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

Project Number: EMSP 55278

Grant Project Officer: Mark Gilbertson

Project Duration: 9/1/96 to 8/31/99
No cost extension: 9/1/99 to 8/31/00

2. Table of Contents

Cover page ……..…….1

Table of Contents ……..…….2

Executive Summary ……..…….3

Research Objectives ……..……..4

Methods and Results ……..7

Relevance, Impact, and Technology Transfer ……...……..….12

Project Productivity ……...…12

Personnel Supported ……..……..13

Publications ……...……..…..13

Interactions ……..…..14

Transitions ……15

Patents ……..……16

Future Work ……16

Literature Cited ……16

Feedback ……..…….19

Appendices ……...…….19

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 $(\gamma\text{-Glu-Cys})_n$Gly, where the number of γ-Glu-Cys units extends up to 11 [11]. Some PC-related peptides lack the carboxyl-terminal Gly or have instead β-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 Cd^{2+} and Cu^{2+}. Several reports show that PCs also chelate Ag^{+}, Hg^{2+}, Pb^{2+} and Zn^{2+} [21,22,23,44]. In the case of 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 ~4:1 to a more economical ~2:1 [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 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* [(26)]. The gene product actively transports cytoplasmic PC-Cd complexes into vacuoles [(27,40)]. 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 [(27,39)].

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 [(41)]. Biochemical studies support this hypothesis. *In vitro*, these enzymes can utilize cysteine sulfinate in place of aspartate [(17)]. 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].

During the funding period, we completed the following:

Mitochondrial gene required for PC production. Mutant JS563 was originally found to be hypersensitive to Cd and deficient in PC peptide accumulation [45, VandeWeghe and Ow, submitted]. Further analysis revealed that this mutant is also hypersensitive to 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 :

S^{2-} + coenzyme $Q_{2(oxid)}$ → $S^0_{(oxid)}$ + coenzyme $Q_{2(red)}$ (Q_2 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 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.

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 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/βNAC family [47,52]. WASP is the only gene that is contained intact within this 7 kb clone. For the BTF3/βNAC 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 guanyltransferase-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 guanyltransferase, 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.

<u>Progress on Strategy #2. Genes expressed during Cd-stress</u>

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 *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 *S. pombe* is a better model system for plant metal tolerance than the more commonly used budding yeast *Saccharomyces cerevisae*. *S. cerevisae* is about 1000-fold more sensitive to Cd than *S. pombe*. Using such a highly sensitive recipient would likely lead to the identification of cDNAs that only marginally increase tolerance. Furthermore, *S. pombe* shares common metal detoxification pathways with higher plants.

Initially, we transformed a cDNA library made from *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 T_1 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 T_1 plants are derived from self-fertilized T_0 (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 *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 (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\cdot$) 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 (*Journal of Biological Chemistry & Molecular Pharmacology*). Another manuscript has been submitted for publication (*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:

1996-1998	Jennifer VandeWeghe, graduated with a Ph.D. degree from U.C. Berkeley, then become a Scientist at MicroGenomics, Inc., San Diego, CA.

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

Ow, D.W. 1996. Heavy metal tolerance genes: prospective tools for bioremediation. *Resources, Conservation and Recycle* 18: 135-149.

Perego, P., VandeWeghe, J., Ow, D.W., Howell, S.B. 1997. The role of determinants of cadmium sensitivity in the tolerance of *Schizosaccharomyces pombe* to cisplatin. *Molecular Pharmacology* 51: 12-18.

VandeWeghe, J., Ow, D.W. 1999. A fission yeast gene for mitochondrial sulfide oxidation. *Journal of Biological Chemistry* 274: 13250-13257.

Ow, D.W. 1998. Prospects of engineering heavy metal detoxification genes in plants. In: *Engineering Crops For Industrial Uses* (Shewry, P., ed.), Portland Press, pp. 111-124.

Ph.D. Thesis

VandeWeghe, J. G. 1997. A mitochondrial sulfide dehydrogenase required for heavy metal tolerance in fission yeast. Ph.D. Thesis,, University of California, Berkeley.

Unreviewed publications

Ow, D.W., Kim, J.H., Fox, T., Sin, M. 2000. Cloning heavy metal tolerance genes. Papers of the Environmental Management Science Program Workshop, Atlanta, GA, April 25-27, 2000. http://home.osti.gov/em52/NWS2000_Posters/id55278.pdf, pages 1-9.

Submitted for publication

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

10. Interactions

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

09/17/96, Biochemical Society Symposium on "Engineering Crops for Industrial Uses", Bristol, England, 09/16/96-09/18/96. "Prospects of engineering plants for metal remediation".

10/09/96, Commenius University, Botany Department, Bratislava, Slovakia, "Prospects of engineering plants for bioremediation"

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

04/07/97, Society for Experimental Biology Symposium on Metals and Genes, Canterbury, England, 04/07/97-04/08/97. "A novel mitochrondrial oxidoreductase required for phytochelatin accumulation and cadmium tolerance in fission yeast". (Lecture delegated to graduate student Jennifer VandeWehge).

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".
04/25/00, Environmental Management Science Program Workshop, Atlanta, GA, April 25-27, 2000. "Molecular Genetics of Metal Detoxification: Cloning Metal Hypertolerance Genes".

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).
Consultant, Joint FAO/WHO (Food & Agriculture Organization/World Health Organization) Expert Consultation on Foods Derived from Biotechnology (May 29-June 2, 2000, Geneva, Switzerland). Committee report: http://www.who.int/fsf/GMfood/FAO-WHO_Consultation_report_2000.pdf

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

1. Baker AJM, Brooks RR (1989) Terrestrial higher plants which hyperaccumulate metal elements - a review of their distribution, ecology, and phytochemistry. *Biorecovery* 1: 81-126.
2. Banuelos GS, Cardon G, Mackey B, Ben-Asher J, Wu L, Beuselinck P, Akohoue S, Zambrzuski S (1993) Boron and selenium removal in B-laden soils by four sprinkler irrigated plant species. *J Environ Qual* 22: 786-7973.
3. Blaylock MJ, Salt DE, Dushenkov S, Zakharova O, Gussman C, Kapulnik Y, Ensley BD, Raskin I (1997) Enhanced accumulation of Pb in Indian mustard by soil-applied chelating agents. *Environ Sci Technol* 31: 860-865.
4. Clemens S, Antosiewicz DM, Ward JM, Schachtman DP, Schroeder JI (1998) The plant cDNA LCT1 mediates the uptake of calcium and cadmium in yeast. *Proc Natl Acad Sci U SA* 95 : 12043-12048
5 Cohen CK, Fox TC, Garvin DF, Kochian LV (1998) The role of iron-deficiency stress response in stimulating heavy-metal transport in plants. *Plant Physiol* 116 : 1063-1072
6. Cunningham S, Ow DW (1996) Promises and prospects of phytoremediation. *Plant Physiol.* 110: 715-719
7. Dameron CT, Reese RN, Mehra RK, Kortan AR, Carroll PJ, Steigerwald ML, Brus LE, Winge DR. (1989) Biosynthesis of cadmium sulphide quantum semiconductor crystallites. *Nature* 338: 596-597.
8 Derry JM, Ochs HD, Francke U (1994) Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell*, 78 : 635-644.
9. Dix DR, Bridgham JT, Broderius MA, Byersdorfer CA, Eide DJ (1994) The FET4 gene encodes the low affinity Fe(II) transport protein *of Saccharomyces cerevisiae*. *J Biol Chem* 269 : 26092-26099.
10. Elmayan T, Tepfar M (1994) Synthesis of a bifunctional metallothionein/beta-glucuronidase fusion protein in transgenic tobacco plants as a means of reducing leaf cadmium levels. *Plant J* 6: 433-440.

11. Grill E, Winnacker EL, Zenk MH (1987) Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. *Proc Natl Acad Sci USA* 84: 439-443.
12. Grotz N, Fox T, Connoly E, Park W, Guerinot ML, Eide D (1998) Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. *Proc Natl Acad Sci U SA* 95 : 7220-7224.
13. Hamer DH. (1986) Metallothioneins. *Ann. Rev. Biochem* 55 : 913- 951.
14. Hattori J, Labbe H, Miki BL (1994) Construction and expression of a metallothionein-beta-glucuronidase gene fusion. *Genome* 37: 508-512
15. Hirschi KD (1999) Expression of *Arabidopsis* CAX1 in tobacco: altered calcium homeostasis and increased stress sensitivity. *Plant Cell* 11 : 2113-2122.
16. Huang JW, Chen J, Berti WR, Cunningham SD (1997) Phytoremediation of lead-contaminated soils: role of synthetic chelates in lead phytoextraction. *Environ Sci Technol* 31: 800-805.
17. Juang R-H, McCue KF, Ow DW (1993) Two purine biosynthetic enzymes that are required for cadmium tolerance in *Schizosaccharomyces pombe* utilize cysteine sulfinate *in vitro*. *Arch. Biochem. & Biophys.* 304: 392-401.
18. Korshunova YO, Eide D, Clark WG, Guerinot ML, Pakrasi HB (1999) The IRT1 protein *from Arabidopsis thaliana* is a metal transporter with a broad substrate range. *Plant Mol Biol* 40 : 37-44
19. Lasat MM, Jiang T, Pence NS, Letham DL.D, Kochian LV (1999) Molecular physiology of Zn transport regulation in the Zn hyperaccumulator, *Thlaspi caerulescens*. ASPP supplement abstract 769.
20. Li L, Kaplan J (1998) Defects in the yeast high affinity iron transport system result in increased metal sensitivity because of the increased expression of transporters with a broad transition metal specificity. *J Biol Chem* 273 : 22181-22187.
21. Maitani T, Kubota H, Sato K, Yamada T (1996) The composition of metals bound to class III metallothionein (phytochelin and its desglycyl peptide) induced by various metals in root cultures of *Rubia tinctorum*. *Plant Phsyiol* 110 : 1145-1150
22. Mehra RK, Kodati VR, Abdullah R (1995) Chain length-dependent Pb(II)-coordination in phytochelatins. *Bioch & Biophy Res Comm* 215 : 730-736.
23. Mehra RK, Miclat J, Kodati VR, Abdullah R, Hunter TC, Muchandani P (1996) Optimal spectroscropic and reverse-phase HPLC analysis of Hg(II) binding to phytochelatins. *Biochem J* 314 : 73-82.
24. Misra S, Gedamu L (1989) Heavy metal tolerant transgenic *Brassica napus* L. and *Nicotiana tabacum* L. plants. *Theor Appl Genet* 78: 161-168
25. Moffat, AS (1995) Plants proving their worth in toxic metal cleanup. *Science* 269: 302-303
26. Ortiz DF, Kreppel L, Speiser DM, Scheel G, McDonald G, Ow DW (1992) Heavy metal tolerance in the fission yeast requires an ATP binding cassette-type vacuolar membrane transporter. *EMBO J* 11 : 3491-3499
27. Ortiz DF, Ruscitti T, McCue K, Ow DW (1995) Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. *J Biol Chem* 270 : 4721-4728
28. Ow DW (1993) Phytochelatin-mediated cadmium tolerance in *Schizosaccharomyces pombe*. *In Vitro Cell Dev Biol* 29P : 213-219

