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# Identification of *SLF1* as a New Copper Homeostasis Gene Involved in Copper Sulfide Mineralization in *Saccharomyces cerevisiae*

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**In *Saccharomyces cerevisiae*, at least 12 genes are important for cells to propagate in medium containing elevated concentrations of copper salts (J. Welch, S. Fogel, C. Buchman, and M. Karin, EMBO J. 8:255–260, 1989). Complementation studies were carried out on a copper-sensitive mutation (*cup14*) from this group. A new yeast gene, designated *SLF1*, was identified as a multicopy suppressor of the *cup14* mutation. Slf1 is important for the physiological process of copper sulfide (CuS) mineralization on the surface of cells cultured in medium containing copper salts. CuS mineralization causes the cells to turn brown. Disruption of *SLF1*, which is located close to the telomere region of chromosome IV, leads to limited copper sensitivity, and the resulting cells lack the normal brownish coloration when grown in $CuSO_4$-containing medium. Overproduction of *Slf1* in wild-type cells confers superresistance to $CuSO_4$ and enhances the coloration of cells cultured in the presence of $CuSO_4$. Upon addition of KCN to Cu-grown cells, the brownish coloration was bleached instantly, and copper ions were solubilized. These data are consistent with Slf1-dependent accumulation of CuS complexes on the cell surface. Disruption of *SLF1* also results in loss of the ability of yeast cells to deplete Cu but not Cd ions from the growth medium, whereas overexpression enhances Cu depletion ability and the resulting deposition of CuS particles. It is proposed that Slf1 participates in a copper homeostasis pathway, distinct from the Cup1 detoxification system, that leads to sulfide generation and CuS biomineralization on the cell surface. This process may coordinate with the Cup1 pathway at different copper concentrations to prevent copper-induced toxicity.**

Copper is an essential element for normal cell physiology. Although it is an essential nutrient, excess copper in the environment can lead to toxicosis (43). Cells have evolved mechanisms to balance intracellular copper levels between nutritious and toxic.

In *Saccharomyces cerevisiae*, one major discriminating factor between copper-sensitive and copper-resistant cells is the protein metallothionein (MT) encoded by the *CUP1* locus (4, 11, 22). Targeted disruption of the *CUP1* gene leads to a copper-hypersensitive phenotype (15, 16). Many laboratory strains of *S. cerevisiae* contain a tandemly amplified *CUP1* locus, and copper resistance correlates directly with the MT gene copy number (49). MT confers copper resistance in yeast cells by sequestering intracellular Cu(I) ions within a single heptacopper proteinaceous cluster (34).

In addition to the correlation of copper resistance and *CUP1* copy number, the expression of *CUP1* is copper regulated. *cis*-acting DNA promoter sequences upstream of the *CUP1* coding region are critical for Cu-induced expression of *CUP1* (12, 45). The *trans*-acting factor that mediates Cu-induced expression of *CUP1* is Ace1 (also designated Cup2) (12, 44, 48). Ace1 is converted to an active transcription factor upon Cu(I) binding (12). Activated Ace1 contains a polycopper cluster which stabilizes a conformation capable of high-affinity binding to *CUP1* DNA promoter sequences (6). The DNA-binding domain of Ace1 is interdigitated within the Cu-binding domain (19).

A careful genetic analysis of copper-sensitive mutations in *S. cerevisiae* by Welch et al. identified at least 12 separate complementation groups (48). *CUP1* and *ACE1* appear to be the dominant genes in copper resistance, as targeted disruption of either gene yields a copper-hypersensitive phenotype. Mutations in other copper resistance genes, which may not be null alleles, confer only limited copper sensitivity (48).

The number of yeast genes identified as important in copper homeostasis is limited. Only one of the original *CUP* mutations besides *CUP1* and *ACE1* has been identified. The *cup5* mutation was shown to be a mutation in the proteolipid subunit, also known as Vma3, of the vacuolar $H^+$-ATPase (9). Mutations in *VMA3* are known to diminish the activity of the ATPase proton pump and result in a phenotype of sensitivity to multiple metal ions, including copper (35). The actual role of yeast vacuoles in copper ion homeostasis remains unclear.

A homeodomain yeast protein, arbitrarily designated Cup9, was shown to confer copper resistance to cells grown on nonfermentable carbon sources. The effect of Cup9 is clearly independent of the effects of Cup1 and Ace1 (24). There is no indication whether copper resistance imparted by Cup9 occurs through altered copper transport, intracellular copper sequestration in a non-MT molecule, compartmentalization within an organelle, or efflux.

A series of genes have been identified as important for copper transport across the plasma membrane. *CTR1* was shown to encode the high-affinity plasma membrane copper transporter (8). Disruption of *CTR1* leads to an aerobic growth defect in medium containing low levels of $CuSO_4$, arising from limiting amounts of active Cu,Zn-superoxide dismutase (7). *CTR1* gene disruption results in hyperresistance to copper salts, whereas overexpression of *CTR1* confers copper sensitivity (7). *CTR1* is only expressed when copper levels are below 10 μM (7). The downregulation of *CTR1* appears to restrict copper uptake by the Ctr1 transporter to Cu concentrations of less than 10 μM. However, copper transport persists at high copper

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concentrations through a putative low-affinity transporter(s). Such a transporter(s) must exist, as *ctr1* cells gain only limited copper resistance (7). Another gene product known to be important in copper uptake in *S. cerevisiae* is Mac1, which regulates expression of the *CTR1* copper transporter and one of the metal reductases, Fre1 (21).

Copper uptake studies in *S. cerevisiae* are complicated by the known precipitation of Cu ions on membranes as copper sulfide (CuS) mineral lattices (13, 23, 33). Sulfide ions produced from sulfate reduction can readily form insoluble copper complexes, which accumulate on the cell surface (2). Although $(CuS)_x$ formation in *S. cerevisiae* has been documented for a long time, the biological basis and the relevant function in copper homeostasis of this process are obscure. Here we describe the identification and characterization of a new yeast gene, designated *SLF1*, that functions in CuS mineralization. This is the first gene identified that controls copper biomineralization.

## MATERIALS AND METHODS

**Yeast culture conditions.** Yeast minimal medium contained 0.67% yeast nitrogen base without amino acids (Difco Laboratories) and 2% glucose. Bactoagar (Difco) is added at 1.5% for solid medium. For certain experiments, nitrogen base lacking ammonium sulfate was used. Ammonium chloride was added at a concentration similar to that of the usual ammonium sulfate. Synthetic complete medium (SC) is minimal medium supplemented with various amino acids and bases as described before (41), as is YPD medium. Sporulation and presporulation media were prepared as described before (41). To determine the metal resistance of yeast strains, drops (13 μl) of cells suspended in water were plated on SC plates containing various concentrations of metals. Metal resistance was determined as the minimum metal concentration which blocked growth after 48 h at 30°C. The concentration of metal ions was analyzed by atomic absorption spectroscopy with a Perkin Elmer 305A spectrometer.

