha curcas* L. Technical plant: biofuel production	Complements Cu(II) and Cd(II) sensitivity of a *cup1* mutant	[31]
*Ha*MT1, *Ha*MT2, *Ha*MT3, *Ha*MT4	*Helianthus annuus* Technical plant: nutritional oil. Seeds tend to accumulate Cd(II), Pb(II), and Hg(II)	Complement Cu (II) (all), Cd(II) (*Ha*MT4-1), and Zn(II) (*Ha*MT3, *Ha*MT4-1) sensitivity	[32, 33]
*Nc*MT1, *Nc*MT2	*Noccaea* (*Thlaspi*) *caerulescens* Cd(II)/Zn(II) hyperaccumulator	*Nc*MT1, and to a lesser extent *Nc*MT2 complement Cu(II), Cd (II), and Zn(II) sensitivity	[35]
*Sv*MT2b, *Sv*MT3	*Silene vulgaris* (Moench) Garcke Cu(II)-hypertolerant plant	Restore Cd(II) and Cu(II) tolerance to yeast sensitive strains	[36, 37]
*Os*MT1-1b	*Oryza sativa*	Confers tolerance to Cd(II), H_2O_2, and ethanol	[38]
*Os*MT1a	*Oryza sativa* L. cv. Iapar 9	Confers tolerance to Zn(II)	[39]
*rg*MT	*Oryza sativa*	Confers vigorous growth under surplus $CuCl_2$, $FeCl_2$, NaCl, $NaHCO_3$, and H_2O_2	[40]
*Put*MT2	*Puccinellia tenuiflora* Alkaline/saline tolerant grass	Tolerance to H_2O_2, NaCl, $NaHCO_3$, Zn(II), Fe(II), Fe(III), Cd(II), Cr(VI), and Ag(I); sensitivity to Mn(II), Co(II), Cu(II), Ni(II)	[41]
*Cv*MT1	*Chloris virgata* Swartz Alkaline tolerant grass	Tolerance to salinity, alkaline conditions, and oxidative stress	[42]
*Th*MT3	*Tamarix hispida* Alkaline/saline tolerant plant	Tolerance to Cd(II), Zn(II), Cu(II), and NaCl stresses; increased accumulation of Cd(II), Zn(II), NaCl, but not of Cu(II)	[43]
*Gint*MT1	*Glomus intraradices* Arbuscular mycorrhizal fungus; confers heavy metal tolerance to exposed plants	Complements Cu(II) and Cd(II) sensitivity of a *cup1* mutant	[44]
*Hc*MT1 and *Hc*MT2	*Hebeloma cylindrosporum* Ectomycorrhizal fungus; confers heavy metal tolerance to exposed plants	Complement Cu(II) and Cd(II) sensitivity of *cup1* and *yap1* mutants	[45]

MT expressed	Source organism	Behavior in *S. cerevisiae*	Reference
*Pi*MT	*Paxillus involutus* Ectomycorrhizal fungus; confers heavy metal tolerance to exposed plants	Complements Cu(II) and Cd(II), but not Zn(II) sensitivity.	[46]
*Dm*MTo, *Dm*MTn	*Drosophila melanogaster*	Complement Cu(II) sensitivity	[47]
sMTIII	*Porcine brain cDNA* Growth inhibitory factor (GIF)	Confers metal resistance to yeast cells	[48]
*Mm*MT2a	*Mus musculus* Canonical Zn(II)-thionein	Complements Zn(II) sensitivity	[33]
*Mm*MT1	*Mus musculus*	Clonable tag for electron microscopy	[82]
*h*MT2, GFP-hMT2	*Homo sapiens*	Increased Cu(II) tolerance and capacity to remove Cu(II) when expressed from yeast *CUP1* promoter	[72]

Table 1. Heterologous expression of MTs in *S. cerevisiae*.

of *At*MTs provided similar levels of Cu(II) tolerance and accumulation to the *cup1Δ* mutant, while the type-4 *At*MTs (*At*MT4a and *At*MT4b) conferred greater Zn(II) tolerance and higher Zn(II) accumulation to the *zrc1Δ cot1Δ* mutant [30]. Metal-gained tolerance was also tested in yeast mutants expressing MTs from technical plants. Thus, the Cu(II) and Cd(II) sensitivity of yeast mutants was complemented by expression of MT2a isolated from *Jatropha curcas* L., a technical plant used for biofuel production [31]. In a different study, expression of MTs from *Helianthus annuus* (sunflower) overcame the Cu(II), Zn(II), or Cd(II) sensitivity, depending on the MT type expressed ([32], **Table 1**). Along with high nutritional value and significant oil content, the seeds of *H. annuus* tend to accumulate Cd(II), Pb(II), and Hg(II) [33], and *Ha*MTs are major candidates to be one of the determinants for the high metal accumulation properties of this plant.

