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Proteomic study of the yeast *Rhodotorula mucilaginosa* RCL-11 under copper stress

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Abstract In order to understand the mechanism involved in *Rhodotorula mucilaginosa* RCL-11 resistance to copper a proteomic study was conducted. Atomic absorption spectroscopy showed that the copper concentration in the medium decreased from 0.5 to 0.19 mM 48 h after inoculation of the yeast. Analysis of one-dimensional gel electrophoresis of crude cell extracts revealed expression of differential bands between cells with and without copper. In order to study this difference, two-dimensional electrophoresis of *R. mucilaginosa* RCL-11 exposed to Cu for 16, 24, and 48 h was carried out. Identification of differentially expressed proteins was performed by MALDI-TOF/TOF. Ten of the 16 spots identified belonged to heat shock proteins. Superoxide dismutase, methionine synthase and beta-glucosidase were also found over-expressed at high copper concentrations. The results obtained in the present work show that when *R. mucilaginosa* RCL-11 is exposed to 0.5 mM copper, differential proteins, involved in cell resistance mechanisms, are expressed.

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Introduction

Copper (Cu) plays an essential role in cellular metabolism due to its versatility as a biological catalyst. It is required as a catalytic cofactor in many enzymes involved in diverse cellular processes, such as radical detoxification, oxidative phosphorylation, and iron metabolism (Puig and Thiele 2002). The two oxidation states of copper (Cu^{+} and Cu^{2+}) make it important to metalloenzymes in many redox-driven reactions (Solioz and Stoyanov 2003). While trace amounts of copper are essential for life, copper can easily react with oxygen or hydrogen peroxide (H_2O_2) generating reactive oxygen species (ROS) that may damage cell constituents through the oxidation of proteins, cleavage of DNA and RNA, and lipid peroxidation (Tamarit et al. 2006). In order to counteract this adverse aspect, all living organisms have developed defense mechanisms against ROS, allowing them to live under different stress situations. Hence, under copper overload, the cells react according to the instability caused by the metal. One of the most efficient ways of preventing Cu toxicity is to sequester the cation in an unchangeable form. Another, more general response is activation of antioxidant enzymes involved in detoxification of O_2^{-} (oxygen superoxide), OH• (hydroxyl radical) and H_2O_2.

Another mechanism involved in stress resistance is the protection of proteins against misfolding and aggregation. Heat-shock proteins (Hsps) are highly conserved proteins within species and carry out essential functions such as protein translocation, folding, and assembly under normal cellular conditions. Hsps play an important role in protection against multiple stressors (heat stress, toxic metals, ionizing, and UV radiation, among others) and act as molecular chaperones assisting in ATP-dependent folding and stabilization of stress-damaged proteins (Parsell and Lindquist 1993). They were first identified due to their increased synthesis following exposure to elevated temperatures (Park et al. 2005). In most cell types, Hsps constitute 1–2% of the total proteins even prior to stress, suggesting their importance in the biology and physiology of the unstressed cell. Hsps are distributed among diverse compartments inside cells and are classified into different groups depending on their molecular weight.

The threat of heavy metal pollution to public health and wildlife has lead to a great interest in the development of effective technologies for heavy metal immobilization in a non-bioavailable form or their conversion into less toxic forms. While the current technologies (precipitation, ion exchange, reverse osmosis, or evaporation) have shown to be inadequate or expensive, the use of biological-based technologies seems to be a very attractive alternative for removal of metal ions (Machado et al. 2009). Organisms subjected to metal (copper) exposure in their natural environments have developed resistance mechanisms such us dedicated components and sophisticated homeostasis allowing them to acquire and maintain adequate intracellular copper concentrations, even under copper overload (Rensing et al. 2000). In this context, yeasts represent an important tool in order to understand cellular mechanisms as a response to high copper concentrations due to their versatility and easy manipulation.

Yeasts with multiple heavy metal tolerance (including Cu (II)) were isolated by Villegas et al. (2005) from a copper filter at a mine plant in the province of Tucumán, Argentina. When cultured with Cu (II), *Rhodotorula mucilaginosa* RCL-11 decreased its growth rate with increasing Cu (II) concentration, and it also absorbed this metal ion from the culture medium. It was shown that *R. mucilaginosa* had increased rates of superoxide dismutase (SOD) and catalase (CAT) as a consequence of oxidative stress due to elevated Cu (II) concentrations (Villegas et al. 2009). However, so far no studies have been conducted to identify novel proteins associated with this resistance. Proteomics is a formalized approach to obtain a rapid 'snap-shot' of the protein complement of a tissue, cell or cell components. It provides an excellent tool for studying variations in protein expression between different states and conditions. Undoubtedly, changes in protein expression are essential in any study aimed at examining cellular networks.