*Escherichia coli* DH5αF′ was used for all DNA manipulations and was grown under standard conditions.

**Yeast vacuolar staining.** Vacuolar staining with fluorescein 5-isothiocyanate (FITC; Molecular Probes Inc.) and quinacrine was carried out as described previously (38).

**Plasmid constructions.** A high-copy genomic library (generously provided by Huaming Wang) of *Sau*3A partial fragments cloned into the *Bam*HI site of plasmid pTF63 (29) was transformed into the *cup14* mutant. The *CUP14*-complementing plasmid pYU400 contains a 9.6-kb insert including the entire *SLF1* locus. pYU401 was generated by inserting the *Pst*I-*Cla*I fragment of *SLF1* into pBluescript SK$^+$ (Stratagene). To create pYU404, pYU401 was digested with *Nco*I filled in with Klenow fragments, and religated. *Pst*I-*Cla*I fragments from pYU401 and pYU404 were then ligated with pTF63 cut with the same enzymes to create pYU405 and pYU406, respectively. As a result, pYU405 contained a wild-type version of *SLF1* on a yeast high-copy vector, whereas pYU406 carried a frameshifted version of *SLF1* on the same vector. The frameshift occurred after codon 38 of *SLF1* by a 4-bp insertion (5′-TGCA-3′), resulting in an early termination at the eighth codon downstream of the insertion site.

pDisruptIII was generated to disrupt *SLF1* in the X2180-1B background with a *hisG-URA3-hisG* cassette (1). An internal *Eco*RI-*Bgl*II fragment of *SLF1* was excised and replaced with an *Eco*RI-*Bam*HI fragment containing a *hisG-URA3-hisG* cassette. An *Xho*I-*Xba*I fragment from pDisruptIII was used to transform X2180-1B-U1 to create a strain carrying a replacement of the *SLF1* gene segment from −4 to +1237 (relative to the ATG start codon) with the *hisG-URA3-hisG* cassette.

To overexpress *SLF1*, pYU421 was constructed, based on the vector pVT102-U (47). An in-frame fusion of the *SLF1* and *HA* (the 12CA5 epitope in the hemagglutinin [HA] protein of human influenza virus) genes was expressed under the control of the *ADH1* (encoding alcohol dehydrogenase) promoter.

**Construction of yeast strains.** Cu-sensitive mutants were obtained from J. Welch and S. Fogel. These were the original isolates from a genetic screen for copper-sensitive mutants by ethyl methanesulfonate mutagenesis (48). The mutants lacked any auxotrophic markers. Ura$^-$ versions of the mutants were generated by plating them on SC plates containing 5-fluoroorotic acid (5-FOA) prepared as described before (42). Colonies from 5-FOA plate (Ura$^-$) were tested for the Ura reversion rate. The most stable Ura$^-$ clones were then transformed with YEp352 (*URA3*) to test their ability to be complemented by an episomally expressed *URA3* gene. Yeast strain X2180-1B (*MAT***a** *SUC2 mal mel gal2 CUP1*$^r$), the wild-type strain congenic to all the Cu-sensitive mutants, was obtained from the Yeast Genetic Stock Center. A *ura3* variant of X2180-1B was prepared and designated X2180-1B-U1. The *SLF1* locus in X2180-1B-U1 was disrupted, and the new strain was designated WY194. The disruption was confirmed by Southern analysis.

**DNA manipulation and sequencing.** Endonuclease restriction enzymes and the Klenow fragment of DNA polymerase were purchased from New England Biolabs. Sequencing reactions were carried out with the Sequenase II dideoxy chain termination kit (U.S. Biochemicals).

**Southern, Northern (RNA blot), and immunochemical analyses.** Yeast total genomic DNA was isolated by the method described before (36). Transfer and hybridization procedures were adapted as described before (39).

To prepare high-quality yeast total RNA, a method developed by Schmitt et al. (40) was used. Total RNA was isolated from 10 ml of mid-log-phase yeast cultures grown in YPD in the absence or presence of $CuSO_4$. For Cu induction, the incubation time was 45 min. For Northern blot analysis, 30 μg of total RNA was subjected to electrophoresis in a 1% formaldehyde–agarose gel and capillary blotted to Nytran membranes (Schleicher & Schuell). Hybridization probes were made by the random priming method with the Random-Prime-It kit (Stratagene).

Amersham's ECL Western immunoblot kit was used for Western analysis with monoclonal antibody (Boehringer Mannheim) against the 12CA5 epitope (50) in the HA protein of human influenza virus. The indirect immunocytochemistry of permeabilized yeast cells was performed as described before (37) with 12CA5 monoclonal antibody.

**Sequencing strategy for the *SLF1* gene.** After subcloning, the complementation ability of the insert was found to reside within the *Pst*I-*Hpa*I fragment of 1.74 kb. Three *Eco*RI fragments, designated E1, E2, and E3 (see Fig. 2), were generated from this insert (E2 has a 0.6-kb overlap with the 1.74-kb insert containing the open reading frame [ORF]) and cloned into pBluescript (Stratagene). The entire ORF for *SLF1* was sequenced from both strands.

**Chromosomal mapping of the *SLF1* gene.** *SLF1* was mapped on an American Type Culture Collection nylon membrane containing DNA from more than 1,000 overlapping recombinant lambda clones with inserts from the genome of *S. cerevisiae* AB972.

**Metal analysis.** To quantify the cell-associated copper levels, cells from a set culture volume were washed with saline and digested with a 5:2 mixture of nitric and perchloric acids prior to analysis on a Perkin-Elmer 305 atomic absorption spectrometer. Cells from a corresponding culture volume were washed similarly and subsequently incubated with 10 mM KCN for 2 min. The copper concentration of the supernatant was analyzed.

To quantify the depletion of metal ions from the culture medium, overnight cultures were diluted to an optical density at 600 nm ($OD_{600}$) of 0.1 in an appropriate medium supplemented with a specified concentration of metal salts. The resulting 20-ml cultures were incubated at 30°C with aeration for various times before being centrifuged to form a cell pellet. The amount of metal ions left in the medium was then measured with an atomic absorption spectrometer. The ability of cells to deplete metal ions is inversely proportional to the fraction of the amount of metal ions left in the medium normalized to the amount in medium lacking cells.

**Nucleotide sequence accession number.** The *SLF1* gene sequence was deposited in GenBank, with accession number U30375.

