FINAL REPORT

U.S. Department of Energy

Molecular Genetics of Metal Detoxification: Prospects for Phytoremediation

Principal Investigator: David W. Ow

Institution: U.S. Department of Agriculture

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3. Executive Summary

Inorganic elements that include toxic metals and radionuclides present an unusual pollution challenge to ecosystems. These elements persist so that even low rates of deposition over time leads to toxic accumulation. Engineering nonfood plants to mine inorganics from contaminated sites offers an economically feasible solution for reclamation. Plants, however, generally have a low tolerance for these toxins. Oxidative stress is induced by high accumulation of heavy metals and radionuclides. Through a first strategy of analyzing fission yeast mutants, we identified and characterized several genes involved in heavy metal tolerance in the fission yeast. Most extensive characterization was conducted on a gene encoding a mitochondrial sulfide dehydrogenase. This gene prevents metal-induced sulfide toxicity in the mitochondria. Interestingly, highly homologous genes of unknown functions are also present in higher eukaryotes including humans.

Through another strategy involving the direct expression and selection of cDNA clones in fission yeast, we identified approximately 50 cDNA clones that confer hypertolerance to cadmium and/or oxidative stress in fission yeast. These cDNAs have been sequenced and many of them have been transferred to vectors for transformation in plant and animal cells. By the end of the funding period, we were ready to test the function of these cDNAs when overexpressed in animal cells and transgenic plants. Functional identification of genes that ameliorate toxic effects of heavy metals and radionuclides will provide vital tools for engineering bioremediating plants. Additionally, these genes provide valuable insights into cellular defense mechanisms against exposure to these harmful oxidants.
4. Research Objectives

Introduction

Promises of phytoextraction

The global industrial revolution has led to an unprecedented dissemination of toxic substances into the environment. Exposure to these pollutants, including dietary intake of plant-derived food and beverages, can have long-term effects on human health. This is of particular concern with substances that have a long biological half-life. The metal cadmium, for instance, can have a half-life of up to 30 years in the human kidney. Chronic bioaccumulation, at low quantities that might otherwise be considered insignificant, could nonetheless contribute to renal failure. Most organic compounds can be inactivated eventually through biotic and abiotic mineralization processes. Inorganic compounds, however, such as most heavy metals and some radionuclides, are practically indestructible. Their persistence in the environment requires that they be removed from the contaminated source or be converted into a biologically inert form. A conversion process is possible for only a few of the toxic elements, such as the biovolatilization of Se, Hg, and $^3$H (2,38,50). For most other toxic metals and radionuclides, effective remediation requires the physical extraction from soil and water systems. Conventional engineering technologies, the “dig and treat”, or “pump and treat” strategies, are prohibitively expensive.

A biological solution has been touted as a promising alternative (6,25). A wide variety of bacterial, fungal, algal and plant systems are capable of concentrating toxic metals from soil or water. However, no cost-effective way exists to retrieve small organisms from the soil. On the other hand, contaminant-laden plants are easy to harvest, especially if the metals accumulate in shoots. Metals may be disposed of after concentrating the plant biomass, or be reclaimed, which may provide an additional incentive for bioremediation.

Phytoextraction is conceptually simple, but plants generally do not accumulate large amounts of toxic metals and radionuclides. This severe limitation begs for greater investment in fundamental research on metal entry and tolerance. The knowledge base gained will have a huge financial side benefit as it is critical to ensure that plants engineered to accumulate essential metals for human consumption do not also accumulate analogous toxic elements.

Breaking the entry barrier

Plant uptake of inorganics is a prerequisite for phytoextraction. All plants have vast root networks that take up and concentrate various minerals. Toxic substances gain entry into the plant nonspecifically through transport processes that recognize chemically similar, but essential nutrients. For example, Cd can be mobilized through transport mechanisms evolved to recognize Zn, Cu, Fe and/or other divalent cations (5,9,12,15,18,20,43,51).

Plants can be artificially induced to accumulate high metal levels by increasing metal bioavailability. This can be done by (i) decapitating roots to allow metals to directly enter the stem from nutrient solutions, (ii) using hydroponics, and (iii) amending soil with metal chelators. Genetic modification can also achieved similar results.
Through engineering high-level production of metal transport proteins, it is possible to create transgenic plants with higher metal content\(^{(20)}\).

**Metal lethality**

Increasing metal uptake is not in itself a sustainable solution for phytoextraction. The plants that take up a high amount of metals lack the mechanisms to tolerate the higher metal content. Consequently, they cease to grow. This has been observed with transgenic yeast and plants that over-produce metal transporters; they become hypersensitive to metal toxicity\(^{(4,15,20,43)}\).

Proponents of the soil amendment strategy have attempted to overcome the metal lethality problem. Metal-sensitive plants were grown to maturity in soil where the bioavailability of toxic metal is low. Upon supplementation with chelating agents, high uptake would occur due to the newly dissolved metal ions. Since the plants would be harvested shortly thereafter, lethality of metal-laden plants would be a moot issue. Indeed, promising results were reported for the initial plantings\(^{(3,16,46,48)}\). This initially promising strategy, however, suffers from two flaws that currently prevents its implementation for phytoextraction. First, the amount of inorganic elements released by chelating agents is difficult to control. If released in excess, subsequent generations of plants (seedlings), which do not possess high metal-tolerance mechanisms, grow poorly on chelator-treated plots. Consequently, the plot can become barren caused by excess bioavailable metals. Second, there is serious concern that dissolved metals would leach down to ground water, spreading the contamination elsewhere.

**Critical role of metal tolerance**

The key for phytoextraction technology is overcoming the inability of plants to detoxify the effects of high metal contents. It is highly likely that most plants already have the genetic capacity for high metal uptake through many nonspecific transport systems and that substrate availability may be more rate limiting than transport. This is supported by (i) hydroponically grown plants take up more heavy metals than the same plants grown in soil, (ii) amending soils with metal chelators dramatically increases plant metal content, and (iii) mine sites are scarcely populated with vegetation. The third observation is consistent with plants capable of metal uptake but inadequate in metal detoxification.

The existence of metal hyperaccumulators reveals that two critical genetic traits exist\(^{(1)}\). First, there is genetic potential for overaccumulating toxic metals. For example, this has been shown for the Zn and Cd hyperaccumulator *Thlaspi caerulescens*, which has a higher Zn influx rate and more ZNT1 Zn-transporter RNA than the nonaccumulator *Thlaspi arvensae*\(^{(19)}\). Second, these plants must also have evolved hypertolerance mechanisms that accommodate their high metal content. It is on this second trait that we have been focusing our research efforts. Our goal is to identify and isolate genes that confer metal hypertolerance to living cells and/or detoxify the oxidative damage. We envision that these genes would provide the genetic hardware to engineer metal-hypertolerant plants.
**Complexation of metals for detoxification**

Unique to plants and certain fungi, such as *Schizosaccharomyces pombe*, metal stress induces the synthesis of metal-binding peptides commonly known as phytochelatins (PCs). Derived from glutathione, PCs have the general structure of \((\gamma\text{-Glu-Cys})_n\text{Gly}\), where the number of \(\gamma\text{-Glu-Cys}\) units extends up to 11 \(^{11}\). Some PC-related peptides lack the carboxyl-terminal Gly or have instead \(\beta\text{-Ala, Ser, or Glu}^{33}\). However, these variant peptides are usually found in lower abundance. Many metals induce PC synthesis, but formation of PC-metal complexes has largely been examined with \(\text{Cd}^{2+}\) and \(\text{Cu}^{2+}\). Several reports show that PCs also chelate \(\text{Ag}^{+}\), \(\text{Hg}^{2+}\), \(\text{Pb}^{2+}\) and \(\text{Zn}^{2+}\) \(^{21,22,23,44}\). In the case of \(\text{Cd}^{2+}\), two PC-metal complexes have been described: a low molecular weight (LMW) PC-Cd complex, and a more stable high molecular weight (HMW) PC-CdS complex that contains additional acid-labile sulfide \(^{7,34}\). Formation of the HMW complex reduces the Cys:Cd ratio from \(\sim4:1\) to a more economical \(\sim2:1\) \(^{42}\). The appearance and the location of the two complexes suggest that PC is a cytoplasmic Cd/metal scavenger that targets metals to the vacuole for storage as a stable HMW PC-CdS complex.

In addition to PCs, plants can synthesize small cysteine-rich proteins known as metallothioneins \(^{37}\). The role of plant metallothioneins in metal tolerance is not entirely elucidated, but the animal and fungal proteins appear to play an important role in metal tolerance\(^{13}\). There have been numerous reports of engineering animal metallothionein production in plants \(^{10,14,24,31,49}\). These proteins increase metal tolerance to varying degrees, but not with substantial increase in metal uptake. In addition, the tolerance effect depends on whether the PCs are given the chance to be synthesized. When a high amount of Cd is suddenly introduced to the seed or the plant, the metallothionein-producing seedling or plants are better able to respond to the toxic metal. This is because of the already accumulated metallothioneins, whereas the control plants would have to induce de novo synthesis of the PC peptides. In field situations, however, plants rarely are challenged with a sudden influx of toxic metals. Rather, they slowly accumulate the metals as their roots extend into the rhizosphere. When given adequate induction of PCs, the additional synthesis of metallothioneins does not confer a significant advantage.

**Original Objective**

Our overall goal was to identify and characterize metal hypertolerance genes. The original focus was on the intracellular metal chelation process in the *S. pombe* fungal model system \(^{28}\). We had planned to characterize several *S. pombe* mutants that are both hypersensitive to \(\text{Cd}^{2+}\) as well as fail to accumulate wild type levels of one or both of the PC-bound Cd complexes. The rationale was to use these mutants to find genes essential for normal metal tolerance, especially genes involved in the PC chelation process. We envisioned that these genes could be used to engineer enhanced metal chelation. This approach was substantiated by previous work on *hmt1*, which encodes a vacuolar membrane transporter that pumps PC-Cd into vacuoles. Overproducing this protein enhanced both vacuolar Cd sequestration and Cd tolerance versus wild type *S. pombe* \(^{26}\).

**Revised Objective**
Midway through this work, three compelling developments prompted us to redirect our emphasis from what we proposed in 1996. First, the genetic approach is rather slow. Characterizing one mutant takes a minimum of 3 years per investigator to clone the responsible gene, sequence both mutant and wild type alleles, and assign functions to the encoded proteins by biochemical experimentation. Second, the hmt1 model proved to be more exception than rule. Several other genes complemented Cd hypersensitive mutants, yet none conferred significant hypertolerance when overexpressed in a wild type strain. This suggests that most pathways might already be optimized. The odds that any basic information generated from these other genes would become useful for phytoextraction are slim. Third, significant advances in genomics research have been made since 1996. With the promise of a whole plant-genome sequence by the end of year 2000, we foresaw the logic of using high-throughput functional-genomic analyses to elucidate genes and their functions.

5. Methods and Results

Progress on Strategy #1. Cd-hypersensitive mutants.

In the original strategy, we mutagenized S. pombe cultures to screen for mutants that are hypersensitive to Cd. Subsequently, we screened for deficiencies in the accumulation of one or both PC-Cd complexes. Finally, we cloned the DNA that restores tolerance to the Cd-hypersensitive mutants.

Prior to the funding period, we had obtained the following results:

**PC-Cd transporter.** From the analysis of mutant LK100, we identified a vacuolar membrane protein encoded by hmt1. The gene product actively transports cytoplasmic PC-Cd complexes into vacuoles. Overproducing HMT1 protein enhances both Cd accumulation and Cd tolerance. Additional Cd and glutathione-conjugate vacuolar transporters exist that are independent of HMT1-mediated PC-Cd transport. The Cd transporters also route Cd into the vacuole for incorporating into HMW PC-CdS complexes.

**Sulfide complex formation.** Through analysis of mutant LK69, a likely source of the S\(^2-\) in the HMW PC-CdS complex appears to be cysteine sulfinate, a sulfur analog of aspartate derived from cysteine oxidation. Genetic data suggest that cysteine sulfinate is incorporated into purine intermediates by two enzymes of the purine biosynthetic pathway, AMP-S synthetase and SAICAR synthetase. Biochemical studies support this hypothesis. In vitro, these enzymes can utilize cysteine sulfinate in place of aspartate. The cysteine sulfinate-derived purine intermediates are probably carriers of another sulfur compound, which then leads to formation of sulfide for the PC-CdS complex.

**Cd-induced sulfide production.** The analysis of DS12 revealed a defect in the sulfite reductase gene. This leaky mutation provides adequate cysteine for normal growth, but insufficient cysteine for Cd-induced PC production. We created a disruption in the
sulfite-reductase gene. As expected, abolishing sulfite-reductase activity is lethal unless cells are fed cysteine. Under these conditions, Cd-induced sulfide production is still operational. This indicates that Cd-induced sulfide production is independent of the assimilatory sulfate reduction pathway, suggesting that a novel pathway may provide S\(^2-\) for the HMW complex. Intriguingly, and in contrast to Cd, Pb-induced sulfide production requires sulfite reductase and the assimilatory sulfate reduction pathway \(^{(29)}\).

