Copper Extrusion after Accumulation during Growth of Copper-Tolerant Yeast *Yarrowia lipolytica*

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The Cu^{2+}-tolerant yeast *Yarrowia lipolytica* accumulated Cu^{2+} until the late logarithmic phase. Thereafter, Cu^{2+} was temperature-dependently extruded into phosphate-limited culture medium containing high concentrations of heavy metal ions but not into 10 mM 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer (pH 6.0). Peptone in the culture medium played an important role in the extrusion, which proceeded even when peptone was substituted with cysteine or histidine, but not with any other amino acid tested.

Key words: Copper, Extrusion, *Yarrowia lipolytica*

Introduction

Fungi develop a variety of mechanisms for adaptation to the presence of heavy metal ions. One is reduced accumulation by the cell either via a defect in the transport system (Joho *et al*., 1991) or via exclusion from cells (Riggle and Kumamoto, 2000; Weissman *et al*., 2000). Another adaptation mechanism is to change the distribution of ions within the cell by binding to specific intracellular molecules such as metallothionein for Cd^{2+} and Cu^{2+} (Puig and Thiele, 2002; Tohoyama *et al*., 1995), the cell wall for Cu^{2+} or Hg^{2+} (Ono *et al*., 1988; Yu *et al*., 1996) and vacuoles for Co^{2+}, Mn^{2+} and Ni^{2+} (Joho *et al.*, 1995; Ramsay and Gadd, 1997).

Aiking *et al.* (1984) reported that the bacterium *Klebsiella* has evolved two cadmium-detoxifying mechanisms depending upon the culture conditions, namely CdS or cadmium phosphate complex formation in phosphate- or sulfate-limited medium, respectively. The tolerance of microorganisms to heavy metals often changes according to nutritional conditions (Osiewacz and Stumpferl, 2001; Saxena and Srivastava, 1998). Furthermore, as the amount of carbon source increases, the apparent toxicity of the metal decreases (Gadd *et al*., 2001). However, little is understood about the nutritional influence on fungal responses towards toxic metals, since most studies have proceeded under relatively nutrient-rich conditions.

We previously reported that the acid phosphatase activity of the Cu^{2+}-tolerant yeast *Yarrowia lipolytica* increases with increasing Cu^{2+} concentrations in the medium (Ito *et al*., 2007). The content of cellular phosphate was decreased by increasing Cu^{2+} concentration, regardless of whether the medium was rich in inorganic phosphate. Since the growth curve of *Y. lipolytica* in medium containing Cu^{2+} positively correlated with the increasing activity of acid phosphatase, we supposed that increased enzyme activity is required for growth in such medium. The amount of acid phosphatase produced in fungi also considerably changes due to the effect of external factors, especially during an inorganic phosphate deficiency (Field and Schekman, 1980; Galabova *et al*., 1993; Hidayat *et al*., 2006). Phosphorus levels can also exert a powerful influence on cellular metabolism and/or structure (Gregory *et al*., 2000). In fact, the composition of the cell wall also considerably varies with changes in physicochemical composition such as a phosphate-limited growth environment (Bhavsar *et al*., 2004; Harder and Dijkhuizen, 1983).

In the present study it was investigated how the yeast *Y. lipolytica* develops tolerance to Cu^{2+} under phosphate limitation *in vitro*. We found that *Y. lipolytica* accumulates Cu^{2+} until a threshold concentration is reached and then extrudes it during growth.

Materials and Methods

Strain and medium

The mutant strain of the dimorphic yeast *Yarrowia lipolytica mhy 1-1* (*MAT A*, *ura3-302, leu2-207, lys8-11, mhy1-1*) was used in this study. This

strain can grow only in the yeast form and was kindly donated by Dr. R. A. Rachubinski of Edmonton University, Alberta, Canada. Cells were routinely maintained on YHG agar of the following composition (g l^{-1}): glucose (20), polypeptone (5), yeast extract (4), KH_2PO_4 (5), $MgSO_4 \cdot 7H_2O$ (2), agar (15) and incubated at 30 °C. For experimental purpose, cells were cultured in phosphate-limited medium containing (g l^{-1}): glycerol (20), polypeptone (5), KCl (5), $MgSO_4 \cdot 7H_2O$ (2) and uracil (0.025). Inorganic phosphate in the medium originated mainly from polypeptone (Wako Chem. Ind. Ltd, Japan). Cells were grown on a reciprocal shaker (120 strokes min^{-1}) at 30 °C. A solution of $CuSO_4 \cdot 5H_2O$ (filter-sterilized) was added to the liquid media to various final concentrations.