The aim of the current study was to determine novel proteins involved in copper tolerance by *R. mucilaginosa* RCL-11 using a proteomic approach. It describes a 2D proteomic approach to study the protein composition of this strain under copper overload.

Materials and methods

Organisms and culture conditions—*R. mucilaginosa* RCL-11

The yeast was isolated from wastewater sediment from a copper plant filter in the province of Tucumán, Argentina, and identified as *R. mucilaginosa* (GenBank AY437842) by sequencing the D1/D2 region of the 26S rDNA gene. Complete identification of the strain was carried out by Villegas et al. (2005) using conventional techniques. Cultures were prepared in Erlenmeyer flasks containing YNB-glucose (Difco), buffered with 50 mM Tris-succinate (pH 5) and supplemented with 0.5 mM Cu^{2+}. Flasks were inoculated at a final concentration of 10^7 cells ml^{-1} with an active overnight pre-inoculum and incubated at 30°C on a rotary shaker at 250 rpm during 72 h. As control, an inoculated flask without copper solution was incubated under the same conditions. Samples of 2 ml were withdrawn periodically. Growth was monitored by measurements of optical density at 600 nm. Total copper concentrations in supernatant were measured by flame atomic absorption spectrophotometry (PerkinElmer).

One-dimensional gel electrophoresis

Yeast cells were harvested by centrifugation and washed twice with water. Cell homogenates were prepared by using glass beads in Tris 50 mM HCl (pH

8.0) with protease inhibitor cocktail I (Calbiochem) in 2 ml Eppendorf tubes (0.5 ml beads and 1 ml cell suspension). Ten pulses of 1 min each on a vortex mixer were used, with 1 min intervals on ice between pulses alternated with ultrasound cycles (5 cycles of 1 min ultrasound and 30 s in off). The homogenates were centrifuged at 10,000×*g* during 5 min at 4°C and supernatants were recovered. Total protein content in the supernatants was determined by the Bradford method with BioRad reagents using bovine serum albumin as reference protein. 5% SDS, 10 mM EDTA and 5% beta-mercaptoethanol were added to the protein homogenates, which were then incubated at 100°C for 5 min. A solution containing sucrose and bromophenol blue was added before rerunning the samples on 10–12.5% polyacrylamide gels. 15–25 μg of total protein were run at 15 and 20 mA. Gels were stained with EZblue (Sigma) or Coomassie Brilliant Blue R-250.

Two-dimensional gel electrophoresis

Cell homogenates were obtained as described above. To minimize SDS concentration, total proteins were precipitated with 9 vol. of acetone for 45 min. Precipitates were washed three times with acetone by centrifugation at 5,000 rpm for 5 min. After acetone elimination, proteins were resuspended in 200 μl of 8 M urea, 4% (w/v) CHAPS, 50 mM dithiothreitol (DTT), 2% (v/v) ampholites (pH 3–10; GE Healthcare Life Science), and bromophenol blue. The suspension was incubated for 3 h on an orbital shaker and centrifuged at 8,000×*g* for 5 min to remove insoluble proteins. Proteins were quantified by the Bradford method (BioRad).

Isoelectric focusing was performed in 11 cm immobilized pH gradient strips (3-11NL, GE healthcare Life Science). The focused strips were stored at −20°C until second-dimension electrophoresis was performed. Thawed strips were equilibrated for 15 min in 370 mM Tris–HCl (pH 8.8) containing 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, and 130 mM DTT, and then equilibrated for 15 min in the same buffer containing 135 mM iodoacetamide instead of DTT. Second-dimension SDS-PAGE was performed on 12.5% polyacrylamide gels. Gels were silver-stained using a PlusOne silver staining kit from General Electric Healthcare or colloidal Coomassie Brilliant Blue R-250. Images were scanned with an Image Scanner III and analyzed with ImageMaster 2D Platinum v 7.0 (both of GE Healthcare Life Sciences).

Proteome analysis

Three replicates of 2-DE gels were obtained for each sample. For matching the detected spots a synthetic master gel was created by matching representative gels with each other (and only maintaining triplicate spots). Spots showing at least a twofold increase in protein abundance between cells growing with and without copper proteomes were digested with trypsin and analyzed in a MALDI-TOF TOF Ultraflex II (Bruker Daltonics). Proteins were identified by peptide mass fingerprinting with MASCOT. Fragmentation was carried out with more intense MS peaks (MS/MS). When possible, MS and MS/MS information was combined for one or more peptide searches. De novo sequencing was inferred from BLAST results when peak fragmentation was allowed. The percentage of protein coverage was determined for each spot using the MASCOT search (Table 1). The induction ratio (the ratio of 0.5 mM Cu^{2+} to 0 mM Cu^{2+}, mean of three independent experiments) was estimated considering all the spots identified as the same protein.