\textit{During the funding period, we completed the following:}

\textbf{Mitochondrial gene required for PC production.} Mutant JS563 was originally found to be hypersensitive to Cd and deficient in PC peptide accumulation \(^{(45, \text{VandeWeghe and Ow, submitted})}\). Further analysis revealed that this mutant is also hypersensitive to \(\text{Hg}^{2+}\), heat shock, hydrogen peroxide, and the thiol-oxidizing agent diamide. It forms yellow colonies in the presence of Cd, suggesting the formation of CdS particles. Indeed the mutant was found to hyperproduce sulfide. The protein, which we have named HMT2, is a sulfide:quinone oxidoreductase \(^{(45)}\). In vitro studies show that it can bind FAD and catalyze the reaction:

\[
\text{S}^{2-} + \text{coenzyme } \text{Q}_{2}^{(\text{oxid})} \rightarrow \text{S}^{0} \text{ (oxid)} + \text{coenzyme } \text{Q}_{2}^{(\text{red})} \quad (\text{Q}_{2} \text{ is a water soluble ubiquinone analog}).
\]

HMT2 is loosely associated with mitochondrial membranes. Our current hypothesis is that during Cd stress, high sulfide levels accumulate, and some of it ends up in mitochondria. HMT2’s role could be to oxidize mitochondria sulfide to protect it from poisoning the electron transport chain and/or to shunt electrons from \(\text{S}^{2-}\) to the electron transport chain. Interestingly, homologous enzymes may be widespread in higher organisms. Sulfide oxidizing activities have been described previously in animal mitochondria, and genes of unknown function, but with similarity \(hmt2^{+}\), are present in the genomes of flies, worms, rats, mice and humans. This suggests a common tolerance mechanism in diverse higher organisms.

\textbf{WASP and a putative P-type ATPase Transporter.} Mutant JS237 was isolated initially as a Cd hypersensitive mutant that lacks the HMW PC-CdS complex. Further analysis revealed that the mutant is also defective in mating, tolerance to \(\text{Ca}^{2+}\), heat shock, and nutrient stress. A 7 kb clone was isolated that complements the mating defect but only partially complements the stress defects. This 7 kb genomic fragment contains the sequence for three genes: a P-type ATPase of the APL/cation transporter family (HMT3), an actin-regulating protein belonging to the Wiskott-Aldrich Syndrome Protein (WASP) family \(^{(8,36)}\), and a putative transcription factor of the BTF3/\(\beta\)NAC family \(^{(47,52)}\). WASP is the only gene that is contained intact within this 7 kb clone. For the BTF3/\(\beta\)NAC gene, only the leader sequence and the first few amino acids is present, and the transporter gene lacks about 1 kb from its 5’-end that includes some coding sequence. Interestingly, the WASP mRNA has two variants that differ depending on alternate splicing of the fourth intron \(^{(30)}\).

When the mutant allele was sequenced, it was found to harbor a mutation in the splice donor sequence of the first intron of the WASP gene. RT-PCR experiments show that this mutation blocks excision of the intron. Since the unspliced message allows translation of only the first 75 amino acids of WASP, the mutation abolishes WASP
function. Consistent with this, JS237 has a severely disrupted actin cytoskeleton, a defect that is exacerbated by stress. GFP-tagging of full-length fission yeast WASP confirms that this protein strictly colocalizes with cortical actin in living cells. Transformation of JS237 with a wild-type sequence containing the intact WASP gene restores a wild-type cortical actin phenotype and mating competence, but does not fully restore cadmium-tolerance.

The single mutation in JS237 may impair the expression of both WASP and the P-type transporter. From Northern blot data, the WASP cDNA detects three transcripts; the expected 2.4 kb WASP, the 0.6 kb BTF3, and a 4.1 kb message. In contrast, the P-type transporter gene only detects the 4.1 kb message. This indicates that the 2.4 kb and the 4.1 kb transcripts overlap. It appears that the single splicing defect has affected the accumulation of the 4.1 kb transcript in addition to the translation of WASP. The Northern data show that the 4.1 kb transcript is upregulated in the mutant during Cd stress, as opposed to decreased expression in wild type cells. In this model, mating and growth defects are ascribed to the loss of WASP function, while stress sensitivities, including Ca$^{2+}$ and Cd$^{2+}$, are ascribed to the altered regulation of the transporter transcript. Many P-type transporters are involved in ion transport. It is unknown but tempting to speculate that HMT3 plays a similar role.

**Mannose-1-phosphate guanylttransferase-like protein.** Mutant JS618 shows deficient accumulation of the HMW complex. The DNA fragment restoring Cd tolerance to this mutant encodes a protein with sequence similarity to mannose-1-phosphate guanylttransferase, an enzyme that converts mannose-1-phosphate to GDP-mannose. GDP-mannose is a substrate for glycosyltransferases in protein glycosylation and polysaccharide biosynthesis. Our current thinking is that the mutation abolishes the glycosylation of a protein needed for formation of the HMW complex, but potential glycosylation targets remain to be determined (29).

**Cd-inducible novel message.** Mutant JS282 hypoproduces phytochelatins. A 7 kb genomic DNA fragment complements the Cd hypersensitivity of the mutant. An 0.8 kb cDNA obtained from the genomic clone is specifically induced at the mRNA level by Cd, but not by other metals (Cu, Hg, Se, Zn), oxidative or heat stress. Expression of this gene is Cd concentration-dependent; increasing Cd exposure induces proportionally higher levels of mRNA accumulation. Removal of Cd from the media leads to a rapid decrease in mRNA abundance. The promoter for this gene contains putative binding motifs for AP-1, an oxidative stress and heavy metal-inducible transcription factor. The 3’ untranslated region contains four AUUUA motifs that are important for regulating mRNA turnover. We assume that these AUUUA motifs are at least partially responsible for the rapid disappearance of the 0.8 kb transcript upon removal of Cd. The genetic signals providing metal-specific transcription initiation and stability may be critical for engineering metal-responsiveness into remediation genes. This will be useful if a gene is found to be toxic when constitutively expressed in plants.

**Progress on Strategy #2.** Genes expressed during Cd-stress
The genome revolution has been progressing with such a rapid pace that whole-genome sequences have been completed for a number of organisms ranging from bacteria, yeast, nematode, fruit-fly, Arabidopsis and human. Biologists will be able to analyze gene function at an unprecedented rate using comparative databases. There are currently two popular approaches for functional genomics. The first relies on mutational analysis. There are multiple libraries of “gene-tagged” plants mutagenized by the random insertion of a defined DNA element. Many are publicly available for screening. A mutant with relevant phenotype caused by the inserted DNA tag allows a facile identification of the “disrupted” gene. Plants can also be screened for disruptions in known genes by PCR for subsequent phenotype analyses. Conceptually, one could search for mutants that are metal hypertolerant. However, a DNA insertion into a known gene will likely disrupt gene function rather than confer a new function, making the plant more likely to be metal hypersensitive. A search for hypersensitive plants would not differ dramatically from our work on metal hypersensitive mutants of \textit{S. pombe}. Therefore, the tagged library approach would not offer a significant advantage. However, we may pursue this approach to probe into possible functions of selected genes that we have already identified in a functional screen (see below).

In the second approach, various high throughput techniques are used to characterize the patterns of mRNA and protein abundance. Identifying the mRNA population that is induced during metal stress might reveal molecular homeostasis mechanisms. We used subtractive hybridization to isolate a collection of ~250 clones that are upregulated by Cd stress. A fifth of these were partially sequenced. This screen was based on expression, not function, and most of the cDNA clones are of partial length. DNA sequencing revealed that many of the deduced peptides share similarity with proteins associated with oxidative stress. A few have metal binding domains and may be involved in Cd complex formation. One clone is similar to ATP binding cassette-type transporters, the class of proteins that includes HMT1. However, induction by Cd indicates neither a “cause and effect” relationship, nor that expression of these cDNAs would increase metal resistance. Since we have identified many genes using a functional screen (see below), this collection of Cd-inducible cDNAs is currently low in priority.

Progress on Strategy #3. cDNAs that confer hypertolerance

Our most promising approach is based on the functional overexpression of cDNAs in fission yeast. Our assumption is that hypertolerance genes that protect this yeast from Cd and oxidative stress agents may also protect higher eucaryotic plants and animal cells. Yeast is an excellent eucaryotic model system with the advantage that DNA transforms these cells at high efficiency. In a single experiment, \(10^5\) - \(10^6\) independent transformants can be readily obtained to screen an entire cDNA library. The fission yeast \textit{S. pombe} is a better model system for plant metal tolerance than the more commonly used budding yeast \textit{Saccharomyces cerevisiae}. \textit{S. cerevisiae} is about 1000-fold more sensitive to Cd than \textit{S. pombe}. Using such a highly sensitive recipient would likely lead to the identification of cDNAs that only marginally increase tolerance. Furthermore, \textit{S. pombe} shares common metal detoxification pathways with higher plants.

Initially, we transformed a cDNA library made from \textit{S. pombe} into wild type cells and screened for Cd tolerance. This allowed us to identify Cd tolerance genes and to
refine the screening protocol prior to testing heterologous cDNA libraries. We subsequently screened plant libraries made from *Arabidopsis*, whose complete genome sequence will be publicly available soon, and *Brassica juncea*, a known metal hyperaccumulator. *B. juncea* and *Arabidopsis* are in the same family of plants, so we expect *B. juncea* genes to be quite homologous to those available in the *Arabidopsis* genome database. Since oxidative damage commonly occurs upon exposure to heavy metals and radionuclides, we also broadened our search to isolate plant genes that confer resistance to oxidizing agents.

By the end of the granting period in 1999, we had collected a number of cDNA clones that confer metal tolerance in *S. pombe*. During the one-year no cost extension period from Sept 1999 to August 2000, we transferred many of these clones to test for function in plants. To date, our collection consists of the following.

From *S. pombe*, we have 18 cDNA clones that confer hypertolerance to Cd. Only two of them are from pathways that are in metal detoxification. A large number of them fall into biosynthetic or regulatory pathways. Some are novel proteins with domains that could have similarity to known proteins. Half of the cDNAs can also confer hypertolerance to oxidative stress in *S. pombe*.

From plants, there are 6 Cd-hypertolerance cDNA clones from *B. juncea*, and 2 from *A. thaliana*. Again, many of these genes were subsequently found to also confer resistance to oxidative stress agents, as well as to other metals. Since oxidative damage commonly occurs upon exposure to heavy metals and radionuclides, a separate search to isolate plant genes that confer resistance to oxidizing agents led to 25 *B. juncea* cDNAs.

**Expression in plants.** Most members of the collection of hypertolerance cDNAs has been introduced into *Arabidopsis*. A few constructs have been tested. In these tests, multiple T1 plants from independent transgenic lines were used. Due to the differences in genome location and pattern of DNA integration, gene expression could vary among different transgenic lines. Hence, some differences among the lines would be expected. In addition, the T1 plants are derived from self-fertilized T0 (primary transformant) plants, which means that the transgenes could be in either a hemizygous or a homozygous state. Despite these possibilities of line to line variation and gene dosage effects, a few cDNAs show promising results. For example, plants transgenic for Bj4 or Bj6 showed greater tolerance to Cd, Zn and diamide compared to empty vector control plants. Bj6 and Bj4 represent two distinct members of the same gene family, as their sequences share extensive homology but are not identical. Independently derived transgenic lines of Bj6 and Bj4 also flowered later than the independent lines of the empty vector control. As early flowering is a sign of stress, late flowering could indicate enhanced stress tolerance.

**Bj4/Bj6 not inducible by Cd.** A popular approach in functional genomics is the use of high throughput methods to detect the appearance and disappearance of mRNA species. This technology has led to the assumption that mRNA abundance would indicate function. Surprisingly, this correlation is not found with Bj4 and Bj6. Using Bj6 as a hybridization probe, which cross hybridizes with Bj4, we failed to detect a difference in steady-state mRNA in the presence or absence of Cd. This indicates that Bj4 / Bj6 would not have been discovered by methods that monitor mRNA abundance.
6. Relevance, Impact, and Technology Transfer

The ability to engineer plants to tolerate the toxic effects of heavy metals and the associated oxidative stress is a prerequisite for re-vegetating toxic waste sites, and for phytoextraction or phytostabilization of the contaminants of concern. We have only begun to examine the first few clones from our cDNA collection. It is likely that some of these clones will not work in a heterologous system. For example, in previous attempts, the \textit{hmt1} gene product (vacuolar transporter of PC-Cd) was not detectable in our transgenic tobacco plants. On the other hand, we are confident that a hypertolerance phenotype will be conferred by a good percentage of these genes, as they were functionally selected for this trait. If this proves true, it would be important to expand our functional tests to other model plants.

The testing for functional expression can also be expanded to animal cell lines. The concern with Cd and other oxidatives stress agents is because of their toxicity to humans. Cd and other toxic metals interfere with many cellular enzymatic activities. They also induce oxidative stress by forming reactive oxygen species (ROS), such as superoxide (\textit{O}_2^\cdot{}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) that oxidize DNA, proteins, lipids, and carbohydrates. Similar to Cd, radionuclides also cause oxidative damage. If the expression of certain cDNAs can alleviate this stress, we may be able to uncover new genetic mechanisms that protect animal cells from harmful effects of toxic metals and radionuclides.

As for impact and technology transfer, this will not be realized until we can show that the genes operate at field levels and that hypertolerance traits can be engineered into plants used for phytoextraction and phytostabilization purposes. This would have been the next phase of research, but unfortunately, the grant was not renewed.

