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The Antifungal Action of 1,10-*o*-phenanthroline and EDTA is Mediated Through Zinc Chelation and Involves Cell Wall Construction

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Summary

Yeast growth was inhibited at 1,10-o-phenanthroline concentrations of 0.1-0.5 mM and EDTA concentrations of 1-2 mM. Growth was most efficiently restored by adding Zn^{2+} to the medium, whereas Fe^{3+} was less effective, and Cu^{2+}, Mn^{2+}, Ca^{2+}, or Mg^{2+} had only a minor or negligible effect. Culturing yeast cells in defined media that lacked individual metal salts showed that zinc ions were indeed needed for growth. These observations indicate that the growth inhibition caused by 1,10-o-phenanthroline and EDTA is largely due to a deficiency in zinc ions. Growth inhibition of filamentous fungi by 1,10-o-phenanthroline and EDTA could also be relieved by adding zinc ions to the medium.

Chelation did not prevent the formation of an osmotically stable cell wall in regenerating yeast spheroplasts indicating that the synthesis of β-glucan and chitin were not affected. Regenerating spheroplasts that expressed α-galactosidase secreted equal amounts of α-galactosidase in the presence of 1,10-o-phenanthroline as control cells indicating that protein synthesis and the secretory pathway were functioning normally in the presence of 1,10-o-phenanthroline. However, regenerating yeast spheroplasts that expressed a cell wall fusion protein containing α-galactosidase as a reporter protein released much less fusion protein into the medium than control cells. We suggest that the processing step responsible for releasing GPI-bound cell wall proteins from the plasma membrane might be affected, resulting in defective cell wall construction.

Keywords: Zn^{2+}, growth, secretion, cell wall, yeast, fungi

Introduction

Chelation of metal ions by agents such as 1,10-*o*-phenanthroline and EDTA has antimicrobial effects *(e.g. 1,2)*. Indeed it is known that a significant number of metal cations are important for fungal growth. Especially, Ca^{2+} and Mg^{2+} (concentrations 10^{-3} to 10^{-4}), and Fe^{3+} and Cu^{2+} (concentration 10^{-6}), have been under investigation *(1,3,4)*. Ca^{2+} is often implicated in cellular signalling *(5–7)*. Intracellular Ca^{2+} influences cell proliferation and stress tolerance. For instance, activation of calcineurin by Ca^{2+} and calmodulin upon heat stress or osmotic stress regulates cell growth. This negative effect is presumably modulated by the *PKC1* pathway which is generally thought to be activated by Ca^{2+} *(8)*. The membrane of yeast cells contains stretch sensitive Ca^{2+} selective channels. Kamada *et al. (6)* have postulated that wall weakening at high temperatures or caused by hypo-os-

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motic stress results in increased membrane stretch, opening of the Ca^{2+} selective channels and thereby activation of the *PKC1* pathway. The *PKC1* pathway is indeed implicated in the regulation of wall β1,3- and 1,6-glucan biosynthesis and also in bud-site localisation and bud-growth after passing START *(7,9–11)*. Mg^{2+} is needed for a proper functioning of various enzymes, most prominently the plasma membrane ATPase *(12)*. Iron is crucial for cytochromes and haem apoenzymes while Cu^{2+} is needed for various enzyme activities and pigments *(4)*.

The micronutrient zinc is nowadays implicated in an ever-growing number of cellular processes. Some proteins with associated zinc include alcohol dehydrogenase, aspartate transcarbamylase, RNA polymerase, reverse transcriptase and a significant number of DNA binding regulatory proteins containing so-called 'zinc fingers' *(13)*. Indeed, it has been said that in early evolution and still in prokaryotes, iron and manganese played the main role of communicating with DNA, while in the evolution of eukaryotes this role has been taken over by cytoplasmic zinc.

We decided to study fungal growth inhibition through metal chelation in both *Sacch. cerevisiae* and filamentous *Ascomycete* fungi. For our studies we used 1,10-*o*-phenanthroline and EDTA. The latter chelator has by far its highest affinity for Fe^{3+} followed by Cu^{2+} and Zn^{2+} with binding constants of several orders of magnitude lower *(14)*. 1,10-*o*-phenanthroline has similar affinities for Fe^{3+}, Cu^{2+} and Zn^{2+} *(15)*. We also performed experiments in which we cultured yeast cells in media with a defined composition of metal salts. Our results indicate that incubation with both chelators leads to growth inhibition and abnormal cell morphology through zinc chelation. A possible mechanism for the deleterious effect of zinc deficiency on fungal growth involving cell wall construction is discussed.

