Appl Biochem Biotechnol (2009) 157:85–97
DOI 10.1007/s12010-008-8253-9

Isolation and Biomass Production of a *Saccharomyces cerevisiae* Strain Binding Copper and Zinc Ions

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Received: 1 February 2008 / Accepted: 18 April 2008 /
Published online: 30 May 2008
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Abstract Copper and zinc are essential trace elements participating in many physiological functions, notably immunity and protection against oxidative stress. Yeasts and the yeast *Saccharomyces cerevisiae*, in particular, possess in their genome tandem repeats of the *CUP1* gene coding for a protein (a metallothionein) capable of capturing and binding toxic elements such as copper ions. The number of copies of this gene in a cell determines its physiological level of resistance to these ions. This paper describes the selection, characterization, and production of a new copper-resistant yeast strain that can bind large quantities of copper and zinc. This approach should lead to increasing the bioavailability of these trace elements and hence to reducing their emission into the environment.

Keywords Selection · Metallothionein · Probiotic · Prebiotic · Yeast · *CUP1* · Bioreactor

Introduction

Public opinion is increasingly concerned about issues such as the quality of consumer products, animal welfare, and environmental protection. In this sustainable development perspective, modern agriculture must reduce or stop the use of antibiotics leading to the emergence of resistant bacteria. It must also be able to supply various trace elements indispensable to animal health while limiting as much as possible their emission into the environment. Aware of this problem, public authorities have prohibited the use of certain

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antibiotics in animal feed and have imposed increasingly restrictive regulations on the use of other food additives. Producers, on the other hand, must maintain a good level of animal performance to ensure the viability of their farms.

The past few years have seen the emergence of prebiotics and probiotics, i.e., food additives capable of improving resistance to disease in both humans and animals and, more generally, of enhancing the well-being and health of individuals [1–5]. Man has used yeasts, particularly *S. cerevisiae*, for centuries. Yeasts are the group of microorganisms to have been the most exploited commercially. Incorporated alive as probiotics into the feed of weanling pigs, yeasts can stimulate digestion, be a source of interesting enzymes (phytases), and help to maintain the microbial balance in the intestine of these animals [6–9]. Dead yeasts can be used also as prebiotics having a non-negligible effect on animal health [providing mannan oligosaccharides (MOS), for instance] [10].

Furthermore, genetic and molecular biological studies conducted by the group of S. Fogel and J. Welch have shown that *S. cerevisiae* cells contain, within their genome, tandem repeats of the *CUP1* gene encoding metallothionein, one of the proteins responsible for copper detoxication and binding during fermentation. This gene, located on chromosome VIII, belongs to a two-gene repeat unit [11, 12]. This structure is depicted in Fig. 1.

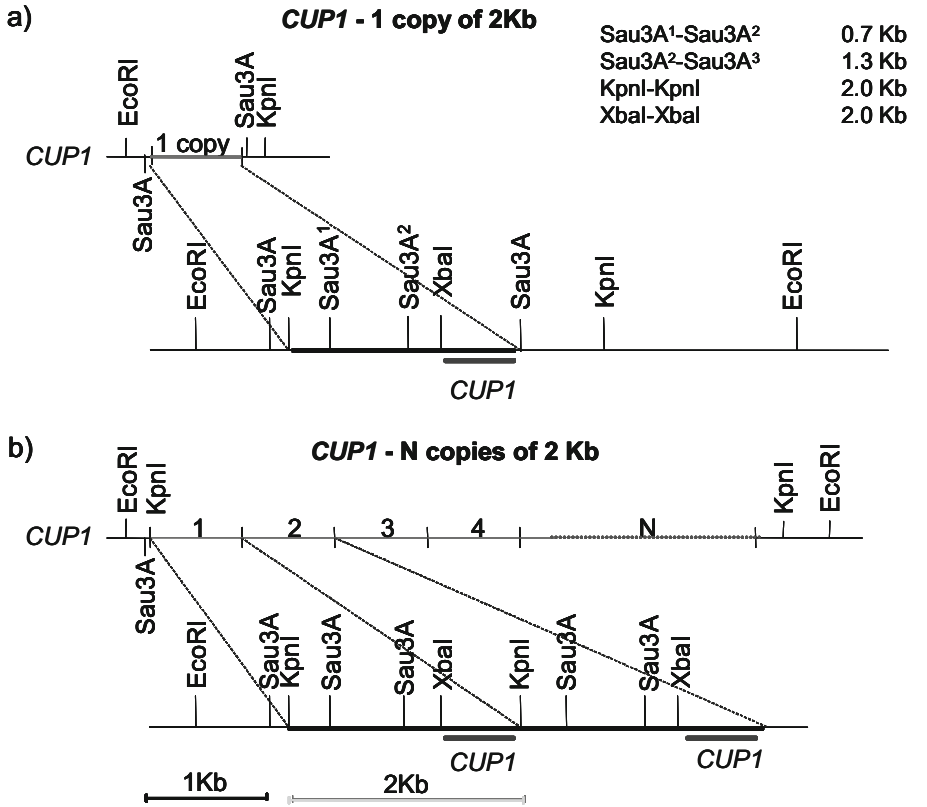

Fig. 1 Structure of the repeat unit containing the CUP1 gene of *S. cerevisiae* chromosome VIII. **a** Strain containing one copy of the CUP1 gene. **b** Strain containing N copies of the CUP1 gene

