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Adaptation of a *Saccharomyces cerevisiae* strain to high copper concentrations

Ileana Sarais*, Marisa Manzano*, Marco De Bertoldi*, Paolo Romandini†, Mariano Beltramini†, Benedetto Salvato† & Gian Paolo Rocco‡

**Department of Food Sciences, University of Udine, Udine, Italy; †Department of Biology, University of Padova, Padova, Italy and ‡CNR Centre for Biochemistry and Physiology of Metalloproteins, Padova, Italy*

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A strain of *Saccharomyces cerevisiae* has been adapted to increasing concentrations of copper at two different pH values. The growth curve at pH 5.5 is characterized by a time generation increasing with the amount of added copper. A significant decrease of cell volume as compared with the control is also observed. At pH 3 the cells grow faster than at pH 5.5 and resist higher copper concentrations (3.8 against 1.2 mM). Experimental evidence indicates that, after copper treatment, the metal is not bound to the cell wall, but is localized intracellularly. A significant precipitation of copper salts in the medium was observed only at pH 5.5. Increased levels of superoxide dismutase (SOD) activity were observed in copper-treated cells and which persisted after 20 subsequent inocula in a medium without added metal. On the contrary, catalase activity was not stimulated by copper treatment and, hence, not correlated with SOD levels. The mechanism of copper resistance, therefore, probably involves a persistent induction of SOD, but not of catalase, and it is strongly pH-dependent.

Keywords: catalase, copper resistance, pH-dependent growth, *Saccharomyces cerevisiae*, superoxide dismutase

Introduction

Copper is an essential heavy metal for the organisms and, like other oligoelements, it becomes highly toxic when its concentration raises the micromolarity. Several authors have obtained *in vitro* microorganisms (yeasts, moulds, bacteria and protozoa) or mammalian cell lines capable of growing at copper concentrations up to the millimolar range. Copper-resistant cells exhibit the induction of metallothionein and copper enzymes (e.g. superoxide dismutase; SOD) (Thiele 1992). The regulation of the synthesis of these metalloproteins is under the control of ACE1 protein (Carri *et al.* 1991, Gralla *et al.* 1991) and other metalloregulatory transcription factors (Thiele 1992). The uptake of copper by the cell probably occurs by a biphasic mechanism: a fast phase consisting of metal binding to the cell surface, followed by slow transport into the cytoplasm (Harris 1991). Copper toxicity may depend on its capability to participate in redox cycles inside the cells, producing activated derivatives of molecular oxygen, the most harmful one being the $OH^{\cdot}$ radical. The induction of different enzymes which are capable of scavenging some of these cytotoxic products is currently viewed as a cell protection mechanism. In particular, a strong stimulation of Cu,ZnSOD (SOD, EC 1.15.1.1) levels by copper has been demonstrated (Galiazzo *et al.* 1991), while the relation with other oxygen scavenging enzymes, like catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase, EC 1.11.1.6) or peroxidases (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7), is still unclear (Freedman *et al.* 1989, Greco *et al.* 1990).

In this work we have studied the relationships among cell growth, metal uptake and cellular localization, and enzymatic activities responsible for the scavenging of toxic oxygen species (superoxide and hydrogen peroxide) using the *Saccharomyces cerevisiae* SS1189 strain. This yeast strain was previously described for the effects of acute exposure to heavy metals (Romandini *et al.* 1992), and for the interaction between methanol and metals (Manzano *et al.* 1993). In this work the *S. cerevisiae* SS1189 strain has been adapted up to 3.8 mM Cu in the culture medium.

Address for correspondence: P. Romandini, Department of Biology, University of Padova, Via Trieste 75, 35121 Padova, Italy. Tel: (+39) 49 8286349. Fax: (+39) 49 8286374.

Materials and methods

Chemicals

All reagents were of the best grade commercially available and were used without further purification.

Yeast strain and media

A *S. cerevisiae* SS1189 strain obtained from Sassari (Italy) collection, and tested by an API 32 ATBC system, was used.

Cells were grown in Yeast Nitrogen Base (Difco, Detroit, MI) liquid medium with 2% (w/v) glucose for the control condition (YNBG) and in YNBG with the addition of copper sulfate for the samples under test. Glucose and copper sulfate solutions were sterilized by filtration with a Minisart filter at 0.2 μm pore size (Sartorius, Göttingen, Germany) and added to the YNBG media previously autoclaved at 121 °C for 15 min.

