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Response of *Schizosaccharomyces pombe* to Zinc Deficiency▽†

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A component of the cellular response to zinc deficiency operates via control of transcript abundance. Therefore, microarray analysis was employed to identify *Schizosaccharomyces pombe* genes whose mRNA levels are regulated by intracellular zinc status. A set of 57 genes whose mRNA levels were substantially reduced in response to zinc deficiency was identified, while the mRNA levels of 63 genes were increased by this condition. In order to investigate the mechanisms that control these responses, a genetic screen was employed to identify mutants with defective zinc-responsive gene expression. Two strains (II-1 and V7) that were identified by this screen harbor mutations that are linked to *zrt1*$^+$, which encodes a putative Zrt/IRT-like protein (ZIP) zinc uptake transporter. Importantly, *zrt1*$^+$ mRNA levels are increased in response to zinc deprivation, and cells lacking functional Zrt1 are highly impaired in their ability to proliferate at limiting zinc concentrations. Furthermore, *zrt1* null cells were found to have severely reduced zinc contents, indicating that Zrt1 functions as a key regulator of intracellular zinc levels in fission yeast. The deletion of *fet4*$^+$, another zinc-responsive gene encoding a putative metal ion transporter, exacerbated the phenotypes associated with the loss of Zrt1, suggesting that Fet4 also plays a role in zinc uptake under limiting conditions.

Zinc is a structural component of numerous transcription factors, enzymes, and cell signaling proteins. Indeed, more than 3% of identified human proteins contain zinc-binding motifs (26), and as a result, a wide variety of cellular processes are dependent upon zinc (1, 32). Therefore, it is not surprising that zinc deficiency in humans is associated with numerous conditions, including impaired immune function, gastrointestinal problems, behavioral abnormalities, growth retardation, delayed wound healing, and dermatitis (29). All organisms must maintain intracellular zinc at an acceptable level, and therefore, cells possess specific zinc uptake systems that mediate its acquisition even when it is scarce.

Studies of the budding yeast, *Saccharomyces cerevisiae*, have provided insight into the molecular basis of zinc uptake. In *S. cerevisiae*, this process is predominantly mediated by two Zrt/IRT-like protein (ZIP) transporters, Zrt1 and Zrt2, which comprise a high-affinity and a low-affinity transport system, respectively (44, 45). Over 90 ZIP or solute carrier 39 (SLC39) family members have now been identified and are present in a wide range of organisms (10, 21). Humans have at least 15 of these transporters, and although the precise biological role of many of them has yet to be determined, at least a subset (human Zip 1 [hZip1], hZip2, hZip3, hZip4, hZip5, and hZip7) has been implicated in zinc transport (8, 10, 18, 21, 37). hZip4 appears to play a major role in dietary zinc absorption, as it is predominantly expressed in the intestine, and mutations in *ZIP4* are responsible for the zinc deficiency disorder acrodermatitis enteropathica (19, 38). ZIP transporters are also implicated in zinc transport in plants because the *Arabidopsis thaliana ZIP1*, *ZIP2*, and *ZIP3* genes all confer zinc uptake when expressed in yeast (14).

The control of zinc uptake is exercised at multiple levels. In zinc-deficient *S. cerevisiae* cells, Zrt1 is stable and located at the plasma membrane, but exposure to elevated zinc concentrations results in rapid endocytosis and degradation in the vacuole (11–13). There is evidence that similar mechanisms operate in mammalian cells because the mouse Zip 1 (mZip1), mZip3, and mZip4 transporters are all subject to zinc-stimulated endocytosis (9, 36). Such mechanisms appear to function to protect cells from the overaccumulation of zinc.

The cellular response to zinc is also controlled at the level of transcript abundance. In response to zinc deficiency, *S. cerevisiae ZRT1* and *ZRT2* mRNA levels are induced by more than 10-fold (46). This is mediated by the Zap1 transcription factor, which binds zinc-responsive promoter elements and induces the coordinate expression of around 40 genes whose products confer an advantage under conditions of zinc limitation (2, 23). The importance of this transcriptional control is underscored by the finding that *zap1* mutants have an impaired ability to grow under conditions of zinc limitation (46). There is evidence that zinc uptake is also regulated at the RNA level in both plant and mammalian cells. In the monocytic cell line THP-1, the level of *hZIP2* mRNA can be markedly induced by zinc depletion and downregulated by excess (4). In addition, the mRNA level of mZip4 has been demonstrated to increase in adult mice fed a zinc-deficient diet and to decrease upon zinc supplementation (9). Furthermore, *A. thaliana ZIP1*, *ZIP3*, and *ZIP4* mRNA levels are increased in zinc-limited plants (14). However, the mechanisms by which these responses are coordinated remain obscure, as homologues of *S. cerevisiae* Zap1 are not present in mammals or plants. Neither are Zap1 homologues present in the fission yeast *Schizosaccharomyces pombe*, which is evolutionarily divergent from *S. cerevisiae* (17). Thus, eukaryotic organisms from fission yeast to

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humans employ alternative mechanisms to regulate transcript abundance in response to zinc deficiency.

As *S. pombe* lacks a Zap1 homologue, we have used this system to investigate the control of mRNA levels in response to zinc limitation. Using RNA blot hybridization and transcript profiling, we have identified sets of genes whose mRNA levels are regulated in response to zinc deficiency. One highly induced gene was *adh4*$^+$, which encodes a putative iron-dependent alcohol dehydrogenase. In order to understand how this response is regulated, we have performed a genetic screen for mutants with aberrant gene expression that is regulated by a low level of zinc. Through this screen, we isolated 19 mutants, 2 of which displayed hypersensitivity to zinc deprivation. This hypersensitivity was found to be linked to the *zrt1* gene, which encodes a putative ZIP zinc uptake transporter. Cells lacking Zrt1 are highly impaired in their ability to proliferate under zinc-limiting conditions and furthermore have severely reduced zinc levels, indicating that Zrt1 mediates zinc uptake under limiting conditions.

MATERIALS AND METHODS

Strains and media. The genotypes of strains used in this study were *h*$^-$ (972), *h*$^+$ *ade6-M210 leu1-32 ura4-D18* (NT4), *h*$^-$ *ade6-M216 leu1-32 ura4-D18* (NT5), *h*$^-$ *ade6-M210 leu1-32 ura4-D18 zip1*::*ura4*$^+$ (*zip1*Δ), *h*$^+$ *ade6-M210 leu1-32 ura4-D18 zrt1*::*ura4*$^+$ (SW227), *h*$^+$ *ade6-M210 leu1-32 ura4-D18 zrt1-III* (SW538), *h*$^+$ *ade6-M210 leu1-32 ura4-D18 V7* (SW542), *h*$^-$ *zrt1-III* (SW511), *h*$^+$ *ade6*$^-$ *leu1-32 ura4-D18 fet4*::*kanMX4* (SW496), *h*$^+$ *ade6*$^-$ *leu1-32 ura4-D18 fet4*::*kanMX4 zrt1*::*ura4*$^+$ (SW500), and *h*$^-$ *ade6-M216 leu1-32 ura4-D18 cta3-lacZ*::*ura4*$^+$ (HAI003). Cell culture was performed in YE5S medium and, where selection was required, EMM (27). EMM is a defined medium whose $ZnSO_4$ concentration is 1.4 μM. Inductively coupled plasma mass spectrometry analysis of the YE5S medium indicated that its zinc concentration is approximately 11 μM. Chelex-treated synthetically defined (CSD) medium, which was used to limit zinc availability, was prepared as described previously (23), with some modifications. All glassware used for the preparation of the CSD medium was pretreated for approximately 12 h with 1% nitric acid and rinsed thoroughly in nanopure H_2O. Twenty grams of glucose and 5.1 g of yeast nitrogen base, without divalent cations or potassium phosphate (Bio 101), were dissolved in 1 liter nanopure H_2O. This was stirred overnight at 4°C with 25 g Chelex-100 ion-exchange resin (Sigma). After the removal of the resin, the pH of the solution was adjusted to 4.0 with HCl, and the following solutions were added: $MnSO_4$ (0.4 mg/ml), $FeCl_3$ (0.2 mg/ml), $CuSO_4$ (0.04 mg/ml), $CaCl_2$ (100 mg/liter), $MgSO_4$ (500 mg/liter), and KH_2PO_4 monobasic (100 g/liter). The resulting solution was then filter sterilized into polycarbonate containers. Inductively coupled plasma mass spectrometry analysis of the filtered CSD medium estimated the final metal concentrations as follows: Zn^{67}, 65 nM; Fe^{57}, 18.5 μM; and Cu^{63}, 174 μM. The culturing of the cells in CSD medium was carried out in polypropylene tubes (Falcon) and polycarbonate flasks (Nalgene). Cells were precultured overnight in CSD medium before being diluted into fresh CSD medium. To create a *zrt1*$^+$ null strain, oligonucleotides Zrt1KOA (5′-GCGTACGTCGACAACCACTTTGGATTCC TAAGG-3′) and Zrt1KOB (5′-CCAGATGGAGATAGCATCC-3′) were used to amplify a 1.5-kb region of the *zrt1*$^+$ open reading frame. The resulting DNA was digested with BamHI and SalI and ligated to the BamHI and SalI sites of pBluescript to yield pBSSK-zrt1. The 1.8-kb *ura4*$^+$ cassette from pRep42 was then cloned into the HindIII site to give plasmid pGEM-zrt1::ura4$^+$. Following digestion with BglII and BamHI, this plasmid was used to transform a strain to Ura$^+$, and correct insertion was confirmed by PCR analysis. A strain from which the *fet4*$^+$ gene was deleted was purchased from Bioneer.