This tandem repeat is 2 kb long and contains many restriction sites. In the presence of increasing concentrations of copper in the growth medium, the number of copies of this unit increases. There exists a direct correlation between the number of repeats of the gene and phenotypic cell resistance to Cu^{2+} ions [13]. Transcriptional regulation of the *CUP1* gene involves the Ace1p (activator of CUP1 expression) protein. Copper present in the cell binds to this protein, altering its conformation so that it can bind to the upstream activator sequence (UAS) of the *CUP1* gene [14, 15]. Other genes, such as *CRS5* (metallothionein non homologous to *CUP1*) and *SOD1* (superoxide dismutase), are also responsible for copper resistance, but to a much lesser extent [16, 17].

As for zinc, the interactions of mineral zinc with other mineral and organic food constituents are mainly responsible for its variability and availability, the main determining factors being the fiber, phytate, and calcium contents. Other factors may be involved, such as the phosphate, copper, cadmium, and iron contents and the protein source and content [18]. These secondary factors often induce a zinc deficiency by preventing zinc absorption at the level of the intestine. By providing zinc in an organic form, it should thus be possible to diminish the interactions of zinc with these other elements/factors.

As copper and zinc are bound to *S. cerevisiae*, they might be administered in organic form and might thus have a direct impact on growth efficiency and on the immune system. Their rational use in a bioavailable form lead to reducing (or eliminating) the use of antibiotics in animal feed and to reduced emission into the environment of unassimilated forms.

The present study focused on selecting and characterizing a new yeast strain capable of binding large quantities of copper and zinc and also on the production of this strain in a fermentor, with a view to its use as a prebiotic or probiotic. The ions and the vector (*S. cerevisiae*) chosen here may have a direct impact on immune system function and may contribute to reducing (or eliminating) the use of antibiotics in animal feed.

Materials and Methods

Yeast Culture Media

The medium used in this study was yeast extract/peptone/dextrose (YPD) medium. This is a rich medium composed of the following ingredients: 1% yeast extract (Gibco, Scotland), 2% peptone (Gibco), and 2% glucose (Gibco).

Storage and Plating of Yeast Cells

The 19 *S. cerevisiae* strains tested in this project are natural strains previously isolated from grapes. They were stored at −70 °C in YPD+20% glycerol. They were grown at 29 °C on Petri plates containing YPD and then purified by spreading onto new plates to obtain isolated colonies. These colonies provided the basis of selection.

Bioreactor Operation

Several working seeds of the previously described strain were produced and stored at −80 °C. These seeds were used to produce precultures. Culture was carried out in a 20-l stirred bioreactor (vessel diameter=0.22 m; Biolaffite, France) equipped with an RDT6 Rushton turbine (impeller diameter=0.1 m) in basic medium containing 5 g/l glucose (initial

concentration before the fed-batch operation), 10 g/l yeast extract, and 10 g/l casein peptone. The initial working volume was 12 l. Regulation of the culture parameters (pH, temperature, etc.) was ensured by a direct control system (ABB). Dissolved oxygen was maintained above 30% saturation by modulating the stirrer speed. Growth of the strain was carried out in fed-batch mode, and both glucose (400 g/l) and the copper solution were added to the bioreactor according to the following exponential feeding algorithm: $F=F_0 \exp(\mu t)$, where F is the feed flow rate (m^3/s), F_0 the initial feed flow rate (m^3/s), μ is the microorganism growth rate (h^{-1}), and t the culture time.

Pulsed Field Gel Electrophoresis

Whole chromosomes of the 19 new strains were extracted and separated by pulsed gel electrophoresis according to Johnston [19] and Friel et al. [20]. The CHEF-DRII apparatus (contour clamped homogeneous electric fields) from Bio-Rad was used to separate the yeast chromosomes.

Selection of the Most Copper-Resistant Strains

The strains were first spread onto YPD containing 100 ppm copper and incubated at 29 °C for 2 days. They were then subjected to successive replatings, at 2-day intervals (incubation at 29 °C), on YPD supplemented with increasing amounts of $CuSO_4$ ranging from 200 to 700 ppm copper (with 100 ppm increments).

Total DNA Extraction from Yeast Cells

Total DNA was extracted by a standard procedure [21] after pre-treatment of the cells with zymolyase 20T.