Growth test

The strain was grown on: (i) YNBG at pH 5.5 and 3.0 (the second broth was brought to the desired pH by the addition of 0.1 N HCl), and (ii) YNBG (pH 5.5 and 3.0) with the addition of a suitable volume of a concentrated solution of $CuSO_4 \cdot 5H_2O$ to reach the maximum final concentration of 3.8 mM.

Cells, activated in the different culture media, were inoculated (5×10^3 cells/ml final concentration) in 250 ml plugged flasks containing 100 ml of the growth medium. The cultures were incubated in a water-bath at 30 °C and shaken at 140 r.p.m. until they reached the stationary phase. Every 6 h a total count with a Burker camera was made. A sample of cell suspension was collected at the beginning of the stationary phase and cell size was measured using a microscope equipped with a micrometer.

After this test, approximately 1 g of cells was collected by centrifugation at $3300 \times g$ at 15 °C for 15 min using a refrigerated centrifuge (ALC model 4233 equipped with a RCF meter). The cells were washed in different media for different purposes: (i) in the culture medium without metal (YNBG) and then in phosphate buffer (0.1 M, pH 7.8) containing 10 mM ethylenediaminetetracetic acid (EDTA), for the analysis of metal uptake, and (ii) in phosphate buffered saline (PBS) solution, for the preparation of cell extracts for enzymatic assays. The cells were stored at −18 °C until biochemical analyses.

Copper determinations

The determination of metal uptake by yeast cells was performed after disruption of cell walls. This was obtained as previously described (Romandini *et al.* 1992) with the only exception being that 0.1 N HNO_3 was used instead of phosphate buffer. The cell extract was collected and pooled with the aliquots of HNO_3 used to rinse the disruption chamber (three times with 10 ml each). The copper content was measured by atomic absorption spectroscopy with a Perkin-Elmer (Beaconsfield, UK) model 4000 spectrophotometer.

Copper determination was also performed in the media (or buffers) utilized for washing the cells and in the culture media at the end of growth after removing the cells by filtration.

The growth media were also analyzed for the oxidation state of copper using the 2,2′-biquinoline assay (Klotz & Klotz 1955). Determination of the copper concentration as well as of its oxidation state in the growth media (before and after cell inoculum) were performed at the beginning of growth and at different days of incubation. The cell-free media were centrifuged at $49000 \times g$ for 1 h; the supernatant and the pellet (when present) were collected and separately assayed for total copper content and for Cu(I) or Cu(II) concentration. Copper was also determined with the same method in cell extracts obtained from cultures at pH 3 and 5.5, before and after 48 h of dialysis against phosphate buffer (containing, in the first 24 h, 10 mM EDTA).

Biochemical determinations

The preparation of crude extracts from cell samples was done as previously described (Romandini *et al.* 1992). In addition the glass beads were washed three times with 5 ml of buffer.

Protein content was measured spectrophotometrically according to Peterson (1977).

SOD activity was determined using a Lumicon (Hamilton, Bonaduz, Switzerland) luminometer thermostated at 20 °C and equipped with an automatic injector. A modification of the method proposed by Puget & Michelson (1974) was used. The sample (up to 800 μl) was added into a luminometer cuvette containing: 20 μl of a 0.7 μM xanthine oxidase solution (grade III, Sigma, St Louis, MI) in 2.3 M ammonium sulfate, 780 μl of 0.15 M sodium carbonate buffer, pH 10.2, containing 150 μM EDTA and 100 μM luminol (5-amino-2,3-dihydro-1,4-phtalazinedione); demineralizated water (milli-Q; Millipore, Milan, Italy) was added to a total volume of 1.6 ml, including the sample. To start the reaction, 800 μl of hypoxanthine (30 μM dissolved in air-equilibrated water and maintained in an ice bath) were injected by the automatic injector of the luminometer, until a total reaction mixture volume of 2.4 ml. The maximum emitted chemiluminescence and the total light emitted over 10 min were recorded in the Repeat Mode (Lumicon user's standard software); in our system 0.1 units of SOD activity corresponded to an inhibition of 50% of the light produced by a blank solution (all reagents without the sample). Several calibration curves were determined utilizing 1–10 ng/ml of a Cu,ZnSOD from bovine erythrocytes and 0.1 units corresponded to about 5 ng Cu,ZnSOD/ml of reaction mixture. To distinguish between Cu,ZnSOD and MnSOD activity, the enzymatic assay was repeated in the presence of 1 mM KCN.