Measurement of dry weight

Cells were harvested and washed twice with distilled water by centrifugation. The cells were weighed after heating for 48 h at 90 °C in glass centrifuge tubes (dry weight, DW).

Copper assay

We determined Cu^{2+} concentrations by atomic absorption spectrometry. Samples (1 ml) in test tubes containing 0.5 ml of 6 M HNO_3 (analytical grade, Wako Chem. Ind. Ltd.) were placed in a boiling water bath for 20 min. Undigested material was removed by centrifugation at $3{,}000 \times g$ for 5 min. The supernatants were diluted in distilled water, and then the Cu^{2+} content was compared using an atomic absorption spectrometer (Hitachi Z-5010, Tokyo, Japan) with a standard Cu^{2+} solution (Wako Chem. Ind. Ltd.).

Results

Fig. 1 shows the effect of various Cu^{2+} concentrations on the growth of *Y. lipolytica*. The cells reached the stationary phase within 24 h regardless of the presence or absence of Cu^{2+} (range 0.2–2.0 mM). Increasing the Cu^{2+} concentration in the phosphate-limited medium caused a reduction in the size of the final biomass. Toxicity became apparent at 1 mM Cu^{2+} as a reduction in the growth rate and a decrease in the final density of the biomass. The phosphate-limited medium contained about 0.4 mM of inorganic phosphate that mainly originated from peptone and was a contaminant of other reagents. When the inorganic phosphate (as 5 g l^{-1} of KH_2PO_4) concentration

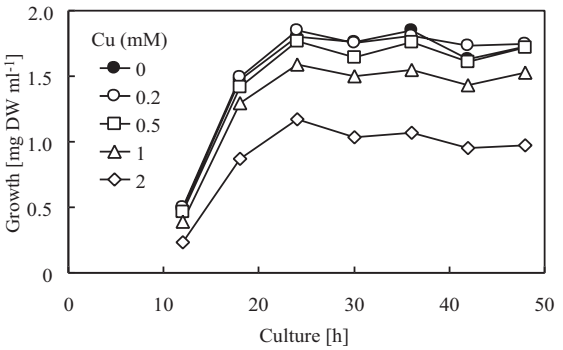

Fig. 1. Effect of Cu^{2+} concentration on growth of *Y. lipolytica*. Cells were cultured in phosphate-limited medium at 30 °C. Results are shown as averages of two replicates.

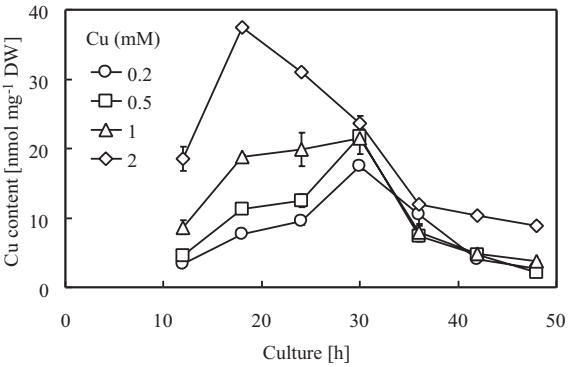

Fig. 2. Cu^{2+} accumulation by *Y. lipolytica.* Cells were cultured in phosphate-limited medium at 30 °C. Results are shown as averages of three replicates with standard error.

in the culture medium was increased about 100-fold of that in the phosphate-limited medium in the absence of Cu^{2+}, the growth rate and the biomass slightly increased (data not shown).