Results and discussion

Decrease in Cu concentration by *R. mucilaginosa* RCL-11

R. mucilaginosa RCL-11 has demonstrated to be able to resist high concentrations of copper by slowing down its growth rate. In previous studies, *R. mucilaginosa* RCL-11 was selected for semi-quantitative and quantitative tolerance of Cu^{2+}, based on its multiple tolerance towards different metals (Ni, Cd, and Cr) (Villegas et al. 2005, 2008). In contrast, *Saccharomyces cerevisiae* viability decreased, showing more sensitivity to copper than RCL-11.

The copper effect on the strain was analyzed during culture growth. Growth rate and the ability of the strain to decrease the copper concentration were determined. Results showed that the copper concentration in the culture medium decreased from 0.5 mM to 0.24 ± 0.061 mM after 24 h and 0.19 ± 0.047 mM after 48 h of incubation (Fig. 1). Lowest copper concentration (0.14 ± 0.038 mM) was obtained after

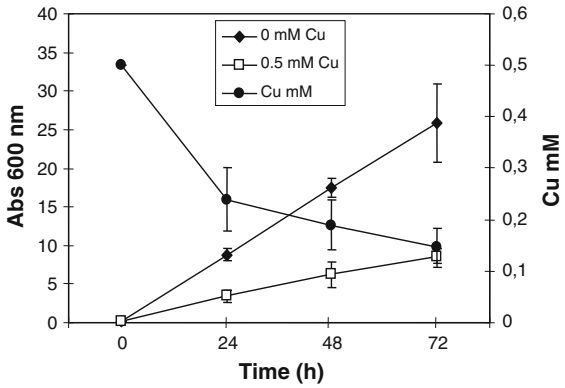

Fig. 1 Effect of 0.5 mM Cu^{2+} on *R. mucilaginosa* RCL-11 growth and the subsequent effect of the yeast on extracellular copper concentration in the culture medium after different incubation times. Absorbance at 600 nm of *R. mucilaginosa* RCL-11 cultures after 24, 48, and 72 h and grown without Cu^{2+} supplement (*filled diamond*) or in the presence of 0.5 mM Cu^{2+} (*open square*). Decrease in copper concentration in the culture medium 24, 48, and 72 h after inoculation of *R. mucilaginosa* RCL-11 cells (*filled circle*). Data are expressed as the mean ± standard deviation of four independent experiments

72 h of incubation, indicating a decrease of 72% in copper concentration.

Copper presence in liquid culture medium had a negative effect on cell viability. Exposure to an initial metal concentration of 0.5 mM reduced both final growth and generation time of yeast cells (Fig. 1). Growth of cells incubated with Cu^{2+} after 72 h was five times lower ($OD_{600} = 5$) than of control cells ($OD_{600} = 25$) and the duplication time (Tg) was 45% higher than the control (Tg without Cu^{2+}= 221.7 min, Tg with Cu^{2+}= 323.0 min).

It is important to highlight that this strain is able to withstand high copper levels without undergoing great physical and physiological changes. Intracellular accumulation of copper and morphological changes were observed, indicating that the resistance mechanism in *R. mucilaginosa* RCL-11 works through sequestration and extrusion of cytoplasmic copper ions (Villegas et al. 2009).

Proteome analysis of *R. mucilaginosa* RCL-11 cells by one-dimensional electrophoresis

In order to understand the mechanisms involved in copper resistance by *R. mucilaginosa* RCL-11 a proteomic approach was conducted. Experiments were carried out with yeast cells grown in minimal medium with glucose as carbon source. The pH is one of the most important environmental factors involved in biosorption of heavy metal ions. The pH of a solution strongly influences the biosorption capacity of metal cations by the biomass surface (Wang and Chen 2006). Our assays were carried out in buffered medium at pH 5 as this is the optimum pH for Cu biosorption by *R. mucilaginosa* RCL-11 (Villegas et al. 2005).

