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The *Schizosaccharomyces pombe* Pccs Protein Functions in Both Copper Trafficking and Metal Detoxification Pathways*[S]

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Because copper is both an essential cofactor and a toxic metal, different strategies have evolved to appropriately regulate its homeostasis as a function of changing environmental copper levels. In this report, we describe a metallochaperone-like protein from *Schizosaccharomyces pombe* that maintains the delicate balance between essentiality and toxicity. This protein, designated Pccs, has four distinct domains. SOD activity assays reveal that the first three domains of Pccs are necessary and sufficient to deliver copper to its target, copper-zinc superoxide dismutase (SOD1). Pccs domain IV, which is absent in *Saccharomyces cerevisiae* CCS1, contains seventeen cysteine residues, eight pairs of which are in a potential metal coordination arrangement, Cys-Cys. We show that *S. cerevisiae ace1*Δ mutant cells expressing the full-length Pccs molecule are resistant to copper toxicity. Furthermore, we demonstrate that the Pccs domain IV enhances copper resistance of the *ace1*Δ cells by an order of magnitude compared with that observed in the same strain expressing a *pccs*$^{+}$*I-II-III* allele encoding Pccs domains I-III. We consistently found that *S. pombe* cells disrupted in the *pccs*$^{+}$ gene exhibit an increased sensitivity to copper and cadmium. Furthermore, we demonstrate that overexpression of *pccs*$^{+}$ is associated with increased copper resistance in fission yeast cells. Taken together, our findings suggest that Pccs activates apo-SOD1 under copper-limiting conditions through the use of its first three domains and protects cells against metal ion toxicity via its fourth domain.

Copper is both an essential and yet toxic cellular constituent (1). As a redox metal, it is used by critical enzymes as a catalytic cofactor (2). However, this same property can render copper cytotoxic because of its ability to participate in Fenton-like reactions that can generate hydroxyl radical, which may in turn cause cellular damage (3). In order to provide organisms with sufficient copper while at the same time preventing copper toxicity, specialized mechanisms have evolved for its uptake, intracellular trafficking, and sequestration within cells (4, 5).

In recent years, the use of yeast as a model organism has revealed a wealth of new information on how cells establish and maintain copper concentrations that are compatible with their needs (6–8). Under copper-limiting conditions and following the reduction of Cu^{2+} to Cu^{1+} by the Fre plasma membrane reductases (9–13), the budding yeast *Saccharomyces cerevisiae* transports copper ions into yeast cells using two separate high affinity permeases encoded by the *CTR1*[1] and *CTR3* genes (14–18). Copper-limiting conditions increase levels of mRNA synthesized from the *FRE1/7*, *CTR1*, and *CTR3* genes, whereas their expression is repressed under copper-replete conditions, thereby preventing copper accumulation during acute copper exposure (12, 19). The copper-responsive regulation of *FRE1/7*, *CTR1*, and *CTR3* gene expression is mediated by the copper-sensing transcription factor Mac1 (12, 19, 20). In addition to transcriptional regulation, copper uptake is further regulated through the copper-dependent degradation of the Ctr1 transporter. Ctr1 is stable under conditions of copper deprivation, but is rapidly degraded in the presence of excess copper (21). Unlike Ctr1, however, the Ctr3 transporter is not affected by increased exogenous copper concentrations (17).

Consistent with the notion that there is little intracellular free copper available in the cytoplasm of the yeast cell (22), specialized metallochaperones have been identified, including Atx1 (23, 24), Cox17 (25–28), and CCS1 (29), that bind copper after it enters the cell. These chaperones subsequently distribute their copper cargo to specific intracellular proteins or compartments (30, 31). Atx1 is a 73 amino acid cytosolic protein with a predicted molecular mass of 8.2 kDa (23). This copper chaperone is known to coordinate a single metal ion via its Met-*X*-Cys-X_2-Cys motif (24). Atx1 shuttles copper from the cytosol to post-Golgi vesicles by specifically docking with the Ccc2 copper-transporting P-type ATPase (32). Once loaded, Ccc2 subsequently pumps copper into the lumen of the Golgi, to metallate the copper-dependent ferroxidase Fet3 (32, 33). Although *ATX1* and *CCC2* gene transcription is unaffected by intracellular copper status, these genes are transcriptionally

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[1] The abbreviations used are: CTR1, copper transporter 1; BCS, bathocuproinedisulfonic acid; CCS, copper chaperone for SOD1; Cuf1, copper factor 1; CuSE, copper-signaling element; MT, metallothionein; Pccs, *S. pombe* copper chaperone for SOD1; PCNA, proliferating cell nuclear antigen; SOD1, copper-zinc superoxide dismutase; YES, yeast extract plus supplements; GFP, green fluorescent protein.

activated in response to iron starvation, illustrating their importance to iron metabolism through the delivery of copper to Fet3 (23, 34). Cox17, a 8.05-kDa copper chaperone, delivers copper specifically to the mitochondria for the assembly of cytochrome *c* oxidase (25, 35). Cox17 binds three Cu^{1+} ions through cysteine residues that are arranged in a Cys-Cys-*X*-Cys configuration (27). Copper-loaded Cox17 has been shown to shuttle in and out of the mitochondrial intermembrane space and is thought to dock to at least one inner mitochondrial membrane protein, Sco1, for subsequent copper donation to the Cu_A site of the cytochrome *c* oxidase (36–38). A third copper chaperone, CCS1, specifically activates SOD1 (29). CCS1 has a predicted molecular mass of 27.3-kDa and possesses three distinct domains (39). Domain I (residues 1–77) resembles the structure of Atx1 (40) and contains a Met-*X*-Cys-X_2-Cys motif (residues 15–20) within its N-terminal portion. This domain has been demonstrated to be important for insertion of copper into SOD1 *in vivo* under conditions of copper deprivation but is not needed for this function under copper-replete conditions (39). Domain II (residues 78–213) strongly resembles the overall structure of SOD1 (40). This domain participates in CCS1-SOD1 protein-protein interactions, and is absolutely required for donation of copper to SOD1 (41, 42). At the C terminus (residues 214–249) a short region, referred to as domain III, contains two conserved cysteine residues arranged in a Cys-*X*-Cys motif that binds copper *in vitro* (39). CCS proteins lacking the C-terminal domain III are unable to activate SOD1, suggesting that the Cys-*X*-Cys site binds copper (possibly with the aid of domain I) and facilitates its insertion into the catalytic site of SOD1 (39, 43). When environmental copper levels are in excess, intracellular buffering of copper is carried out by two metallothionein (MT) proteins, Cup1 and Crs5, as well as by SOD1 (44–48). The copper-dependent up-regulation of *CUP1*, *CRS5*, and *SOD1* gene expression is mediated by the Ace1 copper-detoxifying transcription factor (49, 50).

In the fission yeast *Schizosaccharomyces pombe*, candidate molecules for sequestering excess metal ions have been reported, including the Zym1 MT and the phytochelatins (51, 52). Studies using an *S. pombe* mutant strain that is defective in phytochelatin biosynthesis revealed that phytochelatins play an important role in cadmium and arsenic detoxification but have no apparent clear function in copper detoxification (52). *zym1*$^+$ encodes a MT that has a structure resembling the β-domain of mammalian MTs (51). An *S. pombe* strain harboring a deletion of the *zym1*$^+$ gene exhibits a reduction in zinc accumulation, a slight sensitivity to zinc, and a decrease in cadmium tolerance. Furthermore, *zym1*$^+$ mRNA levels were shown to be induced by zinc and cadmium but not copper (51).

For growth under copper limiting conditions, the molecular mechanisms in the early steps of copper assimilation in *S. pombe* differ from those in *S. cerevisiae* (53). Two integral membrane proteins, Ctr4 and Ctr5, form a two-component copper transporting complex at the cell surface (54, 55). In the absence of Ctr5, Ctr4 is mislocalized within the secretory pathway. Similarly, it was found that in the absence of Ctr4, Ctr5 is retained in the endoplasmic reticulum (55). These results suggest that assembly of a Ctr4/Ctr5 complex is required for either protein to proceed through the secretory pathway to the plasma membrane. Within this complex, the exact function of each protein is currently unclear. Recently, we have identified a gene in fission yeast, *ctr6*$^+$, which encodes an intracellular vacuolar copper transporter (56). In response to copper limitation, Ctr6 appears to mediate the efflux of usable copper from the vacuole into the cytosol (56). Like the *ctr4*$^+$ and *ctr5*$^+$ genes, *ctr6*$^+$ expression is regulated by the Cuf1 transcription factor and is induced in copper-limited cells (54, 56, 57). Cuf1 plays an essential role in coordinating the copper-dependent transcriptional regulation of copper transporter gene expression in *S. pombe*. This regulation involves *cis*-acting copper-signaling elements (CuSEs) found in each of the *ctr4*$^+$, *ctr5*$^+$, and *ctr6*$^+$ promoters (56, 57). Binding studies reveal that the Cuf1 N-terminal 174 amino acids are important for binding to the CuSE (58). A motif containing five clustered cysteine residues near its C terminus constitutes the minimal copper-sensing module of Cuf1 and serves to inactivate Cuf1 function when cells are grown under copper-replete conditions (58).

Upon uptake into fission yeast cells, copper ions are presumably taken up by putative copper chaperones that are as yet uncharacterized at the molecular level. Examination of the *S. pombe* Genome data base suggests that the open reading frame *SPAC22E12.04* encodes a putative ortholog of the *S. cerevisiae* CCS1. Although this putative ortholog bears 30% identity and 47% similarity to its bakers' yeast counterpart, notable differences exist between the two molecules. For instance, the N-terminal domain I of the *S. pombe* CCS ortholog (designated Pccs) lacks the copper-binding Met-*X*-Cys-X_2-Cys motif. In addition, Pccs harbors an extra domain at the C terminus that contains a series of cysteine residues, which are arranged in Cys-Cys configurations. Given these differences between the *S. pombe* and *S. cerevisiae* CCS proteins, we sought to dissect the functional features of Pccs. When the *pccs*$^+$*I-IV* and *pccs*$^+$*I-III* alleles were ectopically expressed in a *S. cerevisae ccs1*Δ strain, we found that cells producing a polypeptide spanning domains I-III displayed nearly wild-type levels of SOD1 activity. Under low basal copper conditions, *S. pombe* strains harboring a deletion of the *pccs*$^+$ gene were defective in SOD1 activity. Transforming this strain with a plasmid expressing the first 222 amino acids of Pccs (domains I-III) restored SOD1 activity to the same level obtained with the full-length Pccs protein. When the full-length *S. pombe* *pccs*$^+$ gene or a cDNA fragment that encodes only the Pccs domain IV was expressed in a *S. cerevisiae ace1*Δ strain, these cells exhibited a copper-resistant growth phenotype in the presence of exogenous copper. Consistently, *pccs*Δ mutant cells were sensitive to copper and cadmium. Furthermore, overexpression of *pccs*$^+$*I-IV* or *pccs*$^+$*IV* alone conferred tolerance to elevated copper levels in fission yeast cells. Taken together, these results reveal that the *S. pombe* Pccs protein function in dual pathways to deliver copper to SOD1 during conditions of copper scarcity and to detoxify metal ions during conditions of metal excess.

