Selection and characterization of a copper-resistant subpopulation of *Schizosaccharomyces pombe*

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Addition of 15 μM - CuSO_4 to *Schizosaccharomyces pombe* 972 growing in a defined medium resulted in a biphasic growth curve and selection for a copper-resistant subpopulation. A clonal isolate of this subpopulation, designated strain SW40, had a decreased copper content per cell compared to the wild-type strain 972. The copper-resistance trait was stable in the absence of selection, and was the result of a single recessive chromosomal mutation. Copper resistance was not the result of metal ion efflux, extracellular chelation or an increased oxidative stress response. The results are consistent with a copper-resistance mechanism in which a modification of the cytoplasmic membrane results in decreased copper accumulation.

Introduction

Copper is an essential element for the proper functioning of biological systems, but it is toxic when present in high concentrations (Lindgren *et al.*, 1958; Martin, 1984; Ohsumi *et al.*, 1988). Toxic effects include disruption of cytoplasmic membranes (Ohsumi *et al.*, 1988), generation of free radicals (Babior, 1988), inhibition of certain enzymes (Moe *et al.*, 1985) and inhibition of respiration (Lindgren *et al.*, 1958; Nagai & Nagai, 1958). In some cases, a number of apparently unrelated events may have a single underlying cause. For example, Cu^{2+} catalyse the formation of cytotoxic reduced oxygen species such as hydrogen peroxide and hydroxyl radicals (Babior, 1988). The latter are highly reactive molecules which inactivate enzymes and catalyse lipid peroxidations, resulting in the disruption of biological membranes (Babior, 1988; Bilinski *et al.*, 1988).

In view of the toxicity of Cu^{2+} , it is not surprising that specific mechanisms for resistance to these ions have evolved. Copper resistance mechanisms that have been identified or proposed are extracellular chelation (Murphy & Levy, 1983), intracellular chelation (Hamer, 1986), increased oxidative stress responses (Naiki, 1980), energy-dependent efflux (Tynecka *et al.*, 1981), and exclusion of Cu^{2+} (Foster, 1977; Rai *et al.*, 1991). In the yeast *Saccharomyces cerevisiae*, copper resistance is usually the result of an increased production of metallothionein, which results in an increased intracellular

chelation capacity (Fogel *et al.*, 1983). Exclusion of copper as a resistance mechanism has been proposed in several algae (Foster, 1977; Rai *et al.*, 1991). Copper-tolerant cells of *Anabaena doliolum*, for example, accumulate less copper than wild type cells (Rai *et al.*, 1991). However, in this instance, copper tolerance is unstable in the absence of selection.

While there have been several studies involving copper uptake (Lin & Kosman, 1990) and resistance (Fogel *et al.*, 1983; Winge *et al.*, 1985) in *Sacch. cerevisiae*, such information has not previously been reported with respect to *Schizosaccharomyces pombe*. We describe here the isolation and characterization of a copper-resistant strain of *S. pombe*. Resistance is the result of a single chromosomal locus (*cur1*) and correlates with decreased copper accumulation by the resistant strains.

Methods

Organism. *Schizosaccharomyces pombe* strains (Table 1) 972 and 975 were obtained from the National Collection of Yeast Cultures (Norwich, UK). Strains FWP15, 165 and 172 were kindly provided by C. Hoffman (Boston College).

Growth media and conditions. Unless otherwise indicated, experiments were done with cells grown in EMM2 (Mitchinson, 1970). Adenine was added to a final concentration of 2 $\mu\text{g ml}^{-1}$ where indicated. Bacto-agar (2%, w/v) was added to EMM2 and PM when solid media were used. PM was made up as described by Beach *et al.* (1985). YPG contains (w/v) 1% yeast extract, 2% Bacto-peptone, 2% glucose and 2% Bacto-agar. MEA contains (w/v) 3% malt extract and 2% Bacto-agar. All cultures were grown at 37 °C without aeration unless otherwise indicated.