One-dimensional analysis of crude cell extracts revealed expression of different bands between cells with and without copper (Fig. 2). Most of the differentially expressed bands in yeast exposed to copper were between 90 and 55 kDa. The degree of difference in expression can be an indication of the type of proteins that cells need to support high copper concentrations. In the presence of Cu^{2+} the protein band profile also depended on the incubation time. The

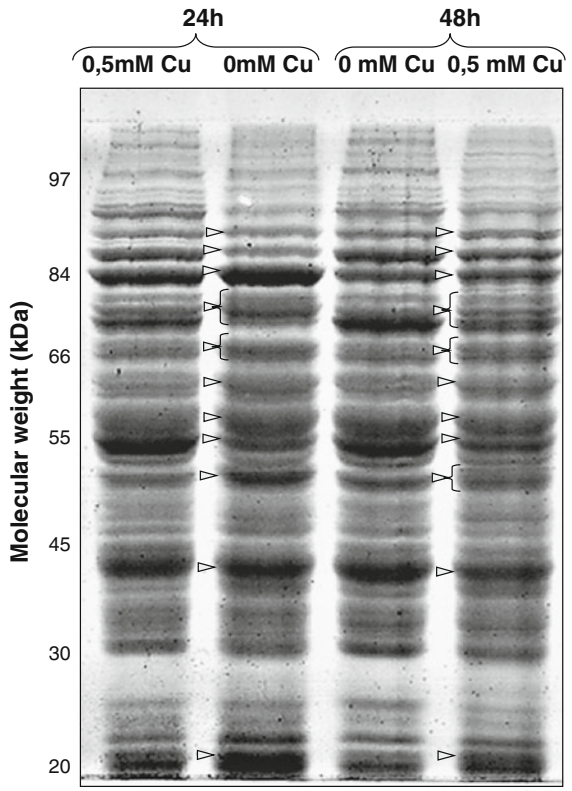

Fig. 2 Differential protein expression of *R. mucilaginosa* RCL-11 under copper overload. Crude extracts from cells grown during 24 and 48 h in medium supplemented or not with copper, were separated on a one-dimensional gel (10% acrylamide:bis-acrylamide 29:1). Protein profile was observed after EZblue staining. Bands differentially expressed under 0.5 mM copper after 24 h are indicated by arrows

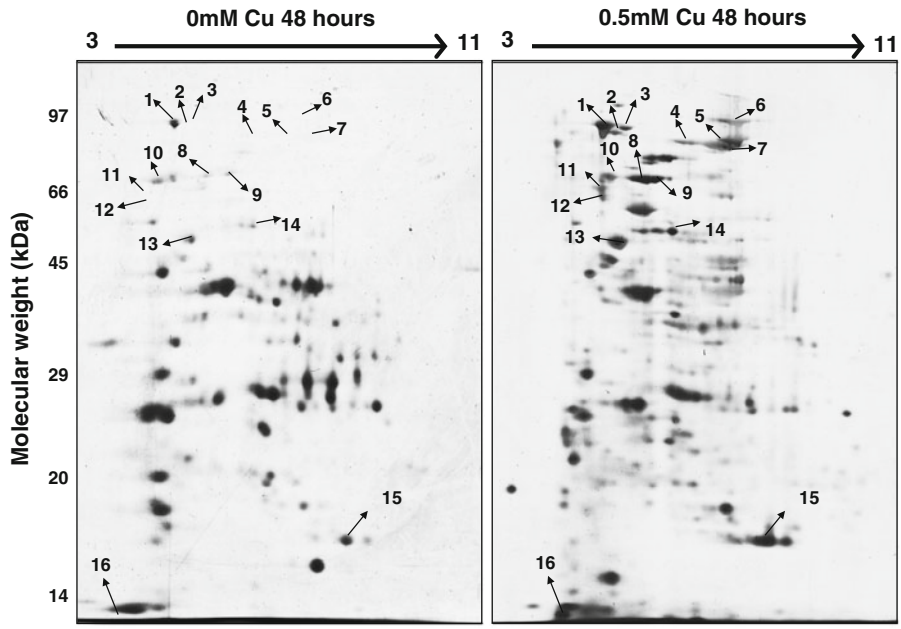

Fig. 3 Analysis of *R. mucilaginosa* RCL-11 proteome in the presence and absence of 0.5 mM Cu^{2+}. Cells were grown in minimal medium in the presence or absence of copper and then 200 μg of total cell lysate were separated by 2D gel electrophoresis. Gels were stained with silver or colloidal Coomassie Brilliant Blue R-250 and analyzed with ImageMaster (GE Healthcare Life Sciences)

differentially expressed proteins may be responsible for the extraordinary cell capacity to resist copper toxicity. In order to identify these proteins, 2D-analysis was carried out.