Analysis of Total Yeast DNA by Southern Blotting

First, the chromosomal DNA was digested with *Eco*RI as recommended by the supplier (Amersham Biosciences, UK). The DNA restriction fragments obtained were separated by electrophoresis through a 0.8% agarose gel as described by Sambrook et al. [22]. The fragments were visualized under UV light after ethidium bromide staining and were transferred by capillarity onto a Hybond N + membrane (Amersham Biosciences). The membrane was heated at 80 °C for 3 h, and hybridization was carried out with a ^{32}P-labelled *CUP1* DNA probe. Labeling was done with the Megaprime DNA Labeling System (Amersham Bioscience) and with [α-P^{32}] 2′-deoxycytidine 5′-triphosphate (3,000 Ci/mol, Amersham Bioscience) as recommended by the supplier. The hybridization and pre-hybridization conditions were as described by Ausubel [20]. A solution of 20× saline-sodium citrate (SSC) was prepared (173.3 g/l NaCl and 88.2 g/l Na citrate, adjusted to pH 7.5 and autoclaved before use), and the membranes were washed for 5 min at room temperature with 4× SSC+0.1% sodium dodecyl sulfate (SDS), 2× SSC+0.1% SDS, and 0.5× SSC+0.1% SDS successively and finally for 10 min with 0.2× SSC+0.1% SDS.

Total RNA Extraction

When the optical density (OD) of the culture reached 0.5, the culture was centrifuged for 10 min at 7,000 rpm and 4 °C. The pellet was resuspended in 1 ml RNA buffer [0.5 mol/

l NaCl, 0.2 mol/l Tris–HCl, 0.01 mol/l ethylenediaminetetraacetic acid (EDTA), pH 7] and 1 ml phenol and mixed for 10 s. To break the yeast cells, 50 μl glass beads (0.45 μm in diameter) were added. The suspension containing the beads was mixed 12 times for 30 s, with a 30-s cooling period (on ice) after each mixing. Three milliliters of phenol, then 3 ml RNA buffer were added and mixed with the solution for 1 min. The resulting mixture was then centrifuged for 10 min at 6,000 rpm and 4 °C. Two to three extractions were carried out with 4 ml phenol. The RNA was precipitated with 2.5 vol. 100% ethanol at 4 °C for 10 min. The mixture was then centrifuged for 20 min at 13,000 rpm and 4 °C. The supernatant was discarded and the pellet resuspended in 2.5 ml buffer (0.01 mol/l Tris–HCl, 0.1 mol/l NaCl, 0.006 mol/l EDTA, 1% SDS, pH 7.5) containing 1 mg/ml proteinase K, incubated for 15 min on ice, and then incubated for another 30 min at 37 °C. The mixture was then extracted with an equal volume of phenol and finally with ether, after which the RNA was precipitated with 2.5 vol 100% ethanol at 4 °C and centrifuged. The pellet was dried and resuspended in 300 μl Na acetate (0.3 mol/l, pH 4.5). Finally, a 15-μl aliquot was diluted in 1.5 ml water. The OD of this mixture was measured at 260 and 280 nm, and the total RNA was divided into 100 μg aliquots and stored at −80 °C.

Northern Blotting

Total extracted RNA was subjected to agarose gel electrophoresis under denaturing conditions (in the presence of formaldehyde) according to Vandenbol et al. [23]. The RNA bands were then transferred by capillarity to a nylon filter and allowed to hybridize with a ^{32}P-labelled *CUP1* probe (Megaprime DNA Labeling System, Amersham Bioscience) [23].

The filters were washed at room temperature for 10 min with 2× SSC+0.1% SDS, 1× SSC+0.1% SDS, and 0.5× SSC+0.1% SDS successively.

To validate the results and to ensure that the same amount of RNA was deposited in each well, the same filters were hybridized with an *ACT1* probe (*ACT1* being the yeast gene coding for actin and whose expression is constitutive).

Quantitative RT-PCR

Relative quantification of *CUP1* mRNA was done by quantitative reverse transcriptase polymerase chain reaction (RT-PCR), with the primers and probes shown in Table 1 (defined with the help of the Primer Express program). First-strand complementary DNA (cDNA) was obtained from total RNA with the help of the First Strand cDNA Synthesis Kit. The PCR reaction was carried out with 5 μl cDNA5, 25 μl TaqMan Universal PCR

Table 1 Names and sequences of the primers and probes used to quantify CUP1 and PDA1.