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Table 1. Description of *S. pombe* strains

Strain	Genotype	Source	Construction method
972	<i>h⁻</i> , wild-type	NCYC	—
975	<i>h⁺</i> , wild-type		—
FWP15	<i>h⁻ ade6-M216</i>		—
FWP165	<i>h⁺ ade6-M216 leu1-32</i>	Charles Hoffman	—
	<i>ura4-D18</i>		—
FWP172	<i>h⁻ ade6-M210 leu1-32</i>		—
	<i>ura4-D18</i>	This study	Exposure of 972 to 15 μ M-CuSO ₄ *
SW40	<i>h⁻ cur1-151</i>		Tetrad dissection
SW41	<i>h⁺ cur1-151</i>		Tetrad dissection
SW4	<i>h⁻ ade6-M216</i>	This study	Tetrad dissection
SW5	<i>h⁻ ade6-M216 cur1-151</i>		Tetrad dissection
SW21	<i>h⁺ ade6-M210</i>		Random spore
SW30	<i>h⁺ ade6-M210 cur1-151</i>		Random spore

*Strain SW40 was isolated as a single colony from a strain 972 culture selected for copper-resistant cells by exposure to 15 μ M-CuSO₄.

Growth and cell viability profiles. Mid-exponential phase (OD₅₉₀ 0.2–0.5) overnight cultures of *S. pombe* were diluted to an OD₅₉₀ of 0.1 and CuSO₄ was added to the concentrations indicated in the figure legends. All copper concentrations were in addition to the 0.2 μ M-CuCl₂ present in EMM2. Growth curves were obtained from OD₅₉₀ measurements in autoclaved Kimax 13 × 100 mm screw-top tubes using a Spectronic 20 spectrophotometer. Anaerobic growth was measured using 13 × 100 mm Pyrex test-tubes with fermentation locks. Stir bars were placed in the bottoms of the tubes to allow for mixing prior to OD₅₉₀ measurements. Cell viability during growth was determined by diluting the culture with sterile deionized water at timed intervals, spreading aliquots on YPG plates and counting colonies after incubation for 3 d. Strain SW40 was isolated as a single colony from a culture of strain 972 from which copper-resistant cells had been selected by exposure to 15 μ M-CuSO₄.

Copper uptake. Mid-exponential phase cultures (OD₅₉₀ 0.2–0.5) were harvested by centrifugation (8800 *g* for 10 min) and washed twice with EMM2. Cells were resuspended to an OD₅₉₀ of 0.1 in EMM2–Cu²⁺ (EMM2 made up without CuCl₂). Cu²⁺ was then added as ⁶⁴Cu²⁺ to a final concentration of 0.2 μ M (approximately 1.5 MBq ml⁻¹). CuSO₄ was added to yield the concentrations indicated in Fig. 2 and Table 1. Samples (100 μ l) were removed at timed intervals and cells were harvested by suction filtration on nitrocellulose filters (0.45 μ m pore size) followed by two 5 ml washes with EMM2. The filters were then counted in a Beckman LS1800 scintillation counter using Aquasol as a scintillant. Total cell counts were estimated from a standard curve comparing cell number with OD₅₉₀.

Removal of bound copper with EDTA. Strains 972 and SW40 were incubated with or without 15 μ M-CuSO₄ as described above. After incubation for 12–25 h, samples (100 μ l) were harvested by suction filtration as above and washed once with 30 ml 0.1 M-EDTA. Radioactivity on the filters was counted as described above.

Random spore analyses and strain construction. Random spore analyses and tetrad dissections were done as described by Gutz *et al.* (1974) and Hoffman & Winston (1990). Colonies generated from the random spore analyses were screened for copper resistance by scoring for growth on EMM2 agar with 120 μ M-CuSO₄. The progeny of all crosses were screened for the desired phenotypes using selective media.

Construction and screening of diploid strains. Strains (Table 1) of opposite mating type containing the *ade6-M210* and *ade6-M216* alleles, which complement intragenically (Hoffman & Winston, 1990), were

mixed on MEA. After 1 d, cells from the MEA were spread on PM agar and the resulting colonies replica-plated onto EMM2 with or without 120 μ M-CuSO₄. Diploid colonies were screened for copper resistance on EMM2 agar with 120 μ M-CuSO₄. All incubations were done at 30 °C.