Materials and Methods

Strains and media

Penicillium roqueforti, Trichoderma harzianum, Paecilomyces variotii, Aspergillus niger, Emericella (aspergillus) nidulans. All mould strains were cultured in diluted malt extract (MEA) medium. The *Saccharomyces cerevisiae* yeast strains used in this study were SU51 (YT6-2-1 L) *MAT***a**, *cir*$^+$, *leu2*-3,112, *his4*-519, *can*1 *(16)*, and X2180-1B (Yeast Genetic Stock Centre, Berkley, California, USA). Yeast strains were routinely grown in YPD (1% yeast extract, 2% Bacto-Peptone, 2% dextrose) or synthetic minimal medium (MM) consisting of 0.7% yeast nitrogen base (YNB), 2% glucose, and amino acids as necessary *(17)*. For the experiments using the Cwp2p-α-galactosidase fusion construct discussed in Fig. 7, YPDG (1% yeast extract, 2% Bacto-Peptone, 0.5% glucose, 5% galactose) was used in yeast precultures and protoplast regeneration. Media samples were prepared using acid extraction, and were then assessed using an atomic emission spectrophotometer. YPD(G) contained 2.3 ppm Fe^{3+}, 0.1 ppm Mn^{2+}, < 0.1 ppm Cu^{2+}, 0.9 ppm Zn^{2+} and 15 ppm Ca^{2+}. MEA medium contained 2 ppm Fe^{3+}, 26 ppm Ca^{2+} and essentially similar concentrations of the other indicated metal ions.

A detailed analysis of metal ion requirement of yeast growth was performed with strain X2180-1B cultured in chemically-defined Yeast Base Medium *(18)*. Media, buffered to pH = 3.5 with 50 mM succinic acid, all contained glucose 20 g/L; ammonium sulphate 1 g/L; potassium orthophosphate 1 g/L; boric acid 0.5 mg/L; sodium molybdate 0.2 mg/L; sodium chloride 100 mg/L; biotin 2 μg/L; pantothenic acid 400 μg/L; folic acid 2 μg/L; inositol 2 mg/L; niacin 400 μg/L; *p*-aminobenzoic acid 200 μg/L; riboflavin 200 μg/L; and thiamine HCl 400 μg/L. Metal salts individually left out of the media were copper sulphate 40 μg/L; ferric chloride 200 μg/L; manganese sulphate 400 μg/L; zinc sulphate 400 μg/L; calcium chloride 0.1 g/L and magnesium sulphate 0.1 g/L. Absence of detectable contamination with the metal salt not included in the culture medium was performed with atomic emission spectroscopy.

Germination/outgrowth inhibition

100 spores of each fungus or a similar number of log-phase yeast cells were inoculated into diluted MEA or YPD, respectively. The incubations contained 0-500 μM 1,10-*o*-phenanthroline or 1–2 mM EDTA sodium salt pH = 5.5 (Boehringer, Mannheim Germany) +/– various relevant metal ions. Zn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{3+} and Mn^{2+} were added as chloride or sulphate salts (see above). 100 μM 1,10-*o*-phenanthroline provides a 100% *in vitro* inhibition of isolated mammalian PI-PLD *(19)*. The highest final ethanol concentration (phenanthroline solvent) did not exceed 0.2%. This ethanol concentration was shown to be non-toxic for all moulds and yeasts tested (Brul and Van der Vaart, data not shown). Growth was evaluated after 2 days of culture at 25 °C. In selected experiments *A. niger* was cultured in the absence or presence of 100 μM 1,10-*o*-phenanthroline and microscopically observed at regular time intervals. Lengths of hyphae, hyphal thickness and branch development were followed by means of a microscopical micrometer.

Scanning Electron Microscopy (SEM)

Yeast cells were washed twice with HEPES buffer (10 mM, pH = 6) and fixed with cold 5% glutaraldehyde in phosphate buffered saline (PBS) at 4 °C for 1 hour. Subsequently, the samples were postfixed with 1% OsO_4 in 0.1 M cacodylate buffer pH = 7.4 for 1 hour. Next, the specimens were dried in a critical-point dryer with CO_2 and examined at 20 kV with a Hitachi S-4100 field emission scanning electron microscope.