Primer/probe	Sequence (5′-3′)
CUP1 F	AGCTGCAAAAATAATGAACAATGC
CUP1 R	GCATTTGTCGTCGCTGTTACA
CUP1 probe	FAM-AATCATGTAGCTGCCCAAC-NFQ-MGB
PDA1 F	CAGGTTCTTTTAGGTGCAGGTT
PDA1 R	AAGAGCAGGCGTCCTCGTT
PDA1 probe	FAM-AGCTTTTGCTCACCAATA-NFQ-MGB

F Forward, *R* reverse

Master Mix (Applied Biosystems, Foster City), 300 nmol/l of each primer, and 200 nmol/l probe in a final volume of 50 μl. Thermal cycling conditions were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min in an ABI Prism 7900 (Applied Biosystems).

Quantification of *CUP1* and of the *S. cerevisiae* housekeeping gene *PDA1* (encoding the E1α subunit of the pyruvate dehydrogenase complex) was done by means of two standard curves (one for each gene). The standard used for *CUP1* (undiluted standard) was that used for Northern blotting. The first point of each straight line represented 300 gene copies, the following points having been obtained by serial tenfold dilutions. Relative *CUP1* expression was determined by dividing the quantity obtained for *CUP1* by that obtained for *PDA1*.

Assays for Soluble Protein-Bound and Total Copper and Zinc in Yeast

To assay the copper and zinc ions contained in yeast, cell lysis is necessary. The assays were done by polarography (ASV). Lysis was carried out at 4 °C as follows. The yeast culture was centrifuged for 5 min at 3,000 rpm. The culture supernatant, containing unbound copper and zinc, was eliminated. The pellet was washed in 500 μl sterile water, centrifuged for 30 s at 13,000 rpm, and then resuspended in 500 μl lysis buffer (0.05 mol/l sodium phosphate, pH 7.4; 5% glycerol) so as to reach an OD between 50 and 100. An equal volume of glass bead slurry was added, and the mixture was vortexed eight times for 30 s with a 30-s pause on ice after each vortexing. The mixture was then centrifuged for 10 min at 13,000 rpm and the supernatant collected. Copper and zinc were assayed in this supernatant and in the initial uncentrifuged lysate (total protein-bound copper and zinc). The concentrations of copper and zinc ions in the supernatant were considered to reflect the amount of soluble protein-bound copper and zinc. They were expressed as percentages of total cell-associated copper and zinc.

Before the assays were performed, the supernatant and the lysate were mineralized as follows. One hundred milligrams of sample was weighed into a Teflon reactor. To this was added 5 ml of 22 mol/l nitric acid and 1 ml of hydrogen peroxide. The reactor was placed in a microwave oven and heated according to the following program: 2 min at 250 W, 2 min at 0 W, 6 min at 250 W, 5 min at 400 W, 5 min at 600 W, and a 10 min ventilation period.

The content of each reactor was poured into an Erlenmeyer flask and carefully rinsed with Milli-Q water. The mixture was heated gently to eliminate excess nitric acid and hydrogen peroxide and thus reduced to a volume of 1 to 2 ml. A second mineralization was carried out by adding a few drops of hydrogen peroxide and 1 ml nitric acid. The mixture was then heated gently until complete evaporation.

Finally, polarographic assays were carried out with a Metrohm 746VA Trace Analyzer, in the differential impulse mode.

This technique makes it possible to measure copper and zinc in samples with a 10 ppm limit of detection. Samples were dissolved in 25 ml 0.5 N HCl. A 15 ml aliquot was taken for analysis, and the pH was adjusted to pH 7 with 1 ml concentrated ammonia solution.

The added-dose technique was used: At regular intervals, an aliquot of copper or zinc sulfate standard solution (1,000 ppm) equal to the volume of the sample was added to compare the heights of the different peaks.

Results and Discussion

Identification and Isolation of *Saccharomyces cerevisiae* Strains from Grape Must and Assessment of their Resistance to Copper

The 19 *S. cerevisiae* strains tested in this study are natural strains isolated from grapes from Italian vineyards (Bari region). Isolation was done by spreading samples of fermenting must on plates of rich medium and incubating the plates at 29 °C.

S. cerevisiae was identified according to techniques and criteria described in "The Yeasts: a Taxonomic Study" [24].

Candidate strains were subjected to morphological tests, genetic tests with analysis of sexual and asexual reproduction, and physiological tests (notably for carbon source assimilation and fermentation, nitrogen source assimilation, etc.). For molecular characterization, whole chromosomes were separated by pulsed-field electrophoresis (Fig. 2).

Next, the 19 isolated *S. cerevisiae* strains were tested for their copper resistance (copper sulfate was added to the culture medium at a concentration of 200 to 700 ppm). The 15 strains that proved resistant to more than 400 ppm copper were retained.