Catalase activity was determined by means of the same luminometer, using an adaptation of the method of

Michelson *et al.* (1977). The sample was brought to 250 μl with water and incubated with 250 μl of H_2O_2 (21 μM in 0.01 M KH_2PO_4, pH 7.8) in a luminometer cuvette for 20 min at 25 °C. After incubation, 1 ml of potassium phosphate buffer (0.1 M, pH 7.8) containing luminol (0.1 mM), EDTA (0.1 mM) and horseradish peroxidase (5 μg) was automatically injected in the sample-containing cuvette. The total chemiluminescence emitted was recorded in the Peak Mode (Lumicon user's standard software) for 90 s and the catalase activity was referred to a calibration curve obtained with 1–12 ng of bovine liver catalase (42 000 U/mg, Sigma) and comparing the inhibition of total light of a blank containing all reagents without the sample.

Results and discussion

The *S. cerevisiae* SS1189 strain has been previously described (Romandini *et al.* 1992, Manzano *et al.* 1993). In this work it has been stepwise adapted to a copper concentration up to 80 μg/ml (i.e. 1.26 mM of Cu^{2+}), by reinoculating the cells in the standard medium, YNBG pH 5.5, in the presence of increasing amounts of the metal. The concentration of 1.26 mM of Cu^{2+} was the highest concentration compatible with cell reproduction in the standard medium. However, as shown in Figure 1, a lower growth rate was observed at this copper concentration as compared with the control: a generation time $G = 10.68$ h ($k = 0.0649$ generations/h) was obtained for Cu_{80}-adapted cultures versus a $G = 2.23$ h ($k = 0.311$ generations/h) for control cultures. At a copper concentration of 1.57 mM (100 μg/ml) no growth was observed within 182 h. The same result was obtained with a 100-fold higher inoculum.

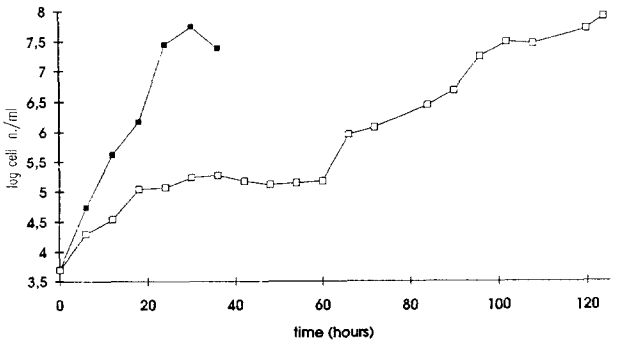

Figure 1. Growth rate for the control (■) and the Cu_{80}-adapted strain (□) in YNBG medium at pH 5.5.

The Cu_{80}-adapted cells were subjected to 20 subsequent passages in standard medium (without copper, ex-Cu_{80} strain) and then reinoculated in the copper-containing medium, to test the maintenance of resistance. Indeed, it was able to reach the stationary phase in the same time (about 120 h) as the Cu_{80}-adapted strain. This result indicates that the induced copper resistance persists for several generations, as expected in the case of genetic adaptation of the yeast rather than in the case of a transitory metabolic response. The time intervals required to reach the stationary phase in the different experimental conditions, together with the corresponding cell volumes, are reported in Table 1. The volume of the copper-treated cells was about 60% less than the control but this effect seems to be independent of the metal dose. The tendency of the cells to aggregate was also enhanced upon copper treatment (Fig. 2A and B). The change of the cell size seems to be a characteristic of metal adapted cells: the cell volume of Cu_{80}-adapted yeasts remained smaller as compared with the control even after 20 subsequent inocula in the copper-free medium (ex-Cu_{80}). Most probably the cell size can be correlated with the genetic changes induced by the adaptation to the metal. This result contrasts with other observations showing that exposure of unicellular eucaryotes to heavy metals caused an increase of cell size (Foster 1977), attributable to the inhibition of the reproductive phase in spite of the increase of cell biomass. In the present case, at high copper concentration, the smaller cell volume suggests a reproductive rate higher than the biomass increment rate, therefore resulting in a further biomass decrease.