7. Project Productivity

The original project goal of characterizing mutants was mostly completed. One Ph.D. dissertation and two refereed manuscripts have been published (\textit{Journal of Biological Chemistry & Molecular Pharmacology}). Another manuscript has been submitted for publication (\textit{Molecular Microbiology}). As for other results, most of them have been described in book chapters. Although there are basic research interests in the genes identified from the mutants, there is little interest from an applied point of view for phytoremediation. This is because they failed to confer metal hypertolerance when over-expressed, even though they are needed for normal metal tolerance. Therefore, in keeping with the goals of DOE-EMSP, we felt that the efforts needed to complete the mutant work just for the sake of additional publications could be better spent hunting for new candidate genes. In redirecting our research emphasis, we have indeed collected about 50 new genes that confer hypertolerance to the fission yeast. If only funding were available, we would have been able to complete the screening of their expression in plants, and possibly also in animal cells. In our view, three years of funding (including the one year no cost extension) is insufficient to start a new research direction as ambitious as ours.
8. Personnel Supported

Graduate Student:


Postdoctoral Scientists:

1996-1997 Todd Zankel, Ph.D., currently a Senior Scientist at Biomarin Pharmaceuticals, Novato, CA
1996-1998 John Henstrand, Ph.D., currently a Scientist at Biomarin Pharmaceuticals, Novato, CA
1996-1999 Sonya Clark, Ph.D., currently a Scientist at Biomarin Pharmaceuticals, Novato, CA
1998-1999 David Finkelstein, Ph.D., currently a Research Associate at the Carnegie Institute, Stanford University.

9. Publications

Peer-reviewed journals and books


Ph.D. Thesis

Unreviewed publications


Submitted for publication

VandeWeghe, J., Ow, D.W. Accumulation of metal-binding peptides in fission yeast requires \textit{hmt2}^*, submitted to *Molecular Microbiology*.

10. Interactions

Participation/presentation at meetings, workshops, conferences, seminars, etc.


10/09/96, Comenius University, Botany Department, Bratislava, Slovakia, “Prospects of engineering plants for bioremediation”

10/15/96, Research Institute of Plant Production, Piestany, Slovakia, “Prospects of phytoremediation”


05/22/97, University of California, Davis, Molecular genetics of metal tolerance.

05/29/97, University of Minnesota Biotechnology Seminar, Minneapolis, MN. “Molecular biology of metal tolerance genes”.

09/26/97, Fifth International Congress of Plant Molecular Biology, 09/21/97-09/27/97, Singapore. “Heavy metal tolerance genes for phytoremediation”

09/29/97, International Symposium on Environmental Engineering, Kyongju, South Korea, 09/28/97-10/1/97. “Heavy metal tolerance genes as tools for phytoremediation.”

10/02/97, Gyeongsang University, Chinju, South Korea. “Recent progress in site-specific recombination and phytoremediation”.

12/16/97, Cold Spring Harbor Meeting on Genetic Approaches to Analyzing Root Physiology, 12/14/97-12/17/97. “Heavy metal transport and sequestration”.

03/12/98, Beltsville Agricultural Research Center, Beltsville, MD. “Heavy metals, recombination and gossypol”.


05/29/98, Institute of Botany, Academia Sinica, Beijing China. “Heavy metal tolerance genes, prospects for bioremediation”.

06/02/98, Peking University, Beijing China. “Heavy metal tolerance genes, prospects for bioremediation”.

06/19/98, University of Connecticut Agricultural Biotechnology Symposium, Storrs, Conn., June 19-20, 1998. “Molecular genetics of heavy metal tolerance”.

08/24/99, National Taiwan University, Taipei, Taiwan. “Molecular genetics of heavy metal tolerance”.


Consultative and advisory functions

Monitoring Editor, Plant Physiology, for the American Society of Plant Physiologists, 1992-1998 (decisions on ~ 30 manuscripts per year).

Panel Member, Review of Department of Energy Biomass Remediation Systems Project (Feb. 1994)

Panel Member, Department of Energy Special Workshop on Phytoremediation Research Needs (July 1994)

Panel Member, Department of Energy Environmental Remediation competitive grants (June 1997)

Member, Plant Science Advisory Board, Biotechnology Research and Development Corporation (BRDC), 1815 N. University St., Peoria, IL (1998)

Invited Participant, DOE Workshop on Phytoremediation (Nov. 30-Dec 2, 1999, Argonne National Laboratory).

Member, Scientific Advisory Board, Icon Genetics, Princeton, NJ (2000-present).


Collaborations

Sulfide-mediated cisplatin resistance. Cisplatin is one of the most widely used cancer drugs. As with many chemotherapeutic agents, acquired resistance to this platinum compound can arise with prolonged use. In a collaborative study with Stephen B. Howell, M.D., we found that while glutathione and PC levels do not correlate with cisplatin resistance, the sulfide levels did, suggesting that sulfide and cisplatin may interact in human cancer cells (32).

11. Transitions

None.
12. Patents

None filed. Additional work is needed to reduce to practice the promising leads made on the collection of hypertolerance genes.

13. Future Work

If funding were available, the collection of hypertolerance gene will be tested in plants and animal cells. Those that function in higher eucaryotic cells will be used to engineer field plants.

14. Literature Cited


15. Feedback

A three year grant is inadequate for research with long-term impact.

16. Appendices

Attachments: electronic files of:


17. Quantifies / Packaging.

None.
Role of Determinants of Cadmium Sensitivity in the Tolerance of Schizosaccharomyces pombe to Cisplatin

PAOLA PEREGO, JENNIFER VANDE WEGHE, DAVID W. OW, and STEPHEN B. HOWELL

Department of Medicine and Cancer Center (P.P., S.B.H.), University of California, San Diego, La Jolla, California 92093, and Plant Gene Expression Center, United States Department of Agriculture-Agriculture Research Service/University of California, Berkeley, California 94720 (J.V.W., D.W.O).

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SUMMARY

The genetic mechanisms underlying cisplatin (DDP) resistance in yeast were investigated by examining the cytotoxicity of DDP to Schizosaccharomyces pombe mutants that were either hypersensitive or resistant to Cd. Despite reports that have linked glutathione (GSH) to DDP resistance in human cancer cells, we found that a mutant of S. pombe that was hypersensitive to Cd by virtue of a 15-fold reduction in GSH level and lack of phytochelatin production was as tolerant as the wild-type strain to DDP. A mutant that harbored a mutation in hmt1, the gene encoding an ATP-binding cassette-type transporter for vacuolar sequestration of a phytochelatin/Cd complex, exhibited only mild hypersensitivity to DDP even though it was 100-fold more sensitive to Cd. Overexpression of hmt1 in wild-type or mutant cells conferred tolerance to Cd but failed to do the same for DDP. However, a strain that produced 6-fold more sulfide than wild-type cells was found to be 6-fold more resistant to DDP and twice as resistant to Cd; an association between DDP resistance and sulfide production was observed in three other strains that were examined, and overproduction of sulfide was accompanied by reduced platination of DNA. These results indicate that GSH and the GSH-derived phytochelatin peptides do not play critical roles in determining sensitivity to DDP in S. pombe but rather identify increased production of sulfide as a possible new mechanism of DDP resistance that may also be relevant to human cells.

The chemotherapeutic agent DDP is widely used for the treatment of ovarian, head and neck, and small cell lung cancer as well as a variety of squamous cell cancers; however, the development of resistance during treatment remains a major obstacle to its effective use. The mechanisms that underlie the development of acquired resistance to DDP in human tumors are not well understood. There have been reports that DDP and Cd share detoxification mechanisms in mammalian cells. Despite the chemical differences between Cd and DDP, both are capable of reacting with nucleophilic sites on small molecules such as thiol compounds and large molecules such as metallothioneins and DNA. Overproduction of metallothioneins protects mammalian cells from Cd toxicity and, under some circumstances, has been reported to protect against DDP toxicity as well (1). GSH also seems to play a role in the defense against Cd and DDP. Depletion of GSH with buthionine sulfoximine increases the sensitivity of human cells to both metals (2, 3), and in some cell lines selected for resistance to DDP, there is an associated increased in GSH content (4). To gain insight into whether the two cytotoxins might share some common defense mechanisms, we examined the link between Cd tolerance and DDP resistance in a fission yeast model system.

In yeasts, several resistance mechanism are known that are activated on exposure to toxic metals, and molecular genetic analysis has been used to identify some specific genes involved in heavy metal detoxification pathways (5). In S. cerevisiae, the CUP1 gene, which codes for a copper-metallothionein, mediates resistance to Cd (6). In contrast, the fission yeast S. pombe lacks metallothioneins (7) but responds to heavy metal stress with increased synthesis of metal-binding peptides enzymatically synthesized from GSH (7). These peptides, known as PCs, are frequently found in the plant kingdom and have the general structure of (γ-Glu-Cys)nGly, where n is usually 2–4. PCs with as many as 11 γ-Glu-Cys units have been reported (8–10). Candida glabrata uses both the metallothionein- and the PC-mediated detoxification pathways and activates copper-metallothionein production in response to Cu and PC production in response to Cd (5).

In the case of Cd, PCs form a loose complex with the metal, which is then sequestered into the vacuoles, where a more stable complex is formed consisting of PCs, Cd, and acid-
labile sulfide (11, 12). Mutations that decrease the production of PCs, including those that affect GSH levels (13), the vacuolar sequestration of the PC/Cd complex (12), or the production of Cd-induced sulfide (14), result in a Cd-hypersensitive phenotype. In addition, certain mutants of C. glabrata and S. pombe can achieve Cd resistance through the anomalous production of high sulfide levels (15).

The aim of the current study was to determine whether alterations that affect the detoxification of Cd in S. pombe would also affect sensitivity to DDP. Our results indicate that the level of GSH, which is critical for Cd tolerance, does not significantly affect DDP sensitivity. The vacuolar sequestration mechanism mediated by PCs likewise does not play a significant role in DDP tolerance. However, the high level of production of sulfide found in one mutant is capable of reducing the sensitivity of the cells to both DDP and Cd. Thus, there is at least one common resistance mechanism shared by both DDP and Cd. Because a defect in the assimilatory sulfate reduction pathway does not affect DDP cytotoxicity, it is possible that the mutation conferring resistance to both Cd and DDP resides in a different sulfide-generating pathway.

Materials and Methods

Reagents. DDP was obtained from Bristol-Myers Squibb (Princeton, NJ). CdCl2 was purchased from Sigma Chemical (St. Louis, MO).

Genetic materials and manipulation. The adenine prototrophic S. pombe strain Sp223 (h− leu1.32 ura4.294 ade6.216) was the wild-type progenitor from which all strains other than DS5 were derived: LK100 is isogenic to Sp223 but is hmt1+ and has been described previously (12). Also, the adenine prototrophic JS21 (h− leu1.32 ura4.294) is the progenitor of LK37, a GSH-deficient mutant; of Y10, a strain that hyperproduces sulfide; and of DS31, a sulfite reductase mutant constructed through gene disruption of the sulfite reductase gene.1 DS1 and DS5, which contain engineered disruptions of the ade2 locus in the Sp223 and B1048 (h+ ura4.294 ade7.50) backgrounds, respectively, produce abnormally low levels of sulfide on Cd induction and fail to accumulate the high-molecular-weight PC/Cd complexes. Based on measurements made using the monooctylglycine (MOG) method (17), the parental JS21 strain. Fig. 1A shows that LK37 contains the first and last peaks shown in Fig. 1A represent the excluded and included volumes, respectively. The middle two peaks contain high- and low-molecular-weight PC/Cd complexes, respectively.

Metal sensitivity assay. To evaluate the toxicity of DDP or Cd, cell survival was determined by clonogenic assay. Specifically, 3-ml cultures containing a total of 2.5 × 10⁶ cells were exposed for 48 hr to graded concentrations of either DDP or Cd and subsequently plated at various dilutions onto 60-mm agar plates lacking DDP or Cd and incubated for 4 days at 30°C. The IC₅₀ was defined as the drug concentration that reduced the number of colony-forming units to 50% of the value obtained from a control culture not exposed to drug. Each experiment was repeated two or three times with triplicate cultures for each drug concentration.

DDP accumulation and DNA platination. The uptake of DDP was measured after a 4-hr exposure of 3-ml cultures containing a total of 1 × 10⁶ cells to 1.6 mM DDP at 30°C. After incubation with DDP, the cells were washed four times with cold phosphate-buffered saline at 4°C, and an aliquot was removed for cell counting using a hemocytometer. The Pt content of the remaining cells was quantified through atomic absorption spectrometry. Pt in DNA was measured using the same technique after extraction of DNA as previously described (17).

Sulfide production. Sulfide production was determined as previously described (14). Briefly, a 50-ml culture was grown in a side-arm flask capped with a rubber stopper through which a glass pipette was inserted. The external pipette was attached to a sterile air filter through which humidified air was supplied to the culture by being bubbled through H₂O. The side arm of the flask was connected to a tube containing ZnOAc to trap the volatile sulfide. After 24 hr, 10 ml of HCl was added to the culture to release intracellular sulfide. Total (extracellular and intracellular) sulfide was measured in a colorimetric assay using N,N-dimethyl-p-phenylene diamine HCl as a substrate, and normalized values were expressed relative to the level for the wild-type strain.

Northern analysis. Total RNA was prepared as described previously (18), and 20 μg was fractionated through electrophoresis on a formaldehyde-containing 1% agarose gel and transferred to a nylon membrane. DNA probes were labeled with a random primer kit (Amersham, Little Chalfont, UK). Hybridization was carried out as previously described using the hmt1 probe retrieved from pDH35 through treatment with EcoRI (12). A leu1 probe (kindly provided by Dr. Paul Russel, Scripps Institute, La Jolla, CA) was used as a control for DNA loading.

In vitro interaction between DDP and sulfide. We incubated 1 mM DDP with different concentrations of Na₂S at room temperature, and the two compounds were allowed to react overnight. After centrifugation, the supernatants were assayed for content of Pt by atomic absorption spectrometry.