The Cu^{2+} content of the actively growing cells was analyzed throughout the growth curve (Fig. 2). The Cu^{2+} content in cells cultured in medium containing 2 mM Cu^{2+} reached a maximum at 37 nmol Cu^{2+} mg^{-1} DW after 18 h of incubation. Thereafter, the cellular Cu^{2+} content rapidly decreased, reaching 7 nmol Cu^{2+} mg^{-1} DW at the end of the incubation period. The accumulation and subsequent exclusion of Cu^{2+} during growth was similar when the medium contained less than 2 mM Cu^{2+}. However, the exclusion of Cu^{2+} from cells during growth in the presence of Cu^{2+} (range 0.2–1.0 mM) began at the stationary phase of culture after a content of about 20 nmol Cu^{2+} mg^{-1}

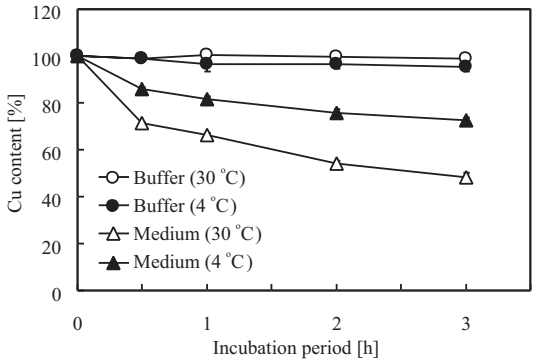

Fig. 3. Effect of temperature and culture conditions on Cu^{2+} extrusion from *Y. lipolytica*. Cells were harvested after 18 h of culture in 2 mM Cu^{2+} medium and transferred into 10 mM MES buffer at pH 6.0 or into culture medium. After incubation at 4 °C or 30 °C, cells were washed twice with distilled water. Metal content was measured as described in Materials and Methods. Results are shown as averages of three replicates with standard error.

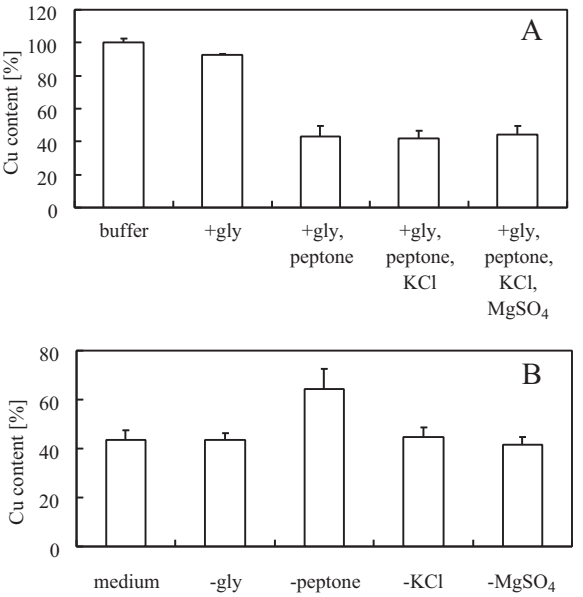

Fig. 4. Effect of medium composition on Cu^{2+} extrusion from *Y. lipolytica*. Cells were harvested after 18 h of culture in medium containing 2 mM Cu^{2+}, transferred into 10 mM MES buffer (pH 6.0) containing individual culture medium ingredients (A) or into culture medium with only one nutritional element omitted (B) at 30 °C for 10 min or 3 h and then washed twice with distilled water. Nutrients were included at following concentrations ($g\,l^{-1}$): glycerol (gly) (20), polypeptone (5), KCl (5), $MgSO_4 \cdot 7H_2O$ (2) as in culture medium. Metal content was measured as described in Materials and Methods. Results are shown as averages of three replicates with standard error.

DW was reached. Furthermore, when the KH_2PO_4 concentration was increased to about 100-fold (40 mM) of the phosphate-limited medium, subsequent Cu^{2+} efflux was inhibited by about 10% (data not shown) in 24 h-cultures containing 2 mM Cu^{2+}.

During growth in medium for at least until 24 h, viability in the presence of 2 mM Cu^{2+} assessed by methylene blue staining did not decrease. Following a further 24 h incubation, non-viable, methylene blue-stained cells accounted for $<5\%$ of the population in the presence of 2 mM Cu^{2+} in medium (data not shown).

Fig. 3 shows the Cu^{2+} efflux after an incubation in 10 mM 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer (pH 6.0) or in nutrient medium in the absence of Cu^{2+}. After 18 h in medium containing 2 mM Cu^{2+}, the cells were transferred to the buffer. Metal release was not significant during a 3 h incubation at 30 °C or at 4 °C. However, when cells were transferred to the culture medium and incubated for 3 h, the Cu^{2+} content decreased by about 50% and 30% of the total metal content at 30 °C and 4 °C, respectively.