Generation and regeneration of protoplasts

Mould protoplasts were made from young mycelium of *A. niger*, and *P. variotii*. Protoplasts were generated by incubating hyphal cells, which had been pretreated with 0.3% β-mercaptoethanol for 30 min, with 10–20 mg/mL Novozym 234 (Novo, Nordisk) for 1 hour at 37 °C. The incubation solutions were osmotically stabilized with 0.6 M KCl. The efficiency of protoplast generation was checked by dilutions of aliquots in distilled water and scoring for protoplast lysis. Protoplasts were diluted in 0.6 M KCl and were allowed to regenerate in diluted MEA osmotically stabilized with KCl. Regenera-

tions were performed with or without 100 μM 1,10-*o*-phenanthroline. At regular intervals samples were taken and 10^2 cells were plated on MEA or MEA + 0.6 M KCl. Colony formation was assessed after 2 days of incubation at 25 °C.

Protoplasts from the yeast SU51 were made as follows. The cells were inoculated in MM and grown for 34 hours at 30 °C. Subsequently, these cells were diluted (1:500) in YPDG (400 mL) and grown for 17 hours until an A_{660} of ±4. These yeast cells were harvested by centrifugation, washed twice in 10 mM Tris-HCl, pH = 7.4 and once in spheroplasting buffer (50 mM Na_2CO_3, pH = 7.4 and 1 M sorbitol). After these washing steps the cells were resuspended in 10 mL spheroplasting buffer and 20 μL β-mercaptoethanol was added. After a 10-min incubation at room temperature 200 μL Zymolyase 100T (5 mg/mL) was added and the incubation was continued for an additional 40 min at 37 °C. Protoplast formation was checked by microscopy. The protoplasts were incubated in 100 mL 'regeneration' buffer (YPDG, 1M sorbitol) with or without 1,10-*o*-phenanthroline (100 and 500 μM) and samples of 10 mL were taken every hour for 5 h.

Construction of fusion fragments and enzyme activity measurement

The 201-bp C-terminal end of *CWP2 (20)* was fused with the coding region of α-galactosidase. An *NheI* recognition sequence was created at the C-terminus of the α-galactosidase gene by insertion of an oligonucleotide linker adapter [5'CAAGGAGCGCTAGCGGTACCGAAAGTTA ACA3' and 5'TCGAACAATTGAAAGCCATGGCGATCGCGAG3'] into the *StyI/HindIII* site of pUR2650 [DNA sequence of invertase signal sequence and α-galactosidase coding region from pUR2740; *(38)*] to give pUR2984. Fig. 1 shows the fusion constructs. The α-galactosidase-*CWP2* fusion was cloned into *SacI/HindIII*-digested pSY16 *(21)*.

The amount of α-galactosidase produced by SU51 expressing free α-galactosidase or the cell wall bound α-galactosidase-Cwp2p fusion-protein was determined by the ability of α-galactosidase to convert the chromogenic substrate PNPG (*p*-nitrophenyl-α-D-galactopyranoside).

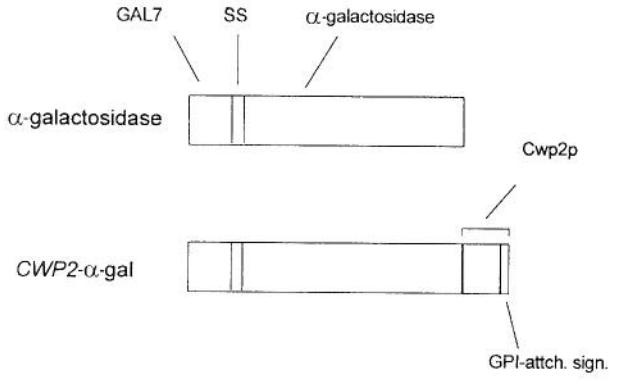

Fig. 1. DNA sequences capable of expressing the α-galactosidase enzyme or the α-galactosidase-CWP2 fusion protein. Both constructs are under control of the GAL7 promoter and contain a signal sequence for ER import.

Fluorescent staining

Cells were stained with Calcofluor White by incubation for 30 minutes at 30 °C in HEPES buffer (10 mM, pH = 6) in the presence of 25 μM Calcofluor White M2R (Molecular Probes, from a 5mM stock in dH_2O). Cells were stained with DAPI (Sigma) by incubation for 15 minutes at room temperature in a 0.02% DAPI solution in HEPES buffer (10 mM, pH = 6). After two washing steps with HEPES buffer the cells were examined with a Zeiss fluorescence microscope.