Plasmids. An *adh4*$^+$ promoter-*lacZ* fusion plasmid was constructed by PCR amplifying a DNA fragment corresponding to positions −1380 to 115 (relative to the predicted ATG start codon) using primers Adh4BamHI (5′-TGGACTGG ATCCCGGTTGATTGATGCTTTAAGCC-3′) and Adh4EcoRI (5′-GCAGCT GAATTCTTACTTTCGATATGATCGAGC-3′). The resulting product was digested with EcoRI and BamHI before being ligated into the BamHI and EcoRI sites of pSPE356 (20) to yield pSPE356-*adh4*.

UV mutagenesis and genetic screens. NT4 cells transformed with pSPE356-*adh4* were subjected to random mutagenesis. Exponentially growing cells were spread onto EMM agar supplemented with 100 μM $ZnSO_4$ at a density of approximately 1×10^3 cells per plate. Cells were then subjected to UV irradiation using a Stratalinker UV cross-linker at a dosage that resulted in approximately 70% killing. Plates were incubated in the dark at 30°C for 4 to 5 days. The resulting colonies were transferred to filters and assayed for β-galactosidase activity as previously described (15). Quantitative β-galactosidase assays were also performed as previously described (34).

RNA analysis. Cell pellets were washed in H_2O and resuspended in 200 μl of RNA buffer (50 mM Tris HCl [pH 8.0], 100 mM NaCl, 50 mM EDTA [pH 8.0], 0.25% [wt/vol] sodium dodecyl sulfate) with 200 μl of phenol-chloroform in a 2-ml screw-cap Eppendorf tube. Cells were ruptured with 0.75 ml of 0.5-mm glass beads (Biospec) in a Ribolyser (Hybaid) using two 10-s bursts at full power. A further 0.75 ml of RNA buffer was added, followed by centrifugation in a microcentrifuge for 5 min. The aqueous layer was subjected to further phenol-chloroform extractions before the RNA was precipitated with 0.1 volume of sodium acetate (pH 5.2) and 0.6 volume of isopropanol. RNA pellets were washed in 70% (vol/vol) ethanol and resuspended in H_2O. A 10- to 15-μg sample of total RNA was denatured with glyoxal, separated on either a 1.2% or 1.4% (wt/vol) agarose gel prepared in 15 mM sodium phosphate (pH 6.5), and transferred to a GeneScreen hybridization membrane (Dupont NEN Research Products). DNA probes were produced by PCR amplification from genomic DNA using the appropriate primers. All probes were labeled with [α-^{32}P]dCTP by use of a Prime-a-Gene labeling kit (Promega).

Microarray analysis. Wild-type (972) and *zrt1-II1* (SW511) cells were grown to the mid-log phase in EMM at 30°C. RNA preparation, RNA labeling, and microarray analysis were performed as previously described (22). Microarray analysis was performed as two independent experiments. A gene was considered to be upregulated if its mRNA level for *zrt1-II1* cells compared to that for wild-type cells was increased ≥1.5-fold in both experiments and had a mean increase of ≥2-fold. A gene was considered to be downregulated if its mean mRNA level (*zrt1-II1*/wild type) was ≤0.5. All normalized data sets are available from our website (http://www.sanger.ac.uk/PostGenomics/S_pombe/).

Metal content analysis. Cell pellets from aliquots (10 ml) of cultures were washed in SSW (1 mM EDTA, 20 mM trisodium citrate [pH 4.2], 1 mM KH_2PO_4, 1 mM $CaCl_2$, 5 mM $MgSO_4$, 1 mM NaCl) and resuspended in 1 ml of 70% (vol/vol) HNO_3. Zinc contents were determined by atomic absorption spectrophotometry.

Microarray data accession number. Microarray data obtained in this study have also been submitted to ArrayExpress under accession number E-TABM-427.

RESULTS

As a first step in the identification of a zinc-sensing system in *S. pombe*, we sought to identify genes whose mRNA levels are increased by zinc deficiency. Transcript profiling of fission yeast stress responses has previously identified genes that are induced by the heavy metal cadmium but not by other environmental insults, such as oxidative and osmotic stress (5). Cadmium is known to displace zinc ions from proteins (33) and therefore may, to some degree, mimic zinc deprivation, suggesting that some of these cadmium-inducible genes may be regulated in response to zinc status. Consistent with this, a number of these genes are homologues of *S. cerevisiae* Zap1 targets. These include SPBC1348.06c, which is homologous to the budding yeast genes *VEL1* and *YOR387C*, *adh4*$^+$ (SPAC5H10.06c), which encodes a putative iron-dependent alcohol dehydrogenase homologous to *S. cerevisiae ADH4* (31), and a gene (SPBC16D10.06) that we named *zrt1*$^+$ because of its similarity to the budding yeast *ZRT1* and *ZRT2* genes (44, 45). We reasoned that the mRNA levels of these fission yeast genes may be regulated in response to cellular zinc status and so analyzed them in more detail. RNA blot hybridization confirmed that SPBC1348.06c, *adh4*$^+$, and *zrt1*$^+$ mRNA levels were induced in response to cadmium. In addition, *adh4*$^+$ and *zrt1*$^+$ transcripts were induced in response to high levels of copper (Fig. 1). Prolonged exposure (30 min) of cells to high levels of copper also resulted in the production of longer

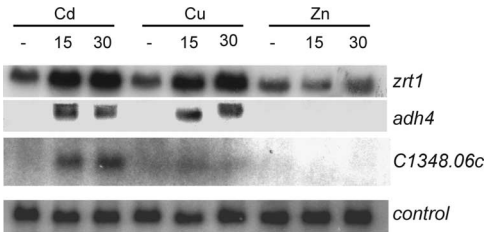

FIG. 1. RNA was prepared from wild-type cells treated with 0.5 mM $CdSO_4$, 2 mM $CuSO_4$, or 2 mM $ZnSO_4$ for 0, 15, or 30 min and was subjected to RNA blot hybridization using *his3*$^+$ (control), *zrt1*$^+$, SPBC1348.06c, and *adh4*$^+$ probes.