Selection of Yeast Strains for High Copper Resistance and High *CUP1* Copy Number

As mentioned in "Introduction," the *CUP1* gene codes for metallothionein, one of the proteins responsible for detoxication and copper binding during fermentation. This gene is part of a repeat unit that increases in number in the presence of copper. There exists a direct correlation in cells between the number of copies of this gene and the level of phenotypic

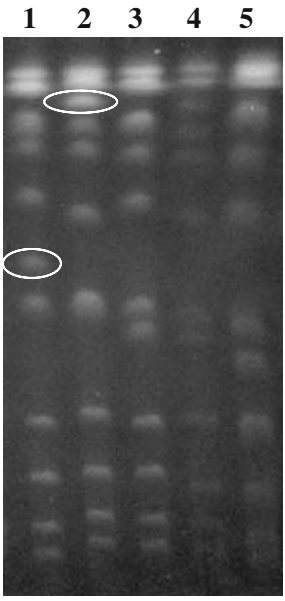

Fig. 2 Separation of whole yeast chromosomes (CHEF technique). All strains displayed an electrophoretic profile typical of *S. cerevisiae*, i.e. 16 chromosomes ranging in size from 2.2 Mb to 200 kb. Several candidates, however, displayed additional bands (see lanes 1 and 2, for example) characteristic of the aneuploid state in natural yeast strains

resistance to Cu^{2+} ions [13]. We therefore estimated the number of *CUP1* gene copies by Southern blotting.

The strains were first grown in YPD supplemented with 400 ppm copper. Total DNA extracted from the cells was cut with *Eco*RI and analyzed by the Southern procedure with the *CUP1* probe. Because *Eco*RI does not cut within the repeat unit (cf. Fig. 1), the length of the locus could be determined on the basis of the migration distance of the band of interest as seen on the autoradiogram. The number of *CUP1* gene copies was estimated by dividing the determined fragment size by that of the repeat unit (2 kb). An illustrative Southern blot is shown in Fig. 3.

It is noteworthy that some strains display two restriction bands. This might be due to differences in chromosome organization, such as the presence of several copies of the chromosome on which the amplification is taking place (aneuploidy) or the presence of a repeat unit of a different length in the same strain.

All of the strains were found to contain more copies of the *CUP1* gene (from 4 to 18) than the copper-sensitive reference strain. On the basis of these results, seven strains were retained for further study: the strains numbered 2, 5, 9, 11, 15, 16, and 19.

Total and Soluble Protein-Bound Copper

Initially, total copper was assayed by polarography in each of the seven strains retained and in a copper-sensitive reference strain. The culture medium used was YPD containing 400 ppm copper. Each assay was performed twice, and the values shown in Fig. 4 are the means of the two measurements.

All of the tested strains were found to retain more copper than the reference strain, which is also the most sensitive to copper in the medium. Two strains, numbered 15 and 16, showed a significantly higher total copper concentration (>7,000 ppm) than the other strains. The higher copper sensitivity of the reference strain was reflected by its slower growth: It took 4 days of culture, instead of 2 days with the resistant strains, to obtain

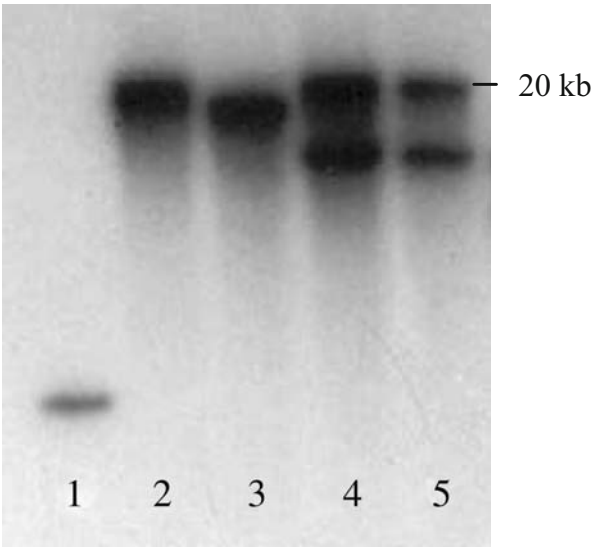

Fig. 3 Southern blot of total DNA from four candidate yeast strains. Total DNA was digested with *Eco*RI and allowed to hybridize with a radioactive probe containing the CUP1 gene. *Lane 1* Copper-sensitive strain. *Lanes 2 to 5* Strains 17, 16, 15, and 14 respectively

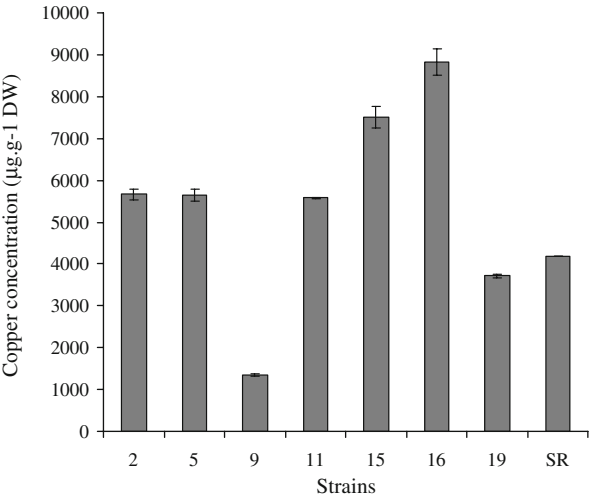

Fig. 4 Total copper in the seven selected strains grown in the presence of 400 ppm copper. Results are expressed in micrograms of copper per gram dry weight (*SR* reference strain, *DR* dry weight)

enough cells for the assay. Furthermore, strain 2 binds copper to a lesser degree than the reference strain. The latter might contain a mutation in the gene coding for the copper transporter. These results led us to retain strains 15 and 16 for further study.