Results

Effects of 1,10-o-phenanthroline and EDTA on fungal growth

Yeast cells were cultured in the presence of various concentrations 1,10-*o*-phenanthroline. Fig. 2 shows a representative example of a SEM micrograph of cells cultured in the presence of 500 μM of the chelator. In these cultures typically large cells with an uneven surface were observed. A similar aberrant morphology of yeast cells was obtained upon culturing cells in the presence of 1 mM EDTA (not shown). Calcofluor White / DAPI staining revealed that, where present, buds contained nuclear structures confirming that DNA duplication had occurred. At 100 μM phenanthroline, growth was severely impaired and at 200 μM growth of the yeast culture ceased. The growth inhibitory effect of incubation of these yeast cells with 1,10-*o*-phenanthroline was relieved most efficiently by the addition of Zn^{2+} in a 1 to 1 molar ratio (Fig. 3). Next, we performed experiments with EDTA. Fig. 4 shows that also in the case of EDTA-mediated growth inhibition Zn^{2+} was the most efficient metal ion in relieving that growth inhibition. Note that this chelator has by far its highest affinity for Fe^{3+}. Table 1 shows that chelation by 1,10-*o*-phenanthroline inhibits also the outgrowth of conidia from filamentous fungi

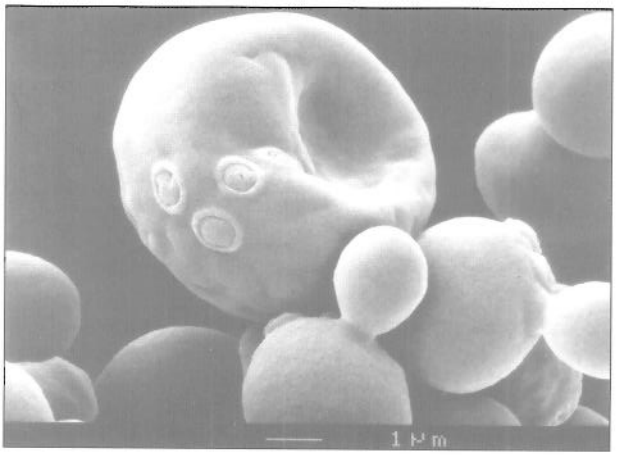

Fig. 2. Scanning Electron Microscopy of yeast cells cultured in YPD in the presence of 500 μM 1,10-*o*-phenanthroline. Bar equals 1 μm.

Table 1. $ZnCl_2$ alleviates the inhibition of germination caused by 100 μM 1,10-*o*-phenanthroline. Diluted MEA containing 100 μM 1,10-*o*-phenanthroline and varying concentrations of $ZnCl_2$ was inoculated with 100 spores of each fungal strain and the plates were incubated at 25 °C for 2 days. The amount of colonies for each mould is given. Blank represents no addition of 1,10-*o*-phenanthroline or $ZnCl_2$. In brackets the colony size is given (cm) after 2 days of culture. Addition of similar amounts of $CaCl_2$, $MgSO_4$, $MnSO_4$ to cultures with 100 μM 1,10-*o*-phenanthroline had no beneficial effect on germination (see text for more details).

μM $ZnCl_2$	*P. roqueforti*	*T. harzianum*	*P. variotii*	*A. niger*	*A. nidulans*
100	111 (0.6 cm)	39 (1 cm)	115 (0.5 cm)	27 (1 cm)	64 (0.3 cm)
50	60 (0.5 cm)	21 (1 cm)	97 (0.2 cm)	36 (1 cm)	90 (0.1 cm)
25	0	20 (1.3 cm)	0	47 (1 cm)	80 (0.1 cm)
10	0	8 (1 cm)	0	57 (0.4 cm)	37 (0.1 cm)
5	0	0	0	63 (0.1 cm)	0
0	0	0	0	44 (0.1 cm)	0
Blank	29 (0.6 cm)	24 (2.5 cm)	83 (0.5 cm)	28 (1.3 cm)	68 (0.5cm)

Table 2. Growth of *Aspergillus niger* at 25 °C in Malt broth in the presence and absence of 100 μM 1,10-*o*-phenanthroline (+,–).

Time/h		Average length of hyphae	Number of branches and average length of hyphal branches
		μm	μm
0	–	9	0
	+	11	0
2	–	17	1 of 0.25
	+	11	0
4	–	34	2 of 2
	+	12	0
6	–	38	5 of 10
	+	9	0
23	–	125	14 of 20
	+	11	0

After 23 hours of incubation in the presence of 100 μM 1,10-*o*-phenanthroline the hyphae started growing again at the tip. However, this newly formed tip was thinner than the old part of the hypha.