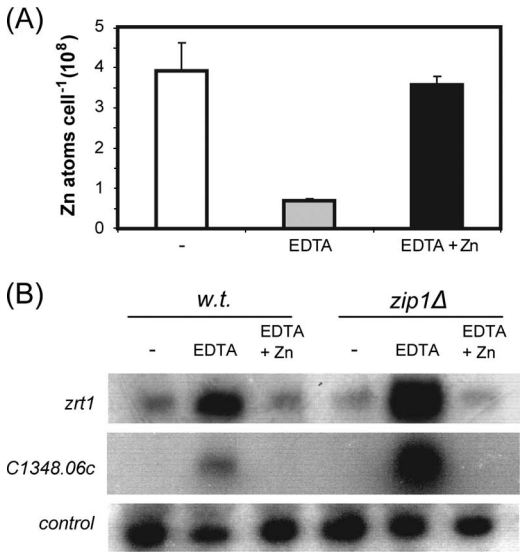

FIG. 2. (A) Wild-type cells were grown to exponential phase in YE5S, YE5S supplemented with 60 μM EDTA, or YE5S supplemented with 60 μM EDTA and 60 μM $ZnSO_4$. Cellular zinc contents were measured by atomic absorption spectrometry. Shown are the mean values from three experiments. Error bars indicate standard deviations. (B) Total RNA was prepared from cells grown as described for panel A and subjected to RNA blot hybridization using *his3*$^+$ (control), *zrt1*$^+$, and SPBC1348.06c probes. w.t., wild type.

adh4$^+$ transcripts. While the reason for this is not clear, it is interesting to note that we have previously observed a similar effect with the *zym1*$^+$ gene (3). In contrast to what occurs with cadmium and copper, none of these genes was induced by high levels of zinc (Fig. 1). In order to test whether their mRNA levels are increased in response to zinc deficiency, intracellular zinc levels were limited by growing cells in rich medium (YE5S) supplemented with the metal chelator EDTA at 60 μM. Metal content analysis revealed that this treatment substantially reduced intracellular zinc levels (Fig. 2A). Furthermore, RNA blot hybridization demonstrated that the mRNA levels of both SPBC1348.06c and *zrt1*$^+$ were significantly increased when cells were grown in the presence of EDTA. Cellular zinc levels were restored by further supplementing the EDTA-containing medium with $ZnSO_4$ at 60 μM (Fig. 2A). Furthermore, this treatment also reduced transcripts to basal levels (Fig. 2B).

The results suggest that the mRNA levels of these genes are regulated by zinc limitation. However, EDTA is not a zinc-specific metal chelator, and thus, we could not exclude the possibility that the observed effects were due to changes in the intracellular concentrations of other cations. Therefore, in order to conclusively demonstrate that the expression of SPBC1348.06c, *adh4*$^+$, and *zrt1*$^+$ is controlled by zinc deficiency, it was necessary to establish conditions for the culture of *S. pombe* cells in which zinc, but not other metal ions, was limited. Therefore, we employed CSD medium, which was originally developed for the culture of *S. cerevisiae* at limiting zinc concentrations (23). In this case, the metal availability of synthetic defined medium is first limited by treatment with Chelex-100 ion-exchange resin. Following this treatment, cations other than zinc (Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, K^+) are added back, resulting in a medium whose Zn^{2+} concentration is submicromolar (23). Indeed, analysis of a batch of CSD medium indicated a zinc concentration of 65 nM (data not shown). We found that CSD medium was able to support the growth of wild-type fission yeast cells without zinc supplementation. However, growth rates were clearly enhanced by the addition of 20 μM $ZnSO_4$, indicating that CSD medium is indeed zinc limiting (Fig. 3A). This is reflected in the very low intracellular zinc content of cells grown in CSD medium compared to that of cells grown in CSD medium supplemented with 20 μM $ZnSO_4$ (Fig. 3B). Growth rates were not further enhanced by the addition of zinc at concentrations up to 60 μM, and above this concentration, zinc impaired growth rates (data not shown). Importantly, the addition of iron or copper did not enhance the growth rate, indicating that this medium is not limited for these metal ions (data not shown). Having established the appropriate conditions, we compared the transcript levels from cells grown at "adequate" (CSD medium plus 20 μM $ZnSO_4$) and "limiting" (CSD medium) zinc concentrations (Fig. 3C). This revealed that the mRNA levels of SPBC1348.06c, *zrt1*$^+$, and *adh4*$^+$ were highly increased by zinc limitation.

In fission yeast, the Sty1/Spc1 stress-activated protein kinase pathway controls the expression of numerous genes in response to a range of adverse conditions (35). However, microarray analysis has suggested that the induction of *adh4*$^+$ and *zrt1*$^+$ transcripts in response to cadmium is independent of this pathway (5), and this was confirmed by RNA blot hybridization (data not shown). This suggests that the increase in mRNA levels in response to zinc deficiency does not require the Sty1/Spc1 pathway. We therefore investigated the role of the Zip1 transcription factor, which mediates the activation of a set of genes in response to cadmium exposure (16). Indeed, the induction of SPBC1348.06c in response to cadmium has previously been shown to be Zip1 dependent (16). However, RNA blot hybridization demonstrated that the increase of SPBC1348.06c mRNA in response to the chelator EDTA occurred in a *zip1*Δ background (Fig. 2B). Furthermore, the deletion of *zip1*$^+$ resulted in an increased level of SPBC1348.06c transcripts, suggesting that Zip1 also has a repressive effect on this gene (Fig. 2B). The induction of *zrt1*$^+$ mRNA levels in response to EDTA was also found to be independent of Zip1.

These findings suggest that Zip1 does not control the response to zinc limitation. Therefore, in order to investigate the mechanisms by which transcript levels are controlled in re-

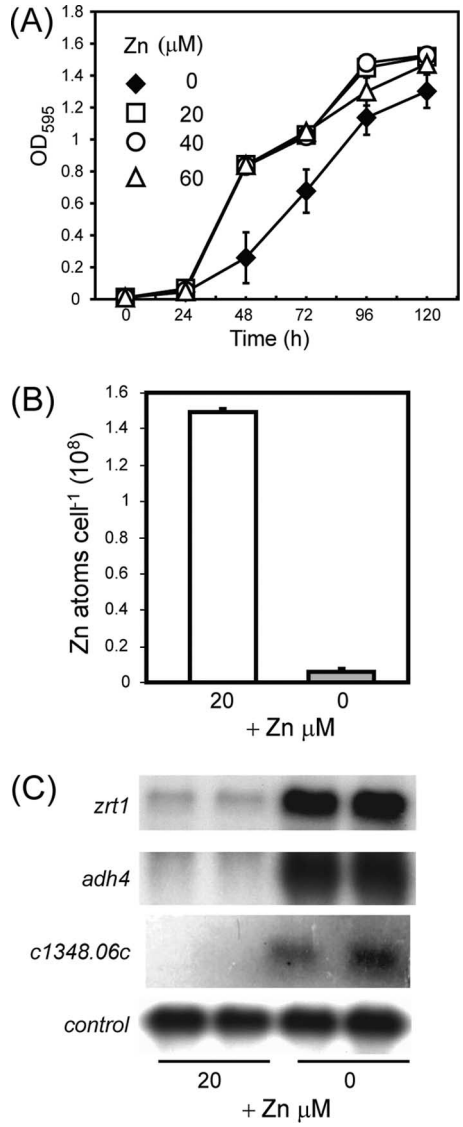

FIG. 3. (A) Growth in CSD medium. Wild-type (wt) cells were precultured in CSD medium and then inoculated into CSD medium supplemented with 20 to 60 μM $ZnSO_4$. Cultures were incubated at 30°C, and the optical densities at 595 nm (OD_{595}) were measured every 24 h over a 120-h period. Shown are the mean values from three experiments. Error bars indicate standard deviations. (B) Zinc contents of wild-type cells cultured in CSD medium. Wild-type cells were cultured overnight in CSD medium and then inoculated into CSD medium with or without 20 μM $ZnSO_4$ and incubated at 30°C until they proliferated exponentially. Total cellular zinc contents were measured by atomic absorption spectrometry. Shown are the mean values from three experiments. Error bars indicate standard deviations. (C) Gene expression in response to a low level of zinc. Wild-type cells were precultured in CSD medium and then inoculated into CSD medium with or without 20 μM $ZnSO_4$. Total RNA was prepared after a 24-h incubation at 30°C and subjected to RNA blot hybridization with *zrt1*$^+$, *adh4*$^+$, SPBC1348.06c, and SPBPB2B2.08 (control) probes.