29. Ow, DW (1996) Heavy metal tolerance genes: prospective tools for bioremediation. *Resources, Conservation and Recycle* 18 : 135-149.
30. Ow, DW (1998) Prospects of engineering heavy metal detoxification genes in plants. In: *Engineering Crops For Industrial Uses* (Shewry, P., ed.), Portland Press, pp. 111-124.
31. Pan A, Tie F, Duau Z, Yang M, Wang Z, Li L, Chen Z, Ru B (1994) Alpha-Domain of human metallothionein I-A can bind to metals in transgenic tobacco plants. *Mol Gen Genet* 242: 666-674
32. Perego P, VandeWeghe J, Ow DW, Howell SB (1997) The role of determinants of cadmium sensitivity in the tolerance of *Schizosaccharomyces pombe* to cisplatin. *Molecular Pharmacology* 51 : 12-18.
33. Rauser W (1995) Phytochelatins and related peptides. *Plant Physiol.* 109 : 1141-1149.
34. Reese RN, Winge DR (1988) Sulfide stabilization of the cadmium-γ-glutamyl peptide complex of *Schizosaccharomyces pombe*. *J Biol Chem* 263: 12832-12835.
35. Reeves RD, Brooks RR (1983) Hyperaccumulation of lead and zinc by two metallophytes from a mining area in central Europe. *Environ Pollution Ser* A 31 :277-285.
36. Rivero-Lezcano OM, Marcilla A, Sameshima JH, Robbins KC (1995) Wiskott-Aldrich syndrome protein physically associates with Nck through Src homology 3 domains. *Molecular & Cellular Biology.* 15 :5725-5731.
37. Robinson NJ, Tommey AM, Kuske C, Jackson PJ (1993) Plant metallothioneins. *Biochem J* 295 : 1-10.
38. Rugh CL, Wilde HD, Stack NM, Thompson DM, Summers AO, Meagher RB (1996) Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana* plants expressing a modified bacterial *merA* gene. *Proc Natl Acad Sci USA* 93:3182-3187.
39. Salt DE, Wagner GJ (1993) Cadmium transport across tonoplast of vesicles from oat roots. Evidence for a Cd2+/H+ antiport acitivity. *J Biol Chem* 268 : 12297-12302.
40. Salt DE, Rauser WE (1995) MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. *Plant Physiol* 107 : 1293-1301.
41. Speiser DM, Ortiz DF, Kreppel L, Scheel G, McDonald G, Ow DW (1992) Purine biosynthetic genes are required for cadmium tolerance in *Schizosaccharomyces pombe*. *Molecular & Cellular Biology* 12 : 5301-5310.
42. Stasdeit H, Duhme A-K, Kneer R, Zenk MH, Hermes C, Nolting H-F (1991) Evidence for discrete Cd(SCys)4 units in cadmium phytochelatin complexes from EXAFS spectroscopy. *J Chem Soc Chem Commun* 16 : 1129-11130.
43. Thomine S, Clemens S, Stephan, A, Kim, EJ, Neumann D, Wang R, Crawford NM, Schroeder J (1999) Molecular analysis of metal transport and metal tolerance in plants. ASPP Supplement abstract 130004
44. Thumann J, Grill E, Winnacker E-L, Zenk MH (1991) Reactivation of metal-requiring apoenzymes by phytochelatin-metal complexes. *FEBS Lett* 284 : 66-69.
45. VandeWeghe, J., Ow, DW (1999) A fission yeast gene for mitochondrial sulfide oxidation. *J. Biol. Chem.* 274:13250-13257
46. Vassil AD, Kapulnik Y, Raskin I, Salt DE (1998) The role of EDTA in lead transport and accumulation by indian mustard. *Plant Physiol* 117: 447-453.

47. Wiedmann B, Sakai H, Davis TA, Wiedmann M (1994) A protein complex required for signal-sequence-specific sorting and translocation. *Nature* 370 : 434-440.
48. Wu J, Hsu FC, Cunningham SD (1999) Chelate-assisted Pb phytoextraction: Pb availability, uptake, and translocation constraints. *Environ Sci Technol* 33: 1898-1904.
49. Yeargan R, Maiti IB, Nielsen MT, Hunt AG, Wagner GJ (1992) Tissue partitioning of cadmium in trangenic tobacco seedlings and field grown plants expressing the mouse metallothionein I gene. *Transgenic Research* 1: 261-267
50. Zayed AM and Terry N (1994) Selenium volatilization in roots and shoots: effects of shoot removal and sulfate level. *J Plant Phys* 143: 8-14
51. Zhao H, Eide D (1996a) The ZRT2 gene encodes the low affinity zinc transporter in *Saccharomyces cerevisiae*. *J Biol Chem* 271 : 23203-23210.
52. Zheng XM, Black D, Chambon P, Egly JM (1990) Sequencing and expression of complementary DNA for the general transcription factor BTF3. *Nature* 344 : 556-559.

15. Feedback

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

16. Appendices

Attachments: electronic files of:

1. Perego, P., VandeWeghe, J., Ow, D.W., Howell, S.B. 1997. The role of determinants of cadmium sensitivity in the tolerance of *Schizosaccharomyces pombe* to cisplatin. *Molecular Pharmacology* 51: 12-18. (pdf file)

2. VandeWeghe, J., Ow, D.W. 1999. A fission yeast gene for mitochondrial sulfide oxidation. *Journal of Biological Chemistry* 274: 13250-13257. (pdf file)

3. VandeWeghe, J., Ow, D.W. Accumulation of metal-binding peptides in fission yeast requires *hmt2*$^{+}$, submitted to *Molecular Microbiology*. (two files: document in Word and figures in pdf)

17. Quantifies / Packaging.

None.

0026-895X/97/010012-07$3.00/0

MOLECULAR PHARMACOLOGY, **51:**12–18 (1997).

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).

Received July 8, 1996; Accepted October 14, 1996

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 $(\gamma\text{-Glu-Cys})_n\text{Gly}$, 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-

This work was supported in part by the Associazione Italiana per la Ricerca sul Cancro (P.P.) and the United States Department of Agriculture Agricultural Research Service Project 5335–21000-009-00D (D.W.O.). This work was conducted in part by the Clayton Foundation for Research, California Division. S.B.H. is a Clayton Foundation Investigator.

ABBREVIATIONS: DDP, cisplatin; PC, phytochelatin; GSH, glutathione; PC/CdS, phytochelatin/cadmium/sulfide.

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). CdCl was purchased from Sigma Chemical (St. Louis, MO).

Genetic materials and manipulation. The adenine prototropic *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 prototropic 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/CdS complex (14). Cultures were grown at 30° in YES medium (consisting of 5 g of Difco yeast extract, 30 g of glucose/liter, 0.075 g of adenine/liter, and 0.075 g of uracil/liter). For construction of strains carrying either the yeast expression vector pART1 or the *hmt1* expression construct pDH35, cells were transformed through electroporation (13), and transformants were selected in minimal medium supplemented with amino acids other than leucine.

Measurement of PC/Cd complexes. PC/Cd complexes were measured as described elsewhere (15). Briefly, LK37 and wild-type cells were cultured in standard medium and induced with 0.2 mM Cd for 30 hr. A cell-free extract containing 2 mg of protein was fractionated on a Sephadex G50 column, and the Cd concentration of the fractions was measured with the use of atomic absorbance spectroscopy. 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, the viability of cells after drug exposure was determined by clonogenic assay. Specifically, 3-ml cultures containing a total of 2.5×10^5 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°. The IC_{50} 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.

GSH level. The intracellular GSH levels in exponentially growing cells were determined through conjugation of GSH with monobromobimane followed by high performance liquid chromatography quantification as previously described (16). Each experiment was performed three times with triplicate samples.

DDP accumulation and DNA platination. The uptake of DDP was measured after a 4-hr exposure of 3-ml cultures containing a total of 1×10^6 cells to 1.6 mM DDP at 30°. After incubation with DDP, the cells were washed four times with cold phosphate-buffered saline at 4°, 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 H_2O. 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 *Eco*RI (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 Na_2S 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/10^6 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

[1] D. M. Speiser, K. F. McCue, and D. W. Ow, unpublished observations.

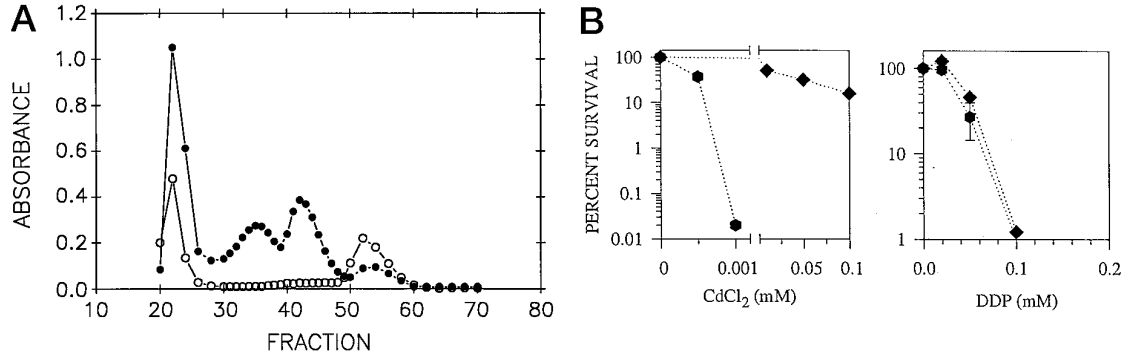

Fig. 1. A, PC profile of LK37 and parental wild-type cells. The middle two peaks represent the high and low molecular weight PC/Cd complexes, respectively. ●, Parental wild-type cells. ○, LK37. B, Plating efficiency (number of colony-forming units) of *S. pombe* strains JS21 (GSH proficient) and LK37 (GSH deficient) after a 48-hr exposure to graded concentrations of $CdCl_2$ and DDP. ♦, JS21 (GSH proficient). •, LK37 (GSH-deficient). *Data points*, mean values from two or three independent experiments performed with triplicate cultures. *Vertical lines*, standard deviation.

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 IC_{50} 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: $IC_{50} = 0.015$ mM; Sp223: $IC_{50} = 0.054$ 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/10^6 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 IC_{50} value for Cd and a 6-fold increase in the IC_{50} value for DDP compared with its parental strain. Examination of a second

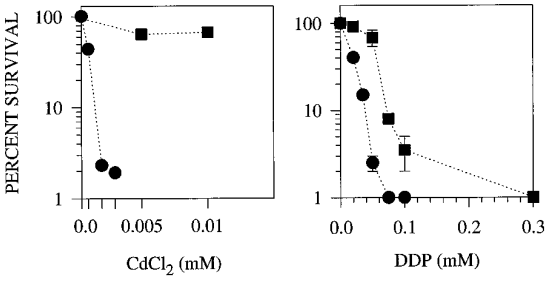

Fig. 2. Plating efficiency of *S. pombe* strains Sp223 (*hmt1*$^+$) and LK100 (*hmt1*$^-$) after a 48-hr of exposure to graded concentrations of $CdCl_2$ and DDP. ●, LK100. ■, Sp223. *Left*, values are from a representative experiment. *Right*, *data points*, mean values from three experiments performed with triplicate cultures. *Vertical lines*, standard deviation (where absent, standard deviation was less than the size of the symbol).

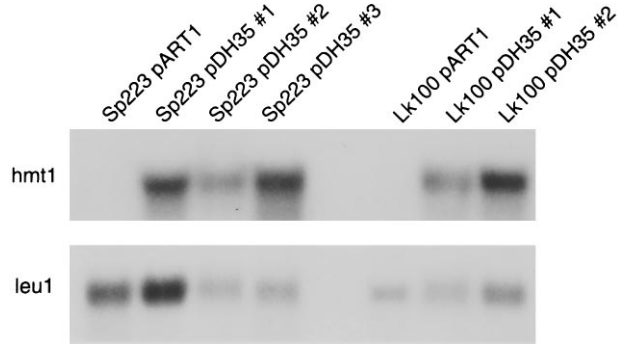

Fig. 3. Northern blot analysis of total RNA from LK100 and Sp223 cells carrying pDH35 (*hmt1*$^+$ cDNA) or pART1 (empty vector). Twenty micrograms of total RNA was loaded in each lane. The *hmt1* cDNA was used as a hybridization probe, whereas the *leu1* gene probe was used as a control to document RNA lane loading. Basal levels of *hmt1* message were detectable when the blot was exposed for longer periods of time.