EXPERIMENTAL PROCEDURES

Strains and Media—Six isogenic *S. pombe* strains, the wild-type FY254 (*h*$^-$ *can1-1 leu1-32 ade6-M210 ura4-D18*) (59), *cuf1*Δ (*h*$^-$ *can1-1 leu1–32 ade6-M210 ura4-D18 cuf1*Δ::*ura4*$^+$) (54), *pccs*Δ (*h*$^-$ *can1-1 leu1-32 ade6-M210 ura4-D18 pccs*Δ::*ura4*$^+$), *sod1*Δ (*h*$^-$ *can1-1 leu1-32 ade6-M210 ura4-D18 sod1*Δ::ura4$^+$), *zym1*Δ (*h*$^-$ *can1-1 leu1-32 ade6-M210 ura4-D18 zym1*Δ::*ura4*$^+$), and *zym1*Δ *pccs*Δ (*h*$^-$ *can1-1 leu1–32 ade6-M210 ura4-D18 zym1*Δ::*ura4*$^+$ *pccs*Δ::*KAN*^{r}) were used in this study. To ascertain that the results seen were not specific to the *S. pombe* FY254 strain, identical experiments were conducted with the FY435 strain (*h*$^+$ *his7-366 leu1-32 ade6-M210 ura4-D18*) (60). The *pccs*Δ (*h*$^+$ *his7-366 leu1-32 ade6-M210 ura4-D18 pccs*Δ::*KAN*^{r}) and *pccs*Δ *zym1*Δ *ctr6*Δ (*h*$^+$ *his7-366 leu1–32 ade6-M210 ura4-D18 ctr6*Δ::*hisG zym1*Δ::*ura4*$^+$ *pccs*Δ::*KAN*^{r}) mutant strains are isogenic to the FY435 strain. *S. pombe* cells were grown in yeast extract plus supplements (YES) or in Edinburgh minimal medium with the necessary auxotrophic requirements (61). When *pccs*Δ and *sod1*Δ mutants were grown, Edinburgh minimal medium was further supplemented with 225 mg/liter of adenine, histidine, leucine, methionine, lysine, and cysteine, unless otherwise stated. Under anaerobic growth conditions, fission yeast cells were grown in culture jars with BD GasPak EZ (BD Diagnostic System, Sparks, MD). For expression of the human *CCS* and *S. pombe pccs*$^+$ genes in *S. cerevisiae*, the *ccs1*Δ (also named *lys7*Δ) mutant strain, denoted EGy103lys7Δ (*MAT*α *leu2-3, 11 his3-1* Δ*trp1-*

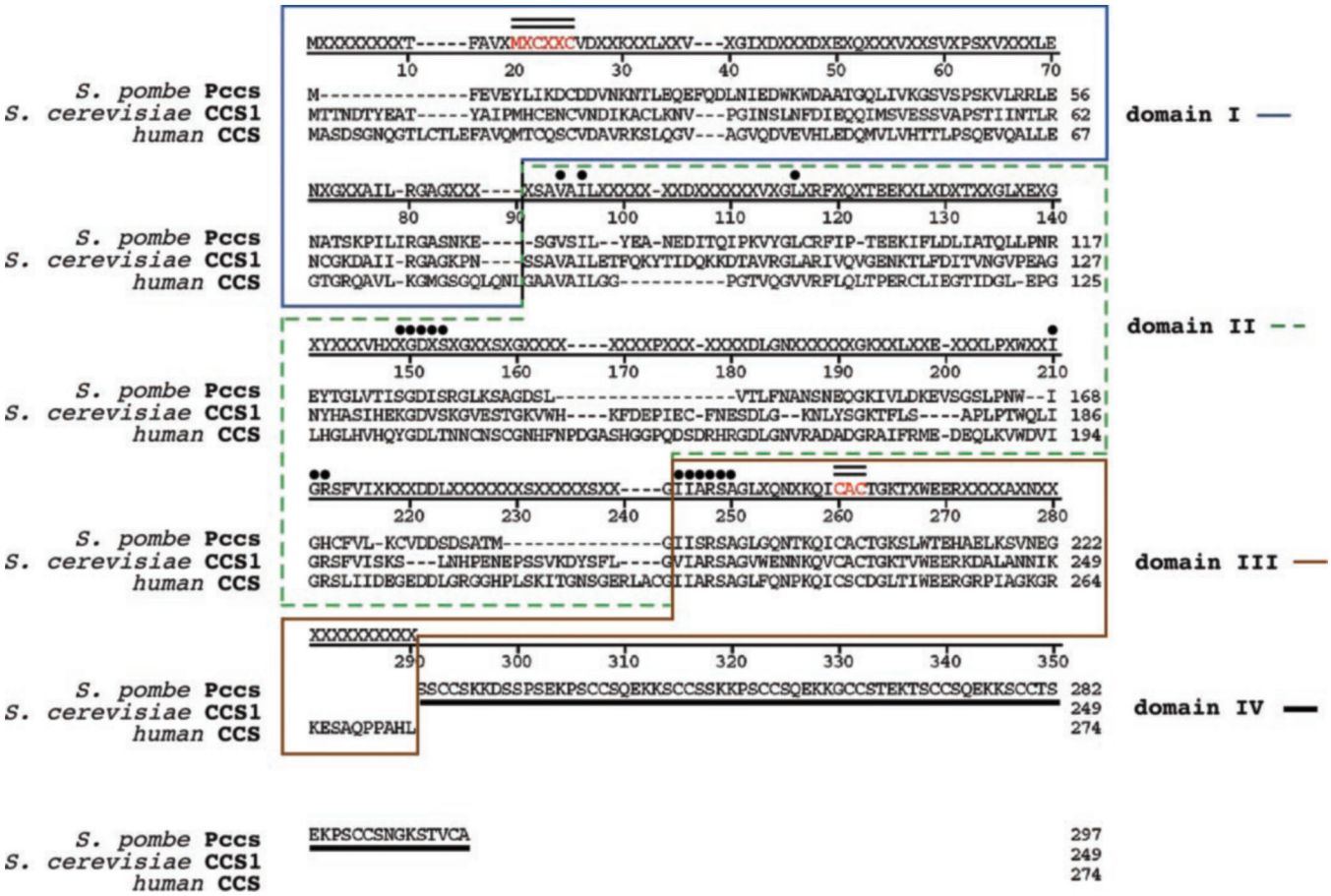

FIG. 1. **Sequence alignment of *S. pombe* Pccs with *S. cerevisiae* and *human* CCS.** Amino acid residues that are identical in at least two of the compared proteins are indicated above the alignment. The domains are numbered I-IV. Domains I, II, and III are *boxed* in *blue*, *green*, and *brown*, respectively. The fourth domain of Pccs is *underlined*. The M*XCXX*C sequence in domain I and the CXC motif in domain III are depicted in *red* and indicated with a *double line* above the sequence. The *dots* indicate residues that are predicted to be involved in CCS-SOD1 protein-protein interactions (40).

289 ura3-52 lys7::*LEU2*) was utilized to ensure that within the cell, only the ectopically expressed hCCS or Pccs was the sole protein with the ability to deliver copper to SOD1. The EGy103lys7Δ strain was derived from the parent strain EGy103 (*MATα leu2-3, 11 his3-1 Δtrp1-289 ura3-52*) (62) by gene deletion and replacement. *S. cerevisiae* strain DTY59 (*MATα his6 leu2-3,−112 ura3-52 ace1-Δ225 CUP1R-3*) (63) was used to test the ability of Pccs domain IV to detoxify copper in bakers' yeast. DTY59 was derived from strain DTY7 (*MATα his6 leu2-3,−112 ura3-52 CUP1R-3*) as described previously (64). *S. cerevisiae* cells were grown in rich medium (1% yeast extract, 2% bactopeptone, 2% dextrose) or synthetic complete medium lacking histidine or uracil for strains transformed with plasmids harboring the *HIS3* or *URA3* gene, respectively.

Plasmids—The *pccs*$^+$ gene was isolated by PCR using primers corresponding to the start and stop codons of the open reading frame from an *S. pombe* cDNA library (ATCC 87284, deposited by S. Elledge) (generous gift of Dennis J. Thiele, Duke University, Durham, NC). To clone the PCR product into the pRS313 vector (65), the EcoRI and BamHI sites found in *pccs*$^+$ were modified by PCR mutagenesis, eliminating both sites without altering the amino acid sequence of the Pccs protein. The modified *pccs*$^+$ allele was re-amplified by PCR using primers designed to generate EcoRI and XbaI sites at the upstream and downstream termini of the open reading frame. The PCR product obtained was digested with EcoRI and XbaI and cloned into the corresponding sites of the centromeric yeast plasmid pRS313 to generate pRS*pccs*$^+$*I-II-III-IV*. Subsequently, the *S. cerevisiae CCS1* promoter up to −395 from the start codon of the *CCS1* gene was subcloned into pRS*pccs*$^+$*I-II-III-IV* at the XhoI and EcoRI sites. Similarly, a 335-bp XbaI-BamHI DNA fragment containing the 3′-untranslated region of *CCS1* was inserted into the same sites of pRS*pccs*$^+$*I-II-III-IV*. The 666-bp EcoRI-XbaI fragment encoding the first three domains of Pccs was used to replace the EcoRI-XbaI fragment from plasmid pRS*pccs*$^+$*I-II-III-IV* to produce the plasmid pRS*pccs*$^+$*I-II-III*. The human and *S. cerevisiae CCS* genes were obtained by PCR amplification using primers that contained EcoRI and XbaI restriction sites using a human HeLa cell cDNA library or genomic DNA from *S. cerevisiae* strain DTY7 as templates, respectively. The purified DNA fragments were digested with EcoRI and XbaI and subsequently cloned into the corresponding sites in plasmid pRS*pccs*$^+$*I-II-III-IV* to replace the *pccs*$^+$*I-II-III-IV* gene.