The findings about the growth rate can be assigned to a specific effect of copper in the growth medium as demonstrated by incubating the cells in a medium where copper was present together with a non-cytotoxic chelating ligand, like sodium citrate. The addition of 10 mM citrate to copper-containing YNBG abolished the effects of the metal on the growth curve: SS1189 control cells inoculated in YNBG or in YNBG with the concomitant presence of copper and sodium citrate (after 20 subsequent inocula in

Table 1. Time required to reach the stationary phase and cell volume of SS1189 strain grown at pH 5.5

Copper (μg/ml)	Growth time (h)			Cell volume (μm³)		
	control	Cu_{80}	ex-Cu_{80}	control	Cu_{80}	ex-Cu_{80}
0	25	25	25	573	120	118
20	NG	48	48	NG	107	129
40	NG	48	72	NG	76	72
60	NG	73	73	NG	149	45
80	NG	120	120	NG	90	41
100	NG	NG	NG	NG	NG	NG

NG, no growth. Control: SS1189 strain not treated with copper. Cu_{80}: SS1189 strain pre-adapted to 80 μg/ml of Cu(II). ex-Cu_{80}: the same strain of Cu_{80} but after 20 successive inocula in YNBG without copper.

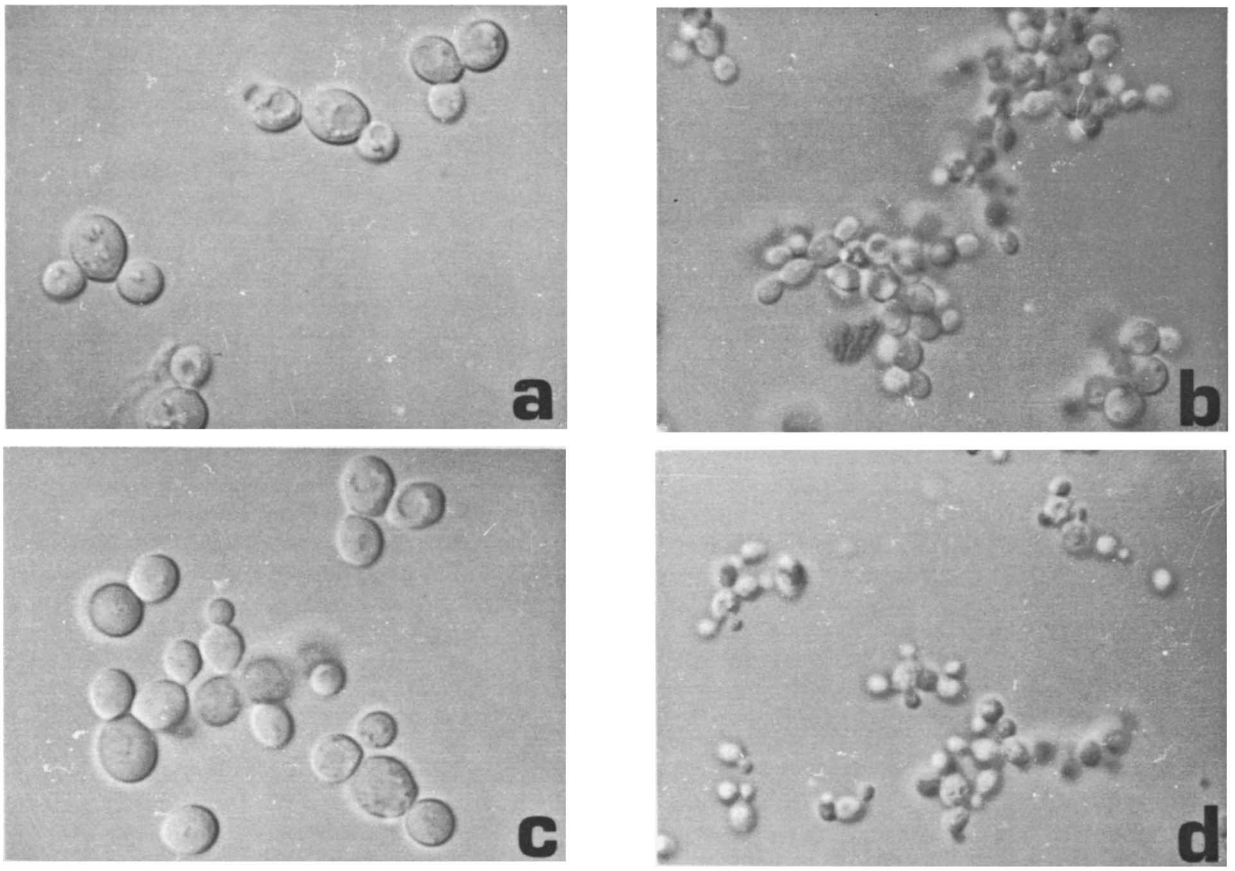

Figure 2. Cells of *S. cerevisiae* SS1189 strain. Total magnification 5200× (Objective: 100×; eyepiece: 10×; photographic enlargement: 5.2×). (**a**) Control in YNBG medium at pH 5.5. (**b**) Cu_{80}-adapted in YNBG, pH 5.5, plus 1.26 mM Cu^{2+}. (**c**) Control in YNBG at pH 3. (**d**) Cu_{80}-adapted in YNBG, pH 3, plus 1.26 mM Cu^{2+}.

the copper plus citrate medium) exhibited the same growth rate. In contrast, when the same cells were reinoculated in YNBG plus copper without citrate no growth was observed (data not shown).

Cu_{80}-adapted cells collected at the stationary phase and reinoculated in a fresh copper-containing medium exhibited a lower growth rate as compared with the parental cells. We therefore considered the possibility that cells modify the composition of the external medium during growth. On this basis we reinoculated the same adapted cells (collected at the stationary phase) in a medium in which the cells were previously grown, after filtration and normalization of the medium to the starting conditions, i.e. addition of a new amount of glucose and copper, and the correction of pH to 5.5. This culture showed a low growth rate coincident with that of a normal culture at pH 5.5 and in the presence of 1.26 mM Cu. On the contrary, when the medium was kept at the pH value reached at the end of the growth (approximately pH 3) and after normalization of the other conditions, the growth rate of the reinoculum was higher.