We examined the effect of the composition of the medium on Cu^{2+} extrusion (Fig. 4). Each ingredient in the medium was individually added to the buffer and incubated at 30 °C for 3 h. The efflux of Cu^{2+} was slight in the presence of 2% of glycerol. In the presence of 0.5% peptone, about 60% of the total Cu^{2+} was lost from cells after a 3 h incubation. In contrast, the further addition of 0.5% KCl and 0.2% $MgSO_4 \cdot 7H_2O$ to the buffer did not affect the Cu^{2+} efflux (Fig. 4A). Fig. 4B shows the effect of eliminating individual components from the culture medium on Cu^{2+} efflux. In the absence of peptone, the amount of Cu^{2+} in the cells decreased by 35% of the initial content. However, the absence of glycerol, KCl or $MgSO_4$ but the presence of peptone could not prevent the loss of Cu^{2+} from cells compared with the complete medium (Fig. 4B). As a result, about 60% of the total cellular Cu^{2+} was released from the cells.

To elucidate which amino acids were effective for Cu^{2+} efflux, cells cultured in medium containing 2 mM Cu^{2+} were harvested at the late logarithmic stage and transferred into 10 mM MES buffer containing 0.5% casamino acid or 0.1% amino acids (Fig. 5). The Cu^{2+} content decreased to 30%

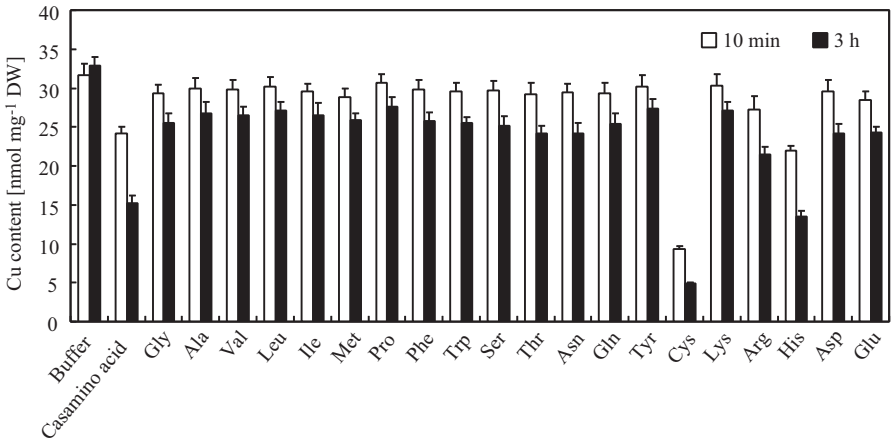

Fig. 5. Effect of amino acids and casamino acid on Cu^{2+} extrusion from *Y. lipolytica*. Cells were harvested after 18 h culture in medium containing 2 mM Cu^{2+} and transferred into 10 mM MES buffer (pH 6.0) containing 0.1% amino acids or 0.5% casamino acid. After incubation at 30 °C for 10 min or 3 h, cells were washed twice with distilled water. Metal content was measured as described in Materials and Methods. Results are shown as averages of three replicates with standard error.

of the total within a 10 min incubation with cysteine. Moreover, 84% and 57% of the total metal content was lost from the cells, respectively, during a 3 h incubation with cysteine and histidine. In contrast, 70% to 80% of the cellular Cu^{2+} content was retained in the presence of other tested amino acids. Casamino acid (0.5%) was also effective for Cu^{2+} extrusion, causing a 50% release of total metal content within a 3 h incubation.

A wash with 10 mM EDTA in 10 mM MES buffer at pH 6.0 resulted in $<10\%$ of the total Cu^{2+} release from cells cultured in medium containing 2 mM Cu^{2+} for 18 h (data not shown).

Discussion

An excess of Cu^{2+} is highly toxic. In general, many fungi prevent Cu^{2+} toxicity either by metal inactivation with chelators such as metallothionein (Wright *et al*., 1988; Winge, 1998) and phytochelatin (Mehra and Mulchandani, 1995) or via efflux with P1-type ATPase (Riggle and Kumamoto, 2000; Weissman *et al*., 2000).