To generate the pSP1*sod1*$^+$ plasmid, a 1598-bp BamHI-NotI PCR-amplified DNA segment containing the *S. pombe sod1*$^+$ locus starting at −860 from the translational start codon up to +273 after the stop codon was inserted into the BamHI and NotI sites of pSP1 (66). The *pccs*$^+$ cDNA was isolated using the *S. pombe* cDNA library described above. The purified DNA fragment that contained flanking BglII and SmaI restriction sites was digested and cloned into the pBluescript SK vector (Stratagene, La Jolla, CA) at compatible BamHI and SmaI sites. To create a plasmid that has the *pccs*$^+$ promoter driving the expression of the *pccs*$^+$ gene, the *S. pombe pccs*$^+$ regulatory region (positions −1105 to −1) was amplified by PCR and inserted just before the ATG codon of the *pccs*$^+$ gene using the NotI and SpeI sites. Subsequently, a SmaI-PstI DNA fragment of the *pccs*$^+$ terminator up to +542 from the stop codon was isolated by PCR from the *S. pombe* FY254 genomic DNA. Once generated and verified by DNA sequencing, the DNA fragment containing the *pccs*$^+$ gene and its regulatory regions was isolated from the pSK*pccs*$^+$*I-II-III-IV* plasmid using NotI and PstI and inserted into the corresponding sites of pSP1. The resulting plasmid was designated pSP1*pccs*$^+$*I-II-III-IV*. To generate the *pccs*$^+$*I-II-III-IV-StuI-BspEI* allele, a 12-bp StuI-BspEI linker was inserted in-frame and downstream of the last codon of the *pccs*$^+$ gene by the overlap extension method (67). The insertion created four extra amino acid residues after the alanine at position 297 (Ala^{297}-Arg-Pro-Ser-Gly-Stop) of Pccs. This allele was found to be functional because of its ability to fully restore SOD1 activity *in vivo*. We used the restriction sites StuI and BspEI created within *pccs*$^+$ to insert a copy of the *gfp* gene (68) or four copies of the *Haemophilus influenzae* hemagglutinin epitope (69). The plasmid, denoted pSP1*pccs*$^+$*I-II-III-IV-GFP*, was used to determine the localization of Pccs-GFP fusion protein in *S. pombe* by fluorescence microscopy. A

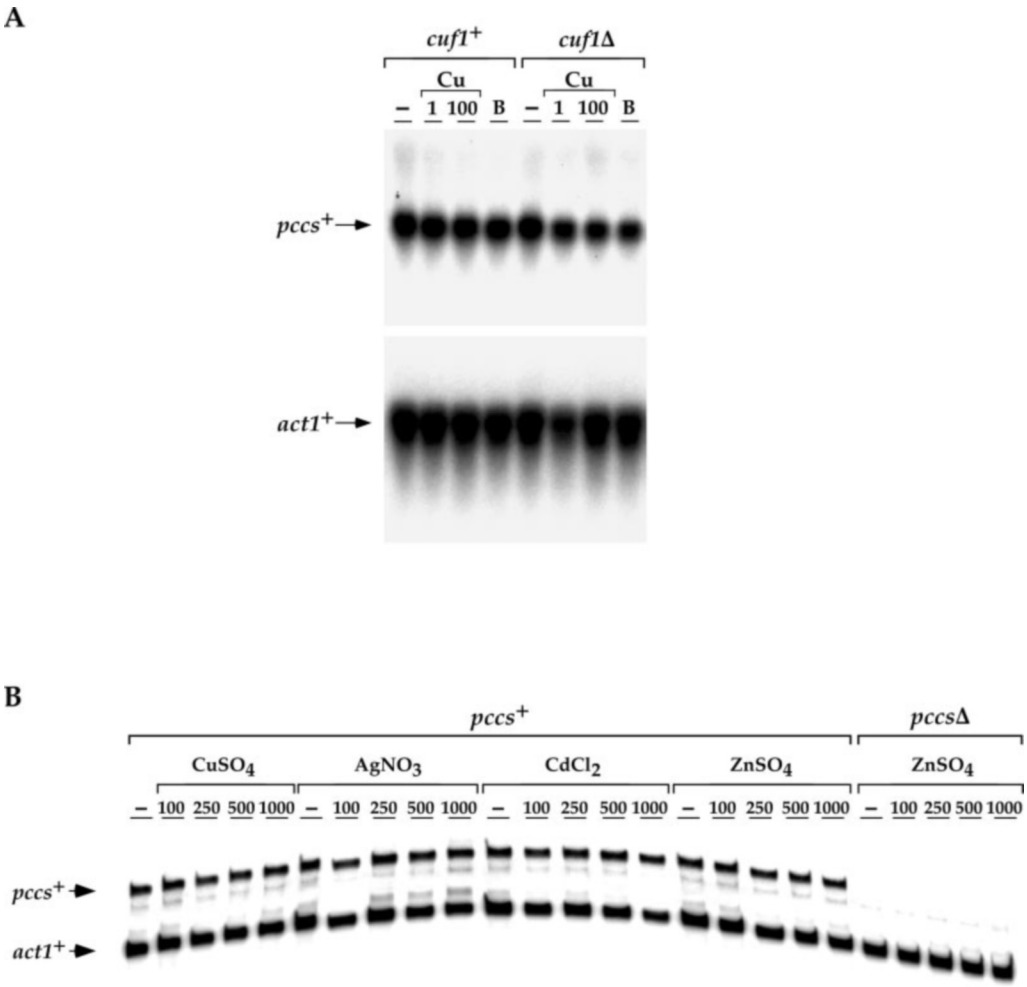

FIG. 2. **Transcription of *pccs*$^+$ mRNA is unregulated by copper, silver, cadmium, and zinc, and a functional *cuf1*$^+$ gene is not required for its expression.** *A*, the isogenic strains FY254 (*cuf1*$^+$) and SPY1 (*cuf1*Δ) were grown to mid-logarithmic phase in YES media. Cultures were incubated in the absence (−) or presence of $CuSO_4$ (1 and 100 μM), or 100 μM BCS for 1 h at 30 °C. Total RNA was isolated and analyzed by Northern blot. *pccs*$^+$ and *act1*$^+$ mRNA steady-state levels are indicated by *arrows*. *B*, total RNA from the wild-type FY254 and the *pccs*Δ disruption strain were analyzed by RNase protection assay. *pccs*$^+$ and *act1*$^+$ mRNA levels are indicated by *arrows*. Total RNA was isolated from control untreated cells (−), and cells treated with $CuSO_4$ (100, 250, 500, and 1000 μM), $AgNO_3$ (100, 250, 500, and 1000 μM), $CdCl_2$ (100, 250, 500, and 1000 μM), or $ZnSO_4$ (100, 250, 500, and 1000 μM). The results shown are representative of three independent experiments.

similar strategy was utilized to generate the *pccs*$^+$*I-II-III-StuI-BspEI* allele, except that the StuI-BspEI linker was placed in-frame at the end of a DNA fragment that encodes only the Pccs domains I, II, and III. Subsequently, the *gfp* or *zym1*$^+$ gene with flanking StuI and BspEI restriction sites was cloned into pSP1*pccs*$^+$*I-II-III-StuI-BspEI* to generate the pSP1*pccs*$^+$*I-II-III-GFP* or pSP1*pccs*$^+$*I-II-III-zym1*$^+$ plasmid, respectively.

To ascertain if the expression of different versions of the *pccs*$^+$ and *zym1*$^+$ alleles contributed to the increased copper resistance of *S. cerevisiae* DTY59 cells, the p4*XXGPD* expression vectors were used as described previously (70). Using appropriate primers that contained SpeI and SmaI sites, the *pccs*$^+$*I-II-III-IV*, *pccs*$^+$*I-II-III*, *pccs*$^+$*IV*, *zym1*$^+$, and *pccs*$^+$*I-II-III-zym1*$^+$ alleles were isolated by PCR from the plasmids pSP1*pccs*$^+$*I-II-III-IV*, pSP1*pccs*$^+$*I-II-III*, pSP1*pccs*$^+$*IV*, pSP1*zym1*$^+$, and pSP1*pccs*$^+$*I-II-III-zym1*$^+$, respectively. The PCR products obtained were digested with SpeI and SmaI and cloned into the corresponding sites of p426*GPD*. To assess if overexpression of *pccs*$^+$ and *zym1*$^+$ alleles can rescue the copper hypersensitivity of a *ctr6*Δ strain overexpressing the wild-type *ctr6*$^+$ gene, plasmids pREP3X-*pccs*$^+$*I-II-III-IV*, pREP3X-*pccs*$^+$*I-II-III*, pREP3X-*pccs*$^+$*-IV*, pREP3X-*zym1*$^+$, and pREP3X-*pccs*$^+$*I-II-III-zym1*$^+$ were constructed as follows. Five DNA fragments encompassing the *pccs*$^+$*I-II-III-IV*, *pccs*$^+$*I-II-III*, *pccs*$^+$*IV*, *zym1*$^+$, and *pccs*$^+$*I-II-III-zym1*$^+$ alleles were PCR amplified with flanking XhoI and SmaI sites from the pSP1*pccs*$^+$ and pSP1*zym1*$^+$ plasmids. The resulting PCR products were digested with XhoI and SmaI and cloned into the corresponding sites of pREP3X (71, 72). For ectopic expression of the *ctr6*$^+$ gene, the thiamine-repressible promoter system was used as described previously (56).

Protein and Enzyme Assays—For Western blotting experiments, *S. pombe* and *S. cerevisiae* cells were grown to OD_{600} of 1.0 in selective medium. Protein extracts were prepared from cells that were untreated or incubated for 3 h (*S. cerevisiae*) or 11 h (*S. pombe*) with either $CuSO_4$ (100 μM) or BCS (100 μM), and then quantitated as described previously (73). The extracts were resolved by SDS-polyacrylamide electrophoresis, transferred to polyvinylidene difluoride Hybond-P (Amersham Biosciences), and the immunoblots analyzed for steady-state levels of SOD1, PGK, and PCNA proteins using antiserum SOD-100 (Stressgen, Victoria, BC), 22C5-D8 (Molecular Probes, Eugene, OR), and PC10 (Sigma), respectively. After a 2-h incubation, the membranes were washed with TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% bovine serum albumin), incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences), and visualized by chemiluminescence. SOD1 activity assays were performed using in-gel nitro blue tetrazolium staining as previously described (54). Spectrophotometric determination of SOD activity was also performed using the protein extracts by measuring the inhibition of the reduction rate of cytochrome *c* by SOD, which competes for reactive oxygen species produced from the xanthine-xanthine oxidase system (74) as described previously (56).