(moulds). Interestingly, *A. niger* forms tiny colonies in the presence of the chelator indicating that growth but not germination is inhibited. Addition of the chelator to *A. niger* hyphae resulted in an impairment in elongation and branching (Table 2). Also for moulds, the inhibition could be relieved effectively by adding Zn^{2+} to the cultures. Addition of similar amounts of Ca^{2+}, Mg^{2+} or Mn^{2+} to cultures with 100 μM 1,10-*o*-phenanthroline had no beneficial effect on spore germination. Even a 10-fold excess of these divalent cations over the chelator had no effect. Upon addition of 100 μM Cu^{2+} or Fe^{3+} to fungal cultures with 100 μM 1,10-*o*-phenanthroline colony formation was restored to a level comparable to the level obtained with Zn^{2+} additions. Colony size, however, was still greatly reduced. A further increase of Cu^{2+} or Fe^{3+} to 1 mM had no effect. On their own $ZnSO_4$, $CaCl_2$, $MgSO_4$, $MnSO_4$, $CuSO_4$ or $FeCl_3$ had at 1 mM no effect on growth.

In summary, we conclude that the changes in cellular morphology and the inhibition of fungal outgrowth by the chelators 1,10-*o*-phenanthroline and EDTA are due to an induced cellular zinc deficiency. In support of this, the experiment shown in Fig. 5 indicates that zinc is after magnesium the most important metal ion for yeast growth. The antifungal mechanism of zinc chelation was subsequently studied in detail.

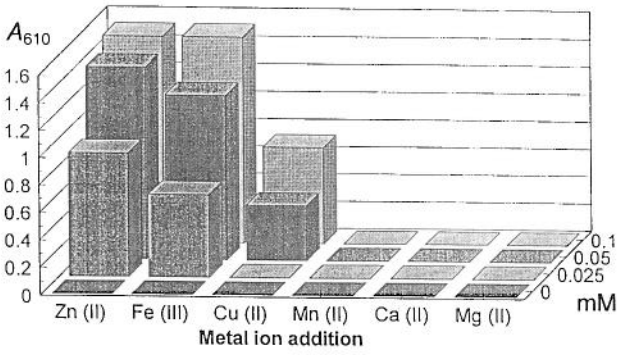

Fig. 3. Yeast growth inhibition by 1,10-*o*-phenanthroline is relieved most efficiently by zinc ions. Yeast cells were cultured in YPD in the presence of 100 μM 1,10-*o*-phenanthroline and the various indicated divalent metal ions. After 48 hours of incubation at 25 °C the absorbance of the cultures was measured.

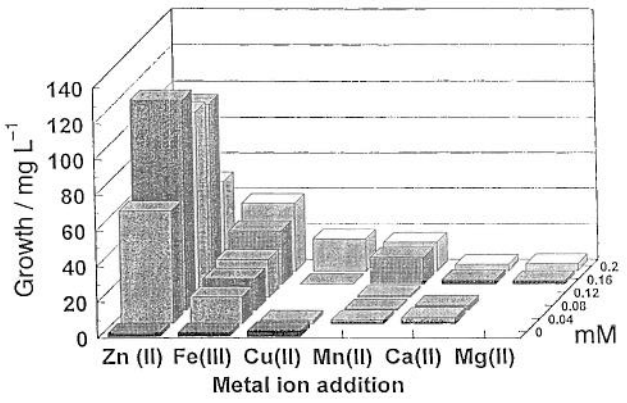

Fig. 4. Yeast growth inhibition by EDTA is relieved most efficiently by zinc ions. Yeast cells were cultured in the presence of 2 mM EDTA and the various metal ions indicated. After 48 hours of incubation at 25 °C the yield of each culture was determined and expressed as mg/L dry weight.

Effect of 1,10-o-phenanthroline chelation on the generation of an osmotically stable cell wall

We asked ourselves whether zinc chelation would have a direct negative effect on the synthesis of an osmotically stable wall. The osmotic stability of regenerating protoplasts of *P. variotii* was investigated to test this (Fig. 6). We hypothesized that if a Zn^{2+} dependent process were involved in wall (sugar) synthesis, inhibition by

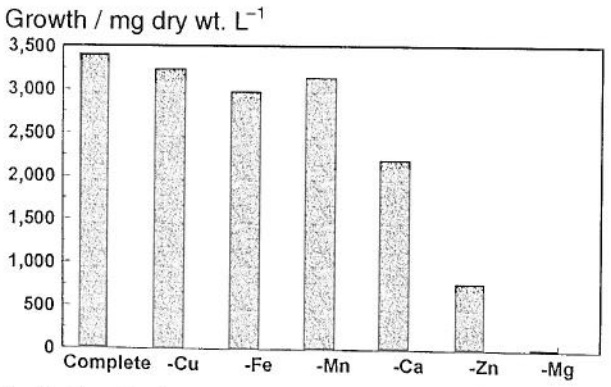

Fig. 5. Growth of *Saccharomyces cerevisiae* X2180-1B after 7 days in Yeast Base media lacking metal salts. In complete Yeast Base Medium these salts are present as $CuSO_4$ 40 μg/L; $FeCl_3$ 200 μg/L; $MnSO_4$ 400 μg/L; $CaCl_2$ 100 mg/L; $ZnSO_4$ 400 μg/L; $MgSO_4$ 500 mg/L.