Cell extracts. All strains were streaked initially on EMM2 agar from liquid cultures grown in EMM2. After 2–3 d, cells were removed from the plates and inoculated in EMM2 at 37 °C. Mid-exponential phase cultures (OD₅₉₀ 0.2–0.5) were diluted to an OD₅₉₀ of 0.1 and CuSO₄ was added to the concentrations indicated in the figure legends. Anaerobic cultures were grown in flasks with fermentation locks. Cell cultures (approximately 200 ml) were grown to mid-exponential phase, harvested by centrifugation, and washed once with 10 ml 10 mM-Na₂HPO₄ containing 1 mM-EDTA (pH 7.8). All subsequent steps were done at 4 °C. Cells were resuspended in 5 ml of the phosphate buffer containing 0.1 % Triton X-100. An equal volume (about 5 ml) of glass beads (450–500 μ m diam.) was added to the tube and the mixture was vortexed at high speed for 10 min. The cell homogenate was centrifuged at 12000 *g* for 30 min. The supernatant was stored at –20 °C. The protein concentration of the supernatant was determined by the Lowry method using bovine serum albumin as a standard.

Enzyme assays. Catalase was assayed at 26 °C according to the method of Aebi (1984). Superoxide dismutase was assayed at 37 °C according to the method of Spitz & Oberley (1989).

Reductase assay. Cu²⁺ reductase activity was measured by a modification of the procedure of Dancis *et al.* (1990). Cultures were grown overnight in EMM2, cells (OD₅₉₀ 0.1–0.25) were harvested by centrifugation, washed once with 0.05 M-sodium citrate (pH 6.5) containing 5% (w/v) glucose and resuspended in the same. After 25 min, CuSO₄ and bathocuproine disulphonate were each added to 1.0 mM at a final cell density of 10⁸ cells ml⁻¹. Cells were harvested by centrifugation after 1 h and the A₄₈₅ of the supernatant was determined. The amount of Cu²⁺ reduced to Cu¹⁺ was determined using a standard curve. The control consisted of reaction mixtures without cells. All incubations were done at 37 °C.

Results and Discussion

Selection for a copper-resistant subpopulation

The growth rate of *S. pombe* strain 972 exposed to CuSO₄ concentrations in the 3–12 μ M range (Fig. 1) decreased

Fig. 1. Growth (OD_{590}) of *S. pombe* strain 972 with various $CuSO_4$ concentrations under aerobic and anaerobic conditions. Aerobic cultures were grown with 0 (●), 3 (○), 9 (□), 12 (■) and 15 (△) μM - $CuSO_4$; an anaerobic culture (X) was grown with 15 μM - $CuSO_4$. Representative results are shown.

Fig. 2. Cell viability and amount of copper bound as a function of time for *S. pombe* strain 972 grown with 15 μM - $CuSO_4$ (a) □, viable cells ml^{-1} ; (a) and (b) ●, OD_{590} . (Representative results.) (b) ○, copper bound per 10^6 cells. (The experiment was done three times; error bars represent one standard deviation).

with increasing Cu^{2+} concentration. At $CuSO_4$ concentrations of 12, 15 μM , biphasic growth profiles were observed. As seen in Fig. 2(a), on addition of 15 μM - Cu^{2+} an initial increase in the number of viable cells was followed by a precipitous drop after about 8 h. At approximately 10 h the number of viable cells reached a

minimum value while the amount of copper bound per cell was at a maximum. Following a lag period which varied in duration from one culture to another (see below), resumption of cell growth was observed as monitored by increases in viable cell counts and OD_{590} measurements (Fig. 2a), while the amount of copper bound per cell decreased during the same period (Fig. 2b). Plate assays indicated that in 1.45×10^4 ($\pm 0.4 \times 10^4$) cells were viable when exposed to 15 μM - $CuSO_4$ in EMM2. Under these conditions approximately 0.007% of the cells in populations of strain 972 were viable, indicating that selection for a copper-resistant subpopulation occurred. Thus the biphasic growth observed with 15 μM - Cu^{2+} (Figs 1 and 2) based on OD_{590} measurements was not the result of an adaptation of cells in the culture to the presence of copper in the medium. These results also indicate that selection for copper-resistant cells results in a cell population which contains a lower copper content than the initial wild-type population (Fig. 2b). This conclusion is supported by the decreased copper content of strain SW40 as compared to strain 972 grown with 15 μM - $CuSO_4$ (Table 2).

Biphasic growth profiles were also obtained for cultures derived from single colonies of strain 972 grown with 15 μM - $CuSO_4$. However, variations in the duration of the lag phase ($SD = 5.4$ h, $n = 8$) were noted in these experiments. Samples of cultures derived from one colony also showed the same variations, indicating that the selection for copper-resistant subpopulations is not simply the result of a heterogeneous cell population in the initial strain 972 culture. The fact that copper-resistant cells arise from cultures of clonal origin strongly suggests that any cell in populations of strain 972 has the potential for becoming copper-resistant.