Analyses of Metal Ion Sensitivity—Cells were grown for 48 h without shaking, re-inoculated to OD_{600} of 0.5, and grown to an OD_{600} of 1.0 ($\sim 1 \times 10^7$) at 30 °C. Each cell culture was diluted ($\sim 2 \times 10^4$) and inoculated into 5 ml of Edinburgh minimal medium containing 50 μM $CuSO_4$ and further supplemented with increasing concentrations of metal ions specified in Fig. 8. After incubation for 7 days at 30 °C without shaking, total growth was measured at OD_{600}.

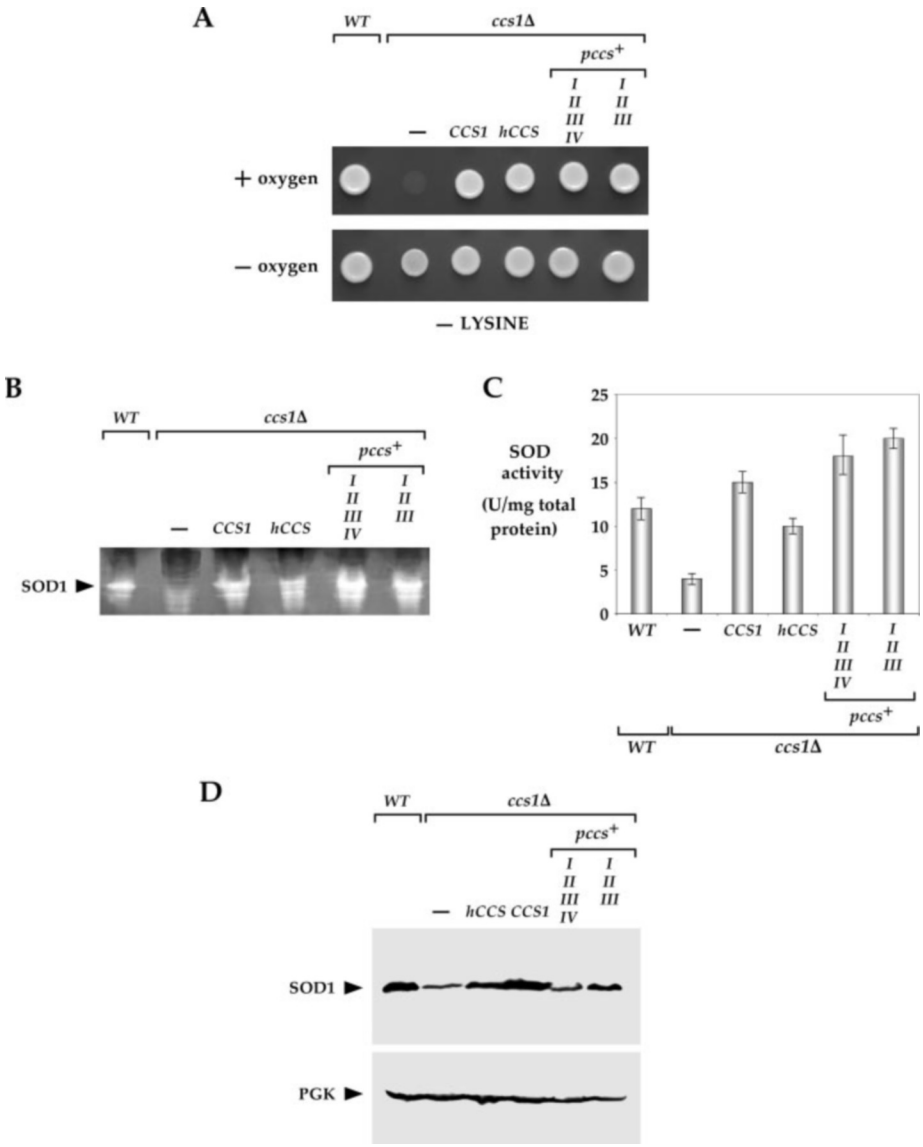

FIG. 3. **Expression of full-length Pccs or Pccs domains I-III complements loss of *CCS1* in *S. cerevisiae*.** *A*, EGy103lys7Δ (also named EGy103ccs1Δ) fails to grow on synthetic complete medium lacking lysine in the presence of oxygen. EGy103ccs1Δ cells were transformed with plasmids expressing human CCS (*hCCS*) or *S. pombe* Pccs (*pccs*$^+$) domains I-IV or domains I-III under the control of the *S. cerevisiae CCS1* gene promoter and assayed for growth in the presence or absence of oxygen. As a positive control, EGy103ccs1Δ cells were also transformed with a plasmid expressing *S. cerevisiae* CCS1 (*CCS1*) under the control of its own promoter and assayed for growth in the same conditions. *B*, EGy103ccs1Δ displays a deficiency in SOD1 activity when cells are grown under copper starvation conditions in the presence of the Cu^+ chelator BCS (100 μM). These cells were transformed with vector only (−) or plasmids expressing the indicated domains of Pccs or CCS1 and hCCS as positive controls. Total extracts from transformed cells were assayed for SOD1 activity using an in-gel activity assay with nitro blue tetrazolium staining. *C*, SOD activity was determined from the cell lysates used in *B* using a spectrophotometric method with cytochrome *c* and xanthine oxidase. The SOD activities reported represent the means of three replicates experiments ± S.D. *D*, Western blot analysis of extracts used in *B* employing an antibody directed against human SOD1 that also recognized the *S. cerevisiae* SOD1 protein (89). Cellular levels of PGK was determined as a load control.

RNA Analysis—The *S. pombe* isogenic strains FY254 (wild type) and SPY1 (*cuf1*Δ) were grown in YES medium. Copper-treated (1 and 100 μM), BCS-treated (100 μM), and control cultures were grown to mid-logarithmic phase (OD_{600} of ~1.0). After a 1-h incubation at 30 °C total RNA was extracted by the hot phenol method (75). RNAs were quantitated spectrophotometrically, and 20 μg of RNA per sample were analyzed by Northern blot using random-primed ^{32}P-labeled DNA probes. For RNase protection analyses (57), two plasmids were used to make antisense RNA probes. The plasmid pSK*act1*$^+$ was described previously (57). The plasmid pSK*pccs*$^+$ was constructed by inserting a 173-bp BamHI-EcoRI fragment of the *pccs*$^+$ cDNA into the same sites in pBluescript SK. The antisense RNA hybridizes to the region between +172 and +345 upstream of the initiator codon of *pccs*$^+$.

RESULTS

The Primary Sequence of Pccs—Analysis of genomic DNA sequences from the *S. pombe* Genome Project revealed an open reading frame (*SPAC22E12.04*) related to two previously identified groups of copper homeostasis proteins: the CCS and MT proteins, which are involved in delivering copper to SOD1 and in copper detoxification, respectively. The *SPAC22E12.04*-encoded protein, which we have denoted Pccs, has four distinct domains. The first three domains of the protein have extended homology to the CCS proteins, including *S. cerevisiae* CCS (CCS1) and human CCS (hCCS) (Fig. 1). Domain I of Pccs (residues 1–72) displays 24 and 18% identity to the same region of the *S. cerevisiae* and human CCS proteins, respectively. Interestingly, domain I of Pccs lacks the Met-*X*-Cys-X_2-Cys motif found in CCS1 and hCCS that is predicted to coordinate a single Cu^{1+} atom under conditions of copper starvation (39). Instead, Pccs has a single cysteine residue at position 11 that aligns with the last cysteine of the Met-*X*-Cys-X_2-Cys sequence (Fig. 1). Domain II of Pccs (residues 73–186) exhibits 28 and 23% identity with the domain II of *S. cerevisiae* CCS1 and human CCS, respectively. Supporting the notion that domain II of Pccs has greater similarity to CCS1 than hCCS, this domain lacks the three histidine and one aspartic acid residues that bind zinc in SOD1 and are preserved in human CCS (76). Furthermore, three of the four histidine residues known to bind copper in SOD1 and are conserved in hCCS domain II are absent in Pccs domain II. However, consistent with the model that domain II is involved in recognizing SOD1 and the subsequent formation of the CCS-SOD1 heterodimer, the residues predicted to be involved in protein-protein interactions between CCS and SOD1 (40) are highly conserved in all three Pccs, CCS1, and hCCS molecules (Fig. 1). Domain III of Pccs (residues 187–222) shows 47% identity with the domain III of both the *S. cerevisiae* and human CCS. This is the most conserved region, with an invariant Cys-X-Cys motif that is capable, in the case of CCS1, of binding copper (39). As shown in Fig. 1, the Pccs protein has an extra domain (residues 223–297) at its C terminus, which is not found in CCS1 or hCCS. The sequence of Pccs domain IV is

highly reminiscent of the metal binding protein, MT (77). This relatively short protein segment contains 17 cysteine residues, eight pairs of which are present in a Cys-Cys configuration. Furthermore, lysine and serine residues represent 19 and 28% of the total Pccs domain IV, respectively. Similar to MTs, domain IV lacks aromatic amino acids. Based on these observations and upon inspection of various sequence databases, the sequence of Pccs domain IV exhibits characteristics that are hallmarks of MTs. Therefore, Pccs bears strong similarity to domains found in the copper delivering CCS proteins, as well as the metal ion buffering MTs.

pccs$^+$ *mRNA Is Moderately Abundant in Wild-type Cells, Not Regulated by Metal Ion Repletion or Starvation, and Present in cuf1*Δ *Cells*—As determined by RNA blotting, the steady-state levels of *pccs*$^+$ mRNA in wild-type strain FY254 are unaffected by either exogenous copper or the copper chelator BCS (Fig. 2*A*). Although the isogenic *cuf1*Δ strain exhibited diminished levels of the *pccs*$^+$ mRNA, no significant copper-dependent changes in *pccs*$^+$ gene expression were observed (Fig. 2*A*). To further examine if *pccs*$^+$ transcription is regulated by metal ions, the wild-type strain was grown in the presence of different metal ions at various concentrations, and the steady-state levels of *pccs*$^+$ mRNA was assayed by RNase protection experiments. We tested the metal ions, Cu^{2+}, Ag^{1+}, Cd^{2+}, and Zn^{2+}, and found no significant alteration in the transcription of *pccs*$^+$ mRNA in response to these metals (Fig. 2*B*). Furthermore, our data indicate that although Cuf1 serves as a transcription factor that is required for expression of genes involved in copper transport, inactivation of the *cuf1*$^+$ locus does not affect the transcriptional competency of the *pccs*$^+$ gene. Consistent with this observation, there were no changes in the *pccs*$^+$ steady-state mRNA levels in response to either metal repletion or starvation.