We then measured both total and soluble protein-bound copper in these two strains grown under identical culture conditions (Table 2). The percentage of soluble protein-bound copper displayed by strain 15 was 25%, and the percentage displayed by strain 16 was 15%. Strain 15 was thus retained as having the best copper-binding potential. We named it MV15-U4. The percentage of protein-linked copper was also measured in the case of the reference strain. The values obtained were very low, so it was hard to get a precise measurement, but they were below the values shown in Table 2.

Estimation of *CUP1* Gene Expression during Growth of Strain MV15-U4

Above, high copper resistance in several selected strains is shown to correlate with amplification of the gene encoding the *CUP1* metallothionein. To confirm that copper can enter cells to induce *CUP1* transcription, the *CUP1* transcript level was measured by northern blotting and RT-PCR in strain MV15-U4 grown in a flask containing YPD medium and 100 to 400 ppm copper.

Table 2 Total and soluble protein-bound copper in the reference strain (SR) and strains 15 and 16 after growth in YPD medium containing 400 ppm copper.

Strains	Soluble protein-bound copper (ppm dry weight)	Total copper (ppm dry weight)	% of soluble protein-bound copper
15	1,555	6,151	25
16	1,374	9,021	15
SR	<200	4,121	<5%

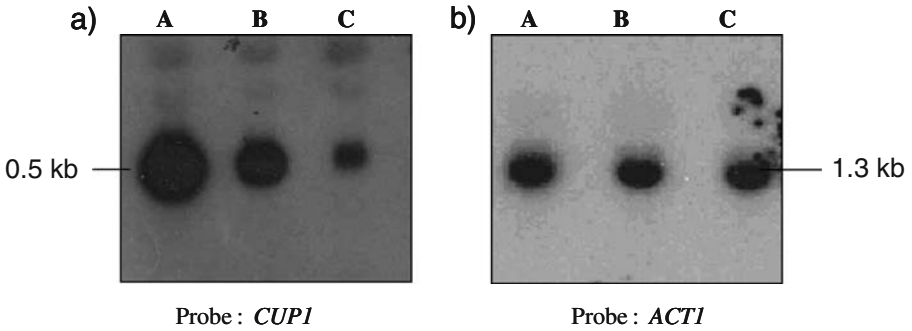

Fig. 5 Northern blots. **a** CUP1 transcripts and **b** ACT1 transcripts in total RNA from strain MV15-U4. **A** Growth in the presence of 400 ppm copper. **B** Growth in the presence of 100 ppm copper. **C** No copper added to the growth medium

Northern Blotting

Test samples were collected at OD=1 (end of the exponential phase). Total RNA was extracted, RNAs were separated on an agarose gel under denaturing conditions, and hybridization was carried out with the radioactively labeled *CUP1* probe. To validate the results, the same samples were hybridized with an *ACT1* probe (yeast gene coding for actin, used as a control gene).

The hybridization bands (Fig. 5) displayed an intensity increasing with the amount of copper added to the YPD medium. This result was validated by the constant intensity of the band corresponding to the *ACT1* transcript, confirming equal loading of the various RNA samples. Thus, the level of *CUP1* correlates with the concentration of copper ions in the external medium.

Quantitative RT-PCR

Strain MV15-U4 was grown in the presence of 400 ppm copper, and relative quantification of the *CUP1* transcript was carried out on six samples collected at different points in time. Results were normalized with respect to the housekeeping gene *PDA1*, coding for the E1α subunit of the pyruvate dehydrogenase complex in *S. cerevisiae.* This gene was preferred to the actin gene because it its expression has been shown to remain more stable than that of actin through the various growth phases of yeast [25].

The results are shown in Fig. 6. A *CUP1* expression peak is observed at the end of the exponential phase, followed by a sudden drop and then a gradual rise. Other tests in flasks confirmed this expression profile. This peak confirms that the strain should have a high binding capacity at the end of the exponential phase. It also confirms the link between the resistance phenotype and amplification of the metallothionein-encoding gene. On the other hand, expression of this gene drops in the stationary phase, where gene regulation and protein stability are known to be variable.