Other MTs studied in yeast were isolated from heavy metal hypertolerant or hyperaccumulating plants. Hyperaccumulating plants belong to a small group of species capable of growing on metalliferous soils without developing toxicity symptoms [34]. The MTs from the intensively studied hyperaccumulator *Noccaea* (*Thlaspi*) *caerulescens* were expressed in yeast, and it was revealed that *Nc*MT1, and to a lesser extent *Nc*MT2, complemented the Cu(II), Cd(II), and Zn(II) sensitive phenotypes [35]. The *Silene vulgaris* (Moench) Garcke population with high levels of copper tolerance was shown to owe this hypertolerance to increased transcripts of *SvMT2b* gene; expression of *Sv*MT2b in yeast restored Cd(II) and Cu(II) tolerance in different hypersensitive strains [36]. In a different study, *Sv*MT3, whose gene has been locally duplicated in a tandem arrangement in *S. vulgaris* genome was shown to restore the Cu(II) tolerance along with increased Cu(II) accumulation in a Cu(II)-sensitive yeast mutant, and that both duplicated genes were functional [37].

Expression of plant MTs in *S. cerevisiae* cells sometimes determined other MTs-related phenotypes, besides metal tolerance and accumulation, indicating that heterologous MTs can be fully functional in yeast cells. Thus a heterologous expression in *S. cerevisiae* of *Os*MTI-1b, a MT isoform from *Oryza sativa* (rice), enhanced Cd(II), H_2O_2, and ethanol tolerance [38], while *Os*MT-1a from a Brazilian variety of rice conferred Zn(II) tolerance [39]; *rg*MT from the same species conferred vigorous growth to transgenic yeast cells when exposed to surplus $CuCl_2$, $FeCl_2$, NaCl (salinity), $NaHCO_3$ (alkalinity), or H_2O_2 (exogenous oxidative stress) [40]. Encompassing a wider range of stresses, expression of *Put*MT2 from the saline/alkaline grass *Puccinellia tenuiflora* increased the tolerance of transgenic yeast cells to H_2O_2, NaCl, $NaHCO_3$, and also to a series of metal ions: Zn(II), Fe(II), Fe(III), Cd(II), Cr(VI), and Ag(I), while conferring sensitivity to Mn(II), Co(II), Cu(II), and Ni(II) [41]. Expression of *Cv*MT1 from the alkaline grass *Chloris virgata* Swartz significantly increased the yeast cell tolerance to salinity, alkaline conditions, and oxidative stress [42]. In the same line of studies, *Th*MT3 isolated from the alkaline/saline-resistant plant *Tamarix hispida* conferred the transgenic yeast cells increase tolerance to Cd(II), Zn(II), Cu(II), and NaCl stresses, triggering increased accumulation of Cd(II), Zn(II), NaCl, but not Cu(II) [43].

Often, plants acquire heavy metal tolerance when growing on contaminated sites due to symbiosis with the radicular, arbuscular mycorrhizal fungi that penetrate the cortical cells of the roots of a vascular plant; one MT isolated from such fungus, *Glomus intraradices*, was also shown to complement the Cu(II) and Cd(II) sensitivity of a *cup1* mutant [44], while MT1 and MT2 from the ectomycorrhizal fungi *Hebeloma cylindrosporum* and *Paxillus involutus* functionally complemented the Cu(II) and Cd(II) sensitivity of yeast mutants [45, 46].

Studies on animal MTs expressed in yeast are less numerous [33, 47, 48, 72] and are used mainly for technical purposes. One notable example though is mouse *Mm*MT1a, a canonical Zn(II)-thionein (yeast Cup1 is considered a canonical Cu(I)-thionein) [3] shown to confer tolerance when expressed in Zn(II)-sensitive yeast mutants [31]. *S. cerevisiae* was also used to express human MTs, but mainly as a host for large-scale production of hMTs [49–51], for which the more productive methylotrophic yeast *Pichia pastoris* is currently preferred [52].