Full-length Pccs or a Polypeptide Harboring the First Three Domains of Pccs Complements S. cerevisiae ccs1-null Phenotypes—To begin to understand the role of Pccs in copper homeostasis and to dissect the functions of its domains, we cloned two different alleles of *pccs*$^+$; one encoding the full-length Pccs protein, and the other encoding its first three domains. For this initial characterization, we expressed these *pccs*$^+$ alleles under the regulation of the *S. cerevisiae CCS1* promoter in a *ccs1*Δ mutant strain of *S. cerevisiae*. In the presence of oxygen, the *ccs1*Δ mutant strain cannot grow on synthetic media without lysine because holo-SOD1 is required for aerobic lysine biosynthesis (78, 79). Transformation of this strain with plasmids expressing Pccs domains I-IV or Pccs domains I-III permitted cell growth under aerobic conditions (Fig. 3*A*). The growth was also restored when either *S. cerevisiae* CCS1 or human CCS proteins were expressed in the *ccs1*Δ cells as shown previously (29) and reproduced here as controls (Fig. 3*A*). To characterize the ability of these alleles to activate SOD1, its activity was assayed in whole cell extracts from wild-type and *ccs1*Δ cells transformed with *pccs*$^+$*I-IV* and *pccs*$^+$*I-III*, grown under low basal copper conditions, using native enzyme polyacrylamide gel electrophoresis. The *ccs1*Δ strain, as observed previously (29), was devoid of detectable SOD1 activity, whereas *ccs1*Δ cells bearing the *pccs*$^+$*I-IV* or *pccs*$^+$*I-III* allele exhibited wild-type levels of SOD1 activity (Fig. 3*B*). In all cases, we also determined SOD activity in native cell extracts by measuring the inhibition of the reduction rate of cytochrome *c* by SOD, which competes for reactive oxygen species produced from the xanthine-xanthine oxidase system (Fig. 3*C*). This assay was conducted on the same cell lysates used for the in-gel staining assay (Fig. 3*B*). Importantly, the SOD activities measured by spectrophotometric analysis (Fig. 3*C*) very closely paralleled the results with the in-gel assay (Fig. 3*B*). When the *ccs1*Δ disruptant was transformed with the full-length

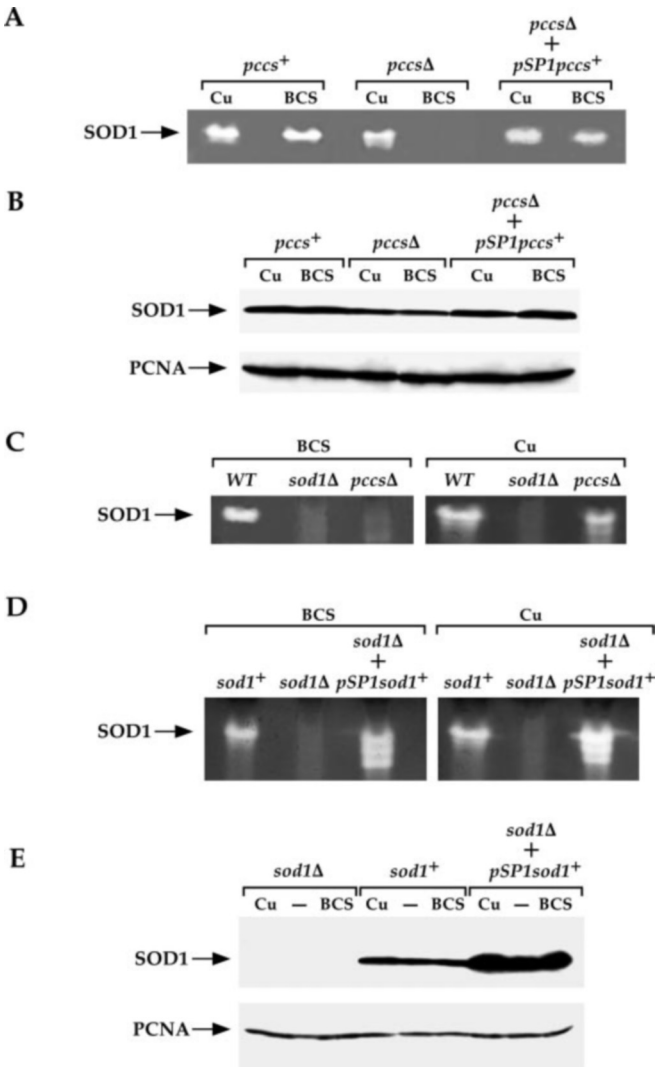

FIG. 4. **An *S. pombe pccs*Δ strain displays a deficiency in SOD1 activity under copper-limiting conditions.** *A*, SOD1 activity was determined in *pccs*Δ cells that were grown under conditions in which copper is required (100 μM BCS), in the presence of exogenous $CuSO_4$ (100 μM) and after transformation with a wild-type copy of the *pccs*$^+$ gene expressed from a plasmid. *B*, aliquots of whole cell extracts used in *A* were analyzed by immunoblotting. The positions of the SOD1 and PCNA proteins are indicated with *arrows*. *C*, SOD1 activity was determined in a wild-type strain of *S. pombe* (*WT*) and an *S. pombe sod1*Δ and *pccs*Δ disruption strains in the presence or absence of exogenous $CuSO_4$ (100 μM). *D*, a plasmid-borne copy of the *sod1*$^+$ gene was tested for its ability to restore SOD1 activity to a *S. pombe* strain with a deletion of the *sod1*$^+$ gene. *E*, the indicated strains were incubated in the absence (−) or presence of $CuSO_4$ (100 μM) or BCS (100 μM). Protein extracts prepared from these strains were analyzed for steady-state protein levels of SOD1 by immunoblotting using either anti-SOD1 or anti-PCNA (as an internal control) antibody.

Pccs and Pccs domains I-III, SOD activity levels were restored to 117 and 133%, respectively, compared with the levels in *ccs1*Δ cells harboring a wild-type copy of the *CCS1* gene expressed from a plasmid (Fig. 3*C*). To verify that the SOD1 protein was present in the wild-type and *ccs1*Δ cells, total protein extracts from cells transformed with plasmids expressing the indicated CCS molecules were analyzed by immunoblotting (Fig. 3*D*). These results showed that detectable levels of SOD1 were present in the *ccs1*Δ strain, indicating that the lack of activity in the *ccs1*-null strain was not due to lack of SOD1 expression. Taken together, these results demonstrate that the full-length *S. pombe* Pccs and Pccs domains I-III polypeptides can substitute for CCS1 in delivering copper to SOD1 in *S. cerevisiae ccs1*Δ cells.

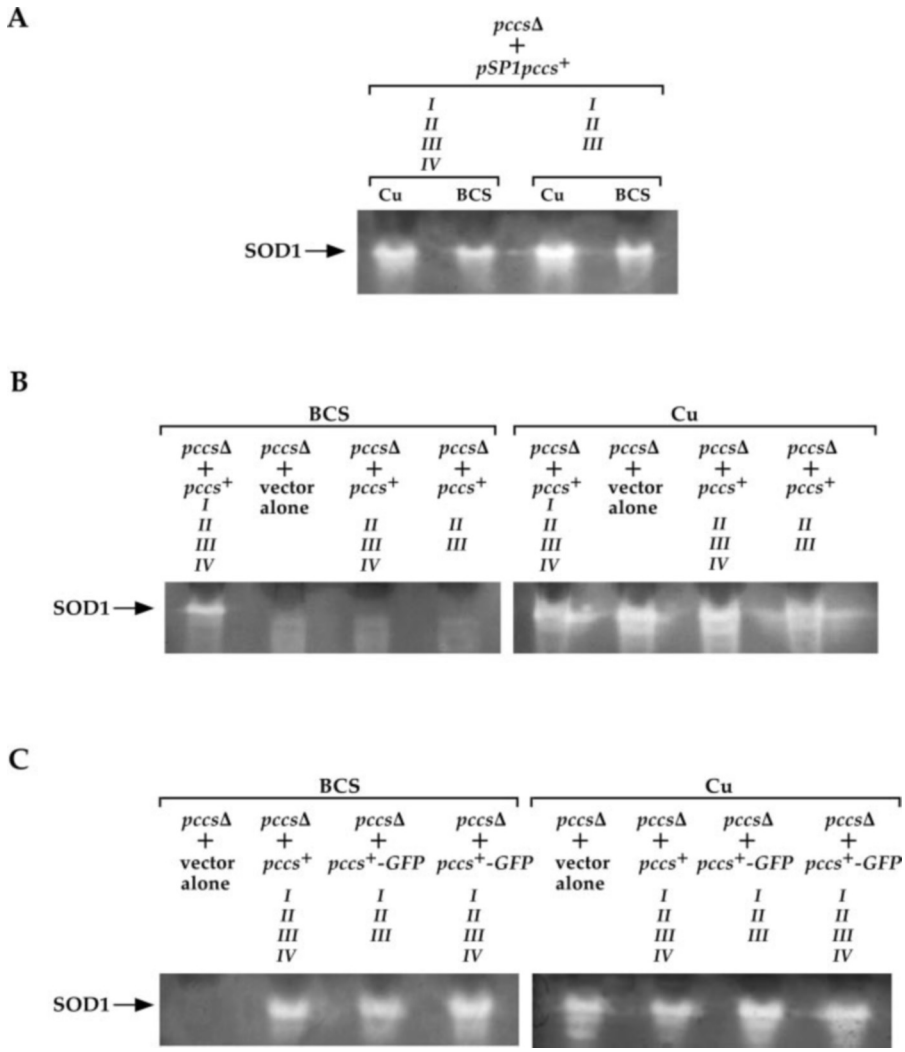

FIG. 5. ***pccs*Δ mutant cells expressing Pccs domains I-III exhibit similar levels of SOD1 activity to that observed for *pccs*Δ cells expressing the full-length Pccs protein.** *A*, exponentially growing cells were treated with $CuSO_4$ (100 μM) or BCS (100 μM) for 11 h. Whole cell extracts were prepared from these cells and analyzed using an in-gel assay for SOD1 activity. *pccs*Δ cells transformed with either *pccs*$^+$*I-IV* or with a plasmid expressing *pccs*$^+$*I-III* were examined for SOD1 activity. *B*, *pccs*Δ cells were transformed with the indicated plasmids and superoxide scavenging activity was monitored by the nitro blue tetrazolium gel assay. *C*, the full-length Pccs and Pccs domains I-III fused to the green fluorescent protein fully complemented the defective SOD1 activity in a *pccs*Δ strain in a manner indistinguishable from the unadulterated Pccs protein.