Results

Role of GSH. GSH plays a major role in the detoxification of xenobiotics in eukaryotic cells. However, other than the associations that GSH depletion sensitizes human cells to DDP and that high GSH content is found in some resistant cell lines (1, 3, 19), the role of GSH in DDP detoxification remains unclear. GSH is known to play an indirect role in Cd tolerance in S. pombe. Because PCs are produced enzymatically from GSH, a reduction in cellular GSH content results in subnormal production of PCs (20, 21). One such mutant is LK37, which was isolated as a Cd-hypersensitive strain from the parental JS21 strain. Fig. 1A shows that LK37 contains no detectable high- or low-molecular-weight PC-bound Cd complexes. Based on measurements made using the monobromobimane conjugation assay, LK37 cells were found to have a 15-fold reduction in GSH level (GSH content was 0.86 ± 0.17 and 0.059 ± 0.012 μmol/10⁶ cells for the JS21 and LK37 cells, respectively). Fig. 1B shows a comparison of the sensitivity of the GSH-deficient LK37 and GSH-replete JS21 cells to DDP; both strains exhibited the same concentration-survival curve. This contrasts with the substantial difference in tolerance to Cd, with LK37 being clearly more sensitive to

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1 D. M. Speiser, K. F. McCue, and D. W. Ow, unpublished observations.
this metal. These results indicate that the wild-type level of neither GSH nor PCs significantly influences the sensitivity of *S. pombe* to DDP.

**Role of PC-mediated sequestration.** After exposure of cells to Cd, the induced production of PCs is followed by the vacuolar sequestration of PC-bound Cd into the vacuole. This process is mediated by an ATP-binding cassette-type transport pump that resides in the vacuolar membrane (12, 22). The gene encoding this transporter is known as *hmt1*, and the mutant harboring a nonsense codon mutation in this gene is LK100. As reported previously, LK100 is hypersensitive to Cd (12). Fig. 2 shows the survival curve for LK100 and its isogenic parent, Sp223, exposed to Cd and DDP. Based on IC50 values of 0.00092 and 0.1 mM (standard deviation, 5%) for *hmt1*− LK100 and *hmt1*+ Sp223, respectively, LK100 is 108-fold more sensitive to Cd. In contrast, LK100 exhibited only 3-fold greater sensitivity to DDP (LK100: IC50 = 0.015 mM; Sp223: IC50 = 0.054 mM; standard deviation < 5%). The slight sensitivity exhibited by the *hmt1*− strain to DDP could indicate a role for this gene in protection against DDP; however, it is also entirely possible that the defect in *hmt1* has disrupted some other aspect of metal homeostasis and reduced the overall fitness of this strain, particularly when subjected to stress.

Overproduction of the *hmt1*-encoded transport pump is known to enhance Cd tolerance, presumably as a result of increased vacuolar sequestration of Cd (12). We sought to determine whether overproduction of *hmt1* would also produce resistance to DDP. Sp223 and LK100 cells were transformed with the *hmt1* cDNA expression construct pDH35 or, as a control, the empty vector pART1. Fig. 3 shows that the pDH35 transformants indeed produced a marked increase in the accumulation of *hmt1* mRNA. However, although Cd tolerance was enhanced, *hmt1* overexpression failed to significantly alter the sensitivity to DDP of either strain (Fig. 4). Thus, despite the important role for *hmt1* in Cd tolerance, neither loss of nor increase in *hmt1* expression had a significant impact on DDP tolerance.

**Sulfide-associated DDP resistance.** Certain mutants that form yellow colonies on Cd plates also show greater tolerance to Cd. Like the mutants reported for *C. glabrata*, these mutants produce anomalously high amounts of sulfide (15). One such mutant is Y10, which had a sulfide production rate that was 5.99 ± 0.85-fold greater than that of the parental strain JS21 (values are given as mean ± standard deviation). The abnormally high sulfide production rate was not accompanied by a correspondingly high level of GSH. The GSH levels found in Y10 and its isogenic parent JS21 were 1.01 ± 0.21 and 0.86 ± 0.17 mmol/10⁶ cells, respectively, reflecting no significant difference in GSH content between the strains. Fig. 5 shows that when Y10 was exposed to Cd or DDP for 48 hr, it demonstrated a 2-fold increase in the IC50 value for Cd and a 6-fold increase in the IC50 value for DDP compared with its parental strain. Examination of a second
strain that had a 3.1 ± 0.3-fold increase in sulfide production demonstrated it to be 2.7-fold resistant to DDP. These findings suggest that sulfide either has a direct role in the protection of *S. pombe* from DDP and Cd cytotoxicity or is a marker for the primary alteration that controls sensitivity. In the case of Y10, the yellow coloration of the colonies on Cd-containing medium suggested that CdS was formed. We observed similar interactions occurring between DDP and sulfide ions (see below). Interestingly, low concentrations of both Cd and DDP stimulated the survival of sulfide-overproducing Y10 cells. A reasonable interpretation is that at concentrations of the metals that are themselves not toxic, the sequestration of the sulfide as metal/sulfide complexes can ameliorate the growth-suppressing effect of the anomalously high sulfide level.

To determine whether DDP could in fact form a complex with sulfide, 1 mM DDP was incubated with concentrations of Na₂S ranging from 0.25 to 4 mM. The formation of a brown precipitate was observed, which was directly proportional to the amount of exogenously provided sulfide. The chemistry of the two reactants suggests that the precipitate consisted of PtS. This interpretation is consistent with atomic absorption measurements presented in Fig. 6 that show a decrease in the concentration of soluble Pt remaining in the supernatant as a function of the concentration of Na₂S.

Despite the lack of detailed knowledge on what seems to be multiple sulfide-generating pathways in fission yeast, much is known about the assimilatory pathway by which sulfate is reduced to sulfite and then sulfide (23). A mutant of this pathway, DS31, fails to convert sulfite to sulfide because of a genetically engineered disruption of the sulfite reductase gene. DS31 cells fail to form the sulfide needed for assimilation into cysteine and are therefore cysteine auxotrophs. Despite cysteine supplementation, however, cysteine auxotrophs are known to be sensitive to Cd and fail to form sufficient amounts of PCs. Presumably, the rate of import of cysteine is inadequate to meet the high cysteine demand for PC biosynthesis. As with other cysteine auxotrophs, Fig. 7 shows that DS31 proved to be hypersensitive to Cd, even when this amino acid was available in the growth medium. However, DS31 was as tolerant as the wild-type strain to DDP. This suggests that the sulfide generated by the assimilatory sulfate reduction pathway is not required for the wild-type level of DDP tolerance. In contrast, two other mutants that have defects in sulfide production were hypersensitive to DDP. These two mutants, DS1 and DS5, are Cd hypersensitive and fail to accumulate the high-molecular-weight PC/CdS complex due to mutations that block inducible sulfide production during Cd stress (14, 24). The Cd-inducible production of sulfide is not attributable to the assimilatory sulfate-reduction pathway but rather to another pathway that involves enzymes of the *de novo* purine biosynthesis pathway. When exposed to DDP, DS1 and DS5 were found to be 4.0- and 3.6-fold more sensitive to DDP, respectively, than the wild-type control. Thus, there seems to be an interesting link between Cd-inducible sulfide production and DDP tolerance.

**Cellular accumulation of DDP.** To determine whether the observed differences in DDP cytotoxicity among the
tested strains could be attributed to differential DDP uptake, accumulation studies were performed. The *S. pombe* strains were exposed to 1.6 mM DDP for 4 hr, a period sufficient for any changes in uptake resulting from exposure to the metal ion to have occurred. Marked differences were not found among the different strains. DDP accumulation in these strains, expressed as micrograms of Pt/10^6 cells (mean ± standard deviation) was as follows: JS21, 0.08 ± 0.01; LK37, 0.13 ± 0.03; Sp223, 0.12 ± 0.02; LK100, 0.17 ± 0.03; and Y10, 0.05 ± 0.01. This lack of significant difference was not surprising for LK37 and JS21, which exhibited no difference in DDP sensitivity. The fact that there was no substantial reduction in Pt accumulation in the quite resistant sulfide-overproducing Y10 strain indicates that the underlying mechanism of DDP resistance cannot be attributed to changes in overall DDP accumulation in this strain either.

The extent of DNA platination produced by a 4-hr exposure to 1.6 mM DDP was compared for the parental JS21 and sulfide-overproducing Y10 strain to determine whether even in the absence of an effect on total cellular accumulation, the sulfide antagonized the ability of the drug entering the cell to platinate DNA. DNA platination was 0.0219 ± 0.0011 nmol of Pt/µg of DNA for the JS21 cells and 0.0138 ± 0.0022 nmol of Pt/µg of DNA for the Y10 cells (p = 0.007), indicating that the sulfide overproduction was associated with a 34% reduction in the ability of DDP to reach the nucleus and react with DNA.

**Discussion**

The aim of this study was to determine whether known mechanisms that yeast cells use to protect themselves against toxic metal ions also play a role in the protection against DDP. Cd is of particular interest in this regard because, like the cytotoxic hydrated form of DDP, it is capable of complexing with other molecules that have nucleophilic sites, particularly thiols. Recent observations suggest that there is cross-resistance in human cells between DDP and a variety of toxic metal ions, including arsenite and antimonite (25). Based on the assumptions that the resistance mechanisms might be conserved at the cellular level among diverse organisms and that other reactive metal ions may share detoxification pathways with DDP, we chose to examine the effect of six different mutations known to alter the tolerance of *S. pombe* to Cd ions.

The role of GSH in the DDP-resistant phenotype remains enigmatic; however, the results obtained in the current study do not support an important role for GSH as a major determinant of DDP sensitivity in *S. pombe* cells. No appreciable difference in DDP sensitivity was found in a strain that had a 15-fold reduction in GSH level and produced no detectable PCs. The fact that Y10 was 6-fold more resistant to DDP but had a GSH content that was similar to its DDP-sensitive wild-type parent lends further support to the idea that there is no correlation between GSH content and DDP resistance in *S. pombe*. This finding was somewhat surprising in view of the fact that reduction in GSH through inhibition of its synthesis with buthionine sulfoximine reliably sensitizes human cells to DDP (2, 3, 26–28). It is noteworthy that although overproduction of GSH has been documented in some DDP-selected human cell lines, this is not a universal observation. Even in cells in which increased GSH levels have been found, it has not been established that GSH *per se* is the proximate cause of the DDP resistance. The increase in GSH level could be the result of a deficit in another pathway whose dysfunction results in dysregulation of both GSH level and DDP resistance.

GSH is needed for Cd tolerance in plants and in *S. pombe* because metal-binding PC peptides are produced through the polymerization of GSH (29, 30). Resistance to Cd in cultured tomato cells has been linked to an increase in PC content (31), whereas hypersensitivity to Cd in *Arabidopsis thaliana* correlates with reduced accumulation of PCs (32).1 The *hmt1* gene product in *S. pombe* mediates vacuolar sequestration of Cd, and abundance of this transport protein shows positive correlation with the degree of Cd resistance (12). Therefore, the PC/*hmt1* pathway is critical for detoxification of Cd. However, although loss of *hmt1* function in *S. pombe* resulted in a 108-fold increase in Cd sensitivity, it produced a minimal effect on DDP sensitivity. Therefore, this excludes the PC/*hmt1* pathway as a major detoxification mechanism for DDP, although it remains possible that it plays a minor role, perhaps serving to sequester DDP/sulfide complexes.

The most interesting observation to emerge from this study is that sulfide overproduction in Y10 was associated with enhanced resistance to both Cd and DDP. Although the magnitude of resistance to Cd was not as great, it was quite substantial for DDP. This shows that the two compounds can be detoxified through a common mechanism involving either sulfide directly or some elements of its biosynthetic pathway.
It is noteworthy that low concentrations of either Cd or DDP effectively increased the survival of Y10, which is consistent with the hypothesis that sulfide overproduction may be harmful to these cells and that metal complexation serves to reduce the concentration of free sulfide.

In plants and in microorganisms, including \textit{S. pombe}, but not in animals, sulfide can be produced by the well-defined assimilatory sulfate reduction pathway, in which inorganic sulfate is added to ATP to produce adenosine phosphosulfate, which is then further converted until sulfide is formed. We determined whether this pathway could affect DDP tolerance by testing a mutant with an engineered defect in the sulfite reductase gene. The results show that a sulfite reductase mutant does not exhibit greater DDP sensitivity. Therefore, this excludes the assimilatory reduction pathway as an important determinant of the wild-type level of DDP tolerance. That is, wild-type cells must possess mechanisms for maintaining normal levels of tolerance that do not depend on sulfide production through assimilatory sulfate reduction, and thus loss of function of this pathway does not result in hypersensitivity. In the sulfide-overproducing variants, however, the increase in sulfide above normal levels could be the basis for a novel "gain of function" mechanism of DDP resistance. Because there are multiple pathways that generate sulfide, it remains possible that a genetic alteration outside the assimilatory pathway is responsible for sulfide overproduction in these mutants. We cannot exclude the possibility that sulfide overproduction is simply a symptom of an underlying metabolic change that directly mediates DDP resistance. However, an investigation of the genetic defect in Y10 should uncover the pathway responsible for its sulfide overproduction, and this pathway would then be the subject of fruitful study. We are currently isolating the gene that causes sulfide overproduction in Y10.