3. Biotechnological relevance of MTs expression in *S. cerevisiae*

The main function of MTs resides in their structure: small proteins with a significant number of cysteine residues (15–30% of the total amino acid number) [53], a characteristic that confers them a remarkable capacity to bind heavy metal ions by forming metal-thiolate clusters. MTs are natively bound to Cu(I) or Zn(II), exhibiting various affinities for the two metals, in between the canonical Cu(I)-thionein (*S. cerevisiae* Cup1) and canonical Zn(II)-thionein (*C. elegans* MT1) [3]. Ag(I) and Cd(II) can be used as respective models of Cu(II) and Zn(II) for the study of the metal-binding sites of MTs, particularly in those techniques that require isotopically active nuclei (note that copper is in the cuprous form Cu(I) when bound to MT, but the environment contains the more stable cupric ion Cu(II); when taken up by the cell, Cu(II) can be reduced to Cu(I) by Fe(III)/Cu(II)-reductases, or simply by the reductive milieu of the cell).

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With high thermodynamic stability combined with kinetic lability, MTs are important candidates for biotechnology applications. In the nonmetalate form, MTs are highly reactive and can virtually bind to any d^{10} metal [53], a trait that makes them interesting candidates for biotechnology. In this case, two aspects of MT reactivity are highly relevant: (1) metal uptake and release and (2) metal exchange [54]. Due to the polydentate thiolate nature of all MTs and their high affinity for most heavy metal ions, there are data available for binding of Cu(I), Cu(II), Cd(II), Hg(II), Ag(I), Au(I), Bi(III), As(III), Co(II), Fe(II), Pb(II), Pt(II), and Tc(IV) [55]. Another important feature of MT reactivity is the dynamic behavior, with metal uptake and release between species of different degrees of metalation. It is widely accepted that the binding of metal ions to MTs occurs rapidly, between 10 and 30 min, although longer stabilization times are required for certain ions, such as Hg(II) or Pb(II) [3].

Studies on metal exchange in MTs have also been done (usually with either Zn(II)- or Cu(I)-thioneins), starting with a metal-loaded MT forced to exchange its initially bound metal ions with other ions. Considering the series of affinity order of heavy metal ions for the thiolate ligands: Fe(II) ≈ Zn(II) ≈ Co(II) < Pb(II) < Cd(II) < Cu(I) < Au(I) ≈ Ag(I) < Hg(II) < Bi(III) [56], the Zn(II)-loaded MTs would be more reactive than Cu(I)-loaded MTs. It was noted that metal exchange occurs at a much slower pace than metal binding to apo-MTs. For example, it was revealed that binding of four equivalents of Cu(I) to Zn(II)-Cup1 required a stabilization time of 24 h to produce a mixture of Cu4-Cup1 and Cu8-Cup1 species by total displacement of the initially bound Zn(II) [3, 57]. It is interesting to note that many xenobiotic metal ions (Cd(II), Pb(II), and Hg(II)) show higher affinity for thiolate ligands than Zn(II) or Cu(I) does, and thus, in case of intoxication, MTs can work as detoxifying agents [53]. This is highly relevant especially when designing a biotechnology system aimed for removal of toxic ions, as in the case of bioremediation. In the following paragraph, studies on metal accumulation by MTs expressing *S. cerevisiae* cells are presented, and also summarized in **Table 2**.

3.1. Display of MTs on the surface of *S. cerevisiae* cells

Cell surface engineering has wide applicability due to the fact that virtually any protein can be produced and autoimmobilized on the cell exterior of an engineered cell (usually a microorganism). *S. cerevisiae* is suitable for this technique by which functional heterologous proteins/peptides can be displayed on cell surface by fusion with parts of cell wall- or cell membrane-anchoring proteins [58–62]. *S. cerevisiae*, generally regarded as safe (GRAS), is a more suitable host for cell surface engineering than other microorganisms in which the cell surface display system has been established, because yeast possesses a quality-control system for proteins and glycosylation systems of secreted proteins. In addition to the general advantages, high-molecular-mass proteins or proteins that require glycosylation modification can be displayed on yeast cell surface with maintenance of their activities, unlike when displaying them on bacteria [63]. Surface engineered cells can be subsequently treated as microparticles covered with the targeted protein [64].