## RESULTS

**Characterization of *CUP* mutants.** From an extensive genetic screen, Cu-sensitive mutants were isolated by ethyl methanesulfonate mutagenesis from the wild-type yeast strain X2180-1A (48). Twelve complementation groups were identified, but only five were mapped (48). Mutations in only three of these genes (*CUP1*, *CUP2*, and *CUP5*) have been characterized to date.

Two other Cu$^s$ mutations, *cup3* and *cup14*, were observed to cause hypersensitivity to copper salts (Fig. 1). Neither of the resulting mutants were sensitive to cadmium salts, and both mutants exhibited wild-type vacuole morphology and vacuolar ATPase activity, as determined by staining with FITC and quinacrine, respectively (data not shown). Vacuolar staining was carried out, as defective vacuoles are known to exhibit metal ion sensitivities (9). Three mutations, *cup5*, *cup10*, and *cup12*, were found to cause sensitivity to both copper and cadmium salts, and *cup5* and *cup10* resulted in fragmented vacuoles indicative of defective organelles. The defective vacuoles may explain the combined Cu and Cd sensitivity (data not shown).

The *cup3* and *cup14* mutants exhibited the most marked phenotype of Cu sensitivity and displayed wild-type vacuole morphology compared with X2180-1A. Of these two mutants, the *cup14* strain was taken for complementation studies.

**Isolation of the *SLF1* gene.** Initial efforts to complement *cup14* by using a yeast DNA library in a centromere-based

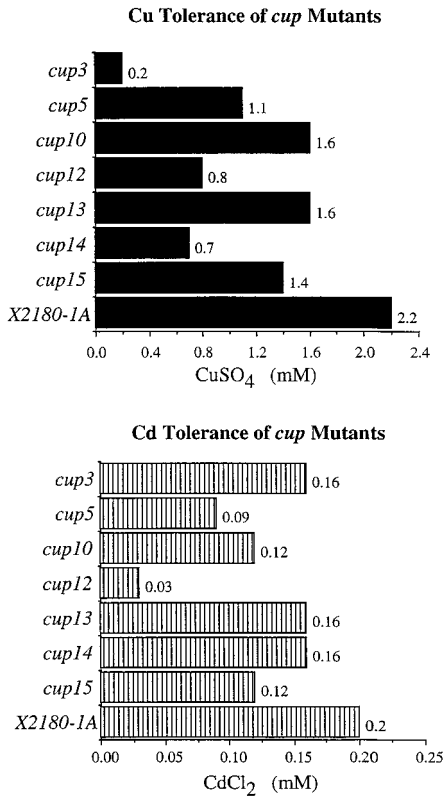


FIG. 1. Copper and cadmium tolerance levels of *CUP* mutants. Cell resuspensions in sterile water (13 μl) were spotted onto SC plates containing the specified concentrations of $CuSO_4$ or $CdCl_2$. The plates were incubated at 30°C for 48 h before results were recorded. X2180-1A is the parental strain from which the *CUP* mutants were derived. The bars show the concentration of $CuSO_4$ or $CdCl_2$ at which growth is inhibited by at least 90% compared with the control plate lacking added metal salts.

vector yielded only clones containing the *CUP1* locus. Overexpression of *CUP1* by virtue of multiple copies and perhaps an increase in plasmid copy number due to selection effectively suppresses the copper sensitivity caused by *cup14*. Subsequently, a yeast genomic library on high-copy plasmid pTF63 was transformed into the *cup14* mutant, and nearly 12,000 transformants were screened. These colonies were replica plated onto SC-uracil plates containing 1.5 mM $CuSO_4$. Copper-resistant colonies were identified, and plasmids from these clones were transformed back into the original *cup14* mutant. The copper resistance of 28 clones was found to be plasmid dependent. Restriction mapping of plasmid DNA from the 28 clones revealed that 27 contained *CUP1*. One clone had an insert of 9.6 kb that was distinct from *CUP1*. Although the plasmid complemented the copper sensitivity defect of the *cup14* mutant, it did not enhance the cadmium resistance of the cell, indicating that complementation was copper specific.

A series of deletion constructs were prepared to delineate the segment of the 9.6-kb fragment responsible for conferring Cu resistance to the *cup14* mutant (Fig. 2). Full complementation activity resided within a 1.74-kb fragment.

**Characterization of *SLF1*.** The DNA sequence of the 1.74-kb complementing DNA was determined and verified by the Stanford yeast genome sequencing project (Fig. 3A). Analysis of the DNA sequence indicated a single extended ORF. To confirm that the single ORF was responsible for conferring Cu resistance to the *cup14* mutant, a frameshift mutation was made by insertion of four nucleotides (5′-TGCA-3′) into the *Nco*I site located at the 38th codon after the putative ATG. The insertion results in an early termination of translation in the putative ORF at the eighth codon downstream of the insertion site (specified by the arrow in Fig. 3A). The pTF63 vector, carrying the frameshifted version of the gene, failed to complement the Cu-sensitive phenotype of the *cup14* mutant. In fact, the Cu resistance level of *cup14* transformants carrying the frameshifted gene was nearly identical to that of transformants containing the pTF63 vector alone (Fig. 4). Thus, the identified ORF is responsible for the complementation of *cup14*.

Examination of the ORF indicates that it has the potential to encode a polypeptide of 447 amino acid residues with a predicted molecular mass of 50,911 Da. The first potential translation initiation ATG triplet is a perfect match with the Kozak consensus in yeast cells, with the sequence (A/C)A(A/C)A**AT G**TC(C/T) (18). Northern analysis revealed an mRNA of nearly 1,500 nucleotides, consistent with a protein of 447 residues. The quantity of the mRNA of the ORF was not altered by the addition of $CuSO_4$ to the medium, suggesting that its transcription was not copper regulated (Fig. 5A).

A fusion gene was constructed in which the putative ORF was fused to DNA sequences encoding a C-terminal epitope tag (nine residues) of the HA protein of the human influenza virus. Slf1/HA was expressed under the control of the *ADH1* promoter. The fusion gene product retained activity in complementation of *cup14*. Expression of the fusion gene in wild-type yeast cells followed by Western analysis with monoclonal antibodies against the HA tag revealed the presence of a protein of the predicted size (54 kDa) for an ORF-HA fusion protein. The presence of the 54-kDa species was dependent on the *SLF1-HA* plasmid (Fig. 5B).