sponse to zinc deprivation, we constructed an *adh4* promoter-*lacZ* fusion reporter and determined whether its expression was regulated in response to zinc availability (Fig. 4). The reporter was expressed at a low (basal) level when cells were grown in liquid medium (EMM). Supplementing the medium with excess zinc ($ZnSO_4$, 200 μM) did not affect the expression of the reporter (see Fig. S1 in the supplemental material), suggesting that wild-type cells are "zinc replete" when grown in liquid EMM. In contrast, the expression of the *adh4-lacZ* reporter was markedly increased by growing cells in medium supplemented with the chelator EDTA, which reduces intracellular zinc levels (Fig. 4A). Consistent with this, when cells were grown in limiting zinc medium (CSD medium), high levels of reporter expression were observed, which were suppressed by the addition of $ZnSO_4$ (20 μM) to the medium (Fig. 4B). Thus, the expression of the *adh4-lacZ* reporter is regulated in response to zinc deprivation. Furthermore, truncation analysis of the reporter indicated that *adh4*$^+$ expression is controlled by a combination of activating and repressing sequence elements (see Fig. S2 in the supplemental material).

We next explored the possibility of using this reporter as the basis of a genetic screen. β-Galactosidase filter assays revealed that colonies of wild-type cells containing this reporter go blue rapidly when grown on minimal agar (EMM). This suggested that EMM agar is somewhat limiting for zinc, which is likely to be due to the ability of the agar to chelate metals and thus restrict zinc availability (Fig. 4C). Consistent with this, we found that supplementing the agar with $ZnSO_4$ at 100 μM repressed the expression of the *adh4*$^+$ reporter, because colonies grown under these conditions remained white for an extended period. It should be noted that wild-type fission yeast cells can tolerate relatively high levels of zinc (up to approximately 2 mM) (3), which suggested that the observed zinc-mediated repression of the *adh4*$^+$*-lacZ* reporter is a specific effect. In support of this, the activity of an unrelated reporter (*cta3-lacZ*) was not detectably influenced by the addition of zinc to the medium (Fig. 4C). Therefore, we next sought to isolate mutants with an impaired ability to mediate the zinc-dependent repression of the *adh4-lacZ* reporter. Cells carrying the *adh4-lacZ* reporter (pSPE356-adh4) were plated onto zinc-supplemented EMM agar and subjected to random mutagenesis, and colonies with high levels of β-galactosidase activity were identified. Approximately 200,000 colonies were screened, from which 19 mutants with increased expression of the *adh4-lacZ* reporter were isolated. An example of some of these mutants is shown in Fig. 4D. Mutant strains were cured of their reporter plasmids, followed by the reintroduction of the reporter. This identified three strains with plasmid-associated (*cis*) mutations. The remaining strains with *trans*-acting mutations were analyzed in order to determine whether any of them possessed altered sensitivity to excess or limiting zinc. None of the strains was sensitive to excess zinc (data not shown). However, two mutant strains, II1 and V7, were hypersensitive to EDTA (Fig. 5A). Importantly, this sensitivity could be rescued by the addition of equimolar zinc but not iron or copper. Genetic analysis of these strains indicated that the EDTA hypersensitivity of these strains resulted from one recessive mutation (or several tightly linked recessive mutations). Quantitative β-galactosidase assays confirmed that the basal expression of the *adh4-lacZ* reporter was vastly increased (approximately 2 orders of magnitude) in these mutants compared to that in the wild-type background. However, the overexpression of the *adh4-lacZ* reporter in the mutant backgrounds was suppressed by supplementing the medium with increasing concentrations of $ZnSO_4$ (Fig. 5B and C). This coupled with the

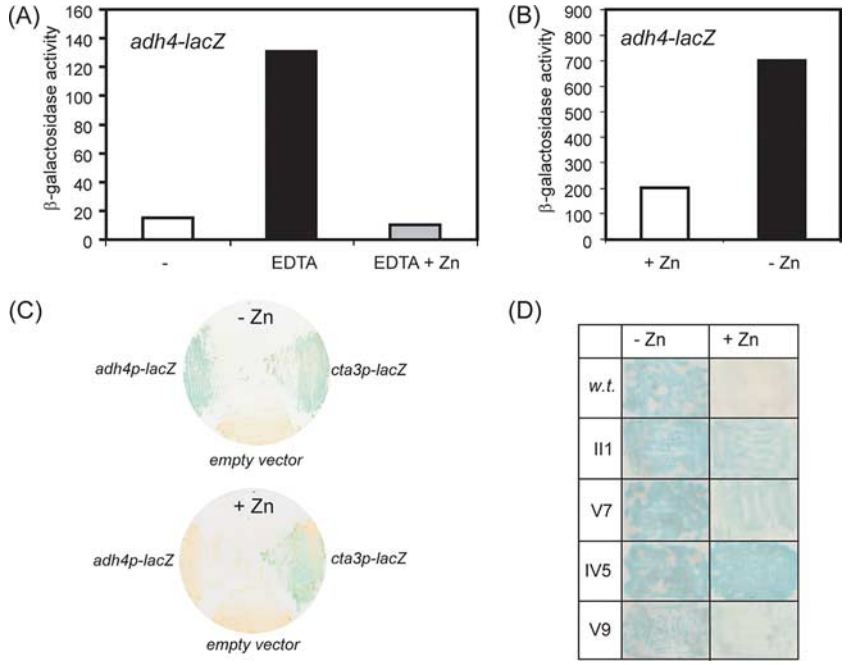

FIG. 4. Expression of an *adh4*$^+$ promoter-*lacZ* reporter in response to limiting zinc. (A) Wild-type cells carrying the *adh4*$^+$ promoter-*lacZ* reporter were cultured at 30°C in EMM (−), EMM supplemented with 10 μM EDTA, or EMM supplemented with 10 μM EDTA and 10 μM $ZnSO_4$ until they proliferated exponentially. Cells were then harvested and processed for liquid β-galactosidase assays. Shown are the mean values (Miller units) from two experiments. (B) Cells were cultured at 30°C in CSD medium with or without 20 μM $ZnSO_4$ and processed as described for panel A. Shown are the mean values (Miller units) from two experiments. (C) Cells containing the *adh4*$^+$ promoter-*lacZ* fusion plasmid, an empty vector (pSPE356), or an integrated *cta3*$^+$ promoter-*lacZ* reporter were cultured onto nitrocellulose membranes on EMM agar (−Zn) or EMM agar supplemented with 0.1 mM $ZnSO_4$ (+Zn) for 2 days at 30°C. Filters were then subjected to β-galactosidase assays as described in Materials and Methods. (D) Examples of mutants isolated from the genetic screen. Cells were cultured onto EMM agar or EMM agar supplemented with 0.1 mM $ZnSO_4$, transferred to filters, and processed for β-galactosidase assays. w.t., wild type.

finding that the V7 and II1 mutants are hypersensitive to EDTA suggested that these strains may have aberrant intracellular zinc contents resulting from impaired zinc uptake. As the *zrt1*$^+$ gene encodes a putative zinc uptake transporter, we reasoned that these strains may harbor mutations in this gene. DNA sequencing revealed that the *zrt1* gene from the II1 strain contained a G-to-A mutation which changes the Trp 141 codon to a stop codon and would therefore be predicted to result in a severely truncated protein. In order to confirm that *zrt1-II1* encodes a nonfunctional protein, we constructed a strain in which *zrt1*$^+$ was disrupted. As expected, *zrt1*Δ cells were also found to be hypersensitive to EDTA, a phenotype that could