The Cu^{2+} concentration in solution is strongly dependent on the pH and the medium composition if potential ligands of this metal are present. As reported by Huges & Poole (1991) the microbial growth in the presence of metal ions at different pH values varies greatly. Out results indicate that the capability of this Cu_{80}-adapted yeast strain to grow in the presence of copper is much higher at pH 3 than at pH 5.5. To test this point SS1189 cells were grown on YNBG at pH 3 and in this medium the metal concentration can be raised as high as 240 μg/ml (corresponding to about 3.8 mM) (Table 2). The different pH of the culture medium has *per se* a marked effect on cell size: the volume of the control cells, grown at pH 3 and in the absence of copper, was 50% bigger as compared with the control cells grown at pH 5.5 (Table 2 versus Table 1). In contrast, the size of Cu_{80}-adapted cells at pH 3 remained in the same range of values as that at pH 5.5 (Figure 2C and D). The cellular properties induced by copper treatment at pH 3 remained even for the ex-Cu_{80} cells (Table 2).

Interestingly, the increased metal resistance of the cells elicited by lowering the pH parallels the effects on metal solubility: the analysis of the growth medium in the two experimental conditions demonstrated the presence of microcrystals formed only in the pH 5.5 medium. In fact only at this pH was a pale blue pellet obtained. On the contrary, no precipitation was observed at pH 3.

The culture media were also controlled for the oxidation state of the metal and for its ligation state. The measure-

Table 2. Time required to reach the stationary phase and cell volume of SS1189 strain grown at pH 3

Copper (μg/ml)	Growth time (h)			Cell volume (μm^3)		
	control	Cu_{80}	ex-Cu_{80}	control	Cu_{80}	ex-Cu_{80}
0	25	25	25	897	92	159
80	NG	48	48	NG	106	142
100	NG	72	72	NG	85	ND
160	NG	72	72	NG	ND	130
240	NG	72	72	NG	ND	127

NG, no growth. ND, not determined. For further details see Table 1.

ment of copper (Table 3) confirmed the better solubility of the metal at the lower pH and the presence of all copper as Cu(II), either in solution as well as in the pellet. The same test was repeated after cell growth and removal of cells by filtration (in both conditions, after cell growth the pH of the medium became approximately 2.8). The amount of pellet recovered from the medium originally at pH 5.5 was very low and copper was all Cu(II) again. On the contrary, when the copper present in cell extracts was analyzed after dialysis against EDTA-containing phosphate buffer, the metal was present almost totally as Cu(I). When the determination was done before dialysis of the extract, however, about 30% of copper was in the form of Cu(II). Furthermore, approximately 50% of the total intracellular copper was found to be resistant to dialysis against EDTA.