Pccs Plays a Crucial Role in Activating S. pombe SOD1 under Conditions of Copper Starvation—To further investigate the role of Pccs in *S. pombe*, the *pccs*$^+$ locus was insertionally inactivated by deletion and replacement with the *S. pombe ura4*$^+$ gene or *kanMX6* genetic marker (80). An *S. pombe* strain bearing the disrupted *pccs*Δ allele displayed a deficiency in SOD1 activity (Fig. 4*A*). As expected, normal SOD1 activity could be rescued either by expressing a wild-type copy of the *pccs*$^+$ gene from a plasmid, or by the addition of $CuSO_4$ to the growth medium at a concentration of 100 μM (Fig. 4*A*). Importantly, Western blot analysis (Fig. 4*B*) of the same lysates shown in Fig. 4*A* revealed that the absence of SOD1 activity in the *pccs*Δ mutant strain was not due to lack of SOD1 expression. To assess the specificity of the non-denaturing gel electrophoresis and nitro blue tetrazolium staining for SOD1 activity, the *sod1*$^+$ gene was inactivated in the *S. pombe* strain FY254 to generate a *sod1*Δ strain. Cell lysates from this strain was analyzed for SOD1 activity. As shown in Fig. 4, *C* and *D*, *sod1*Δ mutant cells were defective in SOD1 activity in the absence or presence of copper. Furthermore, no SOD1 protein was detected in the *sod1*Δ mutant strain, unless a wild-type copy of the *sod1*$^+$ gene expressed from a plasmid was transformed into the cells (Fig. 4*E*). To determine if the first three domains of Pccs is sufficient to activate SOD1, a plasmid expressing the Pccs domains I-III was transformed into a *S. pombe pccs*Δ strain. As a control, the full-length Pccs protein was separately expressed in this strain. As shown in Fig. 5*A*, the levels of SOD1 activity are readily detectable in the *pccs*Δ strain expressing a *pccs*$^+$ allele encoding domains I-III. The levels observed were comparable to those found in the same strain (*pccs*Δ) expressing full-length Pccs. In contrast, a *pccs*Δ mutant strain expressing the *pccs*$^+$*II-III-IV* or *pccs*$^+$*II-III* allele failed to activate SOD1 under copper-limiting conditions (Fig. 5*B*). This lack of superoxide scavenging activity does not reflect low protein levels, because the Pccs protein species were stably expressed in these cells (see supplemental data).[2] Moreover, a *S. cerevisiae ccs1*Δ strain expressing the *pccs*$^+$*II-III-IV* allele displayed a deficiency in SOD1 activity and failed to grow on medium lacking lysine in the presence of oxygen (see supplemental data).[3] These data suggest that the Pccs N-terminal domain I is required to activate SOD1 in conjunction with domains II and III under conditions of copper deprivation. Furthermore, these results suggest that Pccs functions in the same pathway as the *S. cerevisiae* CCS1 protein in providing copper to SOD1.

Based on previous studies that determined ~95–99% of total *S. cerevisiae* CCS1 resides in the cytosol (81, 82), we ascertained the localization of the Pccs protein in *S. pombe*. To ensure that insertion of GFP at the Pccs C terminus did not interfere with its function, we transformed the *pccs*$^+$*I-IV-GFP*

[2] Gel nitro blue tetrazolium assay showing the SOD1 activity of Pccs-HA_4 derivative polypeptides is shown; a Western blot analysis of each polypeptide using an anti-HA antibody.

[3] Spot test for complementation of aerobic lysine auxotrophy is shown; an analysis of SOD1 activity by the nitro blue tetrazolium gel assay.

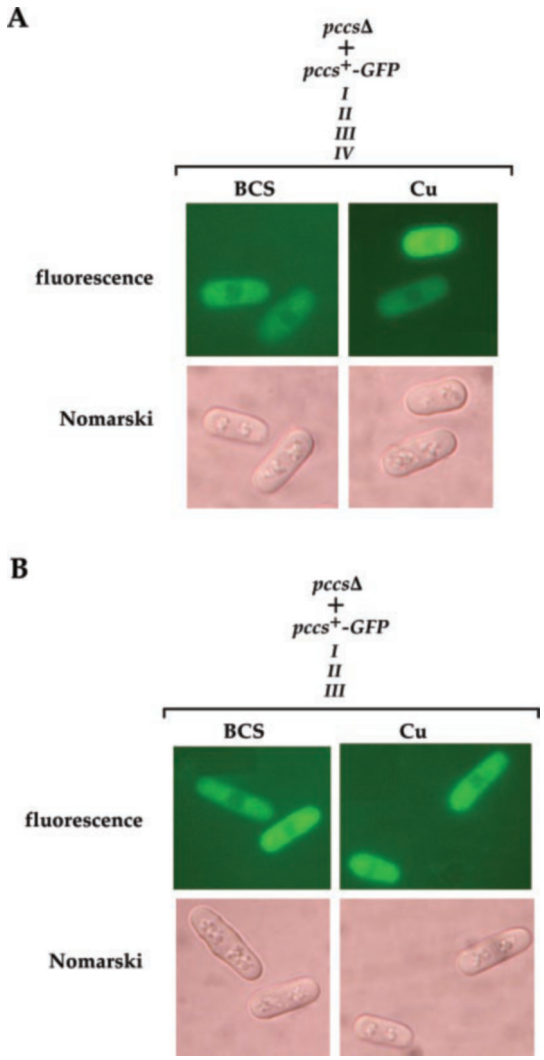

FIG. 6. **Localization of Pccs green fluorescent fusion proteins in *S. pombe*.** *A*, cells expressing the full-length Pccs-GFP fusion protein were grown at 30 °C for 11 h in the presence of BCS (100 μM) or $CuSO_4$ (100 μM) and visualized for GFP by fluorescence microscopy (*top panel*). The cells were also viewed by Nomarski microscopy for cell morphology (*bottom panel*). *B*, cells expressing a GFP protein fused to the C terminus of domain III of Pccs were examined for its cellular location as described in *A*.

and *pccs*$^+$*I-III-GFP* fusion genes into a *pccs*Δ mutant strain, and tested cell lysates from the transformants for the presence of SOD1 activity. As shown in Fig. 5*C*, *pccs*Δ cells expressing plasmids containing the *pccs*$^+$*I-IV-GFP* and *pccs*$^+$*I-III-GFP* genes functionally complemented the *pccs*Δ phenotypes similar to the full-length *pccs*$^+$ and *pccs*$^+$*I-III* genes, indicating that the Pccs I-IV-GFP and Pccs I-III-GFP fusion proteins were functional. The localization of each GFP fusion was determined by fluorescence microscopy. As shown in Fig. 6, *pccs*Δ cells expressing the *pccs*$^+$*I-IV-GFP* or *pccs*$^+$*I-III-GFP* allele accumulated the full-length Pccs-GFP or Pccs domains I-III-GFP protein in the cytosol in the absence or presence of copper. The fluorescence was diffuse and distributed throughout the cytosol. The Pccs I-IV-GFP and Pccs I-III-GFP fusion proteins were predominantly localized in the cytosol, and in most cells, were largely excluded from the nucleus (Fig. 6 and data not shown). Our data do not allow us to establish whether or not a fraction of the total Pccs protein is localized in the mitochondrial intermembrane compartment as reported previously for CCS1 (81). Taken together, these observations suggest that the full-length Pccs and Pccs I-III proteins are primarily cytosolic components similar to SOD1.

*Pccs Domain IV Confers Resistance to Copper When Expressed in an S. cerevisiae ace1*Δ *Strain*—As mentioned above, analysis of the Pccs domain IV sequence revealed that this domain harbors a significant similarity to the MTs. As shown in Fig. 7*A*, several cysteine, serine, and lysine residues in Pccs domain IV align to those of the *S. pombe* Zym1 and human MT-I proteins. Interestingly, Pccs domain IV contains the conserved Lys^{244}-*X*-Ser^{246} motif, which is invariant in the MT structure because of its role in joining the α- and β-domains of the protein (77, 83). Consistent with a role in metal ion sequestration, expression of the *pccs*$^+$*IV*-encoded domain IV in *S. cerevisiae* cells that are hypersensitive to copper toxicity restored the growth of these cells in conditions of copper excess (Fig. 7*B*). To ascertain if functional restoration of copper tolerance in *S. cerevisiae ace1*Δ cells correlated specifically with the presence of Pccs domain IV, cells that were hypersensitive to copper were also transformed with the vector alone or the wild-type *pccs*$^+$*I-IV* or *pccs*$^+$*I-III* alleles. *ace1*Δ cells transformed with the vector alone exhibited no growth on medium supplemented with exogenous copper. Although *ace1*Δ mutant cells expressing the first three domains of Pccs prevented copper toxicity in the presence of 50 μM $CuSO_4$, these cells were sensitive to copper at concentrations of 100 or 500 μM $CuSO_4$ (Fig. 7*B*). Ectopic expression of the full-length *pccs*$^+$*I-IV* gene in the *S. cerevisiae ace1*Δ strain in the presence of 50, 100, or 500 μM $CuSO_4$, allowed the transformed cells to grow in the presence of all elevated copper concentrations (Fig. 7*B*). Analogous to wild-type Pccs or Pccs domain IV, expression of the *S. pombe* Zym1 MT in *S. cerevisiae ace1*Δ cells, allowed these cells to grow in the presence of 500 μM $CuSO_4$. To further investigate the ability of Pccs to confer protection against copper toxicity, we created a chimeric protein containing the first 222 amino acids (domains I-III) of Pccs fused to the residues 1–50 of Zym1. Expression of the chimeric ^{1}Pccs^{222}-^{1}Zym1^{50} protein in the *ace1*Δ disruptant strain allowed these cells to grow when copper was present in excess of physiological requirements, at the same rate as cells expressing the wild-type Pccs protein (Fig. 7*B*). Taken together, these results show that the Pccs protein through its domain IV plays a critical role in cell survival under conditions of copper toxicity.