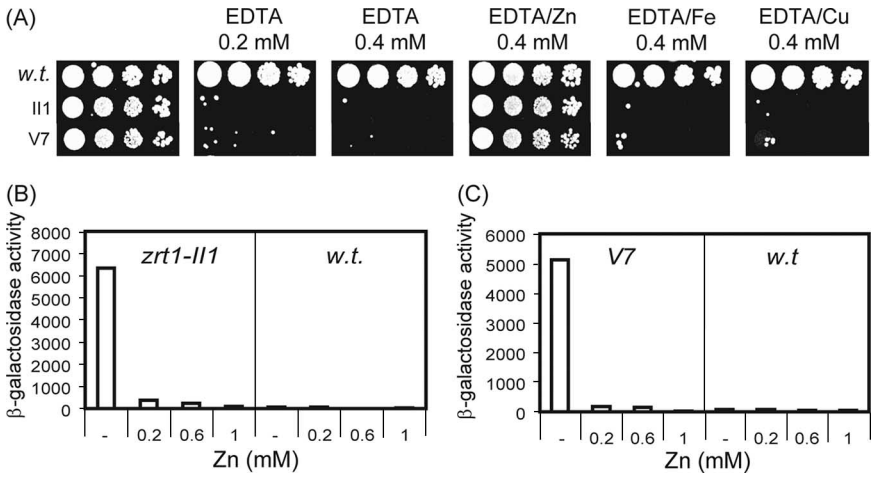

FIG. 5. (A) Wild-type (w.t.), *zrt1-III*, and V7 strains were grown to exponential phase, subjected to fivefold serial dilutions, and spotted onto YE5S supplemented with the indicated concentrations of EDTA, $ZnSO_4$, $FeCl_3$, and $CuSO_4$. Plates were incubated at 30°C for 2 days. (B and C) The indicated strains were grown to exponential phase in YE5S or YE5S supplemented with the indicated concentrations of $ZnSO_4$. Cells were then harvested and processed for liquid β-galactosidase assays. Shown are the mean values (Miller units) from duplicate experiments.

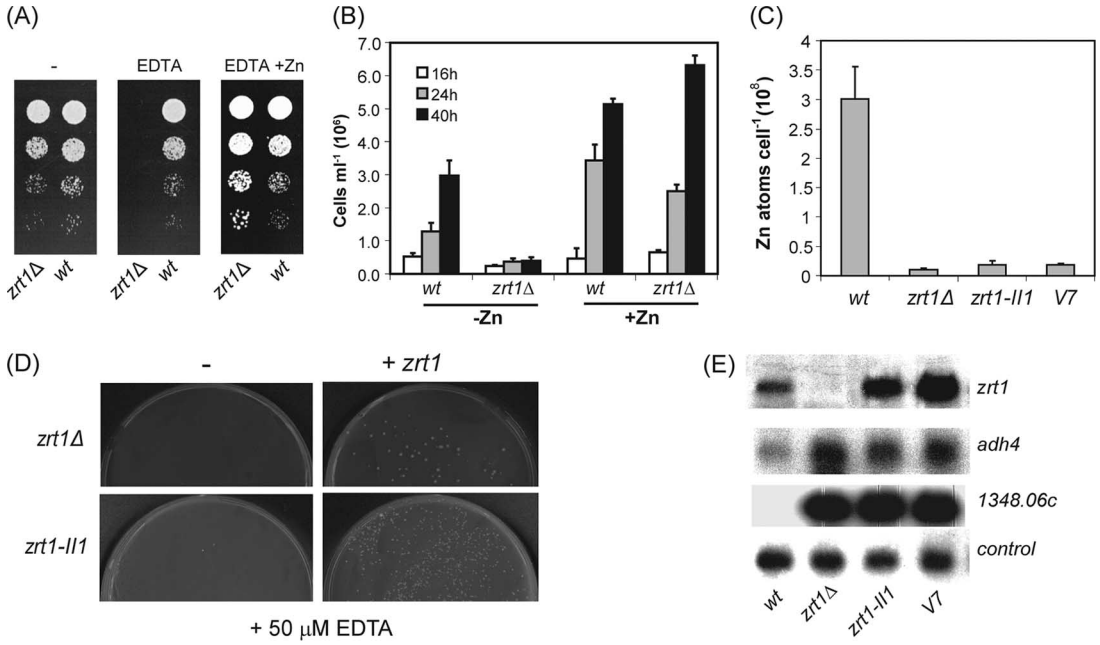

FIG. 6. (A) Wild-type (wt) and *zrt1*Δ strains were grown to exponential phase, subjected to fivefold serial dilutions, and spotted onto YE5S agar supplemented with EDTA (200 μM) and $ZnSO_4$ (200 μM) as indicated. Plates were incubated at 30°C for 2 days. (B) Wild-type and *zrt1*Δ strains were precultured in CSD medium and then inoculated into CSD medium (−Zn) or CSD medium supplemented with 20 μM $ZnSO_4$ (+Zn). Cultures were incubated at 30°C, and cell titers were determined at the indicated time points. Shown are the mean values from three experiments. Error bars indicate standard deviations. (C) Total cellular zinc contents of the indicated strains were measured by atomic absorption spectrometry. Shown are the mean values from three experiments. Error bars indicate standard deviations. (D) Complementation of *zrt1*$^-$ mutants. Cultures of the *zrt1*Δ strain and *zrt1-II1* were transformed with carrier DNA (−) or with carrier DNA and a DNA fragment containing the *zrt1*$^+$ open reading frame (+ *zrt1*). Cells were plated onto EMM agar plates supplemented with EDTA (50 μM) and incubated at 30°C for 3 to 4 days. (E) Total RNA was prepared from the indicated strains and subjected to RNA blot hybridization using *his3*$^+$ (control), *zrt1*$^+$, *adh4*$^+$, and SPBC1348.06c probes.

be rescued by adding back equimolar $ZnSO_4$ (Fig. 6A). Furthermore, the *zrt1*Δ strain showed a very limited ability to proliferate in CSD medium unless it was supplemented with zinc (Fig. 6B), indicating that Zrt1 function is important for viability at limiting zinc concentrations. Indeed, these findings imply that Zrt1 plays a key role in maintaining intracellular zinc levels. Consistent with this, metal content analysis demonstrated that intracellular zinc levels were severely reduced in cells lacking functional Zrt1 (Fig. 6C). Next, we sought to reintegrate the wild-type *zrt1*$^+$ allele into the *zrt1*Δ and *zrt1-II1* mutants in order to complement their phenotypes. Indeed, when cells were transformed with a DNA fragment containing the wild-type *zrt1*$^+$ gene, numerous colonies that had regained the ability to grow in the presence of EDTA were obtained (Fig. 6D). This demonstrates that the EDTA-sensitive phenotype is the result of mutation of the *zrt1*$^+$ gene.

The V7 strain has phenotypes that are indistinguishable from those of the *zrt1*Δ and *zrt1-II1* strains. Indeed, intracellular zinc content was also severely reduced in this mutant. Therefore, it was surprising that the sequencing of V7 did not reveal any mutations within the predicted *zrt1*$^+$ open reading frame in this background. Nonetheless, genetic analysis suggested that the V7 mutation is linked to the *zrt1* locus. Analysis of 13 tetrads resulting from a genetic cross between the *zrt1*Δ strain and V7 revealed that all of the progeny were hypersensitive to EDTA. Furthermore, a *zrt1*Δ/V7 diploid strain was also found to be hypersensitive to zinc limitation.