Analysis of *R. mucilaginosa* RCL-11 by 2D electrophoresis

Two-dimensional-analysis of *R. mucilaginosa* RCL-11 after different growth conditions was performed as described above. The study revealed that when the yeast was exposed to copper for 48 h it produced an over-expression of 16 spots (Fig. 3). Similar results were found after 24 h of exposure (data not shown). Spots were identified by peptide mass fingerprinting or peptide sequencing after MS/MS analysis (Table 1). Cells exhibited an increased number of chaperons when copper was present. Ten of the 16 identified spots corresponded to Hsps: three to Hsp88, six to the Hsp70 family and one to the Hsp60 family (Table 1). Hsps are a family of proteins that act under both normal and stress conditions, but under stress conditions they can be overexpressed. Therefore, overexpression of Hsps in *R. mucilaginosa* RCL-11 would be an adaptive response of the cells to copper exposure stress. There has been a relationship between stress conditions (heat shock, glucose deprivation, exposure to free radicals and heavy metals, etc.) and expression of Hsps in different models and under adverse conditions. Parsell and Lindquist (1993) reported that, depending on the organism, heavy metals induced Hsps. Therefore, Hsps are commonly referred to as stress proteins and constitute an important component of the stress response. Similarly, Cabiscol et al. (2002) found that chaperones helped protect *S. cerevisiae* against oxidative stress.

One protein has been characterized as Hsp88, which appears to be a normal cellular constituent. A several-fold increase in expression of this protein has been observed in response to high temperature stress (Plesofsky-Vig and Brambl 1998). Hsp88 is homologous to several characterized proteins that have been related to heat shock. To date, Plesofsky-Vig and Brambl (1998) are the only authors that have described Hsp88 properties. Most of the induced proteins identified in the present study belong to the 70-kDa Hsp family, which is critical in the cellular response to stress. In fact, these chaperones are among the most abundant cellular proteins protecting against stress-induced damage (Mayer and Bukau 2005). Hsp70 proteins are

Table 1 Identification of differentially expressed proteins in *R. mucilaginosa* RCL-11 after incubation with or without copper

Spot	Protein	Species	NCBI accession number	Identification technique[a]	Peptide	MASCOT Score[b]	Induction ratio[c]
1	Hsp88	[*Puccinia graminis f. sp. tritici* CRL 75-36-700-3]	gil309303163	MS/MS	R.ATPSLVAFGPR.N	40	4.4
2	Hsp88	[*Puccinia graminis f. sp. tritici* CRL 75-36-700-3]	gil309303163	MS/MS	R.ATPSLVAFGPR.N	24	ND in control
3	Hsp88 UM05918.1	[*Ustilago maydis* 521]	gil71023671	MS/MS	K.NALEEMIYDQR.S	22	ND in control
4	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	[*Puccinia graminis f. sp. tritici* CRL 75-36-700-3]	gil309299626	MS/MS	K.AGIFAIQVDEPAIR.E	34	ND in control
5	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	[*Puccinia graminis f. sp. tritici* CRL 75-36-700-3]	gil309299626	MS/MS	K.AGIFAIQVDEPAIR.E	38	ND in control
6	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	[*Puccinia graminis f. sp. tritici* CRL 75-36-700-3]	gil309299626	MS/MS	K.AGIFAIQVDEPAIR.E	60	ND in control
7	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	[*Puccinia graminis f. sp. tritici* CRL 75-36-700-3]	gil309299626	MS/MS	K.AGIFAIQVDEPAIR.E	61	ND in control
8	Hsp70 Ssc1 UM01209.1	[*Ustilago maydis* 521]	gil71005380	MS–MS/MS	R.VINEPTAAALAYGLDR.D K.HAVITVPAYFNDSQR.Q	162	4.16
9	Hsp70 Ssc1 UM01209.1	[*Ustilago maydis* 521]	gil71005380	MS–MS/MS	R.VINEPTAAALAYGLDR.D K.HAVITVPAYFNDSQR.Q R.QATKDAGAIAGLDVLR.V	159	3.4
10	Hsp70	[*Aspergillus fumigatus* Af293]	gil70989489	MS–MS/MS	K.FELTGIPPAPR.G K.DAGTIAGLNVLR.V K.VEIIANDQGNR.I K.VTHAVVTVPAYFNDAQR.Q	75	2
11	Hsp70 Ssa4p	[*Saccharomyces cerevisiae* S288c]	gil6320950	MS–MS/MS	K.FELSGIPPAPR.G K.DAGTIAGLNVLR.I R.LVNFLAEEFK.R R.VEIIANDQGNR.T R.FEELCADLFR.S R.TTPSYVAFTDTER.L	145	2.2
12	Hsp70 Ssa4p	[*Candida albicans* WO-1]	gil238878277	MS–MS/MS	R.NSTIPTKK.S K.FELSGIPPAPR.G R.VEIIANDQGNR.T K.FDDPEVINDAK.H R.FEELCADLFR.S K.SQVEEIVLVGGSTR.I K.MKEIAEGYLGSTVK.D	125	ND in control