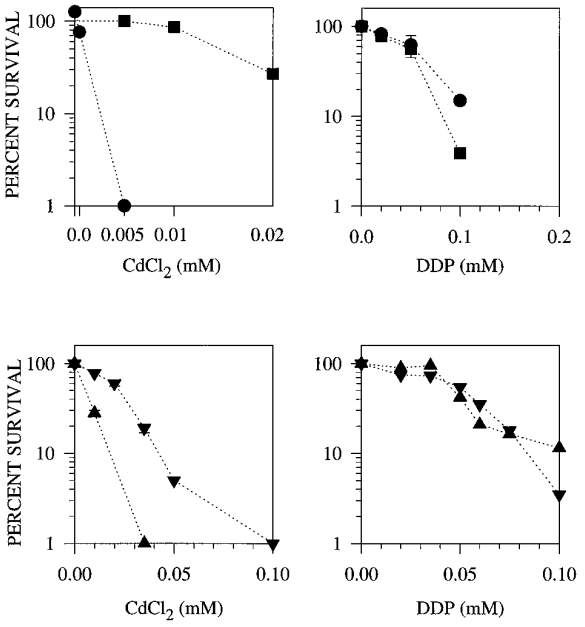

Fig. 4. Clonogenic survival as a function of concentration after a 48-hr exposure to $CdCl_2$ and DDP of *hmt1*$^-$ LK100 and *hmt1*$^+$ Sp223 harboring pART1 (empty vector) or pDH35 (*hmt1*$^+$ cDNA) (Sp223pDH35#1 and LK100pDH35#2). ●, LK100 harboring pART1. ■, LK100 harboring pDH35. ▲, Sp223 harboring pART1. ▼, Sp223 harboring pDH35. *Data points*, mean values from two or three independent experiments performed with triplicate cultures. *Vertical lines*, standard deviation.

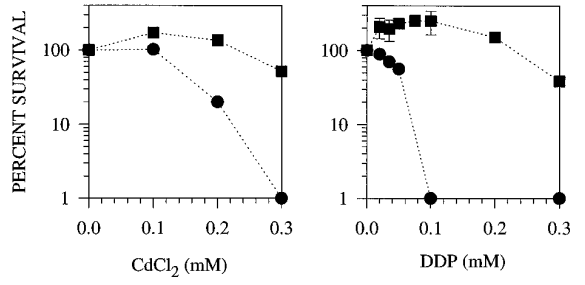

Fig. 5. Clonogenic survival of the *S. pombe* wild-type strain JS21 and the sulfide-overproducing strain Y10 after a 48-hr exposure to $CdCl_2$ and DDP. ●, JS21. ■, Y10. *Data points*, mean values from three to six independent experiments performed with triplicate cultures. *Vertical lines*, standard deviation.

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 Na_2S ranging from 0.25 to 4 mM. The formation of a brown

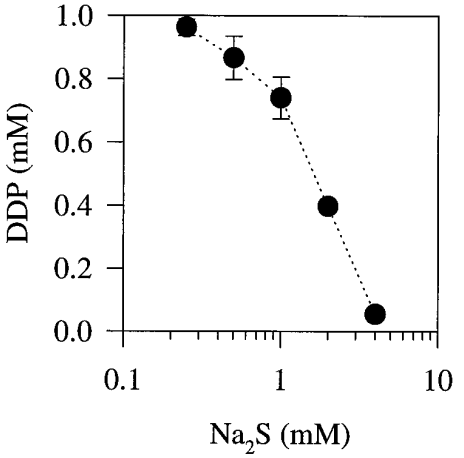

Fig. 6. *In vitro* interaction between DDP and Na_2S. DDP (1 mM) was incubated with different concentrations of Na_2S. The DDP remaining in solution after removal of precipitation is expressed as a function of Na_2S concentration. *Data points*, average values from two independent experiments. *Vertical lines*, standard deviation.

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 Na_2S.

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.[1] 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

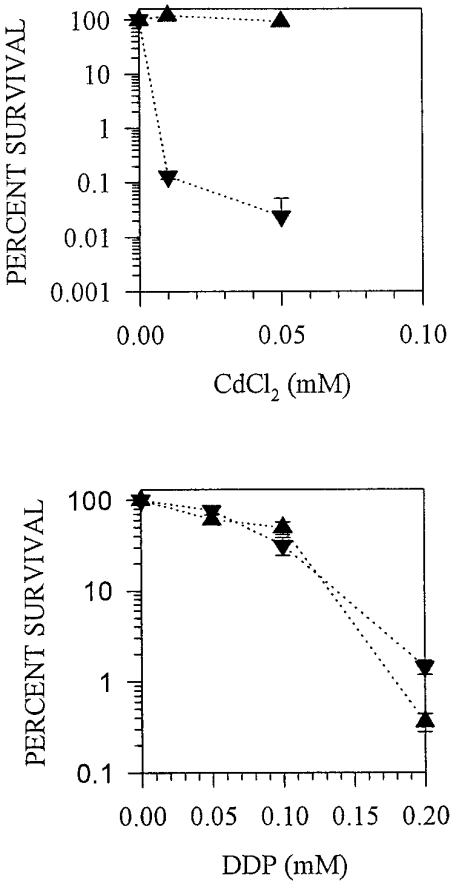

Fig. 7. Plating efficiency of JS21 (wild-type) and DS31 (sulfite reductase mutant) after a 48 hr exposure to $CdCl_2$. ▲, JS21. ▼, DS31. *Data points*, mean values from two independent experiments. *Vertical lines*, standard deviation.

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 *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 generated 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 sulfate 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.[1] Therefore, Cd-induced sulfide production can proceed from a step downstream of the assimilatory sulfate 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 *S. pombe*, homologous pathways in human cells could be revealed as being important in the determination of tumor sensitivity to this important chemotherapeutic agent.

Acknowledgments

We would like to thank Bristol-Myers Squibb for the kind gift of the DDP used in these studies.

References

1. Kondo, Y., S.-M. Kuo, S. C. Watkins, and J. S. Lazo. Metallothionein localization and cisplatin resistance in human hormone-independent prostatic tumor cell lines. *Cancer Res.* **55:**474–477 (1995).
2. Andrews, P. A., M. P. Murphy, and S. B. Howell. Differential potentiation of alkylating and platinating agent cytotoxicity in human ovarian carcinoma cells by glutathione depletion. *Cancer Res.* **45:**6250–6253 (1985).
3. Andrews, P. A., N. P. Murphy, and S. B. Howell. Differential sensitization of human ovarian carcinoma and mouse L1210 cells to cisplatin and melphalan by glutathione depletion. *Mol. Pharmacol.* **30:**643–650 (1986).
4. Godwin, A. K., A. Meister, P. J. O'Dwyer, C. S. Huang, T. C. Hamilton, and M. E. Anderson. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. *Proc. Natl. Acad. Sci. USA* **89:**3070–3074 (1992).
5. Mehra, R. K., and D. R. Winge. Metal ion resistance in fungi: molecular mechanisms and their regulated expression. *J. Cell Biol.* **45:**30–40 (1991).
6. Jeyaprakash, A., J. W. Welch, and S. Fogel. Multicopy CUP1 plasmids enhance cadmium and copper resistance levels in yeast. *Mol. Gen. Genet.* **225:**363–368 (1991).
7. Yu, W., V. Santhanagopalan, A. K. Sewell, L. T. Jensen, and D. R. Winge. Dominance of metallothionein in metal ion buffering in yeast capable of synthesis of (yEC)nG isopeptides. *J. Biol. Chem.* **269:**21010–21015 (1994).
8. Grill, E., E.-L. Winnacker, and M. H. Zenk. Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. *Proc. Natl. Acad. Sci. USA* **84:**439–443 (1987).
9. Robinson, N. J., A. M. Tommey, C. Kuske, and P. J. Jackson. Plant metallothioneins. *Biochemistry* **295:**1–10 (1993).
10. Rauser, W. Phytochelatins and related peptides. *Plant Physiol.* **109:**1141–1149 (1995).
11. Dameron, C. T., R. N. Reese, R. K. Mehra, A. R. Kortan, P. J. Carrol, M. L. Steigerwald, L. E. Brus, and D. R. Winge. Biosynthesis of cadmium sulfide quantum semiconductor crystallites. *Nature (Lond.)* **338:**596–597 (1989).
12. Ortiz, D. F., L. Kreppel, D. M. Speiser, G. Scheel, G. McDonald, and D. W. Ow. Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. *EMBO J.* **11:**3491–3499 (1992).
13. Prentice, H. L. High-efficiency transformation of *Schizosaccharomyces pombe* by electroporation. *Nucleic Acids Res.* **20:**621–624 (1992).
14. Speiser, D. M., D. F. Ortiz, L. Kreppel, G. Scheel, G. McDonald, and D. W. Ow. Purine biosynthetic genes are required for cadmium tolerance in *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **12:**5301–5310 (1992).
15. Mehra, R. K., P. Mulchandani, and T. C. Hunter. Role of CdS quantum crystallites in cadmium resistance in *Candida glabrata*. *Biochem. Biophys. Res. Commun.* **200:**1193–1200 (1994).
16. Miquel, J. *CRC Handbook of Free Radicals and Antioxidants in Biomedicine*. CRC Press, Boca Raton (1993).
17. Perego, P., G. Jimenez, and S. B. Howell. Isolation and characterization of a cisplatin-resistant strain of *Schizosaccharomyces pombe. Mol. Pharmacol.* **50:**1080–1086 (1996).
18. Collart, M. A., and S. Oliviero. *Anonymous Current Protocols in Molecular Biology*. (1994).
19. Hickey, E., S. E. Branden, S. Sadis, B. Smale, and L. E. Weber. Molecular cloning of sequences encoding the human heat-shock proteins and their expression during hyperthermia. *Gene* **43:**147–156 (1986).
20. Mutoh, N., and Y. Hayashi. Isolation of mutants of *Schizosaccharomyces pombe* unable to synthesize cadystin, small cadmium-binding peptides. *Biochem. Biophys. Res. Commun.* **151:**32–39 (1986).
21. Coblenz, A., and K. Wolf. The role of glutathione biosynthesis in heavy metal resistance in the fission yeast *Schizosaccharomyces pombe*. *FEMS Microbiol. Rev.* **14:**303–308 (1994)
22. Ortiz, D. F., T. Ruscitti, K. F. McCue, and D. W. Ow. Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. *J. Biol. Chem.* **270:**4721–4728 (1995).
23. Brzywczy, J., and A. Paszewski. Sulfur amino acid metabolism in *Schizosaccharomyces pombe*: occurrence of two O-acetylhomoserine sulfhydrylases and the lack of the reverse transsulfuration pathway. *FEMS Microbiol. Lett.* **121:**171–174 (1994).
24. Juang, R. H., K. F. McCue, and D. W. Ow. Two purine biosynthetic enzymes that are required for cadmium tolerance in *Schizosaccharomyces pombe* utilize cysteine sulfinate *in vitro*. *Arch. Biochem. Biophys.* **304:**392–401 (1993).
25. Naredi, P., D. D. Heath, R. E. Enns, and S. B. Howell. Cross-resistance between cisplatin, antimony potassium tartrate, and arsenite in human tumor cells. *J. Clin. Invest.* **95:**1193–1198 (1995).
26. Ozols, R. F., P. J. O'Dwyer, T. C. Hamilton, and R. C. Young. The role of glutathione in drug resistance. *Cancer Treat. Rev.* **17:**45–50 (1990).
27. O'Dwyer, P. J., T. C. Hamilton, R. C. Young, F. P. LaCreta, N. Carp, K. Padavic, R. L. Comis, and R. F. Ozols. Depletion of glutathione in normal