We compared the transcript levels in the *zrt1*Δ, *zrt1-II1*, and V7 backgrounds and found, as expected, that *adh4*$^+$, *zrt1*$^+$, and SPBC1348.06c mRNA levels were increased in the absence of functional Zrt1 protein (Fig. 6E). In order to identify other genes whose mRNA levels are regulated in response to zinc deficiency, we used microarray analysis to compare wild-type cells with *zrt1-II1* cells, which have severely reduced intracellular zinc levels. This identified 57 genes whose mRNA levels were reduced by zinc deficiency (Table 1), and 63 genes were found to have increased mRNA levels under these conditions (Table 2). In order to validate the transcript profiling data, a number of genes were also analyzed by RNA blot hybridization (Fig. 7). Prominent among the genes with reduced transcript levels were those encoding proteins involved in protein synthesis, for instance, ribosomal subunits (*rpl3002*$^+$, *rpl31*$^+$, *rpl34*$^+$, *rps403*$^+$, *rpl1101*$^+$, and *rps801*$^+$) and proteins involved in amino acid uptake (SPBC359.03c, SPBC359.01) and biosynthesis (*apt1*$^+$, *eca39*$^+$, SPCC364.07, SPBC428.11, SPBPB2B2.05, SPAP8A3.07c). Furthermore, the mRNA levels of several genes whose products are involved in nucleotide metabolism (SPCC965.14c, SPCC1442.14c, *ura1*$^+$) were also reduced under these conditions. These findings are consistent with the finding that zinc deficiency led to reduced growth rates. Zinc limitation also led to a decrease in the transcript abundance of genes involved in the acquisition of phosphate, such as *pho1*$^+$ (acid phosphatase) and SPBC8E4.01c, which encodes an inorganic phosphate transporter. A further response to zinc deficiency was the downregulation of the mRNA levels of genes whose products are involved in iron and sulfur uptake

TABLE 1. Genes downregulated by zinc limitation

Gene	Function[a]	Relative mRNA level (level in *zrt1-III* cells/level in wild-type cells)		
		Expt 1	Expt 2	Mean
isp3$^+$	Upregulation of meiotic expression	0.196	0.047	0.12
pho1$^+$	Acid phosphatase	0.113	0.176	0.14
SPAC27D7.10c	Similar to SPAC27D7.09c, SPAC27D7.11c, and SPBC3D6.02	0.249	0.178	0.21
str3$^+$	Siderophore-iron transporter	0.218	0.210	0.21
SPBPB10D8.02c	Acylsulfatase (predicted)	0.310	0.144	0.23
SPBC359.03c	Amino acid permease family (predicted)	0.274	0.201	0.24
SPCC965.14c	Cytosine deaminase (predicted) zinc metalloenzyme	0.258	0.240	0.25
SPAC27D7.09c	Similar to SPAC27D7.10c, SPAC27D7.11c, and SPBC3D6.02	0.332	0.169	0.25
wis2$^+$	Cyclophilin	0.311	0.269	0.29
SPAC11D3.18c	Nicotinic acid plasma membrane transporter (predicted)	0.262	0.334	0.30
SPAC27D7.11c	Similar to SPAC27D7.09c, SPAC27D7.10c, and SPBC3D6.02	0.405	0.199	0.30
SPAC869.05c	Sulfate transporter (predicted)	0.367	0.248	0.31
SPBC1347.11	Sequence orphan	0.442	0.207	0.32
SPBC1539.07c	Glutathione-dependent formaldehyde dehydrogenase (predicted)	0.371	0.302	0.34
SPBC3E7.06c	Major facilitator superfamily membrane transporter	0.335	0.355	0.35
pan6$^+$	Pantoate–beta-alanine ligase	0.360	0.336	0.35
SPAC11D3.02c	ELLA family protein	0.368	0.342	0.36
SPBC21C3.08c	Ornithine aminotransferase	0.432	0.294	0.36
SPBPB10D8.01	Membrane transporter	0.405	0.371	0.39
aes1$^+$	Enhancer of RNA-mediated gene silencing	0.460	0.348	0.40
SPCC364.07	D-3 phosphoglycerate dehydrogenase (predicted)	0.464	0.368	0.42
SPBP8B7.05c	Carbonic anhydrase (predicted)	0.501	0.343	0.42
apt1$^+$	Adenine phosphoribosyltransferase (predicted)	0.423	0.422	0.42
SPAC23C4.06c	Methyltransferase (predicted)	0.409	0.450	0.43
SPBC8E4.01c	Inorganic phosphate transporter (predicted)	0.513	0.366	0.44
SPAC11D3.13	ThiJ domain	0.454	0.451	0.45
rpl3002$^+$	60S ribosomal protein L30	0.516	0.391	0.45
rpl31$^+$	60S ribosomal protein L31	0.508	0.399	0.45
SPBC359.01	Amino acid permease family	0.538	0.375	0.46
SPCC330.06c	Thioredoxin peroxidase	0.566	0.349	0.46
eca39$^+$	Branched-chain amino acid aminotransferase	0.486	0.429	0.46
SPCC622.12c	NADP-specific glutamate dehydrogenase	0.462	0.454	0.46
egd2	Nascent polypeptide-associated complex (alpha subunit)	0.549	0.374	0.46
sks2$^+$	Heat shock protein 70 family	0.539	0.387	0.46
SPBPB2B2.05	GMP synthase	0.532	0.399	0.47
rpb8$^+$	DNA-directed RNA polymerase I, II, and III subunit	0.561	0.371	0.47
SPCP20C8.03	Pseudogene	0.491	0.448	0.47
SPAP8A3.07c	Phospho-2-dehydro-3-deoxyheptonate aldolase	0.588	0.360	0.47
SPBC428.11	Homocysteine synthase	0.562	0.389	0.48
SPBC13A2.04c	PTR family peptide transporter	0.518	0.434	0.48
rpl34$^+$	60S ribosomal protein L34	0.591	0.368	0.48
SPBPB2B2.06c	Calcineurin-like phosphoesterase	0.579	0.385	0.48
SPCC330.07c	Membrane transporter	0.437	0.530	0.48
cdb4$^+$	Metallopeptidase	0.537	0.431	0.48
meu19$^+$	Noncoding RNA	0.566	0.403	0.48
hsp90$^+$	Hsp90 family	0.555	0.420	0.49
rps403$^+$	40S ribosomal protein S4	0.598	0.383	0.49
sod1$^+$	Superoxide dismutase	0.525	0.462	0.49
snz1$^+$	pyridoxine biosynthesis protein	0.479	0.513	0.50
rpl1101$^+$	60S ribosomal protein L11	0.533	0.461	0.50
ssa1$^+$	Heat shock protein 70 family	0.563	0.433	0.50
rps801$^+$	40S ribosomal protein S8	0.544	0.452	0.50
ubc15$^+$	Ubiquitin-conjugating enzyme	0.502	0.497	0.50
isu1$^+$	Iron-sulfur cluster assembly scaffold protein	0.638	0.362	0.50
SPCC1442.14c	Adenosine 5′-monophosphoramidase	0.559	0.446	0.50
sua1$^+$	Sulfate adenylyltransferase (ATP)	0.610	0.396	0.50
ura1$^+$	Carbamoyl-phosphate synthase	0.525	0.482	0.50

[a] The descriptions of the protein functions are based on information for *S. pombe* in GeneDB (http://www.genedb.org/genedb/pombe/index.jsp).

(SPAC869.05c, *str3*$^+$) and utilization (*isu1*$^+$, *sua1*$^+$, SPBPB10D8.02c). Thus, zinc limitation impacts a variety of cellular processes.

Interestingly, the *S. pombe* genes whose mRNA levels were most highly increased by zinc deficiency have homologues in *S. cerevisiae* that are similarly regulated. These include SPBC1348.06c and the closely related genes SPBPB2B2.15 and SPAC977.05c, which are homologous to the budding yeast Zap1-regulated genes *VEL1* and *YOR387C*. At present, the functions of the proteins encoded by these genes remain ob-