Table 1 continued

Spot	Protein	Species	NCBI accession number	Identification technique[a]	Peptide	MASCOT Score[b]	Induction ratio[c]
13	Hsp60	[*Pichia pastoris* GS115]	gi|254572906	MS–MS/MS	K.APGFGDNRK.N R.NVLIEQPFGGPK.I K.DRYDDALNATR.A K.NVAAGCNPMDLR.R K.TLEDELEVTEGMR.F	94	2.8
14	Beta glucosidase An11g06090	[*Aspergillus niger*]	gi|145244182	MS/MS	K.GVNVLLGPVVGPTFRKPR.G	28	2.15
15	Superoxide dismutase	[*Rhodotorula glutinis*]	gb|AAL30746.1|	de novo sequencing	VQKDFGGID	48	2.66
16	Hsp70	[*Saccharomyces cerevisiae*]	gi|172714	MS/MS	K.FELSGIPPAPR.G	81	ND in control

[a] MS–MS/MS indicates a combination of MS and MS/MS. De novo sequencing was inferred from and showed by BLAST homology with SOD (identity = 78%)

[b] MS: protein scores >71 were significant ($p < 0.05$). MS/MS: individual ion scores >22 indicate peptides with significant homology and individual ion scores >30 indicate identity or extensive homology ($p < 0.05$)

[c] The induction ratio (0.5 mM Cu^{2+} over 0 mM Cu^{2+}) was estimated considering all spots identified as the same protein. "*ND* in control" indicates that a corresponding spot in cells grown at 0 mM Cu^{2+} was too low to be used as reference value

ubiquitous chaperones, which are essential ATP-binding proteins and involved in cellular functions under heat stress and non-heat stress conditions (Zhang et al. 2006). Hsp70 is the family of universal cytosolic chaperones involved in folding of damaged but repairable proteins and in the degradation of those that are damaged beyond repair (Mayer and Bukau 2005). The present study found members of the Hsp70 sub-family, Ssa proteins that are abundant cytosolic proteins and exhibit the highest identity of the group (76%) to mammalian Hsp70. This class of chaperones was mainly present when cells were exposed to copper (Table 1; spots 11 and 12). They play an essential role in cell viability and can be functionally substituted by one another even though they are differentially regulated (Jarosz and Lindquist 2010). An interesting member of the Hsp70 family is mitochondrial Hsp70 (mtHsp70), which resides in the mitochondrial matrix and is essential for cell viability (Yoneda et al. 2004). A key component, known as Ssc1, functions as a molecular motor to drive translocation of proteins across the inner mitochondrial membrane (Mapa et al. 2010). Two different spots, 8 and 9, were identified as mtHsp70 SSc1 in the present study. A relationship between differential expression of mtHsp70 and response to oxidative stress was observed. (Strain et al. 1998) reported that mitochondrial chaperones are suppressors of SOD1 deficiency by protecting SOD1 enzymes from Fe/S cluster damage through oxidation.

Another essential mitochondrial chaperone, Hsp60, was also overexpressed under copper overload (spot 13, showing a 2.8-fold induction in cells under copper overload). There exists a collective of double-ring assemblies that promote the folding of proteins to their native state, by importing the proteins into the mitochondrial matrix or into the intermembrane space (Raggam et al. 2010). Hsp60 has been directly associated with the response to oxidative stress. It is predominantly found in mitochondria and chloroplasts assisting in the protein folding and stress protection in these organelles.

Cells containing different levels of Hsp60 were obtained by using mutant strains in which the Hsp60 gene expression was under control of a regulable promoter. In this model, cells displaying higher doses of Hsp60s were more resistant to both H_2O_2 and superoxide anions, whereas cells presenting lower levels of Hsp60 showed increased levels of intracellular ROS under oxidative stress (Cabiscol et al. 2002).

Proteomic analysis of yeast cells showed that Hsps are induced in response to H_2O_2 stress, which is important for the protection of cells against this adverse condition. They help abnormal proteins that accumulate under stress conditions regain their proper folding or assist in their proteolytic degradation (Raggam et al. 2010).