and malignant human cells in vivo by buthionine sulfoximine: clinical and biochemical results. *J. Natl. Cancer Inst.* **84:**264–267 (1992).
28. O'Dwyer, P. J., T. C. Hamilton, K. S. Yao, K. D. Tew, and R. F. Ozols. Modulation of glutathione and related enzymes in reversal of resistance to anticancer drugs. *Hematol. Oncol. Clin. North Am.* **9:**383–396 (1995).
29. Grill, E., S. Loeffler, E.-L. Winnacker, and M. H. Zenk. Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). *Proc. Natl. Acad. Sci. USA* **86:**6838–6842 (1989).
30. Hayashi, Y., C. W. Nakagawa, N. Mutoh, M. Isobe, and T. Goto. Two pathways in the biosynthesis of cadystins $(\gamma EC)_nG$ in the cell-free system of the fission yeast. *Biochem. Cell Biol.* **69:**115–121 (1991).
31. Steffens, J. C., D. F. Hunt, and B. G. Williams. Accumulation of non-protein metal binding polypeptides (γ-glutamyl-cysteinyl)n-glycine in selected cadmium-resistant tomato cells. *J. Biol. Chem.* **261:**13879–13882 (1986).
32. Howden, R., P. B. Goldsbrough, C. R. Anderson, and C. S. Cobbett. Cadmium sensitive, cad1 mutants of *Arabidopsis thaliana* are phytochelatin deficient. *Plant Physiol.* **107:**1059–1066 (1995).

Send reprint requests to: Paola Perego, Ph.D., Experimental Oncology B, Istituto Nazionale Tumori, via Venezian 1, 20133 Milano, Italy. E-mail: perego@icil64.cilea.it

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 274, No. 19, Issue of May 7, pp. 13250–13257, 1999
Printed in U.S.A.

A Fission Yeast Gene for Mitochondrial Sulfide Oxidation*

(Received for publication, December 2, 1998, and in revised form, February 24, 1999)

Jennifer G. Vande Weghe‡§¶ and David W. Ow‡‖

From the ‡Plant Gene Expression Center, U. S. Department of Agriculture-Agricultural Research Service, Albany, California 94710 and the §Department of Plant and Microbial Biology, University of California, Berkeley, California 94720

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 (coenzyme Q_2) 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 anoxygenic photosynthesis appeared quite early in evolution. Today, it is widespread in the green and purple phototrophic bacteria and has been reported in cyanobacteria (1). Enzymes mediating sulfide oxidation have been described in the photosynthetic bacteria *Chlorobium limicola* (2, 3), *Oscillatoria limnetica* (4), *Rhodobacter capsulatus* (5), and *Chromatium vinosum* (6); the complete sequence of the latter two proteins has been described.

In recent years, it has become known that sulfide oxidation is not the province solely of bacteria. Some animals from sulfide-rich aquatic sediments, such as the gutless clam (*Solemya reidi*) and the lugworm (*Arenicola marina*) are able to oxidize sulfide within their own tissues without the aid of bacterial symbionts (7–12). This capacity allows them to detoxify sulfide that enters their bodies from the surrounding environment 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 sulfite over several steps, the enzyme sulfite reductase catalyzes the reaction [H_2SO_3 + 3 NADPH + $3H^+ \rightarrow S^{2-}$ + 3 $NADP^+$ + 3 H_2O]. Much of this S^{2-} is incorporated into cysteine, but the cell still accumulates measurable amounts of acid-labile S^{2-} under normal laboratory conditions. 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.

EXPERIMENTAL PROCEDURES

Genetic Materials—S. pombe strains Sp223 (*h*$^-$, *ade6.216, ura4.294, leu1.32*) and B1048 (*h*$^+$, *ade7.50, ura4.294*) have been described (14). JS21 (*h*$^-$, *ura4.294, leu1.32*) and JS23 (*h*$^+$, *ura4.294, 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*$^+$, *ura4.294, leu1.32, hmt2*$^-$) and JV7 (h$^-$, *ura4.294, leu1.32, hmt2*$^-$). JV5 (h$^-$, *ura4.294, leu1.32, hmt2::URA3*$^+$) harbors a homologous insertion, whereas JV11 (*h*$^-$, *ura4.294, leu1.32, URA3*$^+$) harbors a random insertion of an *hmt2*$^-$ disruption construct bearing the *Saccharomyces cerevisiae URA3*$^+$ gene. DS31 (*h*$^+$, *ura4.294, leu1.32, sir1::LEU2*) was generated by disruption of the sulfite reductase gene with a *S. cerevisiae LEU2*$^+$ fragment (D. Speiser, USDA-ARS, Albany, CA). JV3 (*ura4.294, 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.[1] 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,

* This work was supported by U. S. Department of Energy Grant EM96–55278 (to D. W. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number-(s) AF042283.

¶ Present address: MicroGenomics Inc., 11211 Sorrento Valley Rd., San Diego, CA 92121.

‖ To whom correspondence should be addressed: Plant Gene Expression Center, USDA-ARS, 800 Buchanan St., Albany, CA 94710. Tel.: 510-559-5909; Fax: 510-559-5678; Email: ow@pgec.ars.usda.gov.

[1] The abbreviations used are: MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; kb, kilobase(s); IPTG, isopropyl-1-thio-β-D-galactopyranoside.

respectively.

Sulfide Analysis—S^{2-} was collected and assayed as described (14). S^{2-} content was first normalized to the dry weight of the cell culture and, to minimize day-to-day assay variability, was subsequently normalized to each day's value obtained from wild-type cells grown without cadmium (set to 1.0).

For ^{35}S-labeling of sulfide *in vivo*, cells incubated for 20 min in 4 ml of low sulfur medium (MM medium (15) minus sodium sulfate) were spiked with 80 μCi $Na_2{}^{35}SO_4$. After 15 min, the cells were harvested, washed with phosphate-buffered saline, and resuspended in 200 ml of YG containing 200 μM cadmium. Cells at various time points were collected and frozen in liquid nitrogen for high performance liquid chromatography analysis.

For analysis of S^{2-} turnover, labeled cell pellets were homogenized and centrifuged for 2 min at 15,000 $\times$ *g*, 4 °C. Proteins were precipitated with 5% 5-sulfosalicylic acid. After filtration of supernatants, samples were injected onto a Betasil Basic-18 high performance liquid chromatography column (Keystone Scientific) 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 min, at a rate of 1 ml/min. 0.7 mg/ml DTNB in 0.3 M KPO_4, pH 7.8, 7.5 mM EDTA was mixed with post-column effluent at a rate of 0.1 ml/min, and absorption of the derivatized effluent was monitored at 405 nm. Effluent was then mixed with an equal amount of Ultima-Flo M scintillation mixture (Packard Instrument), and radioactive peaks were monitored by flow scintillation counting. Na_2S was used as a standard for peak identification and quantification.

Molecular Cloning—*S. pombe* genomic and cDNA libraries were described previously (16). Transformation of *S. pombe* was performed essentially as described (17). For gene disruption, the *Eco*RV to *Sph*I fragment of pJV1, containing part of the *hmt2*$^+$ coding sequence, was replaced by the *S. cerevisiae URA3*$^+$ gene. A linear 4.1-kb *Xba*I fragment from this pJV17 plasmid was transformed into JS21, and colonies prototrophic for uracil were selected. For Northern analysis of *hmt2*$^+$ expression, RNA was prepared as described (16). Blots were hybridized to random primer-labeled *hmt2*$^+$ cDNA and, following stripping of the blot, were reprobed with an end-labeled oligonucleotide representing a short antisense sequence of the *S. pombe* 18 S rRNA. The mutant *hmt2*$^-$ allele was cloned into pART1 (18) to form pJV30. A 300-base pair *Hin*dIII to *Sca*I fragment containing the mutation was replaced with wild-type DNA to form pJV34.

pJV37 contains the *hmt2*$^+$ coding sequence in the pQE12 *Escherichia coli* expression vector (Qiagen). The *hmt2*$^+$ genomic clone was cleaved with *Pac*I. Ends were made flush with T4 DNA polymerase and ligated to *Bgl*II linkers. The DNA was cleaved with *Bgl*II, followed by *Hinc*II that released a fragment containing all but the first two and the last codons of *hmt2*$^+$. This fragment was ligated to pQE12 that had been cleaved with *Bam*HI, made flush with the Klenow fragment of DNA polymerase I, and subsequently cleaved with *Bgl*II. The resulting construct encodes an *hmt2*$^+$ fusion protein having four new vector-derived N-terminal amino acids and nine new vector-derived C-terminal amino acids, the last 6 of which are histidines. The DNA encoding the entire fusion protein from pJV37 was cloned into pART1 under the control of the alcohol dehydrogenase promoter to form pJV40. This construct was transformed into JS563, and cadmium sensitivity was compared with JS563 and JS23 containing the empty vector pART1.

Cell Fractionation—Cells grown in SG were vortexed with glass beads in an equal volume of 10 mM Tris-Cl, pH 7, 0.15 M NaCl, 0.25 M sucrose, 1 mM each phenylmethylsulfonyl fluoride, EDTA, EGTA, dithiothreitol, and benzamidine HCl. Unbroken cells and debris were removed by centrifugation at 1,000 $\times$ *g* for 10 min. The total protein preparation was further fractionated by centrifugation at 100,000 $\times$ *g* for 1 h. Protein was quantified using an assay kit (Bio-Rad) in the presence of 0.05% CHAPS for membrane-containing samples.

Mitochondria were isolated using published procedures (19, 20). Marker enzymes corresponding to various cell compartments were assayed: cytochrome *c* oxidase (mitochondria), cytochrome *c* reductase (endoplasmic reticulum), α-mannosidase (vacuole), glucose-6-phosphate dehydrogenase (cytoplasm), catalase (peroxisome), and guanosine diphosphatase (Golgi). The results indicated that mitochondria were ~6-fold enriched over their initial abundance in the cell homogenate, whereas the abundance of other organelles was decreased or relatively unchanged. Mitochondrial subfractionation was carried out with minor modifications from a published protocol (21). Activity of the soluble matrix enzyme fumarase (22) was used to monitor mitochondrial breakage. For investigation of the effects of pH on HMT2 solubility, mitochondria were suspended in ice-cold 0.6 M sucrose, 3 mM $MgCl_2$, 20 mM Tris-Cl, pH 7.4, or 0.6 M sucrose, 3 mM $MgCl_2$, 20 mM Na_2CO_3, pH 11. Mitochondria were sonicated for a total of 4 min, with rest periods on ice, then centrifuged for 1 h at 100,000 $\times$ *g*.

Antibody Production and Immunodetection—HMT2 protein was purified under denaturing conditions from XL1-Blue *E. coli* (Stratagene) carrying pJV37, according to the QIAexpress kit protocol (Qiagen). Purified protein was electroeluted from a preparative SDS-polyacrylamide electrophoresis gel, mixed with MPL+ TDM + CWS Emulsion (RIBI ImmunoChem Research), and used to immunize two rabbits. For Western blots, anti-HMT2 antisera were typically used at a dilution of 1/30,000. Chemiluminescent detection of Western blots has been described (23). The polyclonal antisera specifically recognize the recombinant protein in Western blots of extracts from *E. coli* expressing tagged HMT2, but not from *E. coli* bearing the empty expression vector.