TABLE 2. Genes upregulated by zinc limitation

Gene	Function[a]	Relative mRNA level (level in *zrt1-III* cells/level in wild-type cells)		
		Expt 1	Expt 2	Mean
SPBC1348.06c	Similar to SPBPB2B2.15 and SPAC977.05c	165.91	194.24	180.1
SPAC977.05c	Similar to SPBC1348.06C and SPBPB2B2.15	117.46	179.50	148.5
adh4$^+$	Alcohol dehydrogenase	42.52	56.47	49.5
SPBPB2B2.15	Similar to SPBC1348.06C and SPAC977.05c	35.28	22.88	29.1
zrt1$^+$ (SPBC16D10.06)	ZIP zinc transporter	9.72	14.04	11.9
dak2$^+$	Dihydroxyacetone kinase	5.14	10.26	7.7
SPBP26C9.03c	Metal ion transporter similar to *S. cerevisiae FET4*	4.86	7.52	6.2
SPCC18B5.02c	Cinnamoyl-coenzyme A reductase pseudogene	3.27	4.65	4.0
SPBC12C2.03c	Flavin adenine dinucleotide binding protein (predicted)	3.30	4.40	3.9
tlh1$^+$	RecQ type DNA helicase	4.51	2.92	3.7
SPAPB1A10.14	F-box protein	2.73	4.57	3.7
SPAC23H3.15c	Sequence orphan	5.64	1.53	3.6
SPBC1652.01	Predicted Sin3-interacting protein	3.58	3.40	3.5
SPAC977.04	Pseudogene	2.70	3.80	3.3
SPAC212.09c	Pseudogene	2.92	3.45	3.2
SPAC750.08c	NAD-dependent malic enzyme	2.88	3.28	3.1
SPAPB18E9.04c	Glycoprotein (predicted)	2.86	3.02	2.9
mae2$^+$	Malate dehydrogenase	2.71	3.15	2.9
SPCC663.08c	Short-chain dehydrogenase (predicted)	2.51	3.21	2.9
SPAC57A10.06	Sequence orphan	2.54	3.16	2.9
SPBPB7E8.01	Glycoprotein (predicted) neutral zinc metallopeptidases	3.12	2.54	2.8
SPBC1348.05	MFS family membrane transporter	2.25	3.21	2.7
SPAC22A12.06c	Serine hydrolase	1.94	3.43	2.7
obr1$^+$	Flavodoxin-like protein	2.51	2.83	2.7
SPCC663.06c	Short-chain dehydrogenase (predicted)	2.48	2.81	2.6
SPAC2H10.01	Zn_2-Cys_6 transcription factor	3.31	1.90	2.6
SPAC977.07c	Glycoprotein	1.50	3.64	2.6
tpx1$^+$	Thioredoxin peroxidase	2.48	2.60	2.6
php2$^+$	CCAAT-binding factor complex subunit	2.83	2.24	2.5
SPBCPT2R1.07c	Malic pseudogene	2.43	2.59	2.5
SPAC13F5.03c	Glycerol dehydrogenase	2.85	2.17	2.5
SPCC794.01c	Glucose-6-phosphate 1-dehydrogenase	2.27	2.74	2.5
map2$^+$	Pheromone	2.24	2.75	2.5
rds1$^+$	Stress-induced protein	3.42	1.56	2.5
SPAC23C11.06c	Membrane protein	3.13	1.77	2.5
pex7$^+$	Peroxisomal signal receptor	2.43	2.42	2.4
gst2$^+$	Glutathione *S*-transferase	2.36	2.45	2.4
SPAC4F10.06	Conserved fungal protein	2.14	2.63	2.4
srx1$^+$	Sulfiredoxin	3.05	1.72	2.4
SPAC4G8.03c	Pumilio family RNA binding protein	2.31	2.39	2.4
SPBC8E4.02c	Sequence orphan	2.20	2.49	2.3
SPAC22A12.17c	Short-chain dehydrogenase (predicted)	2.36	2.31	2.3
SPAC16.05c	Transcription factor similar to those of *S. cerevisiae* SFP1	2.29	2.32	2.3
SPAC1786.02	Phospholipase (putative)	2.12	2.42	2.3
SPBC1773.13	Aromatic aminotransferase (predicted)	2.34	2.13	2.2
SPCC1235.01	Glycoprotein (predicted)	2.05	2.41	2.2
SPCC569.05c	Spermidine family transporter (predicted)	2.11	2.28	2.2
SPAC5H10.04	NADPH dehydrogenase	2.03	2.31	2.2
SPAP27G11.08c	Meiotic expression upregulated	1.72	2.62	2.2
SPBC16E9.16c	Sequence orphan	2.44	1.88	2.2
SPCC1223.13	Transcription factor (predicted)	2.27	2.05	2.2
meu26$^+$	Conserved fungal protein	2.00	2.29	2.1
SPBPB2B2.19c	Membrane protein	2.01	2.28	2.1
SPAC19B12.10	AMSH protein homolog	2.11	2.16	2.1
pof1$^+$	F-box protein	2.10	2.16	2.1
caf5$^+$	Spermine transporter family	1.89	2.34	2.1
SPBC409.08	Spermine transporter family	1.89	2.22	2.1
tht1$^+$	Nuclear membrane protein involved in karyogamy	2.05	2.05	2.1
gst1$^+$	Glutathione *S*-transferase	2.15	1.93	2.0
atf31$^+$	bZIP transcription factor	2.01	2.02	2.0
spd1$^+$	Ribonucleotide reductase inhibitor	1.96	2.06	2.0
SPCC1020.03	Mitochondrial ion transporter	1.59	2.37	2.0
SPBC1348.02	*S. pombe*-specific protein	1.88	2.08	2.0

[a] The descriptions of the protein functions are based on information for *S. pombe* in GeneDB (http://www.genedb.org/genedb/pombe/index.jsp).

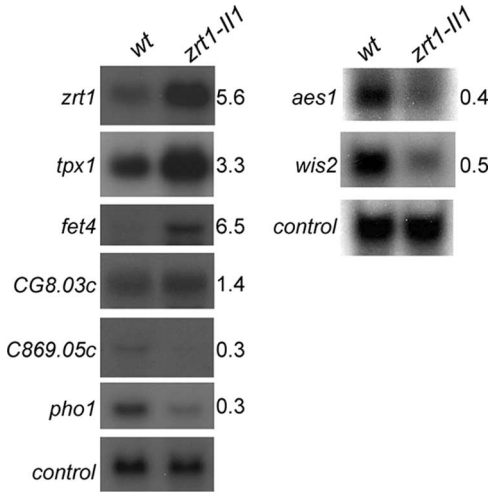

FIG. 7. Genes regulated by zinc deficiency. Wild-type (wt) and *zrt1-II1* cells were grown to exponential phase in EMM medium at 30°C. RNA was prepared and subjected to RNA blot hybridization with the indicated probes, with *his3*$^+$ serving as a loading control. Blots were quantified using a PhosphorImager. The relative mRNA levels (*zrt1-II1* cells compared to wild-type cells) are indicated.

scure, but they are predicted to be located at the cell surface. Many of the upregulated genes identified by this analysis have previously been shown to be induced in response to environmental stresses (5; see also http://www.sanger.ac.uk/PostGenomics/S_pombe/projects/). In particular, the mRNA levels of genes encoding known antioxidants, such as *tpx1*$^+$, *srx1*$^+$, *gst1*$^+$, and *gst2*$^+$, were increased in response to zinc limitation, and consistent with this, a number of limiting zinc-responsive genes are also induced in response to H_2O_2 (e.g., *obr1*$^+$, *rds1*$^+$, and SPCC663.08c) (5). There is evidence suggesting that zinc deficiency results in oxidative stress in mammalian cells (29), and our microarray data suggest that this may also be the case in fission yeast cells.

As expected, *zrt1*$^+$ mRNA was significantly increased by zinc deficiency, as was the mRNA of another gene, SPBP26C9.03c, which we named *fet4*$^+$ based on its homology to *S. cerevisiae FET4*. *S. cerevisiae* Fet4 was originally identified as a low-affinity iron uptake transporter, although more-recent evidence suggests that it is also capable of transporting zinc (39). Therefore, we examined the role of its fission yeast counterpart. Deletion of the *fet4*$^+$ gene alone did not result in any increased sensitivity to limiting zinc concentrations (Fig. 8), nor did it influence intracellular zinc concentrations (data not shown). However, we found that the deletion of *fet4*$^+$ exacerbated the defects associated with the loss of Zrt1. As described above, minimal agar (EMM) is somewhat limiting for zinc. Accordingly, *zrt1*Δ cells grew slowly on minimal agar (EMM), and furthermore a *zrt1*Δ *fet4*Δ double mutant strain was unable to grow on this medium even after an extended period (5 days) (Fig. 8A). Moreover, the deletion of *fet4*$^+$ also exacerbated the slow-growth phenotype of *zrt1*Δ cells in liquid culture (Fig. 8B). In both cases, the phenotypes were suppressed by supplementing the media with zinc. Thus, these findings indicate that Fet4 contributes to viability when zinc is limiting.