The copper-resistance trait: a genetic characterization

Stability in the absence of selection is indicative of a genetic trait. Strain SW40 retained resistance to copper for a minimum of 85 generations in the absence of 15 μM - $CuSO_4$. A stable genetic trait may be the result of chromosomal (Dancis *et al.*, 1990), cytoplasmic (Smith *et al.*, 1990) or plasmid-based inheritance (Tynecka *et al.*, 1981). A genetic trait based on a single chromosomal locus would be expected to segregate with a 1:1 frequency, in contrast to cytoplasmic or plasmid-based inheritance, neither of which would be expected to yield a 1:1 segregation frequency. Random spore analysis of a cross of strain SW40 with wild-type strain 975 resulted in an approximately 1:1 segregation of the copper-resistance trait (217 copper-resistant, 169 wild-type). The copper-resistance trait in strain SW40 is therefore the result of a modification at a single chromosomal locus.

Fig. 3. Growth of diploid cells on EMM2 with (b) and without (a) 120 μM -CuSO₄. The plates were divided into quadrants, each of which contained diploid cells of varying genotypes: upper-left, SW5 $\times$ SW30 (CuR $\times$ CuR); upper-right, SW4 $\times$ SW21 (CuS $\times$ CuS); lower-right SW4 $\times$ SW30 (CuS $\times$ CuR); lower-left, SW5 $\times$ SW21 (CuR $\times$ CuS). The genotypes of the haploid cells strains are described in Table 1. (Representative results.)

Table 2. Binding of copper by cells before and after treatment with EDTA

All measurements were made after 12.25 h growth with or without Cu²⁺ as indicated. Results are means of three determinations, $\pm$ SD.

Strain	CuSO ₄ concn (μM)	Bound copper		
		pg bound per 10 ⁶ cells	pg bound per 10 ⁶ cells after EDTA wash	Percentage removed by EDTA
972	0	3.79 $\pm$ 0.45	3.36 $\pm$ 0.012	11.3 $\pm$ 0.31
SW40	0	2.92 $\pm$ 0.8	2.55 $\pm$ 0.105	12.7 $\pm$ 3.6
972	15	80.0 $\pm$ 1.2	45.7 $\pm$ 4.5	43.4 $\pm$ 3.60
SW40	15	22.8 $\pm$ 0.9	10.1 $\pm$ 9.6	20.5 $\pm$ 2.19

Often, more than one locus is capable of conferring resistance to a toxic agent (Ono *et al.*, 1988; Phelan *et al.*, 1990). This possibility was examined using a series of independently selected copper-resistant strains. Eleven independently isolated *h⁺* strains selected for resistance to 15 μM -CuSO₄ were crossed with strain SW40. When the progeny of these crosses were screened for copper resistance, all were found to be copper-resistant. Thus, in each of these eleven cases, the copper-resistance trait resulted from an effect at the same genetic locus, which we have designated as *cur1*. The number of independently isolated copper-resistant strains examined did not constitute an exhaustive search for genes capable of conferring copper resistance in *S. pombe*. Nonetheless, these results show that cells with a genetic modification at a single chromosomal locus can be selected from wild-type populations by exposure to 15 μM -CuSO₄. Also, this single locus is modified with high frequency [1 in 1.45×10^4 ($\pm 0.4 \times 10^4$)] to generate copper-resistant cells.

Copper has been shown to act as a mutagen in mice (Agarwal *et al.*, 1990). One possibility for the high frequency generation of copper-resistant cells is that copper also acts as a mutagen in *S. pombe*. To examine

this hypothesis, several mutagenic assays were performed including the screening for mutations in the *ade6* and *ade7* genes (Guglielminetti *et al.*, 1967) of over 60 000 colonies derived from cells exposed to copper. In no case was any mutagenic activity identified (data not shown), which makes it unlikely that copper is acting as a mutagenic agent in the generation of resistant cells.

A dominant/recessive test was used to examine expression of the copper-resistance trait in diploid cells (Fig. 3). The progeny of matings grown in the absence of copper served as a control. Resistance to 120 μM -CuSO₄ was observed only in the copper-resistant homozygotes (Fig. 3), indicating that the trait is recessive in the diploid state.