S. cerevisiae cells are very good biosorbents for heavy metal ions due to the cell wall constituents, which readily sequester heavy metals once they encounter them. These constituents possess numerous metal-loving functional groups, including carboxylate, phosphate, sulfate, and

Metal investigated	Expressed MT	MT provenience	Yeast gained characteristics due to expression of MT	Reference
Cd(II)	Cup1/His6 Yeast surface display	*S. cerevisiae*	Cd(II) tolerance, Cd(II) increased adsorption; selectivity against Cu(II)	[66]
Cd(II)	$^{\Delta 1-8}$Cup1 $(^{\Delta 1-8}Cup1)_4$ $(^{\Delta 1-8}Cup1)_8$ Surface display of tandem repeats of head-to-tail yeast MT lacking the first 8 amino acids	*S. cerevisiae*	Cd(II) tolerance and adsorption were dependent on the number of tandem repeats; 4 and 8 repeats determined increased Cd(II) adsorption/recovery 5.9 and 8.9 times, respectively	[67]
Cd(II)	*Sn*MT2a, *Sn*MT2c, *Sn*MT2d, and *Sn*MT2e Yeast surface display	*Solanum nigrum* (Cd(II)/Zn(II) hyperaccumulator)	Increased Cd(II) tolerance and adsorption; concentration of Cd(II) from ultra-trace media; selectivity to Cd(II) against Cu(II) and Hg(II)	[69]
Cd(II)	*Sa*MT2	*Sedum alfredii* Hance (Cd/Zn hyperaccumulator)	Increased Cd(II) tolerance and accumulation	[76]
Cd(II)	*Th*MT3	*Tamarix hispida* Alkaline/saline tolerant plant	Increased Cd(II) tolerance and accumulation	[43]
Cu(II)	hMT2, GFP-hMT2	*Homo sapiens*	Increased Cu(II) tolerance and capacity to remove Cu(II) when expressed from yeast *CUP1* promoter	[72]
Zn(II)	*At*MT4a and *At*MT4b	*Arabidopsis thaliana*	Increased accumulation of Zn(II)	[30]
Zn(II)	*Th*MT3	*Tamarix hispida* Alkaline/saline tolerant plant	Increased Zn(II) tolerance and accumulation	[43]

Table 2. MTs heterologously expressed in yeast that determine increased accumulation of metal ions.

sulfhydryl, which decorate the outer mannan-protein layer of the wall [65]. The metal-binding innate capacity of the cell wall can be substantially increased by expressing metal-binding peptides/proteins at the cell surface [59, 61]. Using this technique, yeast cells were modified for bioremediation of Cd(II) using a cell-surface display system of its own MT, Cup1, fused with a hexahistidyl residue, by using an α-agglutinin-based display system [66]. Surface-engineered yeast cells with Cup1 and hexa-His fused in tandem (Cup1-His6, originally named YMT-hexaHis) showed superior cell-surface adsorption and recovery of Cd(II) under EDTA treatment on the cell surface compared to the His6-displaying cells, through an additive effect on chelating ability. Remarkably, the expression of Cup1-His6 did not have a strong effect on the adsorption of Cu(II). The same study revealed that yeast cells displaying Cup1-His6 exhibited a higher potential for the adsorption of Cd(II) than *Escherichia coli* cells displaying the same constructs. Additionally, cells displaying tandem Cup1-His6 showed increased resistance to Cd(II) through active and enhanced adsorption of the toxic ion, indicating that Cup1-His6-displaying yeast cells are unique biosorbents with a superior functional chelating ability.

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Adsorption of heavy metal ions at the cell surface has certain advantages compared to intracellular accumulation. First, surface adsorption allows recycling of the adsorbed ions, whereas intracellular accumulation necessitates disintegration of the cell for extraction. Second, surface adsorption is possible even in nonviable cells, providing that sufficient biomass can be produced. This is particularly important when cells are used to remove heavy metals from contaminated waters, and the conditions necessary to sustain living cells are difficult to achieve. And third, surface-engineered yeast cells can be used repeatedly as bioadsorbents since the recovery and treatment of the heavy metal ions does not greatly damage the cells [66]. In a sequel study, Cup1 was expressed as tandem head-to-tail repeats of the yeast MT lacking the first 8 amino acids (known to be nonsignificant for metal binding). Three types of constructs that were surface displayed contained 1, 4, and 8 tandem MT repeats [67].