A Role for S. pombe Pccs in Minimizing Metal Ion Cytotoxicity—Because ectopic expression of Pccs or Pccs domain IV in *S. cerevisiae ace1*Δ cells was associated with increased copper resistance, we determined if deleting the *pccs*$^+$ locus increased the sensitivity of *S. pombe* cells to copper. As shown in Fig. 8*A*, the *pccs*Δ mutant strain displayed an increased sensitivity to copper compared with the isogenic *pccs*$^+$ wild-type strain. Whereas the wild-type strain showed 40% inhibition of growth in medium containing ~420 μM $CuSO_4$, the *pccs*Δ mutant was ~2.8-fold more sensitive to copper, exhibiting a similar percentage of inhibition (40%) when grown in medium containing ~150 μM $CuSO_4$. The *zym1*Δ disruption strain also exhibited increased sensitivity to copper, with 40% growth inhibition in medium containing ~200 μM $CuSO_4$. The magnitude of copper sensitivity was more pronounced for the *pccs*Δ *zym1*Δ double mutant strain, with 40% growth inhibition in the presence of ~110 μM $CuSO_4$ (Fig. 8*A*). Deletion of the *pccs*$^+$ gene dramatically lowered resistance to cadmium (Fig. 8*B*). $CdCl_2$ concentrations as low as ~0.6 μM inhibited growth of the *pccs*Δ mutant by 40%. The *pccs*Δ single mutant was ~10-fold more sensitive to cadmium compared with the wild-type strain, which showed 40% growth inhibition in the presence of ~6 μM $CdCl_2$. The *zym1*Δ single mutant strain exhibited 40% growth inhibition in the presence of ~0.75 μM $CdCl_2$. Deletion of both

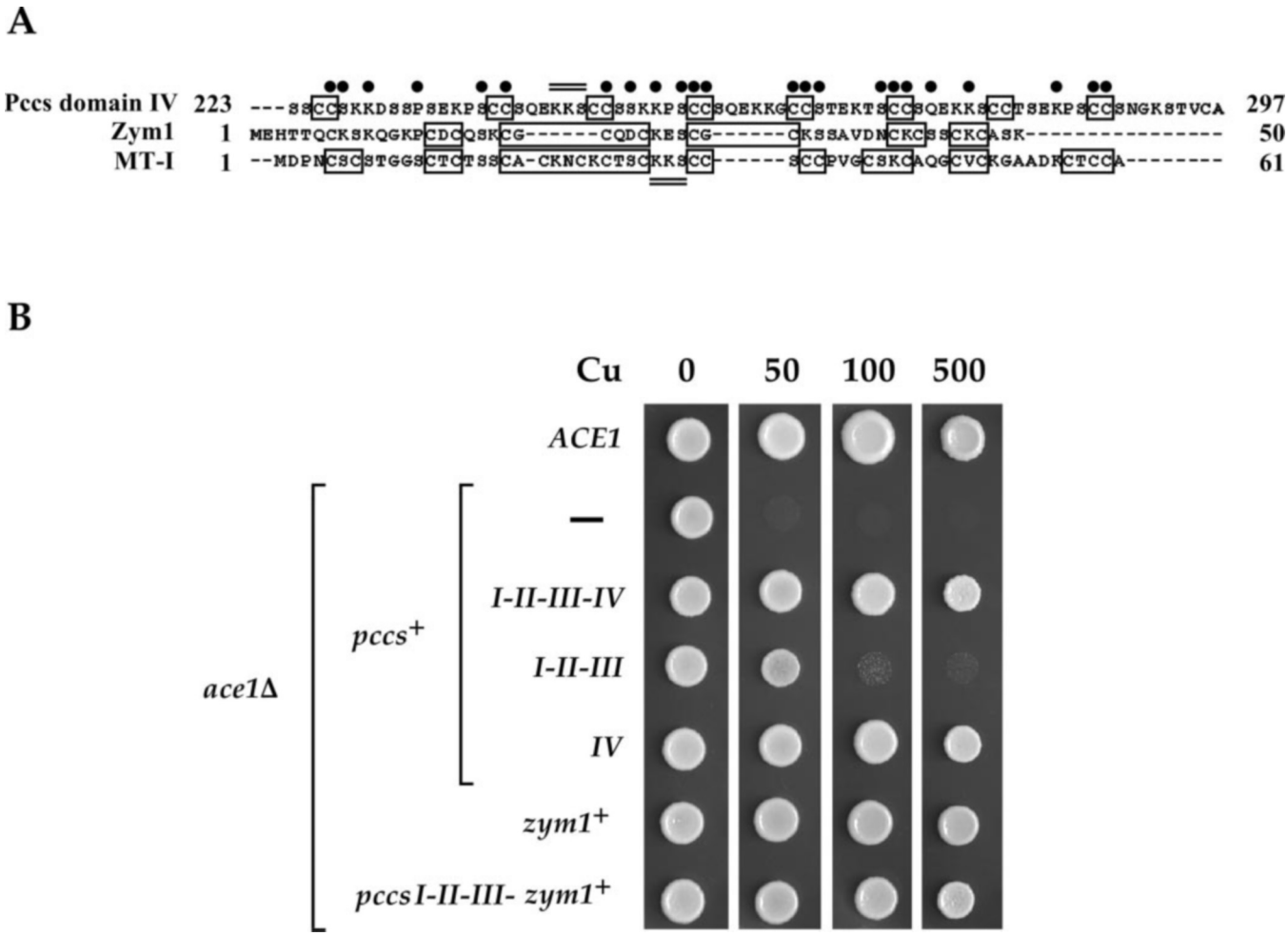

FIG. 7. **Functional comparison of Pccs domain IV with the Zym1 MT of *S. pombe* in an *S. cerevisiae ace1*Δ mutant strain.** *A*, alignment of the amino acid sequences of Pccs domain IV, *S. pombe* Zym1, and mouse MT-I. The *dots* indicate amino acid residues that are identical in at least two of the compared proteins. Cysteine residues that are arranged in Cys-Cys, Cys-*X*-Cys, and Cys-X_2-Cys configurations are *boxed*. The K*X*S motif is shown with a *double line* above or underneath. *B*, an *ace1*Δ mutant strain, transformed with plasmid p426*GPD* alone (−), plasmid p426*GPDpccs*$^+$*I-IV* (*I-II-III-IV*), plasmid p426*GPDpccs*$^+$*I-III* (*I-II-III*), plasmid p426*GPDpccs*$^+$*IV* (*IV*), plasmid p426*GPDzym1*$^+$ (*zym1*$^+$), or plasmid p426*GPDpccs*$^+$*I-III-zym1*$^+$ (*pccsI-II-III-zym1*$^+$), was spotted onto a synthetic complete medium containing the indicated $CuSO_4$ concentrations (0, 50, 100, and 500 μM). The wild-type (*ACE1*) strain DTY7 transformed with p426*GPD* alone was used as a control.

*pccs*Δ and *zym1*Δ slightly increased the sensitivity to cadmium compared with the *pccs*Δ single mutant (Fig. 8*B*). Thus, when environmental metal ion levels are elevated, the Pccs protein appears to play a physiological function in protecting fission yeast cells against both copper and cadmium toxicity.

Overexpression of Pccs Domain IV Suppresses the Copper Toxicity Phenotype Resulting from ctr6$^+$ *Overexpression*—Based on the findings that deletion of the *pccs*$^+$ gene lowered copper tolerance in *S. pombe* and that both Pccs and Pccs domain IV conferred copper resistance when expressed in *S. cerevisiae ace1*Δ cells, we ascertained the ability of these proteins to elicit copper resistance when overexpressed in an *S. pombe* strain that is hypersensitive to copper. Overexpression of *ctr6*$^+$ from the thiamine-regulated *nmt1*$^+$ promoter resulted in an increased sensitivity to copper toxicity when transformed cells were grown on medium containing elevated concentrations of $CuSO_4$ (56). Cotransformation of both the *nmt1-ctr6*$^+$ and *nmt1-pccs*$^+$*I-IV* genes into a *S. pombe ctr6*Δ *pccs*Δ *zym1*Δ mutant strain conferred resistance to 15 μM copper on the transformed cells compared with the same cells that were co-transformed with *nmt1-ctr6*$^+$ and an empty vector (pREP3X) (Fig. 9). Similar to the expression of the full-length *pccs*$^+$ gene, cells expressing the *pccs*$^+$*IV* allele displayed no copper sensitivity. On the other hand, cells expressing the *pccs*$^+$*I-II-III* allele (without domain IV), were sensitive to copper. Consistent with its ability to sequester divalent metal ions, expression of *zym1*$^+$ conferred copper tolerance to the transformed cells. Furthermore, coexpression of the chimeric ^{1}Pccs^{222}-^{1}Zym1^{50} and Ctr6 proteins in the *ctr6*Δ *pccs*Δ *zym1*Δ mutant strain, allowed these cells to grow in the presence of exogenous copper at the same level as cells expressing the wild-type Pccs protein. This finding indicates that the first three domains of Pccs can be fused to a MT-like polypeptide to allow detoxification of excess copper ions. Together with the results from our studies on the *ace1*Δ yeast strain, these data strongly indicate that the role of Pccs in copper buffering is mediated through its fourth domain.

DISCUSSION

In eukaryotes, the CCS proteins function in the delivery of copper to the SOD1 enzyme (31, 84). The copper- and zinc-containing SOD is a free radical detoxifying protein that catalyzes the disproportionation of superoxide ion to yield hydrogen peroxide and dioxygen (85). Genetic, biochemical, and structural data have demonstrated fundamental features of CCS chaperones that revealed a mode of action for both the binding of copper and its specific requirement to incorporate copper into SOD1 (86). Typically, three distinct domains compose the copper carrier CCS (39). Domain I at the N terminus functions in copper binding under conditions of copper deprivation. Domain