The primary response to any kind of environmental oxidative stress like exposure to copper is the use of antioxidant defense, reparation of the system to minimize damage, and removal or reparation of whatever cellular components that got damaged. There is a wide range of enzymatic and non-enzymatic systems involved in cell defense under diverse stress conditions. SOD, CAT, glutathione peroxidase, peroxiredoxin, thioredoxin, and glutaredoxin, among others, represent primary enzymatic defense against ROS. Carotenoids, glutathione, ascorbate, tocopherol, flavonoids, and alkaloids are some of the non-enzymatic antioxidants.

R. mucilaginosa RCL-11 showed induction of an additional protein involved in oxidative stress response, which was identified as SOD by de novo sequencing. This enzyme catalyzes dismutation of superoxide free radicals into O_2 and H_2O_2 (Fridovich 1999). CuZnSOD is predominantly located in the cytoplasm and it can act as an antioxidant and a copper chaperone (Jamieson 1998). Induction of expression of this enzyme in diverse organisms provides them protection against deleterious effects of oxidative stress in various situations. Similarly, a diminished CuZnSOD activity is directly responsible for increased sensitivity of yeast mutant cells to oxidative stress (Irazusta et al. 2010). Studies with *R. mucilaginosa* RCL-11 have demonstrated an increase in SOD activity when cells were exposed to increasing exposure times and copper concentrations (Villegas et al. 2009). In addition, the yeast *Yarrowia lipolytica* was able to grow at high concentrations of copper sulfate and showed higher CuZnSOD activity under these conditions (Ito et al. 2007). An augmented CuZnSOD protein concentration was also demonstrated in a *S. cerevisiae Δyfh1* mutant, whose metal ion homeostasis was deregulated (Irazusta et al. 2006). However, the CuZnSOD activity decreased in these yeast cells (Irazusta et al. 2010). In contrast, the present study showed a positive correlation between CuZnSOD activity and protein concentration when *R. mucilaginosa* RCL-11 was exposed to copper overload. The increased expression of chaperones and SOD indicates an endogenous situation of oxidative stress in the presence of copper.

Two more proteins were identified, methionine synthase and beta-glucosidase. Methionine synthase is involved in the final step of methionine synthesis, and catalyzes the transference of a methyl group from 5-methyltetrahydropteroylpolyglutamate, also called 5-methyl tetrahydrofolate, to L-homocysteine forming L-methionine. From the two major classes of methionine synthases (cobalamin-dependent and cobalamin-independent), only cobalamin-independent methionine synthase has been described in *S. cerevisiae* and *Candida albicans* (Koutmos et al. 2009). To our knowledge there are no reports describing methionine synthase in *R. mucilagenos*a, but we assume it must be similar to that in *S. cerevisiae* and *Candida albicans*. A low expression of this enzyme was reported in *S. cerevisiae* when cells were exposed to H_2O_2 (Godon et al. 1998). Heat and estrogens induced a cobalamin-independent methionine synthase homologue as response by *C. albicans* (Burt et al. 2003).

β-Glucosidase was also overexpressed under copper overload. This is a glucosidase enzyme acting upon *β*-1,4 bonds that link two glucose or glucose-substituted molecules. It catalyzes the hydrolysis of terminal non-reducing residues in *β*-D-glucosides with release of glucose (Jeng et al. 2011). *S. cerevisiae* displays changes in the glycolipid content under ethanol stress. Changes in glucose content can be related to changes in *β*-glucosidase activity in a stress situation. A variation in the proportion of glycolipids during stress might play an important role in both physical and structural stabilization of the membrane (Malhotra and Singh 2006). Thereby, variations in glycolipid content and composition, due to changes in the *β*-glucosidase concentration in cells, may represent another adaptive response to copper stress. Recently, a relation between this protein and several defense mechanisms has been elucidated (Ketudat Cairns and Esen 2010). Yet another relationship between this enzyme and copper stress was demonstrated through a *β*-glucosidase assay to test metal toxicity effects of urban metals on natural communities (Fechner et al. 2010).

Expression of differential proteins under copper overload after different exposure times

Expression of several proteins gradually increased with increasing exposure time to copper (0.5 mM