Concerning the issue of which sulfide-generating pathway is responsible for the enhanced DDP resistance observed in Y10, it is of interest to note that high sulfide production during Cd stress is not attributable to the assimilatory sulfite reduction pathway. During Cd stress, intracellular sulfide levels increase by 5–7-fold concomitant with formation of the vacuolar high-molecular-weight PC/CdS complexes. This Cd-induced sulfide production is also observed in the sulfite reductase mutant DS31 as long as cysteine is available in the growth medium. Therefore, Cd-induced sulfide production can proceed from a step downstream of the assimilatory sulfite reduction pathway. This is consistent with the previously proposed hypothesis that Cd-induced sulfide is generated through a novel pathway. This novel pathway is not well defined but has been postulated to begin with the conversion of cysteine to cysteine sulfinate, which is then incorporated into purine precursors (14, 24).

It is not currently known which sulfide pathway is genetically altered in Y10 to produce the anomalous high level of sulfide. Because humans do not assimilate inorganic sulfate, the generation of sulfide would necessarily be due to pathways that degrade sulfur compounds, such as cysteine or GSH. The findings reported here serve to redirect attention from GSH to other sulfur-containing constituents. Through the deciphering of the pathways in \textit{S. pombe}, homologous pathways in human cells could be revealed as being important in the determination of tumor sensitivity to this important chemotherapeutic agent.

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References


A cadmium-hypersensitive mutant of the fission yeast *Schizosaccharomyces pombe* was found to accumulate abnormally high levels of sulfide. The gene required for normal regulation of sulfide levels, *hmt2*+, was cloned by complementation of the cadmium-hypersensitive phenotype of the mutant. Cell fractionation and immunocytochemistry indicated that HMT2 protein is localized to mitochondria. Sequence analysis revealed homology between HMT2 and sulfide dehydrogenases from photosynthetic bacteria. HMT2 protein, produced in and purified from *Escherichia coli*, was soluble, bound FAD, and catalyzed the reduction of quinone (co-enzyme Q) by sulfide. HMT2 activity was also detected in isolated fission yeast mitochondria. We propose that HMT2 functions as a sulfide-quinone oxidoreductase. Homologous enzymes may be widespread in higher organisms, as sulfide-oxidizing activities have been described previously in animal mitochondria, and genes of unknown function, but with similarity to *hmt2*+, are present in the genomes of flies, worms, rats, mice, and humans.

The oxidation of sulfide can provide energy for chemolithotrophic or photosynthetic growth of bacteria. This capacity allows some bacteria to thrive in such unlikely environments as hot sulfur springs and deep-sea thermal vents. Sulfide-based anaerobic photosynthetic photosynthesis appeared quite early in evolution. Today, it is widespread in the green and purple phototrophic karyotes. Interestingly, potential homologues of this enzyme appear in the genomes of nematodes, fruit flies, mice, rats, and humans. This raises the possibility that sulfide oxidation is more widespread among organisms, and might occur in a wider diversity of habitats, than has previously been imagined.

EXPERIMENTAL PROCEDURES

**Genetic Materials**—*S. pombe* strains Sp223 (h+, ade6.216, ural4.294, leu1.32) and B1048 (h+, ade7.50, ural4.294) have been described (14). JS21 (h+, ural4.294, leu1.32) and JS23 (h+, ural4.294, leu1.32) were derived from the mating of Sp223 with B1048. A cadmium-hypersensitive mutant of JS21 was crossed to JS23 to yield JS563 (h+, ural4.294, leu1.32, hmt2+) and JV1 (h+, ural4.294, leu1.32, hmt2+). JS31 (h+, ural4.294, leu1.32, hmt2::URA3+) harbors a homologous insertion, whereas JV11 (h+, ural4.294, leu1.32, URA3+) harbors a random insertion of an hmt2+ disruption construct bearing the *Saccharomyces cerevisiae* USP1 gene. DS31 (h+, ural4.294, leu1.32, sir1::LEU2) was obtained by disruption of the sulfite reductase gene with a *S. cerevisiae* LEU2+ fragment (D. Spieser, USDA-ARS, Albany, CA). JV3 (ural4.294, leu1.32, sir1::LEU2, hmt2+) was obtained from the mating of DS31 with JV7.

**Growth Conditions**—Cells were grown at 30 °C on complete medium YG (2% glucose, 0.5% yeast extract) or minimal medium SG (2% glucose, 0.67% yeast nitrogen base without amino acids; supplemented with 20 μg/ml uracil and/or 100 μg/ml leucine as needed). JS21 cells were mutagenized by a 45-min exposure to 175 μg/ml MNNG. Media for comparison of growth on glucose or glycerol consisted of either 1% yeast extract, 2% glucose or 1% yeast extract, 4% glycerol, 0.1% glucose, that would otherwise poison aerobic metabolism. The enzymes responsible for sulfide oxidation in these eukaryotes have not been isolated, and their nature and evolutionary origin remain unknown. In particular, it is not clear if sulfide oxidation in this exotic group of organisms bears any relationship with the better-understood pathways of bacteria.

The lower eukaryote *Schizosaccharomyces pombe* (fission yeast) has not been described to live in sulfide-rich habitats, although cells are exposed to sulfide generated internally during assimilation of inorganic sulfur. After reduction of sulfate to sulfide over several steps, the enzyme sulfite reductase catalyzes the reaction \( \text{H}_2\text{SO}_3 + 3 \text{NADPH} + 3\text{H}^+ \rightarrow \text{S}^{2-} + 3 \text{NADP}^+ + 3 \text{H}_2\text{O} \). Much of this \( \text{S}^{2-} \) is incorporated into cysteine, but the cell still accumulates measurable amounts of acid-labile \( \text{S}^{2-} \) under normal laboratory conditions. \( \text{S}^{2-} \) increases during exposure to heavy metals and is involved in resistance to cadmium and cisplatin (13).

In the course of studying the response of fission yeast to heavy metals, we uncovered a mutant with an unusual defect in sulfur metabolism. This led to the cloning and characterization of a new gene encoding a mitochondrial enzyme that can oxidize sulfide. The protein has sequence homology to sulfide-oxidizing enzymes of bacterial photosynthesis, suggesting a common evolutionary origin of sulfide metabolism between prokaryotes and eukaryotes. Interestingly, potential homologues of this enzyme appear in the genomes of nematodes, fruit flies, mice, rats, and humans. This raises the possibility that sulfide oxidation is more widespread among organisms, and might occur in a wider diversity of habitats, than has previously been imagined.
Sulfite Oxidation in Mitochondria

RESULTS

Mutant Phenotype—S. pombe mutant strain JS563 bears a cadmium-hypersensitivity trait that segregates as a single locus. As this locus affects heavy metal tolerance, it was assigned the genetic designation hmt2. When compared with the wild-type parent strain JS21, which can grow at up to 800 μM cadmium, the hmt2 mutant ceases to grow at 100 μM cadmium and shows reduced yield with as low as 5 μM cadmium. A difference in growth rate was not found in the presence of high NaCl or sucrose concentrations, but a reduction relative to the wild type was observed after heat shock at 47 °C or in the presence of hydrogen peroxide, the thiol-oxidizing agent diamide, or the nonfermentable substrate glycerol. Because the catabolism of glycerol requires a functional respiratory pathway, the defect was suspected to be associated with mitochondrial function.

Sulfide Hyperaccumulation and Hypersensitivity—A noticeable trait of the mutant is that the colonies turn bright yellow in the presence of cadmium, suggesting the formation of CdS. Over an 18-h period, JS563 accumulated ~6-fold as much acid-labile sulfide as the wild-type JS23 (Table 1). S. pombe is known to produce sulfide production during cadmium stress and a higher level of sulfide production was observed in both strains grown in 200 μM cadmium, but the hmt2~JS563 continued to exceed wild-type levels. Exogenous sulfide is toxic to S. pombe at concentrations in the 100 μM range. Because the
**hmt2**− mutant already exhibits an elevated level of endogenous sulfide, it could be more sensitive to an exogenous supply of this substance. JS563 showed impaired growth relative to the wild-type strain in the presence of Na2S, with the greatest differential observed at 200 μM Na2S.

**Defect Not in Sulfate Assimilation**—One described route of sulfide production in *S. pombe* is the sulfate assimilatory pathway, where inorganic sulfur is routed from sulfate to sulfite to sulfide and then to cysteine. A defect in this pathway might increase the sulfide pool through either attenuating sulfide incorporation to cysteine or by overproducing sulfide directly. Both possibilities were examined by the following experiments. First, when cultured in minimal medium, JS563 grew as well as the wild type, suggesting that cysteine production is sufficient for cell growth. Further supplementation with cysteine (100 μM) did not increase its growth rate. Therefore, a defect in the incorporation of S2− into cysteine seems unlikely.

Second, if sulfide overaccumulation were because of hyperactivity in sulfate assimilation, a genetic block in this pathway should abolish sulfide hyperaccumulation. The double mutant JV3 contains mutations in both hmt2 and sulfite reductase. Like the parent sulfite-reductase mutant strain DS31, JV3 is unable to convert sulfate to cysteine and therefore requires cysteine supplementation. For this reason, sulfide assays were carried out on cells grown in the complete YG medium. DS31 accumulated far less sulfide than either wild-type or JS563 (Table I), as expected from its lack of sulfite reductase activity. However, DS31 also accumulated ~3-fold less sulfide than JV3, suggesting that the *hmt2* locus in JV3 enhances sulfide accumulation through a pathway separate from inorganic sulfur assimilation.

**Kinetics of Sulfide Consumption**—To address if the hmt2− mutation affects the consumption of S2−, cells were pulse-labeled with 35SO4−. During the brief 15-min labeling period, both wild-type and mutant cells converted 35SO4− into 35S2− (Fig. 1, inset) (Table I), as expected from its lack of sulfite reductase activity. However, DS31 also accumulated ~3-fold less sulfide than JV3, suggesting that the *hmt2* locus in JV3 enhances sulfide accumulation through a pathway separate from inorganic sulfur assimilation.

**Isolation of hmt2**− Gene—JS563 was transformed with a *S. pombe* genomic library. A single plasmid clone, pJV1, with an 8.5-kb insert, restores cadmium tolerance to the mutant (Fig. 2). A deletion analysis of the 8.5-kb insert showed that a 1.9-kb *SacI/ScaI* subclone, in pJV26, complements the poor growth of the mutant on cadmium, hydrogen peroxide, diamide, or glyceral as well as restores wild-type sulfide levels (Table I). The 1.9-kb genomic fragment was used to isolate a 1834-base pair cDNA. In Northern blots, wild-type and mutant cells showed a single band of ~1.9 kb that hybridizes to the hmt2− cDNA (Fig. 3A). Accumulation of this transcript increased by ~2-fold when cells were exposed to 200 μM cadmium (Fig. 3B).

**An Engineered Gene Disruption**—To test whether the cloned DNA represented a wild-type allele of the genetic lesion or an extragenic suppressor, the wild-type allele was disrupted through integration of a *S. cerevisiae* URA3 marker (Fig. 2). Polymerase chain reaction and Southern analysis indicated that, in the disruption strain JV5, the coding region of the gene is intact, but two copies of the disruption construct have integrated in tandem upstream of the *hmt2* coding region, disrupting the promoter and possibly part of the untranslated leader sequence. As a result, the 1.9-kb mRNA is deficient in JV5 (Fig. 3B). A shorter transcript in JV5 can be attributed to the truncated hmt2 gene on the disruption construct itself because this band is also present in JV11, a strain bearing a random integration of the disruption construct. An additional transcript in JV5 of ~4 kb hybridizes faintly to both URA3− (not shown) and hmt2− probes. This band might represent transcriptional read-through from the upstream URA3 gene through hmt2. Neither transcript detected in JV5 is expected to yield a functional hmt2− product.

The disruption phenotype in JV5 is very similar, though not identical, to that of the original mutant. As with JS563, JV5 is hypersensitive to cadmium, hyperaccumulates sulfide (Table I), and exhibits poorer growth after heat shock, or in the presence of hydrogen peroxide or Na2S. Surprisingly, no significant difference is observed in the presence of diamide or with glyceral as the major carbon source. This anomaly could be attributed to a leaky allele. For example, the transcription of a functional mRNA in JV5, though severely reduced, might not
be entirely abolished. When JV5 was crossed to wild-type, the disruption phenotype segregated as a single locus and was linked with the URA3 marker (53 cadmium-hypersensitive, yellow, URA3 progeny: 46 cadmium-resistant, white, URA3 progeny, $x^2 = 0.64$). When crossed to JS563, wild-type recombinants were not recovered (49 cadmium hypersensitive, yellow, URA3 progeny: 51 cadmium hypersensitive, yellow, URA3 progeny, $x^2 = 0.04$). This genetic linkage between the original mutant locus and the disruption locus is consistent with the cloned gene corresponding to the genetic lesion in JS563.

**Sequence Change from hmt2** to hmt2**—**The hmt2** genomic and cDNA clones were sequenced on both DNA strands. hmt2** contains a putative 5' untranslated region of 357 nucleotides, longer than average for S. pombe (24). This region contains multiple poly-T and poly(A) stretches. The putative start methionine occurs at position 358, embedded in the sequence taaaatg, which matches the most commonly observed S. pombe translational start motif at 8 of 9 positions (25). Comparison of genomic and cDNA sequences reveals that hmt2** lacks introns. The deduced hmt2** open reading frame is predicted to encode a protein of 459 amino acids, with a molecular mass of 51,574 Da and an isoelectric point of 9.5–10.