A computer search of the GenBank and EMBL databases did not identify any known protein. Thus, the ORF encodes a novel protein in *S. cerevisiae*. No significant sequence similarity to known proteins was found, and no motif-like sequences were uncovered. The polypeptide is rich in Asn and Gln residues. Glutamine-rich sequences have been implicated in many transcription factors as an important feature for transactivation domains (31). A limited segregation of basic and acidic residues occurs in the molecule (Fig. 3B). If the molecule is a transcription factor, a candidate nuclear localization sequence exists between residues 109 and 114 as a stretch of basic residues (Fig. 3A). Although these features suggest a nuclear func-

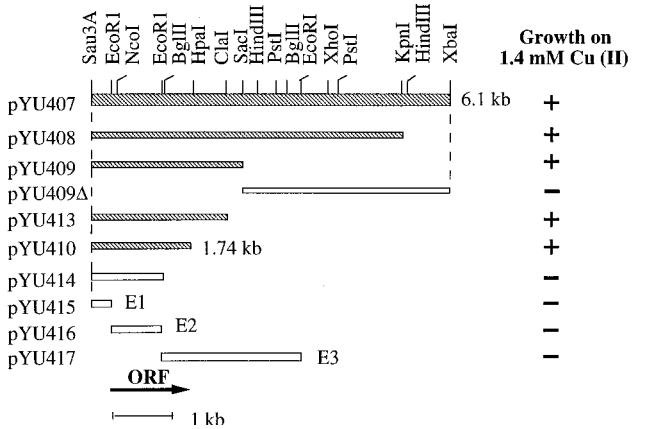


FIG. 2. Subcloning of *SLF1* and restriction map of the *SLF1* locus. Hatched bars indicate DNA fragments that complement the mutation.

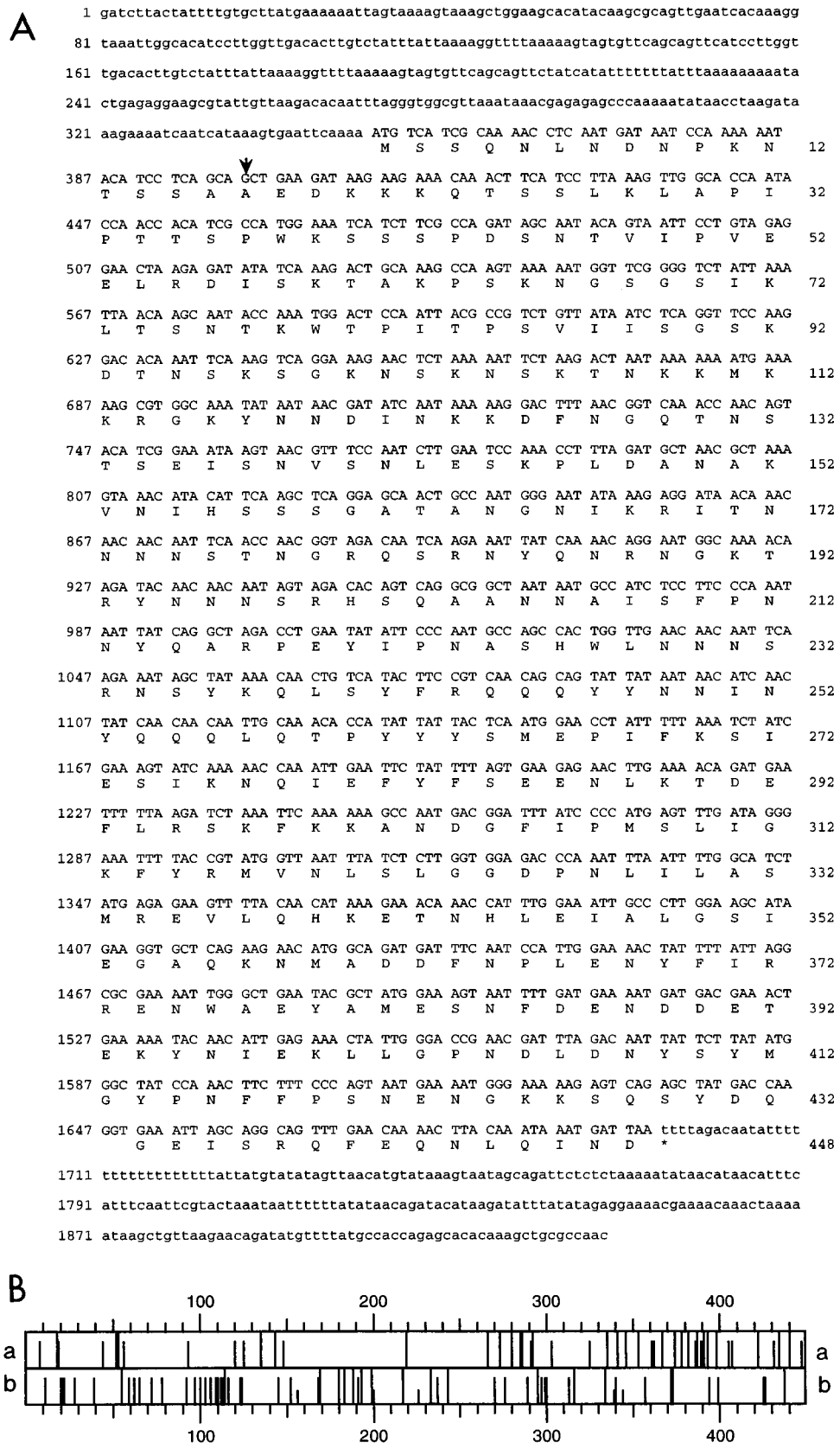


FIG. 3. (A) Nucleotide sequence of *SLF1* and deduced amino acid sequence of Slf1. The arrow marks the position of the frameshift mutation. (B) Distribution of acidic (a) and basic (b) residues of Slf1.

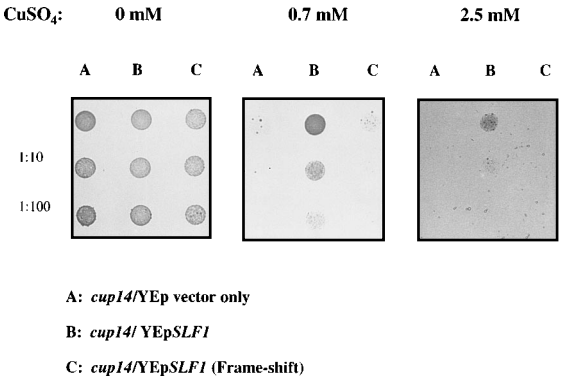


FIG. 4. Complementation of *cup14* by *SLF1* present on a high-copy yeast plasmid. Cell resuspensions in sterile water were serially diluted and spotted onto SC medium containing the specified concentrations of $CuSO_4$. The plates were incubated at 30°C for 48 h before results were recorded.

tion, immunocytochemistry failed to confirm a nuclear localization. Immunofluorescence experiments with antibodies to the epitope-tagged molecule revealed diffuse staining throughout the cell (data not shown). Diffuse staining does not necessarily negate a nuclear function, as overexpression of fusion proteins can result in mislocalization (37). The ORF was designated *SLF1* for reasons outlined below.