Fermenter Production of Copper- and Zinc-Binding Yeasts

Lastly, we undertook to grow strain MV15-U4 in 20-l fermenter cultures. After several inconclusive attempts in which copper was added at 400 ppm at the start of the fermentation, a fed-batch approach was adopted, with copper and zinc added continuously,

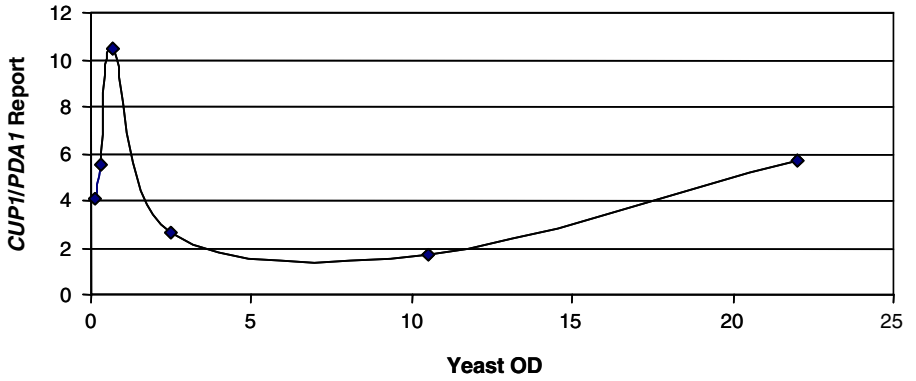

Fig. 6 Quantitative RT-PCR performed on RNA extracted from strain MV15-U4. Relative amount of CUP1 mRNA measured as a function of yeast OD during growth of the strain on YPD + 400 ppm copper

as they were assimilated by the yeast. This was done to minimize their toxicity. Copper, zinc, and glucose were added independently to avoid precipitation of $CuSO_4$ and $ZnSO_4$ by glucose, which would render the ions non-assimilable by yeasts. The optimal conditions of copper and zinc supply were 50 ppm each in the initial broth and a solution of 150 ppm copper and zinc added (flow rate) throughout the fermentation.

After 35 h of fed-batch fermentation, we measured both total and soluble protein-bound copper, and we found that 86% of the zinc detected was soluble protein-bound (Table 3). On the basis of this percentage and given the total concentration of cell-associated zinc, the yeast cells (at the end of fermentation and without lysis) were found to contain 5,739 ppm soluble protein-bound zinc. Soluble protein-bound copper was estimated at 970 ppm in these cells. This copper-resistant yeast strain thus binds high concentrations of both copper and zinc under these conditions.

At the end of the fermentation, the yeasts were washed with sterile water and freeze-dried. The yield of this operation was 350 g freeze-dried ion-binding yeast. Total copper

Table 3 Copper and zinc ions in yeast after growth in the fermenter.

Ions	Concentration in cell lysate (ppm)				Concentration in washed pellet without lysis (ppm DW)	
	Before centrifugation	After centrifugation				
	Total[a]	Supernatant (soluble proteins)[a]	Pellet (unsoluble proteins)[b]	% of soluble protein-bound[b]	Total[a]	Soluble protein-bound[b]
Copper	19	18	1	95	1,021	970
Zinc	200	173	27	86	6,640	5,739

The concentration of soluble protein-bound ion in the washed yeast pellet was calculated by multiplying the total concentration in the pellet by the percentage calculated for the lysate.

DW Dry weight

[a] Polarographic determination

[b] Calculated

and zinc were assayed on ten random dried samples. We measured 7,100 ppm total zinc and 710 ppm total copper. Thus, lyophilization does not seem to affect significantly the amount of ions bound.

Conclusions

This work has yielded a new *S. cerevisiae* strain having a great capacity to accumulate both protein-bound zinc and protein-bound copper. We have also developed a fermentation process where this capacity is maintained. With this strain in lyophilized form, it will be possible to compare the bioavailability of copper and zinc supplied in organic and mineral form. We intend to run such tests on rats receiving a dietary supplement of either ion-binding or non-ion-binding yeast. This will also enable us to test the prebiotic effects of these yeasts in food. We also plan to study parameters and perform analyses to validate these experiments, such as measuring Cu/Zn superoxide dismutase or alkaline phosphatase activity or quantifying *Bifidobacterium* and *Lactobacillus* in feces by qPCR.

Cu–Zn superoxide dismutase is present in all cells, where it prevents cell protein and DNA oxidation and membrane lipid peroxidation [26]. To function, this enzyme requires two atoms of copper and two atoms of zinc. Measurement of its activity will enable us to compare the bioavailability of copper and zinc according to the animals' diet.

Alkaline phosphatase is a zinc-dependent enzyme. Zinc is required to catalyze the hydrolysis of phosphate monoesters to various compounds, including those involved in bone metabolism. Measurement of this activity will provide an estimate of bioavailability sensu stricto, as it will inform on both the absorption of zinc and its use by the organism [18].