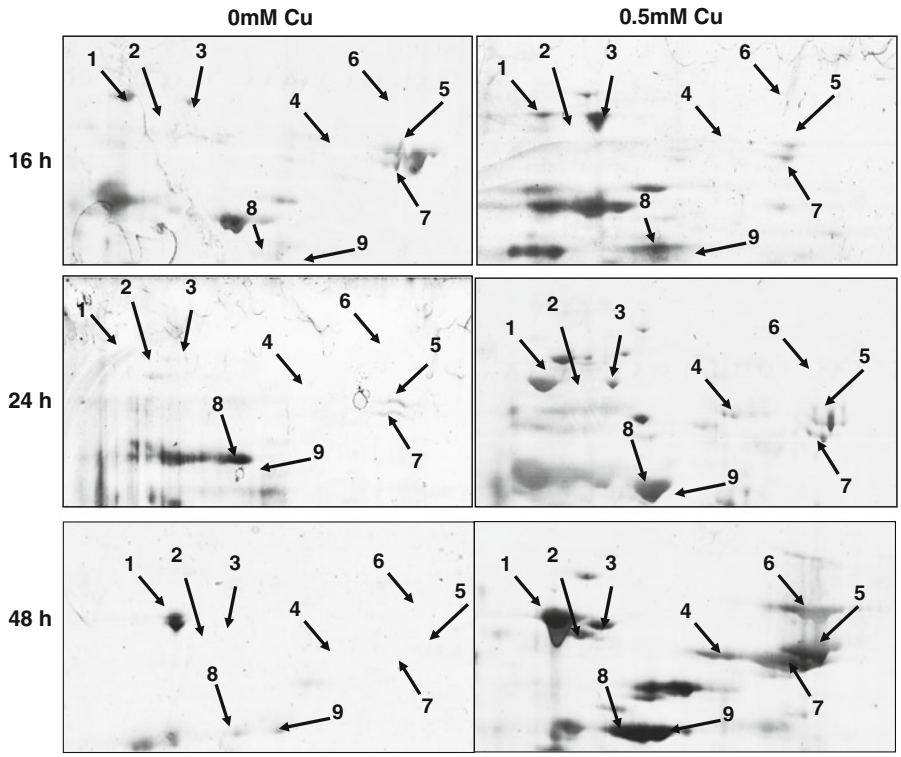

Fig. 4 Analysis of *R. mucilaginosa* RCL-11 proteome in the presence and absence of 0.5 mM Cu^{2+} after 16, 24, and 48 h of incubation. Zoom of gels is showed and proteins are indicated by *numbers* and *arrows*. A variation in the protein profile can be observed after different incubation times with or without the metal

Cu^{2+}) (Fig. 4). This was the case of Hsp88 (spot 1), methionine synthase (spot 5 and 7), and Hsp70 Ssc1 (spot 8). Methionine synthase appeared as an adaptive response to metal exposure stress (Suliman et al. 2005). Our results showed that methionine synthase in *R. mucilaginosa* RCL-11 was only observed under copper overload and not identified in control cells (ND in control, Table 1). In addition, some spots were only observed after prolonged exposure time (spots 2, 6, and 9). Maximum protein expression was obtained after exposure of 48 h. In general, the concentration of Hsps and methionine synthase in gel spots augmented, which corresponded to an increase in protein expression.

In the present study four different spots corresponding to methionine synthase were observed. Spots 4, 5, 6, and 7 showed the same spectra and occupied a similar position in the gel. Each spot revealed four peptides mass after fragmentation: 1,500, 1,041, 1,220, and 1,334. The 1,500 peptide showed the same hit as 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (*Puccinia graminis f. sp. tritici* CRL 75-36-700-3) with scores over 30, indicating identity or extensive homology ($p < 0.05$) (Table 1). Spots differed in molecular weight and isoelectric point, which could be due to oxidative modifications. In fact, methionine biosynthesis in *E. coli* seemed to be deterred under oxidative stress conditions due to modulation of the methionine synthase activity through oxidation (Hondorp and Matthews 2009).

Protein carbonylation is an example of oxidative modification. Yeast cells exposed to metal stress showed an increase in oxidized proteins through addition of carbonyl groups, which produced changes in enzyme features such as molecular weight (Irazusta et al. 2010). One of the explanations for oxidative modifications is metal-catalyzed oxidation (MCO). Through this mechanism, a divalent ion bound to the polypeptide chain promotes the generation of ROS that can damage neighboring amino acids (Irazusta et al. 2010). Gonzalez et al. (1996) demonstrated that zinc is a constituent of methionine synthase and it is required for catalysis. A copper overload would

substitute zinc ions, generating protein oxidation. However, Tamarit et al. 1997 showed that metalloenzymes containing non-redox-active metals such as zinc were highly resistant to MCO.

Conclusions

Our results have demonstrated that *R. mucilaginosa* RCL-11 cells exposed to copper overload, overexpressed proteins such as chaperones and SOD, which are involved in defense against oxidative stress. Consequently, the exposed cells gained advantage of enduring unfavorable conditions, which could allow them to compete successfully with other microorganisms in a contaminated environment.

This is the first work that has comprehensively studied the protein composition to reveal the mechanism through which *R. mucilagenos*a supports high concentrations of metals. Our results are important for future application of this yeast in liquid bioremediation systems.

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