Strouhal *et al*. (2003) reported that Cd^{2+} and Ni^{2+} significantly affect the production of metallothionein in *Y. lipolytica*. However, most of the accumulated metal in that study was detected at the cell wall and as membrane debris, with a small amount being located in the cytoplasm. Therefore, the authors speculated that the detoxification mechanism of Cu^{2+} was deposition to the cell wall or the cell membrane. Here, we found that when cultured in liquid medium containing Cu^{2+}, metal progressively accumulated intracellularly in *Y. lipolytica* until the late logarithmic phase and thereafter was extruded. The accumulation and subsequent extrusion of metal during growth are similar in the fungus *Fusarium* sp., when cultured in medium containing Cd^{2+} (Gharieb, 2001). In that study a simultaneous and significant decrease in the Cd^{2+} content and pH value of the growth medium was found. However, the present study found that regardless of the medium in which *Y. lipolytica* cells were cultured until late logarithmic phase, transfer into fresh medium resulted in Cu^{2+} extrusion without a change in pH value. This evidence indicated that the pH alteration in the growth medium is independent of Cu^{2+} extrusion.

The mechanism of metal extrusion seemed to proceed not only in an active and energy-dependent manner, but also according to nutritional conditions, since the amount of intracellular Cu^{2+} was decreased by incubation in Cu^{2+}-free, fresh medium at 30 °C and slightly decreased at 4 °C, but not in the buffer. In fact, omitting peptone as a nutrient, but not glycerol or other ingredients, from the incubation medium prevented Cu^{2+} efflux. In contrast, Cu^{2+} efflux occurred when the buffer contained only peptone. Therefore, peptone must play an important role not only in nutrition, but also in metal extrusion. Adding casamino acid

to the buffer resulted in a similar effect on Cu^{2+} efflux. Furthermore, both cysteine and histidine obviously increased Cu^{2+} efflux. Cysteine led to Cu^{2+} release from cells within 10 min. Both cysteine and histidine have high affinity for Cu^{2+} (Krämer *et al*., 1996; Kuroda *et al*., 2001; Leberman and Rabin, 1957). These Cu^{2+} extrusion processes are mediated by energy-dependent systems at the plasma membrane. However, amino acids, especially cysteine or histidine, were responsible for the significant amount of metal efflux into the external medium.

Many reports have described that metals locate at the cell wall or cell membrane either in a loosely non-specific bound form or in complexes with carboxy-, amino-groups and other ligands (Brady and Duncan, 1994; Mowll and Gadd, 1984). Therefore, we supposed that metals located near the yeast cell wall were liberated from cells by exchange with external amino acids, especially histidine or cysteine, involving peptone in the medium. However, since Cu^{2+} was slightly washed out by EDTA, we propose that the metal progressively moves from inside the cell to a location near the cell surface where extracellular amino acids are exchanged after the late logarithmic stage of culture. However, we have no other positive evidence to support this notion.

The efflux phenomenon of Cu^{2+} from *Y. lipolytica* cells occurred in phosphate-limited medium. Phosphate limitation has an obvious effect on the production of acid phosphatase by *Y. lipolytica* cultures (Galavoba *et al*., 1993). Acid phosphatase is also involved in heavy metal resistance of the fungus *Pisolithus arrhizus* (Turnau and Dexheimer, 1995) and the bacterium *Citrobacter* sp. (Jeong and Macaskie, 1999). We also found that the acid phosphatase activity of the Cu^{2+}-tolerant yeast *Y. lipolytica* increased with increasing Cu^{2+} concentrations in the medium, regardless of whether the medium was rich in inorganic phosphate. However, the cellular polyphosphate content is decreased by culture in the presence of Cu^{2+} (Ito *et al*., 2007). Under phosphate-limited conditions, increased acid phosphatase activity and/or subsequent decreased cellular polyphosphate content might be involved in the formation of copper(II) phosphate complexes (Roig *et al*., 1995; Turnau and Dexheimer, 1995). Furthermore, these complexes are located near the cell surface and thereafter might be released into the culture medium with the help of some external amino acids.

Moreover, the composition of the cell wall of microorganisms also varies considerably with limitations in nutrients such as carbon, nitrogen and phosphate (Bhavsar *et al*., 2004; McMurrough and Rose, 1967). Increased enzyme production and/or changes in the wall structure caused by culture in phosphate-limited medium might also be sufficient to sustain the life of *Y. lipolytica* through metal efflux in a toxic environment containing high concentrations of Cu^{2+}.

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