Nondenaturing Protein Purification—*E. coli* XL1-Blue carrying pJV37 was grown to A_{600} = 0.6 at 30 °C in 4 liters of LB, 1 M sorbitol, 2.5 mM betaine, 50 μg/ml carbenicillin, and induced for 2.5 h with 1 mM IPTG. Cells were harvested by centrifugation and resuspended in 100 ml of 50 mM $NaPO_4$, pH 7.8, 50 mM NaCl, 10% glycerol, 0.1% Triton X-100. After incubation with 0.1 mg/ml lysozyme on ice for 15 min and three rapid freeze-thaw cycles, extracts were sonicated briefly and centrifuged at 10,000 $\times$ *g* for 20 min. The supernatant was applied to a DEAE-Sephadex column (Amersham Pharmacia Biotech) equilibrated in the same buffer, and the first, highly UV-absorbing flow-through peak was collected. This fraction was stirred with 1 ml of Pro-Bond nickel chelate resin at 4 °C for 2 h. The resin was then loaded onto a column, washed with 10 ml of the same buffer, and then eluted with the same buffer containing 500 mM imidazole. Fractions containing HMT2 were pooled, subjected to several cycles of dilution with imidazole-free buffer, and concentrated by ultrafiltration (Centricon 30). At each step in the procedure, sulfide:quinone reductase activity copurified with anti-HMT2 immunoreactive protein peaks. The final preparation, which showed an ~180-fold enrichment of enzyme specific activity, was adjusted to 50% glycerol and stored at −20 °C.

HMT2 Activity Assay—Sulfide:quinone oxidoreductase activity was measured under air at room temperature. A typical 250-μl reaction contained 20 mM Tris-Cl, pH 7.8, 40 μM coenzyme Q_2 (Sigma), and 0.5 μg of purified HMT2, to which 400 μM Na_2S was added to begin the reaction. Reduction of Q_2 was measured by loss of 285 nm absorption. The decrease in A_{285} was followed for 30 s and was linear during this period for all experiments. The mM extinction coefficient "$\Delta\epsilon$ oxidized-reduced" of Q_2, determined empirically by comparing the absorption of oxidized and $NaBH_4$-reduced Q_2 samples in reaction buffer at 285 nm, was 8.85. S^{2-} from aliquots of reaction mixtures was trapped in 1 M zinc acetate and quantified by the methylene blue assay as described (14). Mitochondria, adjusted to 8 μg of protein in 250 μl, were assayed as above, except reactions contained 3.2 mM Na_2S and 2 mM KCN.

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 given the genetic designation *hmt2*. When compared with the wild-type parent strain JS21, which can grow at up to 800 μM cadmium, the *hmt*$^-$ 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 I). *S. pombe* is known to increase sulfide production during cadmium stress, and a higher level of sulfide production was observed in both strains grown in 200 μM cadmium, but the *hmt*$^-$ JS563 continued to exceed wild-type levels. Exogenous sulfide is toxic to *S. pombe* at concentrations in the 100 μM range. Because the

TABLE I
Genetic control of sulfide accumulation

S^{2-} accumulation over 18 h of growth in minimal (SG) or complete (YG) medium, in the presence or absence of cadmium. Strains used are wild-type (JS23), *hmt2* disruption (JV5) or missense (JS563) mutant, sulfite reductase mutant (DS31), and *hmt2*$^-$/sulfite reductase double mutant (JV3). Where indicated, the strains harbor the empty vector (pART1) or a complementing clone (pJV26). Normalized to the value obtained from the wild type grown in the absence of cadmium.

Strain	Plasmid	Media	Normalized S^{2-}	
			0 μM Cadmium	200 μM Cadmium
JS23	pART1	SG	1	3.2 (±0.8)
JS563	pART1	SG	6.8 (±3.1)	9.8 (±3.8)
JS563	pJV26	SG	1.6 (±0.09)	3.6 (±1.6)
JV5		SG	8.4 (±1.3)	15.2 (±4.0)
JS23		YG	1	
JS563		YG	6.4 (±0.5)	
DS31		YG	0.4 (±0.04)	
JV3		YG	1.1 (±0.05)	

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 Na_2S, with the greatest differential observed at 200 μM Na_2S.

Defect Not in Sulfate Assimilation—One described route of sulfide production in *S. pombe* is the sulfur 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 S^{2-} 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 *hmt*$^-$ 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 S^{2-}, cells were pulse-labeled with $^{35}SO_4^{2-}$. During the brief 15-min labeling period, both wild-type and mutant cells converted $^{35}SO_4^{2-}$ into $^{35}S^{2-}$ (Fig. 1, time = 0 h). Most of this $^{35}S^{2-}$ then disappeared, as it was incorporated into organic sulfur compounds, leaving approximately equal amounts in mutant and wild-type cells after 2 h. Over the next 20 h, wild-type cells gradually turned over this pool of $^{35}S^{2-}$, but in the *hmt2*$^-$ mutant this pool was not depleted. During that time, total (radioactive plus nonradioactive) sulfide levels accumulated more rapidly in the mutant than in wild type (Fig. 1, *inset*). The pattern indicates that the *hmt2*$^-$ defect lowers the consumption of the sulfide pool. This alone may account for the higher accumulation of sulfide, although the data do not rule out the possibility that the mutation also enhanced *de novo* S^{2-} synthesis.

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 *Sal*I/*Sca*I subclone, in pJV26, complements the poor growth of the mutant on cadmium, hydrogen peroxide, diamide, or glycerol 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. 3*A*). Accumulation of this transcript increased by ~2-fold when cells were exposed to 200 μM cadmium (Fig. 3*B*).

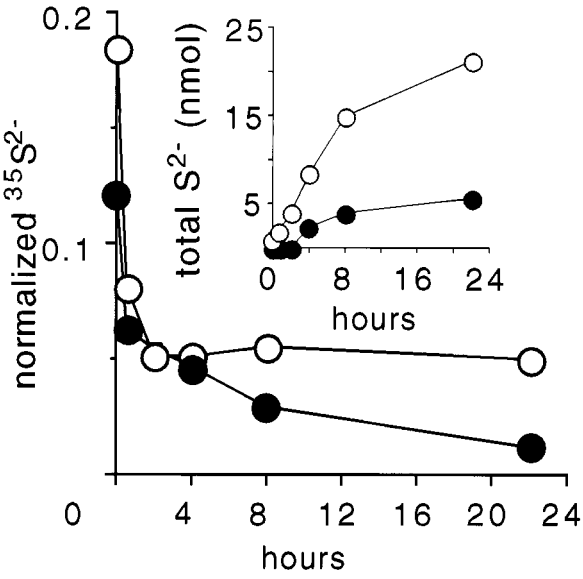

FIG. 1. **S^{2-} turnover and accumulation.** HPLC measurements of extracts from cadmium-induced wild-type (JS23, *filled circles*) and mutant (JS563, *open circles*). At each time point, 4 ml of radiolabeled culture was analyzed simultaneously for radioactive (*main panel*) and total (*inset*) sulfide. Normalized $^{35}S^{2-}$ is the amount of radiolabeled sulfide at each time point divided by total ^{35}S present in the cell at time 0.

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. 3*B*). 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 Na_2S. Surprisingly, no significant difference is observed in the presence of diamide or with glycerol 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

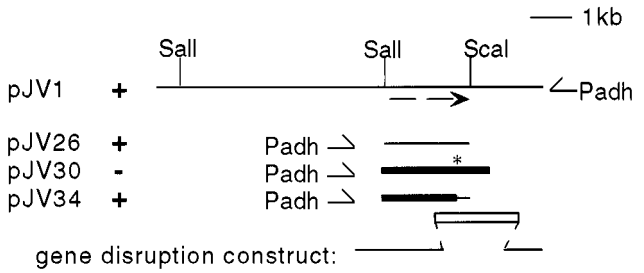

FIG. 2. **Genetic analysis of *hmt2*$^+$.** Deletion derivatives of the complementing genomic clone pJV1 were transformed into JS563 and scored for their ability to complement (+) the mutant phenotype. The alcohol dehydrogenase promoter (Padh) of the pART1 expression vector is indicated, and lies upstream of the *hmt2* coding region in pJV26, pJV30, and pJV34. The gene disruption construct shown is the *Xba*I fragment of pJV17, described under "Experimental Procedures." *Thin line*, wild-type genomic DNA; *thick line*, JS563 genomic DNA; *dashed arrow*, *hmt2*$^+$ cDNA; *open box*, *URA3*$^+$ insert; *asterisk*, site of mutation in JS563.

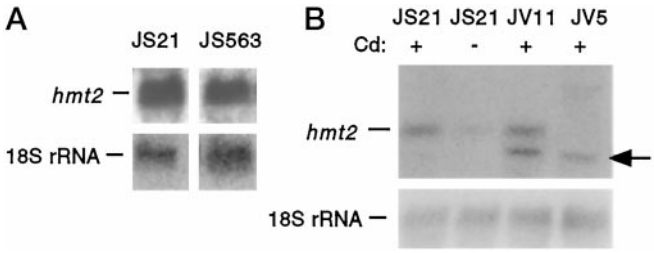

FIG. 3. **Northern analysis of *hmt2*$^+$ expression.** *A*, wild-type (JS21) and mutant (JS563) strains (harboring pART1) were grown in SG and exposed to 200 μM cadmium for 24 h. 30 μg total RNA from these cultures was hybridized with *hmt2*$^+$ cDNA, and rehybridized with a probe specific to the 18S rRNA. *B*, 30 μg total RNA from a wild-type strain (JS21), a strain bearing a random insertion of the *hmt2* disruption construct (JV11), and the *hmt2* disruption strain (JV5) was hybridized to the *hmt2*$^+$ cDNA and to an 18S rRNA probe. Cells were or were not exposed to cadmium (200 μM cadmium for 24 h) as indicated. The *arrow* marks the position of the truncated *hmt2* transcript originating from the disruption construct itself.

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, $\chi^2 = 0.64$). When crossed to JS563, wild-type recombinants were not recovered (49 cadmium hypersensitive, yellow, *URA3*$^+$ progeny: 51 cadmium hypersensitive, yellow, *URA3*$^-$ progeny, $\chi^2 = 0.04$). 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 taaaaatgt, 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 × *g* centrifugation (Fig. 5*A*), 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. 5*B*). 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×) pellet showed increased immunoreactivity for HMT2, suggesting that HMT2 may be membrane-associated (Fig. 5*C*). 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. 5*D*). 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

TABLE II
Sequence similarity to HMT2

Deduced proteins having significant primary sequence similarity to HMT2 ($p < 0.05$ in BLAST searches of the protein and nucleotide data bases) are listed, together with the degree of pairwise amino acid identity with HMT2.