Enzyme activities and oxidative stress response

In *Sacch. cerevisiae* a number of enzymes involved in response to oxidative stress, such as superoxide dismutase and catalase, increase in activity upon exposure to copper salts (Galiuzzo *et al.*, 1988), and a copper-resistant strain of *Saccho. cerevisiae* has been isolated with increased superoxide dismutase activity as compared to wild-type strains (Naiki, 1980). Upstream

Table 3. *Superoxide dismutase, catalase and Cu²⁺ reductase activities of strains 972 and SW40*

Cells were grown aerobically or anaerobically, with or without 15 μM -CuSO₄ as indicated. Results are means of five determinations, $\pm$ SD.

Strain	Superoxide dismutase	Catalase	Cu ²⁺ reductase [nmol Cu ²⁺ reduced per 10 ⁸ cells h ⁻¹]
	[Units (mg protein) ⁻¹]		
<i>(a) Aerobically grown</i>			
972 - Cu ²⁺	2.55 ± 0.34	4.68 ± 0.31	113.2 ± 12.1
SW40 + Cu ²⁺	2.35 ± 0.22	9.12 ± 0.37	61.2 ± 15.0
SW40 - Cu ²⁺	ND	8.42 ± 0.60	79.0 ± 17.6
SW50 + Cu ²⁺	ND	30.80 ± 3.8	ND
SW50 - Cu ²⁺	ND	20.6 ± 3.02	ND
<i>(b) Anaerobically grown</i>			
972 - Cu ²⁺	ND	0.295 ± 0.33	ND
SW40 + Cu ²⁺	ND	15.44 ± 1.72	ND
SW40 - Cu ²⁺	ND	41.66 ± 6.22	ND

activator sequences believed to be components of a copper-dependent transcriptional activation system have been identified for metallothionein and superoxide dismutase genes in *Sacch. cerevisiae* (Carri *et al.*, 1991). In contrast, the superoxide dismutase activity of cell extracts from *S. pombe* strain SW40 grown in the presence of CuSO₄ was not significantly different from that of the wild-type grown in the absence of copper (Table 3).

In contrast to the superoxide dismutase results, catalase activity was elevated in strain SW40, in a manner that was independent of the presence of copper in the growth medium (Table 3). Anaerobic growth conditions repressed catalase activity to undetectable levels in *Sacch. cerevisiae*, whether copper was present or not (Galiasso *et al.*, 1988). Comparable results were obtained with wild-type *S. pombe* grown anaerobically (Table 3). In strain SW40, however, catalase activity increased in cells grown anaerobically (Table 3). The inability of Cu²⁺ to catalyse the formation of reduced oxygen species under anaerobic conditions (reviewed in Bilinski *et al.*, 1988) as well as the toxicity of Cu²⁺ under these conditions (Fig. 1) would seem to rule out a mechanism of copper resistance dependent on increased catalase activity.

Intra- and extracellular chelation as potential resistance mechanisms

Resistance to metal ions conferred by intracellular chelation (e.g. via metallothionein) normally results in metal ion accumulation (Hamer, 1986). Since strain SW40 accumulated less copper than the wild-type strain when grown with 15 μM -CuSO₄ (Table 2), the copper resistance of strain SW40 is unlikely to be the result of

intracellular chelation. In addition, although *S. pombe* produces class III metallothioneins (phytochelatins) when stressed with Cu²⁺ (Grill *et al.*, 1986), these ions are poor inducers of phytochelatin synthesis in comparison to those of cadmium and nickel (Grill *et al.*, 1986). Cell extracts from strain SW40 were assayed for the presence of phytochelatin using reverse phase HPLC according to the methods of Grill *et al.* (1986). Extracts from cultures grown with and without CuSO₄ contained no detectable concentrations of phytochelatin (data not shown). This fact, along with the reduced copper content in the resistant cells, makes it unlikely that intracellular chelation by phytochelatin is a major contributor to copper resistance.

Resistance mechanisms resulting from decreased metal ion accumulation have been shown to result from energy-dependent efflux (Rouch *et al.*, 1989), metal ion exclusion (Foster, 1977; Rai *et al.*, 1991) or extracellular chelation (Murphy & Levy, 1983). Energy-dependent efflux systems that confer resistance to a number of metal ions have been identified (Rouch *et al.*, 1989; Tynecka *et al.*, 1981). These efflux systems are characterized by the exchange of labelled with unlabelled ions as observed by chase experiments (Tynecka *et al.*, 1981). However, labelled Cu²⁺ could not be chased from either *S. pombe* strain 972 or SW40 (data not shown).