Prebiotics are non-digestible food supplements that modify the balance of the intestinal microflora by stimulating the growth and/or activity of beneficial organisms like *Bifidobacterium* or *Lactobacillus* and eliminating potentially undesirable bacteria. In *S. cerevisiae*, components of the cell wall are recognized as having prebiotic effects. This notably concerns cell-wall MOS [10]. qPCR quantification of *Bifidobacterium* and *Lactobacillus* should thus enable us to detect a prebiotic effect of yeasts in rats.

Acknowledgments This work was supported by the "Ministère belge de la Région Wallonne" (contract RW-DGA-31–1111). We wish to thank Anne-Lise Boulvin for providing technical assistance.

References

1. Gournier-Château, N. (1994). *in Les probiotiques en alimentation animale* (pp. 69–85). Paris: Lavoisier Tec/Doc.
2. Fooks, L. J., Fuller, R., & Gibson, G. R. (1999). *International Dairy Journal*, *9*, 53–61.
3. Tannock, G. (1999). *Probiotics: A critical review*. UK: Horizon.
4. Lourens-Hattingh, A., & Viljoen, B. C. (2001). *Food Research International*, *34*, 791–796.
5. Madsen, K., Cornish, A., Soper, P., McKaigney, C., Jijon, H., Yachimec, C., et al. (2001). *Gastroenterology*, *121*(3), 580–591.
6. Kornegay, E. T., Rhein-Welker, D., Lindemann, M. D., & Wood, C. M. (1995). *Journal of Animal Science*, *73*, 1381–1389.
7. Matsui, T., Nakagawa, Y., Tamura, A., Watanabe, C., Fujita, K., Nakajima, T., et al. (2000). *Journal of Animal Science*, *78*, 94–99.
8. Van Heugten, E., Spears, J. W., Kegley, E. B., Ward, J. D., & Qureshi, M. A. (2003). *Journal of Animal Science*, *81*, 2063–2071.

9. Van Heugten, E., Funderburke, D. W., & Dorton, K. L. (2003). *Journal of Animal Science*, *81*, 1004–1012.
10. White, L. A., Newman, M. C., Cromwell, G. L., & Lindermann, M. D. (2002). *Journal of Animal Science*, *80*, 2619–2628.
11. Fogel, S., & Welch, J. W. (1982). *Proceedings of the National Academy of Sciences of the United States of America*, *79*, 5342–5346.
12. Karin, M., Najarian, R., Haslinger, A., Valenzuela, P., Welch, J., & Fogel, S. (1984). *Proceedings of the National Academy of Sciences of the United States of America*, *81*, 337–341.
13. Fogel, S., Welch, J., Cathala, G., & Karin, M. (1983). *Current Genetics*, *7*, 347–355.
14. Thiele, D. (1988). *Molecular Cell Biology*, *8*, 2745–2752.
15. Thorvaldsen, J. L., Sewell, A. K., McCowen, C. L., & Winge, D. R. (1993). *Journal of Biological Chemistry*, *268*(17), 12512–12518.
16. Pena, M. M. O., Koch, K. A., & Thiele, D. J. (1998). *Molecular Cell Biology*, *18*, 2514–2523.
17. Pagani, A., Villarreal, L., Capdevila, M., & Atrian, S. (2007). *Molecular Microbiology*, *63*(1), 256–69.
18. Révy, P. S., Jondreville, C., Dourmad, J. Y., & Nys, Y. (2003). *INRA Prod Anim*, *16*(1), 3–18.
19. Johnston, J. R. (Ed) (1994). *Pulsed field gel electrophoresis in Molecular Genetics of yeast—A practical approach*. Oxford: IRL Press, Oxford University.
20. Friel, D., Vandenbol, M., & Jijakli, H. (2005). *Journal of Applied Microbiology*, *98*, 783–788.
21. Ausubel, F. M., Brent, R., Kingston, R., Moore, D. D., Seidman, J. G., Smith, J. A., et al. (1995). *Short protocols in molecular biology*. New York: Wiley.
22. Sambrook, J., Fritsch, E. F., & Maniatis, T. (2001). *A laboratory manual* (3rd ed.). New York: Cold Spring Harbor Laboratory Press.
23. Vandenbol, M., Jauniaux, J. C., Vissers, S., & Grenson, M. (1987). *European Journal of Biochemistry*, *164*(3), 607–612.
24. Kreger-van Rij, N. J. W. (1987). *The yeasts: A taxonomic study* (3rd ed.). Amsterdam: Elsevier.
25. Wenzel, T. J., Teunissen, A. W., & de Steensma, H. Y. (1995). *Nucleic Acids Research*, *23*(5), 883–4.
26. Pan, Y., & Loo, G. (2000). *Free Radical Biology & Medicine*, *28*(5), 824–830.