Organism	Function	Length (amino acids)	Identity (%)	Accession
R. capsulatus	Sulfide quinone reductase	Full (425)	20.2	EMB X97478
C. vinosum	Flavocytochrome *c*	Full (431)	20.2	SP Q06530
E. coli	NADH dehydrogenase	Full (434)	19.8	EMB V00306
S. cerevisiae	Glutathione reductase	Full (467)	15.6	GB L35342
H. sapiens	Unknown	Full (450)	38.4	Various[a]
M. musculus	Unknown	Full (452)	37.2	Various[b]
Synechocystis sp.	Unknown	Full (409)	36.2	DBJ 90907
C. elegans	Unknown	Full (423)	32.6	Various[c]
R. norvegicus	Unknown	Partial (141)	37.6	DBJ C06990
C. aurantiacus	Unknown	Partial (115)	27.0	EMB Z34000
S. mansoni	Unknown	Partial (61)	21.3	GB T24144
D. melanogaster	Unknown	Partial (143)	20.9	GB AA438562

[a] GB T74801, T64084, T85836, T86387, T95375, R50203, R40496, R13065, N50841, T64005, T74688, T95295, T98225, AA280758, AA281439, W96028, AA100857, AA083029, AA082940, AA057016, AA133917, W94661, AA253283, W15319, N54246, AA056968, N58703, AA486172, AA486109, AA526991, AA527252, AA576059; GB T27349; DBJ D82703, D82662; EMB Z19305; EMB D20113; GB T36144, AA353040, AA359423, AA385681, AA359712, AA353447, AA315963, AA383720, AA361822, AA382189, and EST 112244, 151798, 126925, 172344. The full sequence has been deposited in GenBank, with accession number AF042284.

[b] GB AA222168, AA184667, AA124108, AA266579, AA089176, AA106635, AA146443, AA245215, AA245216, AA208338, AA509391, AA434617, AA544472, AA499804, AA492716, AA473822.

[c] GB T00051, EMB Z82265, DBJ C38797.

initial biochemical characterization. The final protein preparation consisted of two major bands of ~52 and ~50 kDa, both of which were immunoreactive with anti-HMT2 antibodies. The smaller band may result from an alternative translation start site or may be a degradation product of the full-length protein.

Flavin Binding—Sequence analysis predicted that HMT2 might bind FAD. The purified protein from *E. coli* was visibly yellow, and light spectroscopy revealed absorption maxima at ~375 and ~455 nm in addition to the main protein peak at 280 nm (Fig. 6*A*). This profile is characteristic of a flavoprotein. Similar flavin absorption peaks are visible in free FAD (Fig. 6*B*). Free flavins exhibit fluorescence, with excitation maxima at ~375 and ~450 nm and an emission maximum at 520 nm. Although the purified protein was nonfluorescent, boiling for 3 min denatured the protein and released a soluble component having the fluorescence profile expected of a flavin. This indicates that the protein binds flavin noncovalently and that the fluorescence is quenched *in situ*.

Analysis of the fluorescence properties of the dissociated flavin (31) indicated that it consists predominantly of flavin adenine dinucleotide (93%); traces of FMN may represent breakdown products. Although sequence analysis predicts a 1:1 molar ratio of FAD to polypeptide, we experimentally obtained a ratio of ~1:3. This discrepancy may be because of incomplete binding of flavin in the heterologous expression system, losses of flavin during purification, or truncation of the protein.

Sulfide:Quinone Oxidoreductase Activity—The addition of sulfide caused rapid bleaching of the 450-nm absorption peak in HMT2 (Fig. 6*A*), but not in free flavin (Fig. 6*B*) or in another flavoprotein, glutathione reductase (not shown). The bleaching of HMT2 could be reversed when the sulfide was removed by ultrafiltration. This reversible bleaching is consistent with the reduction of the flavin by sulfide. The sequence similarity of HMT2 to sulfide-oxidizing enzymes, and the sensitivity of its absorption profile to sulfide, suggested that it might be capable of using sulfide as a substrate in a redox reaction. By analogy with the activity of the *R. capsulatus* sulfide quinone reductase, we tested the ability of HMT2 to catalyze the reaction S^{2-} + coenzyme $Q_{2(oxidized)} \rightarrow [S_{(oxidized)}]$ + coenzyme $Q_{2(reduced)}$. Coenzyme Q_2 is a water-soluble ubiquinone analogue whose oxidized (Fig. 7*A, a*) and reduced (Fig. 7*A, b*) forms can be distinguished by their absorption profiles in the UV range. Addition of sulfide to a cuvette containing coenzyme Q_2 and purified recombinant HMT2 causes a rapid decrease in the 285-nm peak (Fig. 7*B, b*). The reaction between coenzyme Q_2 and sulfide in the absence of HMT2, or in the presence of an equivalent quantity of free FAD (0.43 ± 0.08 nmol/min), is nearly 8-fold slower than the enzyme-catalyzed rate (3.35 ± 0.54 nmol/min, significant at $p < 0.05$).

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 μM coenzyme Q_2 reduced per s·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-

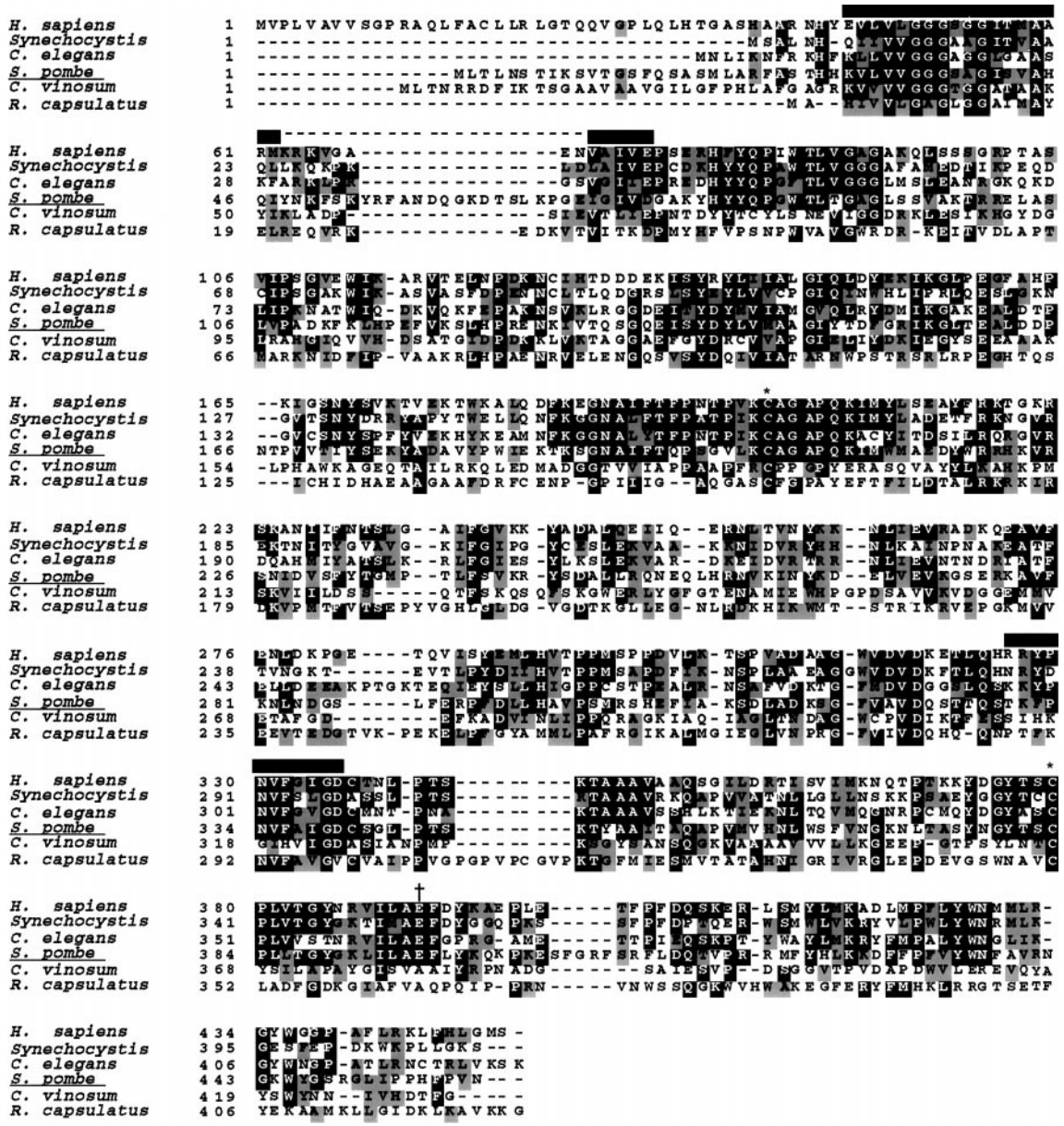

FIG. 4. **Sequence alignment of HMT2 with putative homologues.** Multiple sequence alignment of HMT2 from *S. pombe* (*underlined*) with putative distant homologues from *C. vinosum* and *R. capsulatus* and putative near homologues from *Homo sapiens*, *Synechocystis* sp. PCC6803, and *C. elegans*. Residues that are identical (*black shading*) or similar (*gray shading*) among at least three of the sequences are highlighted. The bipartite FAD-binding motif is indicated by *black bars*; the putative redox-active cysteines, by *asterisks*; and the site of the missense mutation in JS563, by a cross.

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 Q_2 at low rates in the absence of added electron donor (JS23: 75 ± 51 nmol/min·mg; JS563: 68 ± 23 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. 7*C, b*). Sulfide:quinone reductase activity, corrected for the background rate of quinone reduction, is significantly higher in JS23 (144 ± 45 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

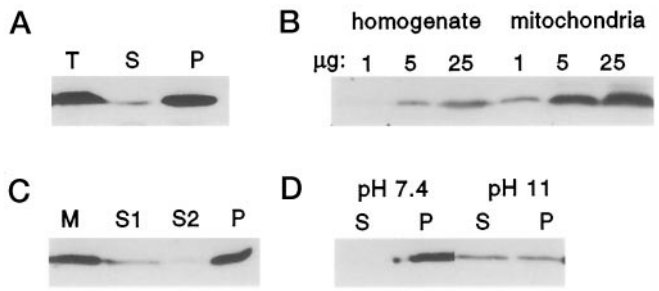

FIG. 5. **Western blots of cell fractions.** *A*, 30 μg total protein (*T*), and the supernatant (*S*) and pellet (*P*) resulting from a 1-h, 100,000 $\times$ *g* centrifugation of an equivalent 30-μg sample. *B*, 1, 5, or 25 μg total protein from an initial *S. pombe* homogenate and from mitochondria purified from the same homogenate were blotted and probed with antibodies to HMT2. *C*, whole mitochondria (*M*) were osmotically shocked and divided into a supernatant fraction (*S1*) and a pellet. The pellet was sonicated and centrifuged to separate a second supernatant (*S2*) and pellet (*P*). Each lane contains 2.5 μg of protein. *D*, whole mitochondria were sonicated at pH 7.4 or pH 11, then separated into supernatant (*S*) and pellet (*P*) fractions by centrifugation at 100,000 $\times$ *g* for 1 h. 2 μg of protein from each fraction was blotted and probed with antibodies to HMT2.

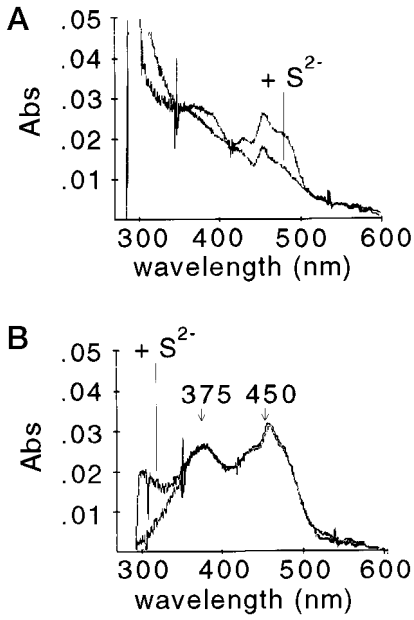

FIG. 6. **Absorbance spectra of HMT2 and FAD.** Absorbance spectra were recorded before and immediately after ($+S^{2-}$) addition of 1.25 mM sulfide to purified HMT2 (*A*) or free FAD (*B*). Indicated are absorbance maxima (375 and 450 nm) of free FAD. Each sample was adjusted to 2.5 μM flavin, assuming a mM extinction coefficient of 11.3 at 450 nm. After addition of sulfide, the increase of absorbance at ~230 nm is because of sulfide itself.

a FAD cofactor via a two-part sequence motif (amino acids 34–64 and 314–324; Fig. 4, *black bar*) (6, 27) that is also present in sulfide quinone reductase and in HMT2. A pair of cysteines (amino acids 191 and 367; Fig. 4, *) forming a disulfide bridge adjacent to the flavin in flavocytochrome c has been implicated to be catalytically involved in the redox reaction (6, 32). Aligning cysteines are also present in sulfide quinone reductase and in HMT2. Finally, the predicted secondary structure of HMT2 bears striking similarities with the known structure of flavocytochrome *c*. The similarity is greater than to any other protein in the Protein Data Bank as judged by the program PHDthreader (33). Previously, it has been suggested that all flavoenzymes known to be involved in sulfur chemistry may be related (27). HMT2 extends this family of related proteins to the eukaryotes.