**Disruption of *SLF1*.** A strain with an *SLF1* gene disruption was constructed by replacing the *Eco*RI-*Bgl*II fragment with a *hisG-URA3-hisG* cassette, deleting the region including the first 297 codons of the *SLF1* ORF. Genomic DNA prepared from the wild-type and the *slf1* disruption strains was digested with *Eco*RI and analyzed by Southern hybridization. A probe consisting of the 0.85-kb *Eco*RI (E2 in Fig. 2) fragment internal to the ORF hybridized to an *Eco*RI fragment of the predicted size (0.85 kb) from genomic DNA of X2180-1B-U1, whereas the fragment was absent, as expected, in DNA prepared from the candidate disruption strain. With 3′-flanking sequences (E3) as a probe, the wild-type strain contained a predicted *Eco*RI fragment of 2.4 kb, whereas the disruption strain generated a fragment of 5.6 kb, exactly the sum of the *hisG-URA3-hisG* cassette and the size of E3 (3.2 plus 2.4 kb). Finally, the disruption was shown to be successful by the absence of any *SLF1* mRNA in Northern analysis of the disruption strain (Fig. 5).

Analysis of the *slf1* disruption strain, WY194, for copper resistance revealed only minimal copper sensitivity. X2180-1B but not WY194 was able to grow on medium containing 2.4 mM $CuSO_4$ (Fig. 6A). Both strains grew in medium containing 1 mM $CuSO_4$. The minimal Cu sensitivity of the *slf1* disruption strain was in obvious contrast to that of the *cup14* mutant, as *cup14* cells fail to grow in medium containing 0.7 mM $CuSO_4$.

Whereas disruption of *SLF1* in a wild-type background yielded limited Cu sensitivity, overexpression of *SLF1* from the aforementioned *ADH1-SLF1* construct present on a high-copy yeast vector conferred hyperresistance to copper salts on the wild-type cells (Fig. 6B) but had no effect on cadmium resistance (data not shown). Furthermore, high-copy expression of *SLF1* conferred limited Cu resistance to *cup1* cells compared with the high-copy *slf1* with the frameshift mutation (Fig. 6C), indicating that *SLF1* can partially suppress the copper-sensitive phenotype of *cup1* cells.

**Evidence that *SLF1* encodes a multicopy suppressor of the *cup14* mutation.** The disruption of the cloned gene produced a copper-hypersensitive phenotype quantitatively different from that of the original *cup14* mutant, which suggests that *SLF1* functions as a suppressor. Further evidence for a suppressor function of *SLF1* was that the *SLF1* locus failed to complement the *cup14* mutation when cloned into a centromere-based plasmid. To confirm that *SLF1* was a multicopy suppressor, we mapped *SLF1*. By using membranes prepared from overlapping recombinant DNA containing inserts from the *S. cerevisiae* genome, a single *S. cerevisiae* clone was shown to be positive for *SLF1*, which corresponded to the end of the right arm of chromosome IV, between *GDR1* and the telomere. Interestingly, the original *cup14* mutation was also mapped by Welch et al. to the same chromosome in proximity to *SLF1* (48). Comparison of the physical and genetic maps suggested that the *cup14* mutation is about 30 kb closer to the telomere than the cloned gene. Chromosomal mapping did not, therefore, produce conclusive proof that *SLF1* was a suppressor.

Genetic linkage analysis was used to determine whether *SLF1* and *CUP14* are allelic. Strain WY194 containing the disrupted *SLF1* locus was crossed with the *cup14* mutant, and the resulting diploid strain was sporulated for tetrad analysis. Of 43 complete sets of tetrads analyzed, 8 sets were parental ditype, 31 sets were tetratype, and the other 4 sets were nonparental ditype, clearly demonstrating that these two genes were at least 45 centimorgans (about 140 kb) apart on chromosome IV. Thus, the original mapping of *CUP14* was incorrect. The other markers in the 43 tetrads segregated in a 2:2 manner (data not shown).

The distinct phenotype of the *slf1* disruption strain, the linkage analysis, and the lack of complementation by single-copy plasmids all indicate that *SLF1* is a multicopy suppressor of the *cup14* mutation.

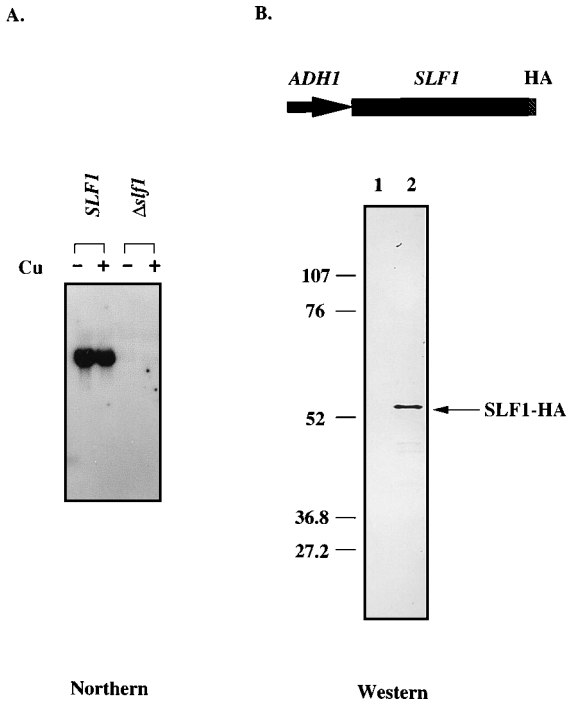


FIG. 5. Northern blot analysis of *SLF1*. (A) A DNA fragment internal to the *SLF1* ORF (IE fragment) was used as a probe for Northern analysis of wild-type and Δ*slf1* cells. (B) Western blot analysis of the Slf1-HA fusion protein. Monoclonal antibodies against the HA tag (HA peptide) were used in Western blot analysis of cells expressing the *SFL1-HA* fusion gene. The positions of protein molecular size standards are shown (in kilodaltons). Lane 1, wild-type cells with no plasmid; lane 2, wild-type cells transformed with the *ADH1-SLF1-HA* fusion plasmid.