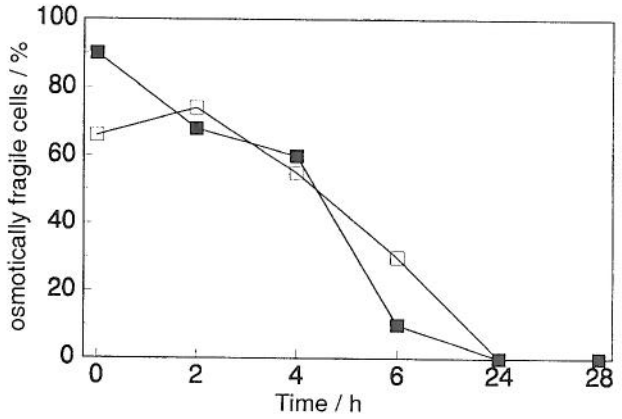

Fig. 6. Zinc chelation does not lead to osmotically fragile walls. Regeneration of protoplasts of *Paecilomyces variotii* obtained as described in Materials and Methods in Malt broth containing 0.6 M KCl (□), or in Malt broth containing 0.6 M KCl and 100μM 1,10-*o*-phenanthroline (■). At various time points of regeneration aliquots were taken and tested for the presence of colony forming units on MEA in the presence or absence of 0.6 M KCl. The % of osmotically fragile cells is given.

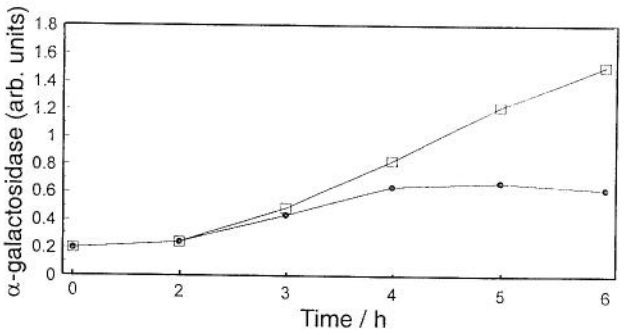

Fig. 7. Zinc chelation prevents membrane processing of glucanase-extractable cell wall mannoproteins. α-galactosidase activities in media of cultures with yeast protoplasts expressing the α-gal-CWP2 fusion protein were measured at various time intervals. The protoplasts were incubated for 6 hours with (●) or without (□) 500 μM 1,10-*o*-phenanthroline.

phenanthroline would perturb regeneration. Consequently, plating on media without an osmotic stabilizer was expected to give a low colony count. No immediate toxic effect of phenanthroline was noted. The results presented in Fig. 6 show that *P. variotii* protoplasts regenerated an osmotically stable wall in the presence of phenanthroline. As regenerated walls initially primarily consist of chitin and β1,3-glucan fibrils this indicates that the synthesis of these components is not affected in the presence of phenanthroline. However, prolonged phenanthroline incubation was clearly cytotoxic (Table 1), a phenomenon apparently not related to osmotic lysis. Similar observations were made with the other moulds and the *Sacch. cerevisiae* yeast strain. Calcofluor White staining showed normal chitin distribution (not shown).

Cell wall mannoprotein synthesis and wall abnormalities upon incubation with 1,10-o-phenanthroline

Glucanase-extractable cell wall mannoproteins (Cwp) are characteristic for yeast and moulds *(20,22–24)*. In yeast it was shown that their biogenesis involves a step at which the plasma membrane bound glycosyl-phosphatidyl-inositol (GPI)-anchored intermediate is processed to a soluble periplasmic form *(25)*. The exact processing step is not known but could involve a phospholipase D (PLD) activity. In mammalian plasma a Zn^{2+} dependent GPI specific PLD has been described *(26)*. Our initial hypothesis was that, by analogy to mammalian cells, in yeast such a GPI-specific PLD might be functional. Inhibition of a crucial Cwp processing step would lead to an impaired wall biogenesis and thereby presumably to abnormalities in fungal growth.