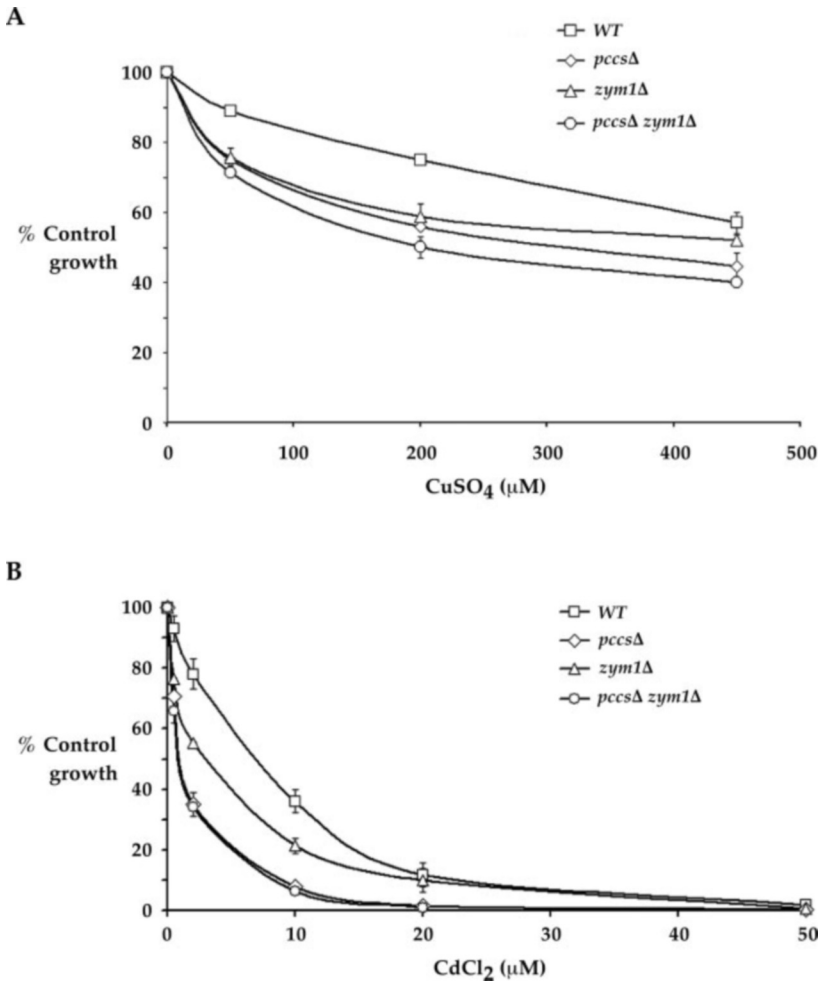

FIG. 8. **Effects of *pccs*Δ, *zym1*Δ, and *pccs*Δ *zym1*Δ gene deletions on tolerance to heavy metals.** Growth of the indicated isogenic yeast strains was assessed in Edinburgh minimal medium that was either untreated (*Control*) or supplemented with increasing concentrations of $CuSO_4$ (*A*) and $CdCl_2$ (*B*). Exponentially growing cells (2×10^4) were inoculated into 5 ml of liquid cultures and were incubated at 30 °C for 7 days without shaking. Total growth relative to that obtained in the absence of the metal ion (% control growth) was determined turbidimetrically at OD_{600}. Each point represents the mean of three separate experiments ± S.D.

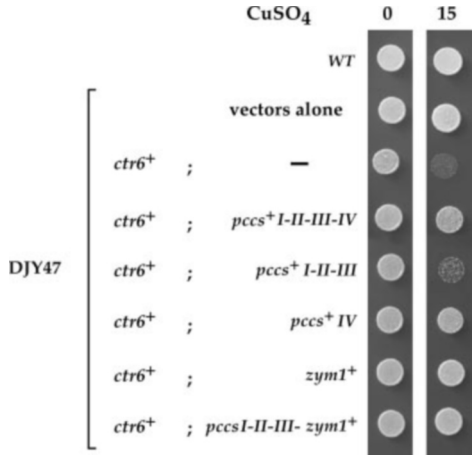

FIG. 9. **Full-length Pccs, Pccs IV, Pccs I-III-Zym1, or Zym1 suppresses the copper toxicity phenotype resulting from *ctr6*$^+$ overexpression.** The isogenic wild type (*WT*) FY435 and *ctr6*Δ *pccs*Δ *zym1*Δ DJY47 strains were co-transformed with the indicated plasmids. As controls, these strains were also co-transformed with the vectors pREP3X*his7*$^+$ and pREP3X*LEU2*. Cells were spotted onto Edinburgh minimal medium supplemented with 0 and 15 μM $CuSO_4$ and were grown at 30 °C for 4 days.

II is required for appropriate docking between CCS and SOD1. Domain III, which is highly conserved among CCS molecules across different species, binds copper and participates in the interaction with SOD1 during the process of copper transfer (39). The existence of a gene in fission yeast that encodes a CCS molecule with an extra domain at the C terminus of the protein raises two important questions: (i) What is the role of the fourth domain with respect to the function of the copper chaperone? (ii) What is the specific contribution of domain IV to cellular metal ion homeostasis in *S. pombe* cells? To address these questions, we first utilized a *S. cerevisiae* yeast system (70) in which the endogenous *CCS1* gene was inactivated. By using this approach, we sought to ensure the presence of Pccs as the sole protein with the ability to activate SOD1. When expressed in *S. cerevisiae ccs1*Δ cells under conditions of copper scarcity, the full-length *S. pombe* Pccs or a Pccs polypeptide spanning domains I to III provided cells with robust levels of activated SOD1. Furthermore, both forms of Pccs complemented the lysine auxotrophy of the *ccs1*Δ mutant cells. Therefore, these data revealed that both full-length Pccs and Pccs domains I-III can substitute for *S. cerevisiae* CCS1 and that domains I-III are sufficient for delivering copper to SOD1. These data further imply that the C-terminal domain IV of Pccs is dispensable with respect to the activation of SOD1 *in vivo*. To ascertain if the presence of the first three domains of Pccs was sufficient for SOD1 activity in *S. pombe*, the chromosomal *pccs*$^+$ gene was deleted by homologous recombination and SOD1 activity evaluated. Deletion of *pccs*$^+$ gene resulted in the loss of SOD1 activity under copper-limiting conditions. Similar to the *S. cerevisiae ccs1*Δ cells, the expression of only the first three domains of Pccs was sufficient to allow *pccs*Δ mutant cells to restore SOD1 activity. Moreover, a *S. pombe pccs*Δ strain expressing the *S. cerevisiae CCS1* gene was also capable of restoring SOD1 activity when copper was limiting.[4] On the basis of these data, we conclude that the presence of the first

[4] J. Laliberté and S. Labbé, unpublished data.

three domains of Pccs is sufficient for normal SOD1 activity in both yeast species.

With respect to the second question, the analysis of Pccs function in a *S. cerevisiae ace1*Δ strain revealed that expression of Pccs domain IV conferred copper tolerance to these cells, protecting them from lethal copper concentrations. Similarly, *ace1*Δ cells expressing the full-length Pccs or Zym1 MT protein acquired copper tolerance. Furthermore, consistent with the hypothesis that Pccs participates in intracellular copper buffering mainly through its fourth domain, we showed that *ace1*Δ cells expressing the first three domains of Pccs are more sensitive to copper by an order of magnitude compared with cells expressing full-length Pccs or Pccs domain IV. The involvement of Pccs in metal ion tolerance was further supported by three additional observations. First, the analysis of a *S. pombe* strain in which $pccs^+$ has been insertionally inactivated clearly demonstrates a phenotype with marked sensitivity to copper and cadmium ions. Second, the primary sequence of Pccs domain IV exhibits extensive homology to MTs and specifically harbors numerous repeats of a putative copper-binding Cys-Cys motif. Third, in a *S. pombe* strain overexpressing the $nmt1^+$-$ctr6^+$ allele that generates a hypersensitivity to copper ions, overexpression of Pccs domain IV, as well as full-length Pccs, protects the cells from the toxic effects of copper ions. Taken together, these data suggest a model wherein the first three domains of Pccs are required to specifically deliver copper to SOD1 under low copper concentrations, while the fourth domain of Pccs functions to sequester metals in the presence of elevated copper concentrations.

In this study, we inactivated the $sod1^+$ locus by deletion and replacement with the *S. pombe* $ura4^+$ gene. Although the *sod1*Δ cells grow poorly in shaken and well-aerated cultures, these cells were viable on standard YES medium or in a modified Edinburgh medium containing supplements of cysteine, methionine, lysine, adenine, histidine, and leucine. It should be noted that recent studies of *S. pombe* with a disrupted $sod1^+$ gene showed that the viability of *sod1*Δ mutants varied depending on the genetic background of the fission yeast strain (87, 88). Although the nature of this genetic variation is not clear, it is possible that the composition of the growth media may be a factor due to the amino acid auxotrophies of *sod1*Δ cells.

Our results thus far support a dual role for Pccs in copper homeostasis. It functions as a copper chaperone when copper concentrations are limiting and as a detoxifier when copper concentrations are in excess. Because its function is required in both high and low levels of copper, it is not surprising that the steady-state levels of $pccs^+$ mRNA is constitutive and unaffected by changes in copper concentrations. Consistently, expression of $pccs^+$ mRNA at steady-state levels, was independent of $cuf1^+$, a gene encoding the nutritional copper sensing *trans*-inducer of the copper transport genes $ctr4^+$, $ctr5^+$, and $ctr6^+$ in fission yeast. Interestingly, $pccs^+$ mRNA was fairly abundant in wild-type *S. pombe* cells possibly to maintain adequate intracellular levels of Pccs for both copper distribution and detoxification pathways.

Two notable differences exist between the *S. pombe* and *S. cerevisiae* CCS chaperones. First, the N-terminal domain I of the *S. pombe* Pccs lacks the copper-binding Met-*X*-Cys-X_2-Cys motif, except for the last cysteine residue, and second, Pccs contains a fourth domain at its C terminus. Given that Pccs lacking the N-terminal domain I cannot activate the SOD1 under copper-limiting conditions, it is likely that other residues within domain I besides the Met-*X*-Cys-X_2-Cys site that is found in CCS1 but not Pccs, are important for its metallochaperone-like activity *in vivo*. Efforts are currently under way to identify the residues within domain I that may be important for this function. Based on computer algorithm analysis, the C-terminal 75 amino acids of Pccs exhibited sequence similarity to MTs. Thus far, a single MT encoded by the $zym1^+$ locus has been described in *S. pombe* (51). In fission yeast cells, $zym1^+$ is transcriptionally induced by zinc and cadmium, but not copper (51). Although Zym1 suppresses zinc toxicity (51), our findings showed that *S. cerevisiae ace1*Δ cells expressing Zym1 were protected against copper toxicity. Deletion of $zym1^+$ ($zym1$Δ) from fission yeast resulted in reduced in copper tolerance in the mutant strain. However, as reported previously (51), the steady-state levels of $zym1^+$ mRNA were unaffected by cellular copper status.[5] Interestingly, deletion in the $pccs^+$ gene ($pccs$Δ) in *S. pombe* cells also resulted in increased sensitivity to copper toxicity. In a heterologous strain, expression of $pccs^+$*I-IV* or $pccs^+$*IV* in the copper sensitive *S. cerevisiae ace1*Δ cells conferred significant levels of resistance to these cells. Using this strain, we further demonstrated that a chimeric Pccs protein harboring the *S. pombe* metallothionein Zym1 instead of Pccs domain IV allowed detoxification of excess copper at the same level as the wild-type protein. Thus, the fact that there is no known MT in *S. pombe* whose expression is induced by the presence of copper, fission yeast cells may circumvent this situation by the use of two genes, $pccs^+$ and $zym1^+$, to protect cells against copper poisoning.

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