We conclude therefore that at pH 3 copper may be present in the medium bound to different ligands, in a form less toxic than at pH 5.5, e.g. at the latter pH, copper could precipitate on the cell wall as insoluble copper carbonate (J. Peisach, personal communication) and so interfere with cellular diffusion mechanisms of solutes.

The stability of copper binding to the cell surface was tested by measuring the copper content of the cells after two cellular wash steps, i.e. with YNBG medium and with EDTA-containing buffer. The double washing with the same solution did not improve metal removal. When the Cu_{80}-adapted cells were grown in the presence of different amounts of copper (Table 4), they accumulated about 8- to 9-fold more metal as compared with the control cells, independently of both the pH and the concentration of

Table 3. Copper content in the culture media and in the cellular extract of the SS1189 strain grown in 100 ml of medium (80 μg/ml Cu)

Sample	pH 5.5		pH 3.0	
	total Cu (mg)	Cu(I) (%)	total Cu (mg)	Cu(I) (%)
Media before growth				
supernatant	6.535	2.2	8.556	0.9
pellet	1.297	1.5	0	0
Filtered media after growth				
supernatant	6.137	0.5	6.886	0.9
pellet	0.146	7.8	0	0
Cellular extract				
before dialysis	0.739	75.4	0.785	66.0
after dialysis[a]	0.338	93.8	0.252	77.0

[a]Dialysis was kept overnight against phosphate buffer (0.1 M, pH 7.8), containing 10 mM EDTA, and for a further 24 h against the same buffer but without EDTA.

Table 4. Copper content in media or cells, determined by atomic absorption spectrophotometer (μg or μg/mg of total protein, for cell extracts)

Sample and treatment	Total copper	Culture medium	Medium		EDTA		Cell extract
			wash I	wash II	wash I	wash II	
Control pH 5	41.6	29.4	—	—	0.0	—	6.0
Control pH 3	39.6	31.1	—	—	1.6	—	6.0
Cu_{20} pH 5	1605.0	1352.0	69.0	—	47.4	—	136.6
Cu_{80} pH 5	6522.3	6291.7	—	—	51.9	—	114
Cu_{80} pH 5	7068.9	6569.8	127.2	29.6	28.1	11.3	303.0
Cu_{80} pH 3	6546.0	6139.0	—	—	179.7	—	220.2
Cu_{160} pH 3	12928.5	12495.5	—	—	108.4	—	249.1

Table 5. Cu,ZnSOD and catalase activity levels in SS1189 cells treated with 1.2 mM of Cu(II) or not treated

Enzymatic activity	Treatments		
	control	Cu_{80} pH 5.5	Cu_{80} pH 3.0
SOD			
(U/mg proteins)	13.65	127.50	154.45
(standard error)	3.36	1.98	3.15
Catalase			
(U/mg proteins)	38.20	20.80	13.45
(standard error)	2.19	3.96	0.39

Control: SS1189 strain not treated with Cu. Cu_{80}: SS1189 strain preadapted to 80 μg/ml of Cu(II), at pH 5.5 or 3, respectively.

copper in the culture medium. The stable copper can be thought of as being firmly bound to the cell wall or more probably localized in the inner compartment of the cell bound to specific proteins like metallothioneins (Butt *et al.* 1984) and other copper proteins (Thiele 1992).

The intracellular localization of the copper was supported by the finding that the copper treatment resulted in a strong (about 10-fold) stimulation of the level of Cu,ZnSOD activity (Table 5). This stimulation was observed for cells grown both at pH 5.5 and 3, indicating that the process was fairly independent of the pH of the medium. Quite interestingly, the ex-Cu_{80} cells maintained the increased level of Cu,ZnSOD activity after 20 subsequent inocula in fresh medium without added copper (about 150 U/mg of proteins).

In contrast, the catalase activity was not stimulated by copper treatment (Table 5). A preliminary assay of glutathione peroxidase activity, which was previously recovered by Galiazzo *et al.* (1987) from copper-treated yeasts, showed that in all the conditions we tested the enzyme is below the detection limits of the method used (Lawrence & Burk 1976) (data not shown).

Therefore the process of Cu,ZnSOD stimulation in the presence of copper could be linked not only to oxygen metabolism but it could be also dependent on the overlapping of the induction process between metallothionein and Cu,ZnSOD via a common copper-sensitive cytoplasmic factor, as previously reported (Carri *et al.* 1991).

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