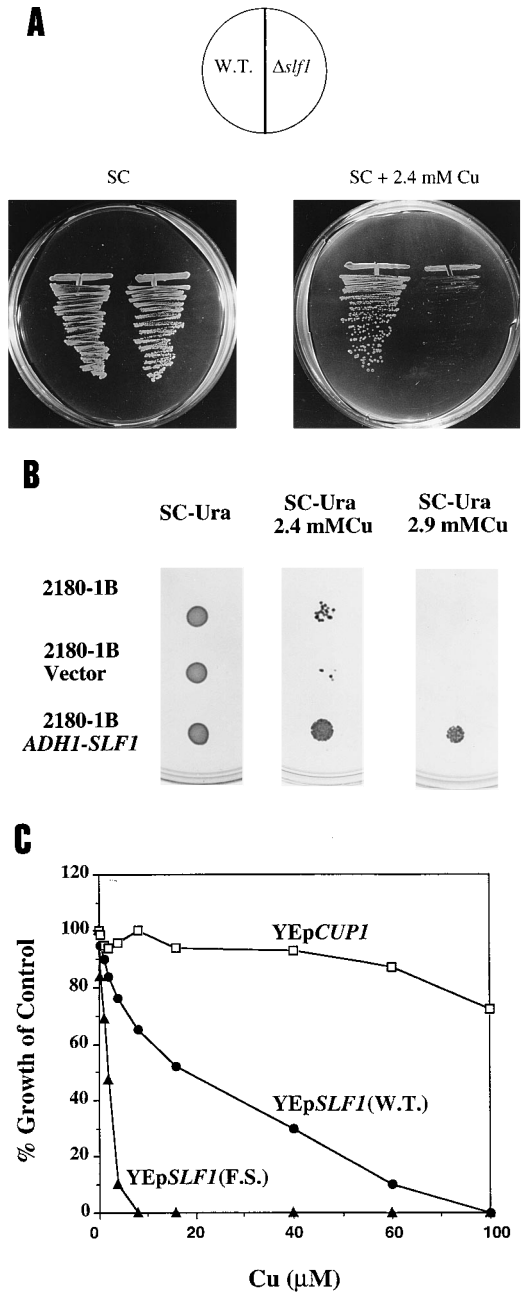


FIG. 6. Copper tolerance of *SLF1* and Δ*slf1* strains. (A) Cu sensitivity of the *SLF1*-disrupted strain. Wild-type (W.T.) and *SLF1*-disrupted (Δ*slf1*) cells were streaked onto SC plates with and without 2.4 mM $CuSO_4$. (B and C) Copper tolerance of wild-type and *cup1* cells. The *ADH1-SLF1-HA* construct was transformed into wild-type (B) and *cup1* (C) cells. For wild-type transformants, cell resuspensions in sterile water (13 μl) were spotted onto SC plates with and without $CuSO_4$. The plates were incubated at 30°C for 48 h before results were recorded. For *cup1* transformants, overnight cultures were diluted to an $OD_{600}$ of 0.02 with selective medium. The relative growth of cells at 24 h is expressed as a percentage of that of control cultures without metal ions.

**Mechanism of suppression by *SLF1*.** *SLF1* functions independently of the *CUP1* system. A Northern analysis of the X2180-1B, WY194 (Δ*slf1*), and *cup14* strains with *CUP1* as a probe revealed little difference in *CUP1* message levels in Cu-containing medium (Fig. 7), suggesting that Slf1 is not likely to be involved in Cu-induced *CUP1* expression via Ace1.

In the analyses of the *slf1* disruption strain, we observed that the colonies lacked any coloration when cultured on medium containing $CuSO_4$. Wild-type yeast cells normally exhibit a brownish color when grown on Cu-containing SC medium. In contrast, overexpression of *SLF1* from the *ADH1* promoter (plasmid pYU421) resulted in enhanced coloration of the transformants grown on medium containing $CuSO_4$ (Fig. 8). The differential coloration was also apparent in liquid cultures (data not shown). It is known that yeast cells typically display a brownish hue when cultured in the presence of copper salts because of the formation of copper sulfide $(CuS)_x$ mineral lattices adsorbed on the cell surface (2, 23, 33). Since the cloned gene was associated with CuS formation, this gene was designated *SLF1*, indicating a function in sulfide production. Slf1 appears to be a critical factor in the formation of $(CuS)_x$ particles.

To confirm that the brownish pigment arose from surface CuS crystallites, wild-type X2180-1B cells cultured in medium containing 0.5 mM $CuSO_4$ were harvested and resuspended in 1 ml of water prior to the addition of 10 μl of either 1 N HCl or 10 mM KCN. CuS crystallites are stable to dilute acid but are dissociated with KCN as a result of the formation of copper cyanide complexes (46). Cell pellets instantly lost the brownish color upon addition of KCN, whereas 1 N HCl had no effect. The effect of KCN on cell coloration was not due to a pH elevation, as 2 N NaOH enhanced coloration, consistent with accretion of the CuS particles. Cu-grown cells overexpressing *SLF1* were also bleached with KCN. In addition to bleaching coloration, KCN solubilized copper ions from saline-washed pellets of *SLF1* cells. The addition of 10 mM KCN to pellets of *SLF1* cells cultured overnight in medium containing 0.5 mM $CuSO_4$ resulted in solubilization of between 60 and 70% of the total cell-associated copper, whereas only 8% of cell-associated copper was solubilized with 10 mM KCN in *slf1* cells.

The source of the sulfide ions that precipitate copper ions is not fully known. Sulfate reduction is involved, in part, as the brown coloration of Cu-treated cells was attenuated but not abolished when ammonium chloride was substituted for ammonium sulfate in the growth medium. Cu-induced coloration in *SLF1* cells was likewise inhibited when extra methionine (1.2 mM final concentration) was added to the SC growth medium. The presence of excess methionine also greatly diminished the quantity of Cu ions solubilized from cells with 10 mM KCN. Excess methionine can elevate levels of intracellular *S*-adenosine methionine, which is a corepressor of genes involved in sulfate reduction. Methionine levels did not affect the coloration observed in medium containing ammonium chloride in

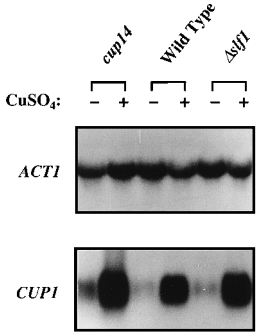


FIG. 7. Northern analysis of *CUP1* in wild-type cells, *cup14* mutant cells, and Δ*slf1* cells in the presence and absence of $CuSO_4$. An actin probe was used as a control.

FIG. 8. Coloration of wild-type (2180-1B) and Δ*slf1* yeast cells on medium containing 0.7 mM $CuSO_4$.

place of ammonium sulfate. Thus, an additional source of sulfide ions must exist for $(CuS)_x$ formation.