The mutant allele was cloned and sequenced across the entire coding region. Comparison with the wild-type sequence revealed a G to A transition that changed amino acid 396 from glutamate to lysine. When the mutant allele, in the vector pART1, is reintroduced into JS563, it is unable to complement (Fig. 2, pJV30). When a 300-base pair region surrounding amino acid 396 is replaced by wild-type sequence, the hybrid allele is fully functional (Fig. 2, pJV34). This result demonstrates that this single point mutation is sufficient to account for the mutant phenotype.

**Similar Genes—**BLAST searches (26) of protein and nucleotide sequence data bases found sequence similarity between the encoded protein, HMT2, and a variety of oxidoreductases, some of which are listed in Table II. The two best matches, C. vinosum flavocytochrome c (6, 27) and R. capsulatus sulfide quinone reductase (28), are bacterial enzymes that oxidize sulfide. Overall sequence identity between HMT2 and these two enzymes is low (~20%). However, potentially functional features are conserved among these sequences (Fig. 4; and see the Discussion).

The sequences with greater similarity to HMT2 have unknown function. Apparent full-length genes from human, mouse, Caenorhabditis elegans, and the cyanobacterium Synechocystis sp. PCC6803 all appear to have >30% overall sequence identity with HMT2. A fragmentary expressed sequence tag (EST) from rat appears similarly well conserved. ESTs from Drosophila, Chloroflexus, and Schistosoma all have >20% identity with HMT2. These proteins are predicted to have unusually high pi: human, pi ~ 9.5; mouse, pi ~ 9.1; C. elegans, pi ~ 9.8; Synechocystis, pi ~ 8.9. This feature is shared with HMT2 (pi ~ 9.9), sulfide quinone reductase (pi ~ 9.5), and NADH dehydrogenase (pi ~ 9.4).

**Mitochondrial Localization—**The N-terminal 24 amino acids of HMT2 display features characteristic of mitochondrial targeting sequences (29). The PSORT protein-targeting analysis program predicted with up to a 59% probability that HMT2 might be directed to mitochondria (30). In fission yeast extracts, a single anti-HMT2 immunoreactive band shows an apparent molecular mass of ~48 kDa. Abundance of this protein band correlates to hmt2 mRNA accumulation. It is found in both hmt2** and in hmt2** (JS563) cells, is more abundant in cells containing hmt2** on a multicopy plasmid, and is barely detectable in cells containing a disruption of the hmt2** promoter (JV5). The data are consistent with the 48-kDa band being the translation product of the hmt2** gene. The ~3-kDa reduction from the predicted size of HMT2 is consistent with the presumed cleavage of the putative mitochondrial targeting sequence during translocation from cytoplasm to mitochondria.

The bulk of HMT2 immunoreactive protein is pelleted by 100,000 x g centrifugation (Fig. 5A), suggesting that HMT2 is membrane-associated or enclosed within an organelle. In purified mitochondrial fractions (6-fold enriched in cytochrome oxidase specific activity), HMT2 is ~5-fold enriched (Fig. 5B). A mitochondrial localization for HMT2 was supported by immunofluorescence microscopy experiments (not shown). The mitochondrial fractions were further subfractionated at pH 7.4. The mitochondrial membrane-enriched, fumarase-depleted (0.5 x g) pellet showed increased immunoreactivity for HMT2, suggesting that HMT2 may be membrane-associated (Fig. 5C). HMT2 is not predicted to have any hydrophobic regions capable of forming transmembrane helices. However, subfractionation at pH 11 increases the yield of soluble HMT2 (Fig. 5D). This property is characteristic of some peripheral membrane proteins.

**HMT2 Purification—**To facilitate purification of HMT2, a gene fusion construct, pJV40, was made to encode a modified HMT2 protein with additional histidine residues. This construct was able to complement fully the hmt2** mutant phenotype, suggesting that the additional amino acid residues do not interfere with the in vivo activity of the protein. We therefore assumed that the His$_6$-tagged protein is suitable material for...
Sulfide Oxidation in Mitochondria

TABLE II
Sequence similarity to HMT2

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*a* GB T74801, T64084, T85836, T93837, T95375, R50203, R40496, R13065, N50841, T64005, T74688, T95295, T98292, AA280758, AA281439, W96028, AA100857, AA083029, AA082940, AA057016, AA133917, W94661, AA253283, W15319, N54246, AA056968, N58703, AA466172, AA485681, AA359712, AA354477, AA165963, AA383720, AA381822, AA381829, and EST 112444, 12744, 12744. The full sequence has been deposited in GenBank, with accession number AF042284.

**Substrate Specificity**—We tested the ability of other electron acceptors to replace coenzyme Q$_2$ in the HMT2-catalyzed reaction. Sulfide reacts rapidly and spontaneously with cytochrome c, 2,6-dichloroindophenol, and ferricytochrome, and the addition of HMT2 did not increase reaction rates. Sulfide fails to reduce menadione, NAD$^+$, or NADP$^+$ spontaneously, and HMT2 was unable to stimulate these reactions. Likewise, sulfite, thiosulfate, cysteine, glutathione, β-mercaptoethanol, succinate, and pyridine nucleotides were all unable to replace sulfide in the HMT2-catalyzed reduction of Q$_2$. Therefore, HMT2 appears to possess a specific sulfide:quinone oxidoreductase activity.

**Reaction Stoichiometry**—Sulfide and oxidized quinone concentrations were determined independently at the end of a 10-min reaction of 50 nmol each of sulfide and coenzyme Q$_2$ with 5 μg of purified HMT2. Sulfide and oxidized quinone were consumed in an approximately 1:1 molar ratio (7.85 ± 1.45 nmol S$_2$ /7.6 ± 0.99 nmol quinone, $n = 2$). Because flavoproteins and quinones both commonly carry two electrons, the stoichiometry of the reaction suggests that sulfide is oxidized to elemental sulfur and donates two electrons to coenzyme Q$_2$.

**Kinetic Parameters**—The sulfide-quinone reaction catalyzed by HMT2 is saturable with increasing concentrations of substrate; the apparent $K_m$ for coenzyme Q$_2$ is 2 mM. It is important to note that coenzyme Q$_2$ is a water-soluble analogue of the more likely physiological electron acceptor, ubiquinone, and therefore the $K_m$ obtained may not reflect the affinity of HMT2 for its true substrate. Additionally, because of the insolubility of high quinone concentrations in the reaction buffer, it was not possible to assay $K_m$ for sulfide under saturating concentrations of quinone. At lower quinone concentrations (40 μM), $K_m$ for sulfide was calculated to be 2 mM. Using saturating S$^2$ (3 mM) and the highest concentration of quinone (1.5 mM) that can be assayed, we obtained an apparent $V_{max}$ of 1.36 μmol coenzyme Q$_2$ reduced per μg of enzyme, and $k_{cat}$ of 52/sec (assuming an active enzyme concentration of 22.3 nM, based on incomplete FAD binding).

### Sulfide:Quinone Oxidoreductase Activity in Mitochon-
dria — We tested the native protein in its physiological context for the ability to carry out the same reaction. Mitochondria were isolated from wild-type (JS23) and mutant (JS563) cells. The specific activity of fumarase, an enzyme of the mitochondrial matrix, was equivalent in the two preparations. Mitochondria from both strains were able to reduce exogenous coenzyme Q2 at low rates in the absence of added electron donor (JS23: 75 ± 65 nmol/min·mg; JS563: 68 ± 623 nmol/min·mg).

The addition of sulfide significantly increased the rate of quinone reduction in JS23 mitochondria, and the spectrum of the reaction mixture showed a rapid decrease in the 285-nm absorption peak (Fig. 7C, b). Sulfide:quinone reductase activity, corrected for the background rate of quinone reduction, is significantly higher in JS23 (144 ± 65 nmol/min·mg) than in JS563 mitochondria (21 ± 12 nmol/min·mg, p < 0.05). Therefore, fission yeast mitochondria possess a sulfide:quinone oxidoreductase activity that correlates to the presence of functional HMT2 protein.

**DISCUSSION**

Purified recombinant HMT2 protein is ~25-fold enriched over isolated mitochondria in both protein abundance and in sulfide:quinone oxidoreductase-specific activity. This suggests that the activity of HMT2 itself could account for all of the sulfide:quinone oxidoreductase activity measured in fission yeast mitochondria. The apparent $K_m$ for sulfide is relatively high (2 mM), when determined with a water-soluble analogue of the more likely electron acceptor, ubiquinone. Assuming this value is physiologically meaningful, HMT2 could nonetheless be adequately supplied with substrate under such conditions as heavy metal exposure, when whole-cell sulfide levels can exceed 1 mM. Quinone should also be available, as ubiquinone is abundant in the mitochondrial inner membrane.

Key features are shared among HMT2 and two sulfide dehydrogenases, flavocytochrome c from *C. vinosum* and sulfide quinone reductase from *R. capsulatus*. Flavocytochrome c binds...
Mitochondrial sulfide oxidation is not unprecedented. Mitochondria of several species of marine animals have been shown to couple sulfide oxidation to the production of ATP (7, 9–12), but the proteins or genes responsible for the initial oxidation step have not been purified or cloned. In the lugworm Arenicola marina, electrons from sulfide appear to enter the electron transport chain at the level of ubiquinone (12), and the authors postulated that the enzyme involved might be similar to the sulfide quinone reductase of R. capsulatus. HMT2, a mitochondrial protein with homology to the R. capsulatus sulfide quinone reductase, strengthens this hypothesis by providing a genetic link between mitochondrial and bacterial sulfide oxidation. Intriguingly, genes similar to hmt2" appear in worms, suggesting a conserved role in detoxifying sulfide during cadmium tolerance. It is possible that HMT2 functions in the detoxification of endogenous sulfide. Not all sulfide produced during the assimilation of inorganic sulfur is immediately incorporated into amino acids, and we can detect a low level of acid-labile sulfide even in wild-type cells. Sulfide is a potent inhibitor of cytochrome c oxidase (34), and accumulation of sulfide in mitochondria would be expected to poison respiration. Therefore, a mitochondrial sulfide dehydrogenase might play a role in ensuring that local sulfide concentration near cytochrome c oxidase is kept low. Consistent with this hypothesis, hmt2" cells grow poorly on a nonfermentable carbon source, suggesting that unchecked accumulation of sulfide may interfere with respiration. The exact role of HMT2 in cadmium tolerance is not yet clear, but a likely possibility is to detoxify excess sulfide generated during cadmium stress.
flies, mice, rats, and humans. A heat-labile sulfide oxidizing activity has been reported in rat liver mitochondria (35, 36). It is possible that the machinery for capturing electrons from sulfide has been conserved in evolution, although it has been adapted to new physiological roles.

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Accumulation of metal-binding peptides in fission yeast requires $hmt2^+$

Jennifer G. Vande Weghe and David W. Ow*

Plant Gene Expression Center, U.S. Department of Agriculture - Agricultural Research Service, 800 Buchanan Street, Albany, CA 94710, and Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720

* To whom correspondence should be addressed.
phone: (510)559-5909
fax: (510)559-5678
email: ow@pgec.ars.usda.gov

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Summary

The fission yeast *Schizosaccharomyces pombe* detoxifies cadmium by synthesizing phytochelatins, peptides of the structure \((\gamma\text{-GluCys})_n\text{Gly}\), which bind cadmium and mediate its sequestration into the vacuole. The fission yeast protein HMT2, a putative sulfide-oxidizing enzyme of the mitochondria, appears to be essential for tolerance to multiple forms of stress, including exposure to cadmium. We found that *hmt2* strains are unable to accumulate normal levels of phytochelatins in response to cadmium. Specifically, the high sulfide levels accumulated in the absence of HMT2 function appear to block the activity of phytochelatin synthase, perhaps by precipitating the free cadmium ions required for enzyme activity. Although sulfide is required for phytochelatin-mediated metal tolerance, aberrantly high sulfide levels inhibit this pathway. Thus, precise regulation of sulfur metabolism, mediated in part by HMT2, is essential for metal tolerance in fission yeast.
Introduction

Heavy metals such as Cd$^{2+}$, Cu$^{2+}$, and Pb$^{2+}$ are toxic, and different organisms have evolved a variety of mechanisms to detoxify metals. In eukaryotes, the route of detoxification involves synthesis of chelating molecules that bind to the metal ions and mediate their transport out of the cells or their sequestration into subcellular compartments. The fission yeast *Schizosaccharomyces pombe* has become a well-developed model system for understanding metal detoxification pathways common to fungi and plants.

*S. pombe* responds to the presence of cadmium, and some other heavy metals, by producing phytochelatins, peptides of the structure ($\gamma$-EC)$_n$G, n=2-11, which chelate and sequester free metal ions (Murasugi et al., 1981; Kondo et al., 1984; Grill et al., 1986; Rauser, 1995). During cadmium exposure, apo-phytochelatin peptides are synthesized from glutathione by the enzyme phytochelatin synthase, a dipeptidyl transpeptidase, for which the gene has recently been cloned (Ha et al., 1999; Clemens et al., 1999; Vatamaniuk et al., 1999). This enzyme is present constitutively in the cell, but requires heavy metal ions for activity. Direct binding of cadmium to phytochelatin synthase has been demonstrated (Vatamaniuk et al., 1999), and presumably occurs through the multiple cysteines in the C-terminal end of the protein.

Once synthesized, apo-phytochelatins bind cadmium ions to form a low molecular weight complex, which is transferred to the vacuole by the ABC-type transporter HMT1 (Ortiz et al., 1992; 1995). In the vacuole, stoichiometric addition of sulfide and additional free cadmium ions leads to the formation of a high molecular weight cadmium-sulfide-phytochelatin complex, which the metal is bound with high affinity (Murasugi et al., 1983; Reese and Winge, 1988). The additional cadmium appears to enter the vacuole via a Cd$^{2+}$/H$^+$ antiporter (Ortiz et al., 1995), whereas the sulfide ions appear to be generated by a pathway involving intermediates of purine biosynthesis (Speiser et al., 1992; Juang et al., 1993).