The transgenic cells obtained were tested against excess Cd(II), and it was revealed that the adsorption and recovery of Cd(II) on the cell surface was increasingly enhanced with increasing the number of tandem repeats under conditions that allowed complete occupation of the Cd(II)-binding sites in the MT tandem repeats. Considering the relationship between cell-surface adsorption and protection against heavy metal ion toxicity, the tolerance of these surface-engineered yeasts to Cd(II) was found to be also dependent on the number of displayed MT tandem repeats, indicating that the characteristics of surface-engineered yeasts as a bioadsorbents correlated with the ability of the displayed proteins to bind metal ions [67]. Unfortunately, these promising studies soon came to a halt and no other metal ions or other MTs were taken into consideration to be used in this technique. It took ten years before another group displayed at the surface of yeast cells four type-2 MTs from *Solanum nigrum* (*Sn*MT): *Sn*MT2a, *Sn*MT2c, *Sn*MT2d, and *Sn*MT2e [68]. *S. nigrum* is an ornamental shrub (nightshade) and a Cd(II)/Zn(II) hyperaccumulator, apparently due to the four *Sn*MTs subtypes (*Sn*MT2a, *Sn*MT2c, *Sn*MT2d, and *Sn*MT2e) shown to have an important role in metal detoxification [69]. Yeast strains displaying the *Sn*MTs specified above on the cell surface were obtained, and these strains were shown to develop both Cd(II) tolerance and increased Cd(II) adsorption, exhibiting a higher affinity for Cd(II) than for Cu(II) or Hg(II) [68]. Notably, these displaying strains could effectively adsorb ultra-trace Cd(II) and accumulate it under a wide range of pHs (between 3 and 7), without disturbing the co-exising Cu(II) and Hg(II) [68]. Moreover, apart from showing a high potential for removing Cd(II) from contaminated waters, the yeast-surface engineered strains expressing *Sn*MT showed a remarkable resistance to Cd(II): while the nonengineered cells were stopped from dividing by 80 μM Cd(II), the engineered strains could live in 500 μM Cd(II) [68], a very high concentration for aqueous environments. While the study does not present accumulation data on other heavy metal ions, it is notable that the engineered strains expressing *Sn*MT could concentrate ultra-trace Cd(II) on the cell surface, encouraging further attempts to display other MTs on yeast surface (from hyperaccumulating species) with the final scope of concentrating rare metal ions from ultra-traces environments.

3.2. MT-expressing *S. cerevisiae* cells for bioremediation

Heavy metal bioremediation is an appealing approach for decontaminating polluted environments, especially because standard physico-chemical methods are ineffective and very often a source of pollution themselves [5]. An ideal heavy metal bioremediator would have certain metal-related characteristics: tolerance to high concentrations, increased accumulation, and

substantial biomass production for effective removal of heavy metal ions from the contaminated sites. These traits fall into the characteristics of the heavy metal hyperaccumulating plants, with the exception that they usually do not produce sufficient biomass [70]. *S. cerevisiae* is an example of an organism that could be engineered for bioremediation purposes. The cell surface display of metal-binding peptides/proteins presented above may not be the best approach for obtaining hyperaccumulating yeasts, since the metal binding is restricted to cell surface. Rather, (over)expressing nontoxic metal-binding proteins within yeast cell may increase the chances of obtaining hyperaccumulating strains fit for bioremediation purposes. *S. cerevisiae* is not a heavy metal accumulator due to a very active excretion system which extrudes excess metal ions from the cell [71, 72]. However, the excess free ions could be retained within the cell in a nontoxic form through sequestration by an abundant metal-binding protein, such as an overexpressed MT. Recently, an increased Cu(II) bioremediation ability of new transgenic and adapted *S. cerevisiae* strains was described [73]. In this study, *S. cerevisiae* cells were manipulated to integrate human MT2 (hMT2) and GFP-hMT2, expressed from either the constitutively p*ADH1* yeast promoter or the Cu(II)-inducible p*CUP1* yeast promoter. It was shown that only cells that expressed hMT2 from the *CUP1* promoter exhibited both increased Cu(II) tolerance and capacity to remove Cu(II) ions from growth media [73].

3.3. Heterologous expression of MTs from heavy metal hyperaccumulators

The natural heavy metal hyperaccumulators, mostly belonging to a small group of plants [34, 70], are the species whose metal-related characteristics initially prompted the ideas of bioremediation, biomining, and bioextraction. To accumulate heavy metals without developing toxicity symptoms, these organisms utilize a variety of chemical ligands capable of coordinating the metal ions in a nontoxic form. Although MTs are important candidates for sequestering heavy metal ions, the studies relevant for correlating MT expression with heavy metal accumulating phenotype are scarce and hardly encouraging [74, 75]. The examples of MT from hyperaccumulating organisms expressed in yeast are few, and they mainly focus on functional complementation tests [33, 36–38, 69, 76]. One example is worth mentioning here though, as it deals with an unusual hyperaccumulating phenotype: Ag(I)-hyperaccumulation due to three distinct MT genes of the ectomycorrhizal fungus *Amanita strobiliformis* [77, 78]. Although expressed in *S. cerevisiae* only to test the restoration of Cd(II), Cu(II), and Zn(II) tolerance to sensitive mutants, further employment of *As*MT for cellular handling of Ag(I) is worth considering.