We aimed at testing a PLD involvement in Cwp GPI-anchor processing using *Sacch. cerevisiae* as a model. In recent years, a number of new glucanase-extractable cell wall mannoproteins have been identified. The gene for one of them Cwp2p (Cell Wall Protein 2; *(20)*) is located on chromosome XI *(27)*, and represents a transcript capable of encoding 94 amino acids. This transcript contains a putative GPI attachment signal at the C-terminus. A fusion construct consisting of the guar α-galactosidase gene, and the C-terminal half of *CWP2* was created (Fig. 1). This fusion protein is incorporated into the cell wall *(28)* and was used as a marker protein to assess the influence of 1,10-*o*-phenanthroline on processing and subsequent secretion of wall proteins by regenerating protoplasts. As a control on the efficiency of secretion we assessed the secretion of free α-galactosidase in the presence of 500 μM 1,10-*o*-phenanthroline during a 24 hour-culture. No negative effect of the chelator on secretion was noted.

Although *Sacch. cerevisiae* is not capable of regenerating a normal wall in the usual liquid media *(29)*, regenerating spheroplasts do release cell wall proteins into the medium indicating that normal processing of cell wall proteins takes place. If 1,10-*o*-phenanthroline were able to inhibit processing of these proteins, it should be possible to detect this by adding 1,10-*o*-phenanthroline to the 'regeneration' medium and monitoring the inhibition of release of the α-galactosidase-Cwp2 fusion pro-

tein. Fig. 7 shows that secretion of the fusion protein was negatively affected by the presence of the chelator. In agreement with these observations we found that after a 24-hour incubation of intact yeast cells with 1,10-*o*-phenanthroline there is a 10-fold lowered mannoprotein concentration in isolated cell walls compared with the control cells (not shown). We concluded that general protein synthesis and secretion of proteins were not affected upon a 24-hour incubation of yeast cells in the presence of 500 μM 1,10-*o*-phenanthroline, while proper wall incorporation of glucanase-extractable mannoproteins was seriously reduced.

Discussion

1,10-o-phenanthroline and EDTA affect fungal morphology and inhibit growth through zinc chelation

Yeast cell walls are known to bind various metal ions. For instance, zinc, calcium, magnesium, manganese, copper and iron have all been studied extensively *(1)*. The current study shows that both 1,10-*o*-phenanthroline and EDTA prevent growth of yeast and moulds in a zinc-dependent fashion. The chelators themselves do not accumulate intracellularly. It is known from radioactive EDTA uptake studies that this chelator does not pass the membrane of intact fungal cells (Stratford & Hilles, personal communication) while 1,10-*o*-phenanthroline is pumped out of cells by the multidrug resistance pump *(30)*. SEM studies show that yeast cellular morphology is significantly altered upon culturing cells in media containing 1,10-*o*-phenanthroline or EDTA. What are potential targets of Zn^{2+} chelation?

Targets for zinc chelation in cells

DNA synthesis and processing are possible targets for zinc chelation. From the DAPI staining it is clear, however, that nuclear division occurred. Correct DNA transcription might be impaired. Indeed, it is known that Zn^{2+} is present in RNA polymerase. However, our studies in yeast show that in the time frame of the experiments protein synthesis and secretion were unaffected by the chelation of zinc ions. Nonetheless, correct regulation of RNA transcription and translation could still be affected. In cells, various Ca^{2+} dependent signal transduction cascades are involved in cellular morphogenesis *(5–7,10)*. Both nitrogen starvation and incubation with fusel alcohols are culture conditions which also lead to marked morphological changes in yeast cells *(31,32)*. The way in which fusel alcohols influence cellular morphology at the molecular level is still a complete enigma while for the formation of pseudohyphae in diploids upon nitrogen starvation a Ca^{2+} dependent MAP kinase route is thought to be responsible. Zinc ions might also play a role in pathways which are involved in cellular morphogenesis. The synthesis and addition of GPI anchors to newly synthesized mannoproteins which are to be covalently linked to the cell wall may be affected by an intracellular zinc deficiency. As GPI anchors are essential for wall incorporation of the proteins a major consequence is that fungal walls will become deficient in such proteins. Below we will argue that this can lead to abnormal cellular morphology and growth inhibition. Alternatively, the release of cell wall proteins from the plasma membrane, necessary for their proper incorporation into the cell wall, might be affected.