Extracellular chelation of Cu²⁺ should result in the immobilization and consequent detoxification of those ions by the copper-resistant cells. When copper-resistant cells were grown with CuSO₄ in EMM2 to stationary phase, the Cu²⁺ in the resulting conditioned medium remained toxic to copper-sensitive cells (data not shown). Hence, extracellular chelation as a resistance mechanism does not appear likely.

Cu²⁺ reductase activities of strains 972 and SW 40

Copper uptake by *Debaryomyces hansenii* (Wakatsuki *et al.*, 1988) and *Sacch. cerevisiae* (D. Hamer, personal communication) depends on the reduction of Cu²⁺ to Cu¹⁺. Alterations in Cu²⁺ reduction rates leading to decreased copper uptake might account for the decreased copper content of strain SW40 and the recessiveness of the *curl* allele. A comparison of Cu²⁺ reductase activity, using a whole cell assay, showed a decrease of approximately 30% in strain SW 40 as compared to 972 (Table 3). A decrease in reductase activity of this magnitude is probably not sufficient to account for the observed copper resistance for several reasons. First, in a comparable study of a copper-resistant strain of *Sacch. cerevisiae* there was no detectable cell-surface-associated Cu²⁺ reductase activity in the resistant strain (D. Hamer, personal communication). Second, it is unlikely that a 30% decrease in Cu²⁺ reductase activity would result in the large differences in copper accumulation between strains 972 and SW40 (Table 2).

The Cu²⁺ reductase activity of *S. cerevisiae* has been shown to decrease as a function of increasing Cu²⁺ concentration (Dancis *et al.*, 1990; D. Hamer, personal communication). The reductase activity of strain SW40, however, is not significantly decreased by growth with 15 µM-CuSO₄ (Table 3).

Copper exclusion and resistance to membrane damage as potential resistance mechanisms

Apart from the studies of De Vos *et al.* (1991), Foster (1977), and Rai *et al.* (1991) little information is available on the exclusion of copper as a resistance mechanism. One reason for this may be the difficulty in interpreting data on metal ion content of cells in which membrane damage has occurred.

The results in Table 2 are indicative of a resistance mechanism dependent on decreased accumulation of copper. This could be the result of protection from copper-induced membrane disruption (Ohsumi *et al.*, 1988). Such a mechanism has been proposed for the higher plant *Silene cucubalus* (De Vos *et al.*, 1991), for which it was demonstrated that the cytoplasmic membranes of copper-resistant populations were less susceptible to copper-induced ion leakage than copper-sensitive populations. In *S. pombe*, a mechanism of this sort could account for the difference in copper content of the wild type as compared to the resistant strain. Cu²⁺ bound to cells with damaged cytoplasmic membranes would probably be more accessible to EDTA than Cu²⁺ taken up by cells with intact membranes. Similar percentages (approximately 10%) of bound Cu²⁺ were removed by EDTA from strain SW40 grown with or without added

copper and strain 972 grown without added copper (Table 2). However, approximately 46% of the Cu²⁺ bound by strain 972 grown with added copper were accessible to EDTA. This suggests that cytoplasmic membrane disruption occurs when strain 972, but not strain SW40, is exposed to 15 µM-CuSO₄ (Table 2).

It has been proposed that Cu²⁺ damage cytoplasmic membranes via the catalysis of lipid peroxidations (De Vos *et al.*, 1989). However, only fatty acids with at least two double bonds undergo peroxidation under physiological conditions (Bielski *et al.*, 1983). Since *Sacch. cerevisiae* and *S. pombe* contain only saturated and monounsaturated fatty acids (Bilinski *et al.*, 1989; Johnston & Coddington, 1984), the membrane alterations observed with *Sacch. cerevisiae* and *S. pombe* would not seem to be the result of lipid peroxidation. This conclusion is supported by the observation that Cu²⁺ remain toxic to strain 972 under anaerobic growth conditions (Fig. 1), where the oxygen-requiring process of lipid peroxidation could not occur (Bielski *et al.*, 1983).