Metal sulfide mineralization also occurs in fungi and plants in intracellular complexes with phytochelatin isopeptides (5). Two enzymes in the adenine biosynthetic pathway are critical for generation of the sulfide ions important in cadmium sulfide particles coated with phytochelatin isopeptides (20). There is no indication that the sulfide ions important in CuS complex formation arise from this pathway, as *slf1* cells are not auxotrophic for adenine.

**Effect of *SLF1* on Cu depletion ability of yeast cells.** During normal growth, yeast cells accumulate copper ions from the medium. Accumulation of copper ions is the summation of Cu adsorbtion on the cell surface, Cu uptake through transporters, and intracellular Cu binding by MT and copper metalloenzymes. Thus, a steady depletion of copper from the medium occurs at low levels of added copper ions.

With the addition of 50 μM $CuSO_4$ to the culture medium, we readily detected a diminution in the Cu ion concentration, as quantified by atomic absorption spectroscopy, remaining in the conditioned medium after culturing X2180-1B cells for 30 h compared with the Cu ion concentration in medium lacking cells. Cells lacking *SLF1* (WY194) depleted only limited amounts of Cu ions relative to X2180-1B cells, whereas *SLF1* transformants containing the *ADH1-SLF1* construct (pYU421) depleted more Cu(II) from the medium than transformants containing the vector (pVT102-U) alone (Fig. 9). Interestingly, when these strains were cultured for a prolonged period (60 h), Δ*slf1* cultures retained 68% of the original Cu(II) in the growth medium. This process appeared to be specific for copper ions, since Slf1 had no effect on depletion of either Zn(II) or Cd(II) from the growth medium (Fig. 9). The question arose whether cells exposed to copper salts would also codeplete other metal ions. Such codepletion could not be observed in wild-type cells grown in medium containing $CuSO_4$ plus either $ZnSO_4$ or $CdSO_4$, as the presence of either 50 μM Zn(II) or 50 μM Cd(II) in cultures actually inhibited the normal depletion of Cu ions from the growth medium.

## DISCUSSION

Although *CUP1* and *ACE1* (*CUP2*) are two of the most important determinants in the copper-resistant phenotype in *S. cerevisiae*, additional genes were also implicated in yeast copper tolerance in an early genetic study (48). The recent identifications of the copper transporter Ctr1 (8), the homeodo-

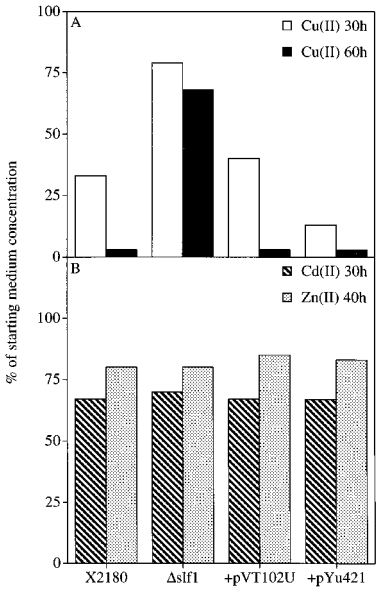


FIG. 9. Slf1-dependent depletion of copper ions from the growth medium. Overnight cultures of yeast strains were used to inoculate SC medium containing specified concentrations of metal ions to a final $OD_{600}$ of 0.02. Cultures were incubated at 30°C with aeration for the specified time before removal of cells by centrifugation. Metal ions left in the conditioned medium were analyzed with an atomic absorbtion spectrometer and expressed as a percentage of the metal concentration in the control medium that had not been inoculated with yeast cells.

main protein Cup9 (24), the copper accumulation protein Bsd2 (27), and the Ace1-like transcription factor Mac1 (21) suggest that maintenance of a balanced intracellular pool of copper ions in yeast cells is a complex process. In order to uncover new mechanisms through which yeast cells regulate the intracellular copper ion concentration, one effective strategy has been to isolate genes that, when mutated, confer copper sensitivity. The $Cu^s$ mutants (48), from which *CUP2* (*ACE1*) and *CUP5* were identified, provide unique opportunities for finding new genes in yeast copper homeostasis.

Using classical complementation cloning and yeast genetics, we have identified a new yeast gene, *SLF1*, as a multicopy suppressor of a *CUP14* defect. The amino acid sequence of Slf1 reveals limited information on its function. Several lines of evidence strongly implicate a role for Slf1 in yeast copper homeostasis: (i) overexpression of *SLF1* suppresses the Cu-sensitive phenotype of *cup14* and *cup1* cells; (ii) overexpression of *SLF1* confers hyperresistance to copper salts on wild-type cells; and (iii) the disruption of *SLF1* in a strain wild type for *CUP1* and *ACE1* leads to limited copper sensitivity. Although Slf1 apparently plays a role in metal ion homeostasis, it exhibits none of the classical metal-binding motifs previously described for *S. cerevisiae* Cu-binding proteins.

Slf1 appears to be a determining factor for $(CuS)_x$ biomineralization. Cells lacking a functional *SLF1* remain white on the SC plates containing $CuSO_4$. In contrast, wild-type cells exhibit a brownish hue on this medium. This coloration directly correlates with their ability to deplete copper ions from the growth medium. Wild-type cells that show a brown coloration in Cu-containing medium have a greater ability to deplete copper from the medium than Δ*slf1* cells, which develop no coloration in Cu-containing medium. Moreover, overproduction of Slf1 enhances both the brownish hue in cells grown in the presence of copper salts and depletion of copper from the medium. These results confirm the direct involvement of Slf1 in the physiology of coloration in Cu-treated yeast cells.

It has been long known that *S. cerevisiae* cells develop a brownish color in the presence of copper salts as a result of the formation of CuS mineral complexes on the cell surface (2, 23, 33). Electron microscopic studies established that the electron-dense particles present in Cu-treated yeast cells are located mainly inside the yeast cell wall (2). X-ray powder analysis confirmed that the electron-dense particles contain copper and sulfur (2). The sulfur is likely present as sulfide anions. The sulfide ions (hydrosulfide anion at physiological pH) arise only in part from reduction of sulfate, as cells growing in medium lacking ammonium sulfate retain limited capacity to form CuS complexes, as determined by their brownish hue.

In a systematic study on the fate of newly arrived radioactive $^{64}Cu$ in wild-type yeast cells, it was found that the cell association of $^{64}Cu$ could be divided into two distinguishable pools (26). The pool that is exchangeable with exogenous Cu(II) is believed to be the CuS complexes on the cell surface; the unexchangeable pool is believed to be the cytoplasmic copper pool (26). The exchangeable pool accounts for more than 70% of the Cu associated with yeast cells (26). Although the phenomenon of copper sulfide precipitation around the yeast cell surface has been observed for many years, the functional significance of this pathway was not understood until now.