3.4. Metallothionein as clonable tags

Due to their small size and metal-binding capacity, metallothioneins may be interesting candidates for tagging proteins for imaging, especially by electron microscopy (EM) [79–81]. Localization of proteins in cells or complexes using EM relies upon the use of heavy metal clusters, which can be difficult to direct to sites of interest. For this reason, a metal-binding clonable tag, such as it is green fluorescent protein (GFP) for light microscopy, has been pursued for a long time, and would be unvaluable for imaging by EM techniques. In this respect, MT is a very good candidate, because instead of fluorescing like GFP, it would initiate formation of a heavy metal cluster adjacent to the protein to be analyzed. A suitable clonable tag for EM is expected to have certain properties: small size and low molecular weight, so

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as not to disrupt protein kinetics/function *in vivo*. Using MTs as clonable tag implies working on either macromolecular assemblies or cells, but avoiding issues of heavy metal toxicity by delaying the addition of metal until the samples that include a protein-MTH chimera are in preparation for EM. Two successful procedures: (1) adding heavy metal to sections of samples that have already been rapidly frozen, fixed by freeze substitution, and embedded in a hydrophilic plastic and (2) adding metal during the process of freeze substitution have been described [82]. Using *S cerevisiae* as an expression system, it was shown that MT can be localized in the complex environment of a cell, and with a very good signal-to-noise ratio [83]. Thus, mouse MT1 used to tag the yeast centrosomal protein Spc42 allowed the localization the MT-tagged Spc42 in the outer layer of the central plaque of the mature yeast spindle pole body. Nevertheless, although very promising, MT tagging for protein localization may not be universally applicable as this approach did not work with protein components of the nuclear pore complex [82]. Another potential use of MT as clonable tag for imaging would be the yellow luminescence observed for Cu(I)-MT complexes [84, 85].

4. Concluding remarks

The numerous studies on MTs stand for the uniqueness of these small proteins whose undisputed trait is binding to heavy metal ions. This is evidently due to the cysteinyl residues, which represent more than 20% of the total number of MT amino acids, whereas the usual percentage of cysteinyl residues seldom surpasses 5% in most proteins. The intrinsic characteristic of sequestering metal ions in thiolate clusters make MTs very interesting biomolecules for various biotechnological application. Since *S. cerevisiae* represents a very good cellular system for heterologous expression of MTs from virtually any species (including itself), the use of MT-(over)expressing yeasts is a promising starting point for biotechniques such as heavy metal bioremediation and bioextraction. The data summarized in **Table 1** reveal that until now MT-expressing *S. cerevisiae* cells have been used for functional complementation studies (mainly MTs from plants and mycorrhizal fungi) rather than to investigate their biotechnological potential. Moreover, most of the studies concern the ions that naturally bind to MTs, Cu(I) and Zn(II), and their nonessential counterparts Ag(I) and Cd(II), along with few studies on sulfur-loving metal ions such as Hg(II) and Pb(II). Although other interesting noncanonical metal ions such as Mn(II), Ni(II), and Co(II) have been shown to strongly bind to MTs [55, 56], very few studies actually determined the MT binding to these ions *in vivo* (**Table 2**), and MT-expressing yeast cells would be very good models for filling this gap. Especially, obtaining heavy metal hyperaccumulating yeast cells by heterologous (over)expression of recombinant MTs would open new opportunities for bioremediation, bioextraction, and for emerging techniques, such as synthesis of clonable heavy metal nanoparticles [86]. Another promising biotechnique involving MTs is obtaining new clonable tags for cell imaging. While some timid progress has been reported on imaging by EM of proteins tagged with MT in yeast cells [82], the possibility to use the yellow luminescence of Cu(I)-MT for imaging MT-tagged proteins is largely unexplored. In this direction, construction of a systematic collection of *S. cerevisiae* strains that express *all* the MTs identified so far would be not only a challenge but also a prerequisite for systematic investigation of MTs for various biotechnology purposes.

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