The copper-resistance trait identified in this paper possibly involves modification of the cytoplasmic membrane resulting in decreased copper accumulation. This modification could confer copper resistance either by lowered activity of a copper transport system or increased stability of cytoplasmic membranes with respect to Cu²⁺. Either of these potential membrane modifications could result from decreases in the number or affinity of cell-surface copper-binding sites. The finding that copper resistance is attributable to a single genetic locus argues in favour of a single rather than a dual mechanism.

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References

- AEBI, H. (1984). Catalase *in vitro*. *Methods in Enzymology* **54**, 121-126.
- AGARWAL, K., SHARMA, A. & TALUKDER, G. (1990). Clastogenic effects of copper sulfate on the bone marrow chromosomes of mice *in vivo*. *Mutation Research* **243**, 1-6.
- BABIOR, B. (1988). Microbial oxidant production by phagocytes. In *Oxy-Radicals in Molecular Biology and Pathology*, pp. 39-51. Edited by P. Cerutti, I. Fridovich & J. McCord. New York: Alan R. Liss.
- BEACH, D., RODGERS, L. & GOULD, J. (1985). *Ran1⁺* controls the transition from mitotic division to meiosis in fission yeast. *Current Genetics* **10**, 297-311.
- BIELSKI, B., ARUDI, R. & SUTHERLAND, M. (1983). A study of the reactivity of HO₂/O₂-with unsaturated fatty acids. *Journal of Biological Chemistry* **258**, 4759-4762.
- BILINSKI, T., LITWINSKA, J., BLASZCZYNSKI, M. & BAJUS, A. (1989). Superoxide dismutase deficiency and the toxicity of the products of autooxidation of polyunsaturated fatty acids in yeast. *Biochimica et Biophysica Acta* **1001**, 102-106.
- BILINSKI, T., KRAWIEC, Z., LITWINSKA, J. & BLASZCZYNSKI, M. (1988). Mechanism of oxygen toxicity as revealed by studies of yeast mutants with changed response to oxidative stress. In *Oxy-Radicals in Molecular Biology and Pathology*, pp. 109-123. Edited by P. Cerutti, I. Fridovich & J. McCord. New York: Alan R. Liss.