HMT2, a mitochondrial sulfide dehydrogenase (Vande Weghe and Ow, 1999), also appears to play an important role in metal tolerance. The *hmt2* mutant is extremely cadmium-
sensitive; exposure to concentrations as low as 5 µM reduces its growth rate relative to wild type. Mutant cells accumulate an abnormally high amount of sulfide, which further increases during cadmium stress. This association between high sulfide levels and metal sensitivity was surprising. It contrasts with previous studies in *S. pombe* (Perego et al., 1997) and *Candida glabrata* (Mehra et al., 1994). In those studies, mutants producing high amounts of sulfide form correspondingly high amounts of cadmium-sulfide-phytochelatin high molecular weight complex, and are hypertolerant to cadmium.

HMT2 appears to contribute to metal tolerance by playing an essential role in the phytochelatin-mediated metal detoxification pathway. We sought to determine whether HMT2’s effect on the pathway is direct—whether it is itself involved in the production and maintenance of phytochelatins—or indirect, through its effects on cellular sulfur metabolism. In the latter case, a better understanding of HMT2 function might clarify how the regulation of sulfur metabolism controls the cell’s ability to respond to metal stress.

**Results**

**Phytochelatin deficiency in the *hmt2* mutant**

The growth of the *hmt2* mutant is not more sensitive than wild type to NiCl$_2$ (1-800 µM), Bi(NO$_3$)$_2$ (1µM-2mM), or Pb(NO$_3$)$_2$, (1µM-4mM) across the range of concentrations tested. However, it is more sensitive to CdSO$_4$ (>5 µM), HgCl$_2$ (>50 µM), CuSO$_4$ (>2mM), and ZnSO$_4$ (>4 mM). Sensitivity to multiple heavy metals could indicate a defect in the phytochelatin-mediated metal detoxification pathway. Cadmium-induced wild-type cells, such as JS23, produce two characteristic cadmium-containing peaks on gel filtration chromatography, representing high- and low-molecular weight phytochelatin-cadmium complexes (Figure 1A). A strain bearing a point mutation in the *hmt2* coding sequence, JS563, produces neither phytochelatin-cadmium complex. A second *hmt2* strain, JV5, in which an engineered disruption of the *hmt2* promoter causes reduced gene expression, forms only low levels of the high
molecular weight phytochelatin-cadmium complex. Correspondingly, overexpression of \( hmt2^+ \) on a multicopy expression plasmid (pJV26) restores the ability of an \( hmt2^- \) strain to accumulate phytochelatin-cadmium complexes (Figure 1B).

To determine whether failure to produce phytochelatin-cadmium complexes resulted from deficient synthesis of the phytochelatin peptide or from a problem in Cd complex assembly, we assayed for the presence of apo-phytochelatins. Extracts of cadmium-induced cells were fractionated by reversed-phase HPLC. Thiol-containing molecules were detected after post-column derivatization with 5,5'-dithiobis-[2-nitrobenzoic acid] (DTNB), which reacts specifically with -SH groups to create a yellow compound. Wild type cells exposed to cadmium accumulate large amounts of the phytochelatins (\( \gamma\)-EC)_2G and (\( \gamma\)-EC)_3G (referred to as PC_2 and PC_3, respectively) (Figure 2A); mutant cells accumulate only minor amounts of these peptides (Figure 2B). Since \( hmt2^- \) cells lack a mitochondrial sulfide dehydrogenase activity (Vande Weghe and Ow, 1999), a possibility could exist that the redox balance in mutant cells was altered such that phytochelatins accumulated in an oxidized form, which would be nonfunctional as well as undetected by the DTNB assay. To see if this could be the case, NaBH_4 was added to chemically reduce extracts prior to HPLC. As a control, NaBH_4 was tested on a sample of cell extract spiked with 10 nmol oxidized glutathione. The addition of NaBH_4 caused a quantitative conversion of the oxidized glutathione to the reduced form (data not shown). When NaBH_4 was added to extracts from \( hmt2^- \) cells, however, the amount of phytochelatins detected by DTNB failed to increase (Figure 3).

We also searched for oxidized phytochelatins using ^{35}S labeling. Extracts from radiolabeled \( hmt2^- \) cells were examined simultaneously by DTNB detection (Figure 4A) and flow scintillation counting (Figure 4B). While oxidized phytochelatins would escape DTNB detection, ^{35}S-labeled peptides should not. Due to formation of interchain and intrachain disulfide bonds (Meuwly et al., 1995), retention times of oxidized phytochelatins should be slightly altered from those of the reduced forms. Therefore, if the \( hmt2^- \) mutant possesses a pool
of oxidized phytochelatins, it should display $^{35}\text{S}$-labeled peaks at unusual retention times, and those peaks should be absent from the DTNB-detection profile. This was not the case; with extracts from $hmt2^-$ cells, each radiolabeled HPLC peak corresponded to a peak visible by DTNB. Likewise, the $^{35}\text{S}$-labeled peaks at the retention times of reduced PCs were smaller sizes relative to those from the Cd$^{2+}$-exposed wild-type strain (not shown.) Since an $^{35}\text{S}$-labeled peak with a novel retention time was not found, we conclude that there is not a significant amount of oxidized phytochelatins in $hmt2^-$ cells.

**Position of the defect in the phytochelatin biosynthetic pathway**

*S. pombe* and *A. thaliana* mutants lacking normal levels of glutathione, the biosynthetic precursor of phytochelatins, fail to produce phytochelatins and are hypersensitive to heavy metals (Glaeser et al., 1991; Howden et al., 1995a; Mutoh and Hayashi, 1988). However, $hmt2^-$ cells grown in the absence of cadmium have normal levels of reduced glutathione, as measured by HPLC (Figures 2A, 2B), and of total glutathione, as measured by the glutathione reductase recycling assay (wild type: 19.0 nmol GSH/ mg protein, $\pm$ 1.5 S.E.; mutant: 18.6 nmol GSH/ mg protein, $\pm$ 2.6 S.E.; n=8.). Therefore, the $hmt2^-$ mutant phenotype is not due to a basic deficiency in glutathione production.

It remained possible that mutant cells can synthesize phytochelatins at normal rates, but quickly run out of substrate, or degrade the phytochelatins soon after synthesis. To evaluate these possibilities, we examined the time course of cadmium induction. Cells were fed $^{35}\text{SO}_4^{2-}$ in the absence of cadmium to label the glutathione pool. Excess unincorporated label was then removed and cells were placed in media containing unlabeled $\text{SO}_4^{2-}$ along with 200 $\mu$M cadmium. Prior to cadmium induction, mutant and wild type cells have comparable amounts of glutathione (Figure 5A and 5B), which is consistent with previous assays. However, exposure to cadmium induces synthesis of additional glutathione in wild type cells but not in $hmt2^-$ cells.
The two strains also differ in rates of glutathione turnover. The $^{35}$S-labeled glutathione peak diminishes in wild-type cells after two hours of cadmium exposure, while $^{35}$S-phytochelatins rise steadily (Figure 5B). In the mutant, glutathione levels decrease only slightly, while phytochelatins increase much more slowly than in the wild type. The slow rate of phytochelatin accumulation in the mutant could be consistent with either slower rates of phytochelatin synthesis, or normal rates of synthesis coupled with higher rates of degradation. If the latter explanation were true, we would expect a pulse of $^{35}$S label to enter the phytochelatin pool at equal rates in the mutant and wild-type cells, but to “chase” out of the pool faster in the mutant than in wild type. In fact, the converse is true: $^{35}$S-labeled phytochelatins appear more rapidly in the wild type cells than in the mutant, and levels in both strains remain elevated, with no evidence of differential degradation rates.

Phytochelatin synthase activity was assayed in extracts from wild type and mutant cells. Both samples were capable of producing phytochelatins in vitro when supplied with excess glutathione and 500 µM cadmium. The reaction failed to occur in the absence of added heavy metal (not shown). The activity appears to be rather low compared to synthesis rates in vivo, but is not significantly different between the two strains (wild type: 6.25 nmol phytochelatin (as GSH equivalents)/ mg protein•hr ± 2.77 S.E.; hm2− mutant: 5.91 nmol phytochelatin/ mg protein•hr ± 1.39 S.E.; n=3). Since hm2 cells possess a phytochelatin synthase that appears fully active in vitro, the HMT2− protein is not likely to be directly involved in PC biosynthesis. This leaves us to postulate that the HMT2+ protein may be indirectly required for the reaction to occur in vivo, either by activating phytochelatin synthase or by removing an inhibitor of the reaction.

Possible mechanism of phytochelatin synthase inhibition

We hypothesized that the high level of sulfide accumulated in hm2− cells might be responsible for inhibition of phytochelatin synthesis. To test this possibility, phytochelatin
synthase activity of wild-type extracts was tested in the presence of increasing levels of sodium sulfide and either 500 µM or 2 mM cadmium. When added sulfide was at least equal molar to added cadmium, phytochelatin synthase activity was inhibited by over 90% (Figure 6). To determine the relevance of these in vitro data to actual conditions in cells, we measured cadmium and sulfide concentration in metal-exposed cell (Table 1). Wild-type cells accumulated cadmium and sulfide at a ratio of approximately 2:1. In contrast, mutant cells accumulated cadmium and sulfide at a ratio of 1:1 or lower. Based on in vitro data, in wild-type cells, the moderate sulfide levels might be expected to inhibit phytochelatin synthase by about 50%. In mutant cells, however, the high sulfide levels should almost completely inhibit phytochelatin synthase activity. High sulfide levels, therefore, may be sufficient to account for the low accumulation of phytochelatins in hmt2− cells.

The 1:1 stoichiometry of cadmium and sulfide required for inhibition suggested that cadmium and sulfide might be directly interacting. Formation of an insoluble CdS complex might deprive phytochelatin synthase of the free cadmium needed for enzyme activation, and thus prevent synthesis of phytochelatins. Metal-sulfide complexes appear to form in hmt2− cells, as the colonies turn bright yellow in the presence of cadmium, brown in the presence of copper, and gray in the presence of lead or bismuth (not shown). Fluorescence microscopy, using UV illumination, showed that when hmt2− cells are exposed to cadmium for a few hours, a faint, diffuse orange material on the cell surface is seen. This material is not seen with wild-type cells. After longer exposure of hmt2− cells to cadmium, the diffuse staining pattern gives way to discrete, apparently intracellular bodies that luminesce white, blue, yellow, or orange (Figure 7). Wild type cells contain a small number of similar, but much fainter, fluorescent bodies. DAPI staining of these samples gives better visualization of the cells, but the cadmium-specific fluorescence patterns could also be seen in the absence of DAPI (not shown).

The bodies that are visible in cadmium-exposed hmt2− cells are likely to consist of or contain cadmium sulfide because of the following: 1) they occur only in the presence of
cadmium, 2) they disappear upon treatment with 0.05N HCl that would liberate sulfide, and 3) they are reactive with a silver stain for sulfide. Silver staining also quenches the previously observed fluorescence (data not shown). These objects appear to pellet with the insoluble fraction of a cell-free extract after centrifugation at 15,000x g. Fluorescent particles were partially purified from cadmium-exposed hmt2- cells (Figure 7C) by differential centrifugation in the presence of 1% Triton X-100. This fluorescent material contains cadmium and sulfide in an approximately 1:1.45 ratio. All indications, therefore, suggest that in hmt2- cells, cadmium and sulfide interact to form insoluble cadmium-sulfide complexes.

Discussion

A defect in sulfide dehydrogenase activity causes hypersensitivity to Cd²⁺, Hg²⁺, Cu²⁺, and Zn²⁺ in hmt2- cells. The spectrum of metal sensitivity suggested the failure of a phytochelatin-mediated tolerance pathway, since Cd²⁺, Hg²⁺, Cu²⁺, and Zn²⁺ all induce phytochelatin synthesis (Grill et al., 1987). Phytochelatins have been shown to bind these metals (Maitani et al., 1996; Mehra et al., 1996a; Mehra et al., 1996b); and phytochelatin-deficient Arabidopsis thaliana mutants are hypersensitive to these metals (Howden and Cobbett, 1992; Howden et al., 1995b). Recent studies of S. pombe phytochelatin synthase mutants have confirmed the hypersensitivity to cadmium, disagreed on copper, and failed to show hypersensitivity to zinc or mercury (Ha et al., 1999; Clemens et al., 1999). Differences in concentrations and specific growth conditions tested could potentially account for the discrepancies.

HMT2 deficiency is associated with deficient accumulation of phytochelatins. Our initial hypothesis was that this oxidoreductase, in addition to acting on sulfide, might be capable of reducing glutathione or phytochelatins. Therefore, the absence of this function might result in an accumulation of an oxidized form that would fail to bind metals. However, we found no evidence for perturbation of the redox state of thiol pools in hmt2- cells, and have been unable to demonstrate direct reduction or oxidation of thiols by the HMT2 protein. Instead, pulse-chase
labeling experiments suggest an indirect effect that operates at two different levels. First, the 
*hmt2* mutant fails to up-regulate production of glutathione when exposed to cadmium. Second, 
it fails to initiate phytochelatin synthesis, as judged by both a lack of newly made phytochelatin 
peptides and a lack of glutathione turnover. It is known that the *S. pombe* phytochelatin synthase 
requires activation by metals such as Cd$^{2+}$, Cu$^{2+}$, Ag$^{+}$, Hg$^{2+}$, Zn$^{2+}$ or Pb$^{2+}$ (Ha et al., 1999). In the 
*hmt2* mutant, high levels of intracellular sulfide appear to precipitate out large amounts of 
cadmium as cadmium sulfide. Therefore, the free cadmium concentration may be too low to 
activate phytochelatin synthase or to induce additional glutathione synthesis.