Precipitation of copper ions as copper sulfide in bacteria was reported as one mechanism of copper detoxification (10). Copper-tolerant *Mycobacterium scrofulaceum* was shown to contain a plasmid that conferred resistance (10). The cells turned black when grown in the presence of copper salts. No dark coloration was observed in the absence of copper or in cells lacking the plasmid. The black color, characteristic of $(CuS)_x$, disappeared when the cells were treated with NaCN. Cells containing the plasmid concentrated nearly fivefold more copper than cells lacking the plasmid. Plasmid-bearing cells effectively depleted the medium of Cu(II) (10).

Sulfide-mediated biomineralization of metal ions is not confined to copper ions in bacteria. Deposition of Cd(II) ions into cadmium sulfide mineral particles occurs in certain strains of bacteria. X-ray analysis of extracellular cadmium sulfide crystallites in *Klebsiella aerogenes* revealed particles ranging in size from 20 to 200 nm, and the crystallites contained cadmium and sulfur in a 1:1 ratio (18).

CuS biomineralization in yeast cells is dependent on Slf1. This pathway is clearly of importance in copper detoxification, since disruption of *SLF1* results in limited Cu sensitivity. In addition, overexpression of *SLF1* confers limited resistance and superresistance to copper salts on *cup1* cells and wild-type cells, respectively. Overexpression or disruption of *SLF1* cells has no effect on tolerance of cells to cadmium salts.

There are indications that the Slf1-dependent sulfide generation pathway is copper dependent. No depletion of Zn(II) or Cd(II) ions occurs in the medium of cells overexpressing *SLF1*. The lack of involvement of Slf1 in cadmium tolerance is consistent with a previous study that demonstrated that sulfide ions are not involved in the acquired resistance of yeast cells to cadmium ions (30). Copper regulation of some component in the CuS deposition pathway may be advantageous to cells. In the absence of metalloregulation, the Slf1-dependent generation of hydrosulfide ions may result in constitutive secretion of hydrosulfide ions, which could precipitate multiple metal ions in the growth medium. A methionine-auxotrophic strain of *Candida glabrata* that we isolated previously showed unregulated sulfate reduction to sulfide ions, and these cells exhibited intense black coloration on medium containing copper salts and yellow coloration on medium containing cadmium salts (3). These cells were hyperresistant to both cadmium and copper salts. The absence of a cadmium sensitivity phenotype in cells with various *SFL1* expression levels suggests that *SLF1* cells do not generate hydrosulfide ions constitutively.

It is believed that the external Cu ions must reach a certain threshold level before precipitation of copper sulfide can occur on the cell surface (23). Slf1 may exert its effect only above a certain threshold concentration of intracellular Cu(I). A putative metalloregulation of CuS deposition may be one of several responses to high external copper levels. When the extracellular copper concentration exceeds 10 μM, expression of *CTR1* is repressed (7). Repression of *CTR1* expression leads to inhibition of the high-affinity transport pathway. Copper ions are still able to be taken up through one or more putative low-affinity transporters. The secretion of hydrosulfide ions and subsequent mineralization of CuS on the external cell surface may reduce the effective concentration of copper ions that reach the putative low-affinity transporter(s). Moreover, CuS mineralization on the plasma membrane may also alleviate the deleterious effects of Cu ions on the cellular membranes, which contributes to overall copper-induced cytotoxicity (28).

Similar copper detoxification mechanisms may also exist in animal species known to have copper-loaded livers which are black in appearance. Dominican toads and mute swans have been reported to have blackened livers in cases in which the hepatic copper concentration exceeded 1,000 μg/g (dry weight), a level that is detrimental in humans (14, 32). In both species, the excess copper is deposited in electron-dense granules, with no evidence of hepatocellular degeneration. The nature of the copper components in the granules is unknown, but the black coloration suggests the presence of CuS mineral lattices. Further characterization of the copper detoxification

pathway involving Slf1 will be needed to determine whether a similar pathway exists in higher eukaryotes.

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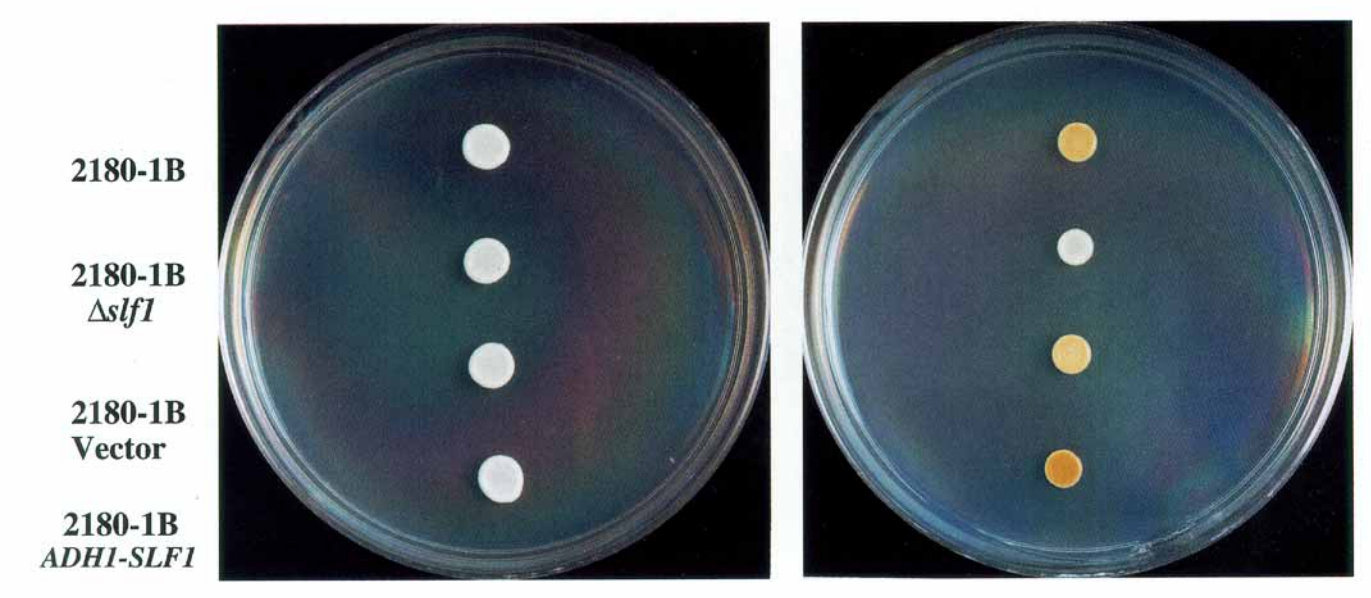
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