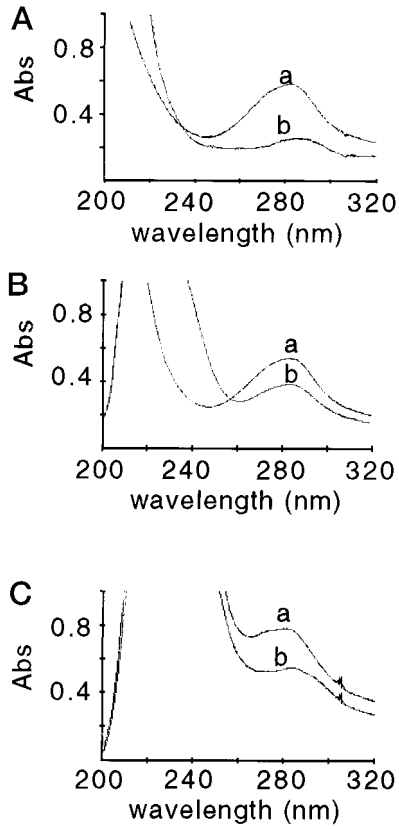

FIG. 7. **Absorbance spectra of oxidized *versus* reduced coenzyme Q_2.** *A*, absorbance spectra of 40 μM coenzyme Q_2 before (*a*) and after (*b*) chemical reduction with $NaBH_4$. *B*, absorbance spectra of reaction mixture containing 40 μM coenzyme Q_2 and 0.5 μg of HMT2 before (*a*) and after (*b*) addition of 400 μM sulfide. The additional absorbance at ~230 nm is because of sulfide itself. *C*, absorbance spectra of a sulfide:quinone reaction catalyzed by mitochondria. Spectra were taken immediately after the addition of sulfide (*a*) and 4 min later (*b*).

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.

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,

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.

Acknowledgment—We thank H. A. Koshinsky for assistance with the preparation of figures.

REFERENCES

1. Brune, D. C., and Truper, H. G. (1986) *Arch. Microbiol.* **145,** 295–301
2. Shahak, Y., Arieli, B., Padan, E., and Hauska, G. (1992) *FEBS Lett.* **299,** 127–130
3. Van Beeumen, J., Van Bun, S., Meyer, T. E., Bartsch, R. G., and Cusanovich, M. A. (1990) *J. Biol. Chem.* **265,** 9793–9799
4. Arieli, B., Shahak, Y., Taglich, D., Hauska, G., and Padan, E. (1994) *J. Biol. Chem.* **269,** 5705–5711
5. Shahak, Y., Klughammer, C., Schreiber, U., Padan, E., Herrman, I., and Hauska, G. (1994) *Photosynth. Res.* **39,** 175–181
6. Chen, Z.-W., Koh, M., Van Driessche, G., Van Beeumen, J. J., Bartsch, R. G., Meyer, T. E., Cusanovich, M. A., and Mathews, F. S. (1994) *Science* **266,** 430–432
7. Oeschger, R., and Vismann, B. (1994) *Ophelia* **40,** 147–158
8. Oeschger, R., and Vetter, R. D. (1992) *Mar. Ecol. Prog. Ser.* **86,** 167–179
9. Lee, R. W., Kraus, D. W., and Doeller, J. E. (1996) *Biol. Bull.* **191,** 421–430
10. Bagarino, T., and Vetter, R. D. (1990) *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **160,** 519–527
11. Powell, M. A., and Somero, G. N. (1986) *Science* **233,** 563–566
12. Voelkel, S., and Grieshaber, M. K. (1996) *Eur. J. Biochem.* **235,** 231–237
13. Perego, P., Vande Weghe, J., Ow, D. W., and Howell, S. B. (1997) *Mol. Pharmacol.* **51,** 12–18
14. Speiser, D. M., Ortiz, D. F., Kreppel, L., Scheel, G., McDonald, G., and Ow, D. W. (1992) *Mol. Cell. Biol.* **12,** 5301–5310
15. Alfa, C., Fantes, P., Hyams, J., McLeod, M., and Warbrick, E. (1993) *Experiments with Fission Yeast: a Laboratory Course Manual*, pp. 134–135, Cold Spring Harbor Laboratory Press, Plainview, NY
16. Ortiz, D. F., Kreppel, L., Speiser, D. M., Scheel, G., McDonald, G., and Ow, D. W. (1992) *EMBO J.* **11,** 3491–3499
17. Beach, D., and Nurse, P. (1981) *Nature* **290,** 140–142
18. McLeod, M., Stein, M., and Beach, D. H. (1987) *EMBO J.* **6,** 729–736
19. Jault, J.-M., Comte, J., Gautheron, D. C., and Di Pietro, A. (1994) *J. Bioenerg. Biomembr.* **26,** 447–456
20. Moore, A. L., Walters, A. J., Thorpe, J., Fricaud, A.-C., and Watts, F. Z. (1992) *Yeast* **8,** 923–933
21. Daum, G., Bohni, P. C., and Schatz, G. (1982) *J. Biol. Chem.* **257,** 13028–13033
22. Bergmeyer, H. U., and Gawehn, K. (eds) (1974) *Methods of Enzymatic Analysis*, pp. 452–453, 2nd Ed., Vol. 1, Academic Press, Inc., New York
23. Schneppenheim, R., Budde, U., Dahlmann, N., and Rautenberg, P. (1991) *Electrophoresis* **12,** 367–372
24. Russell, P. (1989) in *Molecular Biology of the Fission Yeast* (Nasim, A., Young, P., and Johnson, B., eds), pp. 243–271, Academic Press, San Diego
25. Zhang, M. Q., and Marr, T. G. (1994) *Nucleic Acids Res.* **22,** 1750–1759
26. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410
27. Van Driessche, G., Koh, M., Chen, Z.-w., Mathews, F. S., Meyer, T. E., Bartsch, R. G., Cusanovich, M. A., and Van Beeumen, J. J. (1996) *Protein Sci.* **5,** 1753–1764
28. Schutz, M., Shahak, Y., Padan, E., and Hauska, G. (1997) *J. Biol. Chem.* **272,** 9890–9894
29. von Heijne, G., Steppuhn, J., and Herrmann, R. (1989) *Eur. J. Biochem.* **180,** 535–545
30. Nakai, K., and Kanehisa, M. (1992) *Genomics* **14,** 897–911
31. Faeder, E. J., and Siegel, L. M. (1973) *Anal. Biochem.* **53,** 332–336
32. Meyer, T. E., Bartsch, R. G., and Cusanovich, M. A. (1991) *Biochemistry* **30,** 8840–8845
33. Rost, B. (1995) in *The Third International Conference on Intelligent Systems for Molecular Biology (ISMB)* (Rawlings, C., Clark, D., Altman, R., Hunter, L., Lengauer, T., and Wodak, S., eds), pp. 314–321, AAAI Press, Menlo Park, CA
34. National Research Council, Division of Medical Sciences, Subcommittee on Hydrogen Sulfide (1979) *Hydrogen Sulfide*, University Park Press, Baltimore
35. Bartholomew, T. C., Powell, G. M., Dodgson, K. S., and Curtis, C. G. (1980) *Biochem. Pharmacol.* **29,** 2431–2437
36. Baxter, C. F., Van Reen, R., Pearson, P. B., and Rosenberg, C. (1958) *Biochim. Biophys. Acta* **27,** 584–591

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

Running title: Phytochelatin accumulation in *S. pombe*

Key words: sulfide, cadmium, metal, phytochelatin, *Schizosaccharomyces pombe*

Total word count: 5,400 (including Table)

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 $(\gamma\text{-EC})_nG$, 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 $(\gamma\text{-EC})_2G$ and $(\gamma\text{-EC})_3G$ (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}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}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}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 $^{35}SO_4^{2-}$ in the absence of cadmium to label the glutathione pool. Excess unincorporated label was then removed and cells were placed in media containing unlabeled SO_4^{2-} along with 200 μM 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 $\pm$ 2.77 S.E.; *hmt2*$^-$ mutant: 5.91 nmol phytochelatin/ mg protein•hr $\pm$ 1.39 S.E.; n=3). Since *hmt2*$^-$ 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 *hmt2*$^-$ 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 Cd^{2+}, Hg^{2+}, Cu^{2+}, and Zn^{2+} in *hmt2*$^{-}$ cells. The spectrum of metal sensitivity suggested the failure of a phytochelatin-mediated tolerance pathway, since Cd^{2+}, Hg^{2+}, Cu^{2+}, and Zn^{2+} 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 KPO_4 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)$_2$G, (γ-EC)$_3$G, and sulfide were used as standards for the identification of peaks; purified GSH was used for quantitation of peaks.

For reduction of thiols with $NaBH_4$, 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. $NaBH_4$ 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 *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 μCi $Na_2{}^{35}SO_4$ 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 μM 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 μl 10mM $NaPO_4$, pH 7.0/ 1mM EDTA. A 10 μl 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 *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 μM $CdCl_2$. 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% $AgNO_3$, 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 $CdCl_2$ 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

This work was funded by Grant #55278 from the Department of Energy Environmental Management Science Program.

References

Akerboom, T.P.M. and Sies, H. (1981) Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. In *Methods in Enzymology, Vol. 77. Detoxication and Drug Metabolism: Conjugation and Related Systems.* Jakoby, W.B. (ed). San Diego: Academic Press, pp. 373-382.

Alfa, C., Fantes, P., Hyams, J., McLeod, M. and Warbrick, E. (1993) *Experiments with fission yeast: a laboratory course manual.* Plainview, N.Y.: Cold Spring Harbor Laboratory Press.

Clemens, S., Kim, E.J., Neumann, D., and Schroeder, J.I. (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *EMBO J.* **18:** 3325-3333.

Glaeser, H., Coblenz, A., Kruczek, R., Ruttke, I., Ebert-Jung, A. and Wolf, K. (1991) Glutathione metabolism and heavy metal detoxification in *Schizosaccharomyces pombe*. *Curr. Genet.* **19:** 207-213.

Grill, E., Winnacker, E.-L. and Zenk, M.H. (1986) Synthesis of seven different homologous phytochelatins in metal-exposed *Schizosaccharomyces pombe* cells. *FEBS Lett.* **197:** 115-120.

Grill, E., Winnacker, E.-L. and Zenk, M.H. (1987) Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. *Proc. Natl. Acad. Sci. U.S.A.* **84:** 439-443.

Ha, S.-B., Smith, A. P., Howden, R., Dietrich, W.M., Bugg, S., O'Connell, M.J., Goldsbrough, P.B., and Cobbett, C.S. (1999) Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe.* *Plant Cell* **11:** 1153-1163.