If the above model is correct, and most of the cadmium in mutant cells is present as an 
insoluble CdS precipitate, it begs the question on why the cells would be sensitive to cadmium? 
One explanation may be that unregulated intracellular deposition of CdS itself could interfere 
with cell metabolism. Although wild-type fission yeast cells also form intracellular cadmium 
sulfide, it is less luminescent. Previous studies of *Candida glabrata* have correlated the 
luminescence intensity of cadmium sulfide with particle size (Mehra et al., 1994). Mutant 
cadmium sulfide particles may not only be larger, but because of the failure to produce 
phytochelatins, the complex would also remain unshielded. In wild-type cells, cadmium-sulfide 
complexes are surrounded by a shell of phytochelatin peptides, and this entire high molecular 
weight complex is enclosed within the vacuole (Mehra et al., 1994; Ortiz et al., 1992; Vogeli-
Lange and Wagner, 1990). Vacuolar sequestration would minimize potential toxic effects of the 
precipitate on the rest of the cell.

Previous studies have shown that the ability to reduce sulfur is absolutely required for *S. 
pombe* to achieve cadmium tolerance. A sulfite reductase mutant that cannot assimilate the 
sulfur required to produce cysteine, glutathione, and phytochelatins, is cadmium-hypersensitive. 
The demands for reduced sulfur during cadmium detoxification are such that even supplying 
exogenous cysteine in the medium fails to restore cadmium tolerance to the mutant (D. Speiser, 
this laboratory, unpublished observation). Similarly, strains with certain mutations in the purine
biosynthesis pathway are unable to increase sulfide production during cadmium stress. These strains fail to accumulate the high molecular weight cadmium-sulfide-phytochelatin complex and are likewise cadmium-hypersensitive (Speiser et al., 1992). In the same vein, some *S. pombe* and *C. glabrata* mutants that hyperaccumulate sulfide have been shown to be cadmium hypertolerant, accumulating large amounts of high molecular weight cadmium-sulfide-phytochelatin complex (Mehra et al., 1994; Ow lab, unpublished observation).

The present study demonstrates that although sulfide production is necessary for cadmium tolerance in *S. pombe*, it is not sufficient. In fact, the ability to appropriately modulate sulfide levels, controlled by *hmt2*+, is required in order for normal phytochelatin-mediated metal detoxification to proceed. The observation that high sulfide levels do not preclude phytochelatin synthesis in the cadmium-hypertolerant mutants described above, whereas sulfide does block synthesis in *hmt2*− cells, suggests that the cell may finely control the timing, level, or location of sulfide production. If sulfide production were increased only within the vacuole, or only after phytochelatin synthesis was initiated, the sulfide could be used productively to form the high-molecular-weight complex. Early, excessive, or inappropriately located sulfide production in the *hmt2*− strain could lead to abnormal CdS deposits in the cell. This model would indicate that spatial and temporal regulation of sulfur metabolism plays a key role metal tolerance.

**Experimental Procedures**

**Genotypes, plasmids, and culture conditions:**

JS23 (wild-type) is *ura4.294, leu1.32 h*+; JS563 is *ura4.294, leu1.32, h*, *hmt2*+; JV5 is *ura4.294, leu1.32, h*, *hmt2::URA3* (Vande Weghe and Ow, 1999). pJV26 contains the minimal *hmt2*+ genomic clone in the pART1 (McLeod et al., 1987) fission yeast expression vector. Cells were grown at 30° C in YG (2%glucose, 0.5% yeast extract) or in SG (2%glucose, 0.67% yeast nitrogen base without amino acids) supplemented with 100 µg/ml leucine and/ or 20 µg/ml uracil as appropriate.
Analysis of phytochelatins:

Protein content of samples was measured by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Phytochelatin-Cd complexes were separated by gel filtration chromatography as described (Ortiz et al., 1992), and the cadmium content of resulting fractions was measured by atomic absorption spectroscopy (Model 3110 atomic absorption spectrometer, Perkin Elmer Corp., Norwalk, CT). For HPLC analysis of apo-phytochelatins, cell pellets resuspended in 100 µl distilled water were vortexed with glass beads, then centrifuged at 15,000xg, 4°C for 2 minutes. An equal volume of ice-cold 10% 5-sulfosalicylic acid was added. After a 5-minute incubation on ice, centrifugation was repeated and supernatants filtered through a 0.2 µm syringe filter. Nonradioactive samples contained 200 µg protein prior to deproteinization; radioactive samples corresponded to 4 ml of the radiolabeled cultures. 50 µl samples were injected onto a Betasil Basic-18 HPLC column (Keystone Scientific, Bellefonte, PA) equilibrated in 5% acetonitrile/95% (0.05% trifluoroacetic acid in water), and eluted by a linear gradient to 12.5% acetonitrile/87.5% (0.05% trifluoroacetic acid in water) over 20 minutes, at a rate of 1 ml/minute. 0.7 mg/ml DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] in 0.3M KPO₄ pH7.8/ 7.5 mM EDTA was mixed with post-column effluent at a rate of 1 ml/minute (for non-radioactive samples) or 0.1 ml/minute (for radioactive samples), and absorption of the derivatized effluent was monitored at 405 nm. For radioactive samples, effluent was then mixed with an equal amount of Ultima-Flo M scintillation cocktail (Packard Instrument Co., Meriden, CT), and radioactive peaks were monitored by flow scintillation counting. Purified GSH, (γ-EC)₂G, (γ-EC)₃G, and sulfide were used as standards for the identification of peaks; purified GSH was used for quantitation of peaks.

For reduction of thiols with NaBH₄, cell-free extract corresponding to 2 mg protein was adjusted to 1 M HCl and centrifuged to remove precipitated protein. The supernatant was adjusted neutral pH with NaOH and brought to 10 mM Tris-Cl pH 7.0. NaBH₄ was added to 0.3
M and extracts were incubated at 37°C for 30 minutes. 5-sulfosalicylic acid was then added to 5% and extracts were analyzed by HPLC.

For \textit{in vivo} ^{35}S labeling, 200 ml YG cultures at A_{550}= 0.25 were centrifuged at 4000 x g for 5 minutes. Pellets were washed once in 1x phosphate-buffered saline and incubated for 20 minutes in 4 ml low-sulfur medium (MM medium (Alfa et al., 1993) minus sodium sulfate). After adding 80 \mu Ci Na_2^{35}SO_4 for 15 minutes, cells were harvested and washed once with 1x phosphate-buffered saline, and a time=0 sample was collected. The remaining cells were diluted into 200 ml YG containing 200 \mu M cadmium, and at various time-points, aliquots were spun down and frozen in liquid nitrogen for subsequent HPLC analysis.

**Enzyme assays:**

For the glutathione reductase recycling assay, cells grown to saturation in SG were harvested at 4000 rpm, 4°C and washed once with 1x phosphate-buffered saline, then resuspended in 1x phosphate-buffered saline to a total volume of approximately 0.8 ml. Cells were frozen in liquid nitrogen and ground with 1.2 g alumina in a mortar and pestle, then resuspended in 500 \mu l 10mM NaPO_4, pH 7.0/ 1mM EDTA. A 10 \mu l aliquot was reserved for protein quantitation, and the remainder was deproteinized with 2 ml 3% 5-sulfosalicylic acid. After a 5 minute incubation on ice and a 5 minute centrifugation at 15,000xg, aliquots of the supernatant were adjusted to pH 7.0 with NaOH and assayed for glutathione as described (Akerboom and Sies, 1981).

Phytochelatin synthase assays were performed essentially as in Howden \textit{et al.} (1995b). A typical assay contained 4 mg protein extract from uninduced cells, 0.2M Tris pH 8, 10 mM beta-mercaptoethanol, 3.3 mM glutathione, and 500 \mu M CdCl_2. The reaction mixture was incubated at 30°C, and aliquots were removed at various time-points for analysis of phytochelatin production by HPLC.
Measurement of sulfide and cadmium:

YG-grown cells exposed to 200 µM cadmium for 5 hours were harvested, washed once in 1x phosphate-buffered saline, resuspended in 10mM Tris-Cl pH 8.0, and broken by vortexing with glass beads. Extract amounts were normalized by total cell number, cell pellet size, and soluble protein content; all three methods yielded the same conclusion. The extract was divided in half, and cadmium concentration of one half was measured by atomic absorption spectroscopy; sulfide concentration of the other half was determined by the methylene blue assay as described in Speiser et al. (1992), after deproteinization with 5% 5-sulfosalicylic acid and centrifugation.

The silver stain for sulfide is a modification of the procedure in Pearse (1972). Cells were incubated briefly in 3% AgNO₃, washed once with water, mounted in a mixture of .0185% formaldehyde/0.005% citric acid, and examined under UV and visible light.

Fluorescent particles were prepared from JS563 cells grown to saturation in YG and induced with 800 µM CdCl₂ for 48 hours. Cells were washed once in distilled water, and lysed by vortexing with glass beads followed by 2 minutes of sonication. The cell lysate was subjected to three rounds of differential centrifugation, in which the fraction that pelleted between 1,000 x g and 15,000 x g was collected. For the last round of centrifugation, Triton X-100 was added to a final concentration of 1%. The final 15,000 x g pellet was resuspended in distilled water and analyzed for cadmium and sulfide content.

Acknowledgment

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References


Table 1. Cadmium and sulfide content of cadmium-induced cells.

Wild-type and \textit{hmt2} \textsuperscript{-} mutant cells were exposed to cadmium for 5 hours, and intracellular cadmium and sulfide were assayed independently. Concentrations were calculated based on the wet cell pellet volume of 75 ml.

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<th>Strain</th>
<th>Trial</th>
<th>mM Cd</th>
<th>mM S\textsuperscript{2-}</th>
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Figure legends

Figure 1. Gel filtration analysis of cadmium-binding complexes.
A. JS23 (wild type), JS563 (hmt2<sup>−</sup> mutant), and JV5 (hmt2 disruption mutant) were grown in rich (YG) media with 200 µM cadmium for 30 hours. Extracts containing 2 mg protein were fractionated on a G-50 column. B. JS563 bearing the empty vector pART1 or the complementing hmt2<sup>+</sup> plasmid pJV26 were grown in minimal (SG + uracil) media and induced with 200 µM cadmium for 24 hours. Extracts containing 0.6 mg protein were fractionated on a G-50 column. Cadmium content was measured by atomic absorbance spectroscopy. Peaks corresponding to high- and low-molecular weight phytochelatin-cadmium complexes are marked HMW and LMW, respectively. Free Cd appears after fraction 50.

Figure 2. HPLC analysis of acid-soluble thiols.
Extracts from wild-type JS23 (A) and hmt2<sup>−</sup> JS563 (B) cells grown in rich (YG) media in the absence of cadmium (foreground) or with 200 µM Cd for 24 hours (background) were separated by reverse-phase HPLC and detected by reaction with DTNB. GSH: glutathione; S<sup>−</sup>, sulfide; PC<sub>2</sub>, PC<sub>3</sub>: (γ-EC)<sub>2</sub>G and (γ-EC)<sub>3</sub>G phytochelatins, respectively.

Figure 3. HPLC analysis of thiols after chemical reduction.
Extracts from wild-type JS23 and hmt2<sup>−</sup> JS563 cells were induced by cadmium and analyzed by HPLC with DTNB detection before (left) and after (right) NaBH<sub>4</sub> reduction.

Figure 4. Detection of radiolabelled thiols.
An extract from <sup>35</sup>S-labeled hmt2<sup>−</sup> JS563 cells induced with 200 mM cadmium for 8 hours was separated by HPLC. Peaks were detected simultaneously by reaction with DTNB (A) and flow scintillation counting (B).
Figure 5. Time course of phytochelatin synthesis.
Radiolabeled wild-type JS23 and hmt2− JS563 cells were exposed to 200 µM cadmium. At the designated time-points, aliquots were analyzed for thiol content by HPLC with DTNB detection (A) and flow scintillation counting (B). Values in (B) were normalized to the total acid-soluble radioactivity of the sample at time=0.

Figure 6. Phytochelatin synthase activity in the presence of sulfide.
Extracts from wild-type cells grown in minimal (SG) media without cadmium were assayed for phytochelatin synthase activity in the presence of various concentrations of Na₂S and 0.5 mM (left) or 2 mM (right) CdCl₂. Each column represents net PC synthesis in 4 hours by an extract corresponding to 50 ml (left) or 10 ml (right) of cell culture.

Figure 7. Fluorescence micrographs of cadmium-exposed cells.
Wild-type JS23 (A) and hmt2− JS563 mutant (B) cells were grown in rich (YG) media with 1 mM cadmium for 24 hours. Whole, unfixed cells were counter-stained with DAPI (1 µg/ml) mounted in glycerol containing phenylene diamine (1 mg/ml), and viewed by fluorescence microscopy with UV excitation. Deep blue staining is due to DAPI. In the mutant (B), cadmium exposure gave rise to diffuse pale orange fluorescence and to discrete luminescent bodies (arrowheads). Particles (C) prepared from cadmium-exposed mutant cells by differential centrifugation were viewed by fluorescence microscopy.