- CARRI, M., GALIAZZO, F., CIRIOLO, M. & ROTILIO, G. (1991). Evidence for co-regulation of Cu,Zn superoxide dismutase and metallothionein gene expression in yeast through transcriptional control by copper via the ACE 1 factor. *FEBS Letters* **278**, 263–266.
- DANCIS, A., KLAUSNER, R., HINNEBUSCH, A. & BARRIOCANAL, J. (1990). Genetic evidence that ferric reductase is required for iron uptake in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **10**, 2294–2301.
- DE VOS, C., SCHAT, H., DE WAAL, M., VOOIJS, R. & ERNST, W. (1991). Increased resistance to copper-induced damage of the root cell plasmalemma in copper tolerant *Silene cucubalus*. *Physiologia Plantarum* **82**, 523–528.
- FOGEL, S., WELCH, J., CATHALA, G. & KARIN, M. (1983). Gene amplification in yeast: *CUP1* copy number regulates copper resistance. *Current Genetics* **7**, 347–355.
- FOSTER, P. (1977). Copper exclusion as a mechanism of heavy metal tolerance in a green alga. *Nature, London* **269**, 322–323.
- GALIAZZO, F., SCHIESSER, A. & ROTILIO, G. (1988). Oxy-independent induction of enzyme activities related to oxygen metabolism in yeast by copper. *Biochimica et Biophysica Acta* **965**, 46–51.
- GRILL, E., WINNACKER, E. & ZENK, M. (1986). Synthesis of seven different homologous phytochelatins in metal exposed *Schizosaccharomyces pombe* cells. *FEBS Letters* **197**, 115–120.
- GUGLIELMINETTI, R., BONATTI, S., LOPRIENO, N. & ABBONDADOLO, A. (1967). Analysis of the mosaicism induced by hydroxylamine and nitrous acid in *Schizosaccharomyces pombe*. *Mutation Research* **4**, 441–447.
- GUTZ, H., HESLOT, H., LEUPOLD, U. & LOPRIENO, N. (1974). *Schizosaccharomyces pombe*. In *Handbook of Genetics*, pp. 395–446. Edited by R. King. New York: Plenum Press.
- HAMER, D. (1986). Metallothionein. *Annual Review of Biochemistry* **55**, 913–951.
- HOFFMAN, C. & WINSTON, F. (1990). Isolation and characterization of mutants constitutive for the expression of the *fbp1* gene of *Schizosaccharomyces pombe*. *Genetics* **124**, 807–816.
- JOHNSTON, P. & CODDINGTON, A. (1984). Drug resistance in the fission yeast *Schizosaccharomyces pombe*: pleiotropic mutations affecting the oleic acid and sterol composition of cell membranes. *Current Genetics* **8**, 37–43.
- LIN, C. & KOSMAN, D. (1990). Copper uptake in wild-type and copper metallothionein-deficient *Saccharomyces cerevisiae*: kinetics and mechanism. *Journal of Biological Chemistry* **265**, 9194–9200.
- LINDEGREN, C., NAGAI, S. & NAGAI, H. (1958). Induction of respiratory deficiency in yeast by magnesium, copper, cobalt, and nickel. *Nature, London* **182**, 446–448.
- MARTIN, B. (1984). Bioinorganic chemistry of metal ion toxicity. In *Metal Ions in Biological Systems*, vol. 20, pp. 22–65. Edited by H. Sigel. New York: Marcel Dekker.
- MITCHINSON, J. (1970). Physiological and cytological methods for *Schizosaccharomyces pombe*. *Methods in Cell Biology* **4**, 131–165.
- MOE, O., PHAM, S., SELINSKY, B. & DANG, T. (1985). A kinetic study of the effects of Co^{2+} , Cd^{2+} , Cu^{2+} , and Ni^{2+} on yeast inorganic pyrophosphatase. *Biochimica et Biophysica Acta* **827**, 207–214.
- MURPHY, R. & LEVY, J. (1983). Production of copper oxalate by some copper tolerant fungi. *Transactions of the British Mycological Society* **81**, 165–168.
- NAGAI, S. & NAGAI, H. (1958). Relation of the respiratory competence in yeast to color development and resistance to copper. *Experimental Cell Research* **15**, 623–624.
- NAIKI, N. (1980). Role of superoxide dismutase in a copper-resistant strain of yeast. *Plant and Cellular Physiology* **21**, 775–783.
- OHSUMI, Y., KITAMOTO, K. & ANRAKU, Y. (1988). Changes induced in the permeability barrier of the yeast plasma membrane by cupric ions. *Journal of Bacteriology* **170**, 2676–2682.
- ONO, B., OHUE, H. & ISHIHARA, F. (1988). Role of the cell wall in *Saccharomyces cerevisiae* mutants resistant to Hg^{2+} . *Journal of Bacteriology* **170**, 5877–5882.
- PHELAN, A., THURMAN, D. & TOMSETT, A. (1990). The isolation and characterization of copper-resistant mutants of *Aspergillus nidulans*. *Current Microbiology* **21**, 255–260.
- RAI, L., MALLICK, N., SINGH, J. & KUMAR, H. (1991). Physiological and biochemical characteristics of a copper tolerant and a wild type strain of *Anabaena doliolum* under copper stress. *Journal of Plant Physiology* **138**, 68–74.
- ROUCH, D., LEE, B. & CAMARKARIS, J. (1989). Genetic and molecular basis of copper resistance in *Escherichia coli*. In *Metal Ion Homeostasis: Molecular Biology and Chemistry*, pp. 439–446. Edited by D. Winge & D. Hamer. New York: Alan R. Liss.
- SMITH, M., DUCHESNE, L., BRUHN, J. & ANDERSON, J. (1990). Mitochondrial genetics in natural populations of the plant pathogen *Armillaria*. *Genetics* **126**, 575–582.
- SPITZ, D. & OBERLEY, L. (1989). An assay for superoxide dismutase activity in mammalian tissue homogenates. *Analytical Biochemistry* **179**, 8–18.
- TYNECKA, Z., GOS, Z. & ZAJAC, J. (1981). Energy-dependent efflux of cadmium coded by a plasmid resistance determinant in *Staphylococcus aureus*. *Journal of Bacteriology* **147**, 313–319.
- WAKATSUKI, T., IBA, M. & IMAHARA, H. (1988). Copper reduction by yeast cell wall material and its role in copper uptake in *Debaryomyces hansenii*. *Journal of Fermentation Technology* **66**, 257–265.
- WINGE, D., NIELSON, K., GRAY, W. & HAMER, D. (1985). Yeast metallothionein: sequence and metal binding properties. *Journal of Biological Chemistry* **260**, 14464–14470.