DISCUSSION

Here we have employed both transcript profiling and a genetic screen to characterize the response of fission yeast cells to zinc deficiency. We have identified sets of genes whose mRNA levels are regulated by zinc availability, and we have also identified Zrt1, a ZIP transporter that is a central component of zinc homeostasis. The importance of Zrt1 is underscored by the inability of cells lacking this transporter to proliferate when zinc is scarce. Furthermore, cells lacking Zrt1 function have severely reduced intracellular zinc contents compared to wild-type cells. These findings suggest that *S. pombe* Zrt1 is a high-affinity zinc uptake transporter that is analogous to *S. cerevisiae* Zrt1. Sequence analysis of the *S. pombe* genome indicates that it lacks an obvious counterpart to *S. cerevisiae* Zrt2, the low-affinity ZIP zinc uptake transporter. However, *S. pombe* does encode a homologue of the *S. cerevisiae* Fet4 transporter, and our data are consistent with a role for this transporter in zinc uptake. Furthermore, there are a number of uncharacterized proteins (such as SPCC126.09, SPAP8A3.03, and SPAC17D4.03c) that have the potential to contribute to zinc uptake.

S. cerevisiae cells also respond to zinc deficiency by increasing the expression of Zrt3, another ZIP transporter that is responsible for the mobilization of zinc from the vacuole (24). Paradoxically, budding yeast cells also upregulate the expression of Zrc1, a vacuolar zinc influx cation diffusion facilitator transporter, and it has been suggested that zinc flux through the vacuole is important under conditions of deficiency (23). It is therefore intriguing that while *S. pombe* encodes homologues of Zrt3 and Zrc1 (SPCC126.09 and Zhf1, respectively),

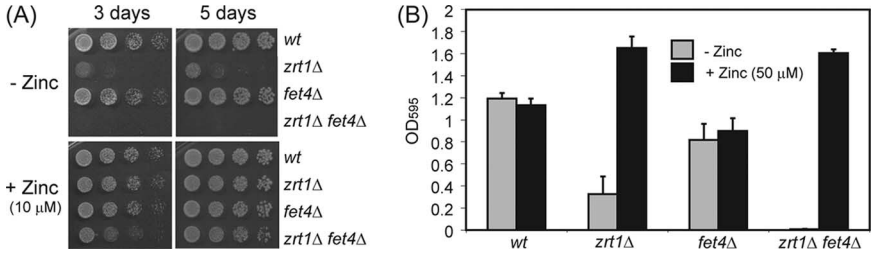

FIG. 8. (A) The indicated strains were grown to exponential phase, subjected to fivefold serial dilutions, and spotted onto EMM agar or EMM agar supplemented with $ZnSO_4$ (10 μM) and incubated at 30°C for the indicated times. (B) The indicated strains were grown for 25 h at 30°C in EMM or EMM supplemented with $ZnSO_4$ (50 μM). Shown are the mean values from three experiments. Error bars indicate standard deviations. Note that the wild-type (NT4) and *fet4*Δ (SW496) strains are *ura4*$^-$, whereas the *zrt1*Δ (SW227) and *fet4*Δ *zrt1*Δ (SW500) strains are *ura4*$^+$. wt, wild type.

our microarray data indicate that the mRNA level of neither of these fission yeast genes is substantially influenced by intracellular zinc status. However, it is possible that these transporters are subject to zinc-dependent, posttranslational regulation.

Differential gene expression is predicted to aid the adaptation to the stress imposed by zinc deprivation. A potential example of this is the mitochondrial alcohol dehydrogenase Adh4 (6), which is similar to an alcohol dehydrogenase from *Zymonas mobilis* (and also *S. cerevisiae* Adh4) and is predicted to employ iron rather than zinc as a cofactor. As such, it has been suggested that the upregulation of this iron-dependent isoform may compensate for the loss of zinc-dependent alcohol dehydrogenase activity (23). However, the nature of the cofactor employed by these enzymes remains controversial. Despite being closely related to bacterial iron-dependent alcohol dehydrogenases, the activity of *S. cerevisiae* Adh4 was found to be stimulated by zinc rather than iron (7). Other genes that are upregulated by zinc-limiting conditions are also known to be induced in response to other adverse environmental conditions. These include a number of antioxidants and also other genes that are known to be induced upon exposure to hydrogen peroxide. Consistent with this, a number of studies have linked zinc deficiency to increased levels of reactive oxygen species in mammalian systems (28, 29, 42, 43). Furthermore, recent evidence suggests that zinc deficiency also results in oxidative stress in budding yeast (41). Although zinc is not redox active, a number of possible roles for zinc in antioxidant defense have been postulated. These include being a constituent of antioxidant enzymes, replacing redox-active metals from membrane binding sites, and protecting sulfhydryls (29). It is also noteworthy that work with human neuroblastoma cells has demonstrated that zinc status influences their sensitivity to iron-induced oxidative stress (25). It is therefore interesting that our data indicate that fission yeast cells downregulate some genes involved in iron uptake and utilization in response to zinc deficiency.

The function of other genes whose mRNA levels are increased under conditions of zinc deficiency is less obvious. SPBC138.06c, SPAC977.05c, and SPBPB2B2.15 are closely related genes that have arisen through the duplication of subtelomeric regions. These genes are homologues of the *S. cerevisiae* genes *VEL1* and *YOR387C*, which are regulated by Zap1. Although the functions of the proteins encoded by these genes are not understood, they are predicted to be cell surface glycoproteins. Whether or not they confer any selective advantage under zinc-limiting conditions remains to be determined.

The requirement of zinc for numerous cellular processes dictates that zinc deficiency will have an adverse impact upon growth rate. This clearly has been observed, as the doubling time of zinc-limited (*zrt1-II1*) cells in minimal medium (EMM) is increased relative to that of wild-type cells. This effect of zinc deficiency is reflected at the level of mRNA because a large number of downregulated genes have products that are involved in cell growth, including ribosomal proteins and amino acid transporters, and in nucleotide metabolism. A potential candidate for mediating this process is SPAC4G8.03c, a Pumilio family RNA binding protein, which is induced by low intracellular zinc concentrations (Table 2). Pumilio family members are known to negatively regulate gene expression either through inhibition of translation or by enhancing mRNA turnover (40). It will be interesting to identify the mRNAs that are regulated by this RNA binding protein.

Our studies indicate that 2.5% of *S. pombe* genes have mRNA levels that are regulated in response to zinc deficiency. Comparison studies of other organisms have revealed much more profound changes in global transcript profiles. For instance, the study of Lyons et al. revealed that zinc limitation led to changes in more than 15% of *S. cerevisiae* genes (23). It is possible that some of this difference may reflect different approaches used to induce zinc deficiency. Whereas Lyons et al. employed Chelex-treated (CSD) zinc-limiting medium, we have exploited the *zrt1-II1* mutation. However, a recently published study also finds substantial differences in the responses to copper and iron between budding and fission yeast (30). Nonetheless, there is a significant overlap between the *S. pombe* genes that are highly induced (greater than sixfold) by zinc deficiency and the *S. cerevisiae* Zap1 regulon. All but one ($dak2^+$) have *S. cerevisiae* homologues that are upregulated by zinc limitation. For instance, both organisms strongly increase the mRNA levels of genes encoding zinc uptake transporters, mitochondrial alcohol dehydrogenases, and cell surface proteins. However, *S. pombe*, like mammals and plants, does not encode a homologue of the *S. cerevisiae* zinc-sensing transcriptional activator Zap1. Therefore, different regulatory mechanisms must be used by *S. pombe* to regulate mRNA levels in response to zinc availability. Indeed, preliminary evidence suggests that the expression of $adh4^+$ is regulated by a combination of activating and repressing promoter elements. Furthermore, it is possible that posttranscriptional controls may also play a role in the response to zinc deprivation.

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