Howden, R. and Cobbett, C.S. (1992) Cadmium-sensitive mutants of *Arabidopsis thaliana*. *Plant Physiol.* **100:** 100-107.

Howden, R., Andersen, C.R., Goldsbrough, P.B. and Cobbett, C.S. (1995a) A cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*. *Plant Physiol.* **107:** 1067-1073.

Howden, R., Goldsbrough, P.B., Andersen, C.R. and Cobbett, C.S. (1995b) Cadmium-sensitive, *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. *Plant Physiol.* **107:** 1059-1066.

Juang, R.-H., McCue, K.F. and Ow, D.W. (1993) Two purine biosynthetic enzymes that are required for cadmium tolerance in *Schizosaccharomyces pombe* utilize cysteine sulfinate *in vitro*. *Arch. Biochem. Biophys.* **304:** 392-401.

Kondo, N., Imai, K., Isobe, M., Goto, T., Murasugi, A., Wada-Nakagawa, C. and Hayashi, Y. (1984) Cadystin A and B, major unit peptides comprising cadmium binding peptides induced in a fission yeast-- separation, revision of structures and synthesis. *Tetrahedron Lett.* **25:** 3869-3872.

Maitani, T., Kubota, H., Sato, K. and Yamada, T. (1996) The composition of metals bound to class III metallothionein (phytochelatin and its desglycyl peptide) induced by various metals in root cultures of *Rubia tinctorum*. *Plant Physiol.* **110:** 1145-1150.

McLeod, M., Stein, M. and Beach, D.H. (1987) The product of the *mei3*$^{+}$ gene, expressed under control of the mating-type locus, induces meiosis and sporulation in fission yeast. *EMBO J.* **6:** 729-736.

Mehra, R.K., Mulchandani, P. and Hunter, T.C. (1994) Role of CdS quantum crystallites in cadmium resistance in *Candida glabrata*. *Biochem. Biophys. Res. Commun.* **200:** 1193-1200.

Mehra, R.K., Miclat, J., Kodati, V.R., Abdullah, R., Hunter, T.C. and Mulchandi, P. (1996a) Optical spectroscopic and reverse-phase HPLC analysis of Hg(II) binding to phytochelatins. *Biochem. J.* **314:** 73-82.

Mehra, R.K., Tran, K., Scott, G.W., Mulchandani, P. and Saini, S.S. (1996b) Ag(I)-binding to phytochelatins. *J. Inorg. Biochem.* **61:** 125-142.

Meuwly, P., Thibault, P., Schwan, A.L. and Rauser, W.E. (1995) Three families of thiol peptides are induced by cadmium in maize. *Plant J.* **7:** 391-400.

Murasugi, A., Wada, C. and Hayashi, Y. (1981) Cadmium-binding peptide in fission yeast, *Schizosaccharomyces pombe*. *J. Biochem.* **90:** 1561-1564.

Murasugi, A., Wada, C. and Hayashi, Y. (1983) Occurence of acid-labile sulfide in cadmium-binding peptide 1 from fission yeast. *J. Biochem.* **93:** 661-664.

Mutoh, N. and Hayashi, Y. (1988) Isolation of mutants of *Schizosaccharomyces pombe* unable to synthesize cadystin, small Cd-binding peptides. *Biochem. Biophys. Res. Commun.* **151:** 32-39.

Ortiz, D.F., Kreppel, L., Speiser, D.M., Scheel, G., McDonald, G. and Ow, D.W. (1992) Heavy metal tolerance in fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. *EMBO J.* **11:** 3491-3499.

Ortiz, D.F., Ruscitti, T., McCue, K.F. and Ow, D.W. (1995) Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. *J. Biol. Chem.* **270:** 4721-4728.

Pearse, A.G.E. (1972) *Histochemistry: Theoretical and Applied.* London: Longman Group Limited.

Perego, P., Vande Weghe, J., Ow, D.W. and Howell, S.B. (1997) Role of determinants of cadmium sensitivity in the tolerance of *Schizosaccharomyces pombe* to cisplatin. *Mol. Pharmacol.* **51:** 12-18.

Rauser, W.E. (1995) Phytochelatins and related peptides: structure, biosynthesis, and function. *Plant Physiol.* **109:** 1141-1149.

Reese, R.N. and Winge, D.R. (1988) Sulfide stabilization of the cadmium-.gamma.-glutamyl peptide complex of *Schizosaccharomyces pombe*. *J. Biol. Chem.* **263:** 12832-12835.

Speiser, D.M., Ortiz, D.F., Kreppel, L., Scheel, G., McDonald, G. and Ow, D.W. (1992) Purine biosynthetic genes are required for cadmium tolerance in *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **12:** 5301-5310.

Vande Weghe, J. and Ow, D.W. (1999) A fission yeast gene for mitochondrial sulfide oxidation. *J. Biol. Chem.* **274:** 13250-13257.

Vatamaniuk, O.K., Mari, S., Lu, Y.-P., and Rea, P.A. (1999) AtPCS1, a phytochelatin synthase from *Arabidopsis*: isolation and *in vitro* reconstitution. *Proc. Natl. Acad. Sci. U.S.A.* **96:** 7110-7115.

Vogeli-Lange, R. and Wagner, G.J. (1990) Subcellular localization of cadmium and cadmium-binding peptides in tobacco leaves. Implication of a transport function for cadmium-binding peptides. *Plant Physiol.* **92:** 1086-1093.

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

Wild-type and *hmt2*$^{-}$ 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.

Strain	Trial	mM Cd	mM S^{2-}	Cd:S ratio
JS23	I	3.1	1.3	2.38
JS563	I	1.8	2.0	0.9
JS23	II	2.7	1.6	1.69
JS563	II	3.3	5.7	0.58

Figure legends

Figure 1. Gel filtration analysis of cadmium-binding complexes.

A. JS23 (wild type), JS563 (*hmt2*$^{-}$ 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*$^{+}$ 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*$^{-}$ 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^{2-}, sulfide; PC_2, PC_3: $(\gamma\text{-EC})_2G$ and $(\gamma\text{-EC})_3G$ phytochelatins, respectively.

Figure 3. HPLC analysis of thiols after chemical reduction.

Extracts from wild-type JS23 and *hmt2*$^{-}$ JS563 cells were induced by cadmium and analyzed by HPLC with DTNB detection before (left) and after (right) $NaBH_4$ reduction.

Figure 4. Detection of radiolabelled thiols.

An extract from ^{35}S-labeled *hmt2*$^{-}$ 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 Na_2S and 0.5 mM (left) or 2 mM (right) $CdCl_2$. 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 (1μg/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.

Figure 1

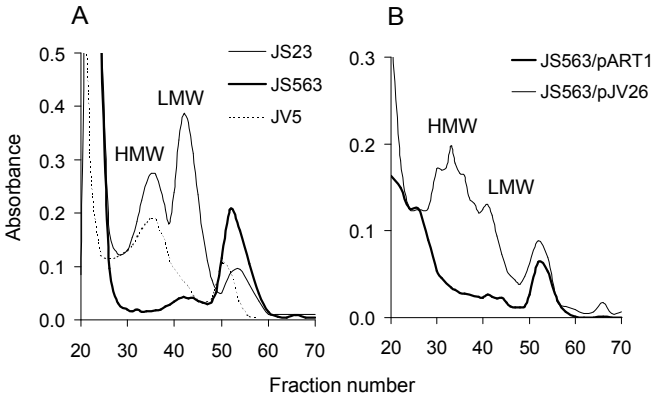

Figure 2

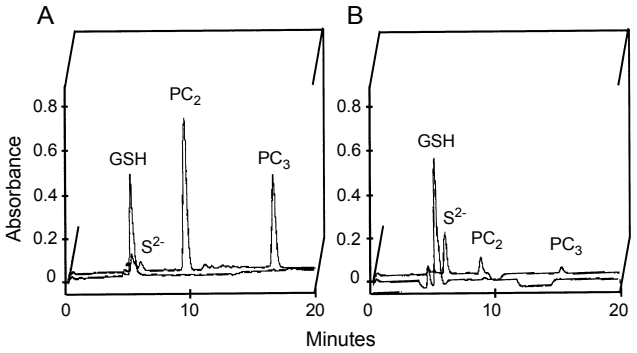

Figure 3

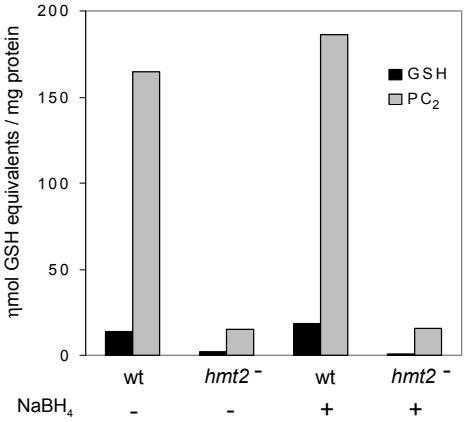

Figure 4

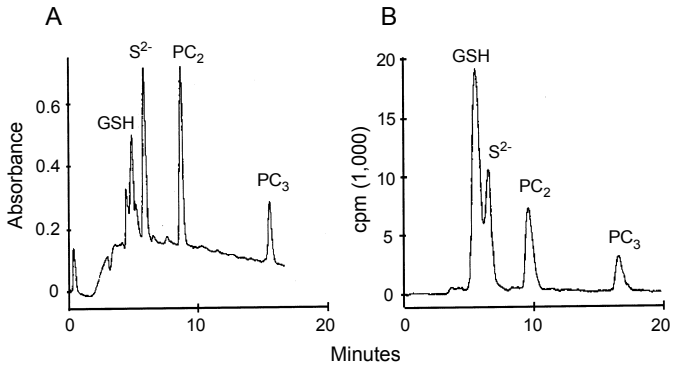

Figure 5

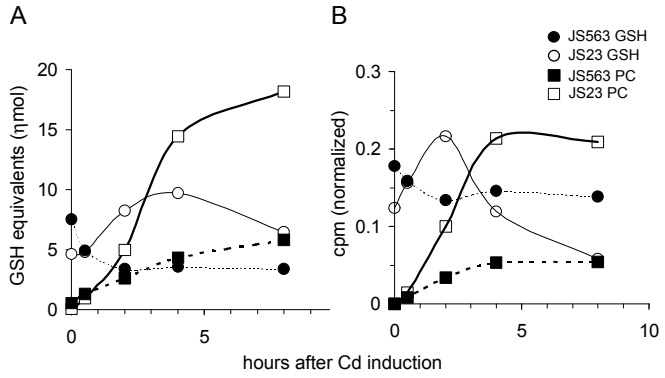

Figure 6

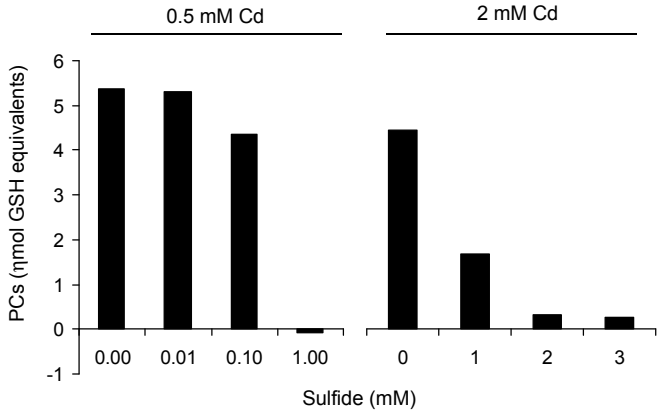

Figure 7