Targets for zinc chelation on the outside of cells

Yeast cell walls consist of an inner layer of N-acetylglucosamine (chitin) polymers linked to a β1,3-glucan polymer network *(23,33)*. Genes encoding plasma membrane proteins involved in the biosynthesis of these wall structures have recently been cloned. The genes currently known are the gene for a subunit of the glucan synthase complex *(11,34)*, the Gas1 protein with an as yet unknown role *(11)* and 3 chitin synthases of which one is present at the plasma membrane *(33,35)*. None of these proteins seem to have a requirement for Zn^{2+} ions. In accordance, the experiments reported in this paper indicate that zinc is not essential for the regeneration of an osmotically stable wall.

Cell wall glucanase-extractable mannoproteins are synthesized *via* a glycosyl phosphatidyl inositol (GPI) intermediate membrane-bound state. This form of the protein might be processed in analogy with an enzyme present in mammalian systems by a GPI specific phospholipase D *(19)*. The mammalian enzyme is dependent on zinc ions and is *in vitro* inhibited by the presence of 1,10-*o*-phenanthroline. Our experiments reported in Fig. 7 show that, under conditions where the mammalian GPI-PLD enzyme is inhibited *in vitro*, the secretion of cell wall proteins from yeast protoplasts was impaired. In agreement with this, proper wall incorporation was also impaired. This suggests that the changes in cellular morphology induced by 1,10-*o*-phenanthroline may result from inhibition of normal incorporation of glucanase-extractable mannoproteins in yeast walls caused by inhibition of a Zn^{2+} dependent enzymatic process. This may be the activity of a PLD. However, Brul and co-workers recently also suggested that a PLC activity may be involved in the biogenesis of glucanase-extractable cell wall mannoproteins in fungi *(22)*. Further experiments are under way to assess the involvement of phospholipase C and/or D in the membrane processing of cell wall mannoproteins. Interestingly, Vossen *et al. (36)* postulated that the concentration of glucanase extractable mannoproteins in the cell wall is an important regulatory factor for cellular growth rate. Additionally, Gentzsch and Tanner *(37)* reported some experimental data which support an involvement of these proteins in wall morphology and cell division. They suggest that an impaired incorporation of mannoproteins into cell walls may be involved in the observed wall abnormalities in O-glycosylation mutants. As the biogenesis of glucanase-extractable wall proteins in filamentous fungi is analogous to the mechanisms operative in yeast *(22)*, similar consideration for involvement of Zn^{2+} ions in the biogenesis of glucanase-extractable wall mannoproteins are valid in filamentous *Ascomycetes*.

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Antifungalno djelovanje, 1,10-*o*-fenantrolina i EDTA omogućeno je helacijom sa cinkom, a obuhvaća i izgradnju staničnog zida

Sažetak

Koncentracija od 0,1 do 0,5 mM 1,10-o-fenantrolina i 1-2 mM EDTA inhibira rast kvasca. Dodatkom Zn^{2+} u podlogu omogućen je najbolji rast, dok je udjel Fe^{3+} bio manje djelotvoran, a mali ili neznatni učinak postignut je sa Cu^{2+}, Mn^{2+}, Ca^{2+} ili Mg^{2+}. Uzgojem stanica kvasca u definiranoj podlozi bez pojedinih metala utvrđeno je da su cinkovi ioni uistinu potrebni za rast. To pokazuje da je inhibiciju rasta, u prisutnosti 1,10-o-fenantrolina i EDTA, uglavnom uzrokovao nedostatak cinkovih iona. Inhibicija rasta filamentoznih gljiva zbog 1,10-o-fenantrolina i EDTA mogla se ukloniti dodatkom cinkovih iona u podlogu. Helacija nije spriječila stvaranje osmotski stabilnog staničnog zida u regeneriranim sferoplastima kvasca, što pokazuje da je sinteza β-glukana i hitina bila neometana. Regenerirani sferoplasti, koji su izlučivali α-galaktozidazu, izdvajali su istu količinu α-galaktozidaze

u prisutnosti 1,10-o-fenantrolina kao i kontrolne stanice, što znači da su sinteze proteina i put sekrecije funkcionirali normalno u prisutnosti 1,10-o-fenantrolina. Međutim, regenerirani kvaščevi sferoplasti, što su sintetizirali protein staničnog zida koji je sadržavao α-galaktozidazu izlučivali su manje tog proteina nego kontrolne stanice. Držimo da je došlo do ometanja mehanizma odgovornog za izlučivanje proteina staničnog zida, nastalih preko glikozil-fosfatidil-inozitola (GPI) intermedijara, što ima za posljedicu manjkavu izgradnju staničnog zida.