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Proteomic and enzymatic response under Cr(VI) overload in yeast isolated from textile-dye industry effluent

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ABSTRACT

Cyberlindnera jadinii M9 and *Wickerhamomyces anomalus* M10 isolated from textile-dye liquid effluents has shown capacity for chromium detoxification via Cr(VI) biological reduction. The aim of the study was to evaluate the effect of hexavalent chromium on synthesis of novel and/or specific proteins involved in chromium tolerance and reduction in response to chromium overload in two indigenous yeasts. A study was carried out following a proteomic approach with *W. anomalus* M10 and *Cy. jadinii* M9 strains. For this, proteins extracts belonging to total cell extracts, membranes and mitochondria were analyzed. When Cr(VI) was added to culture medium there was an over-synthesis of 39 proteins involved in different metabolic pathways. In both strains, chromium supplementation changed protein biosynthesis by upregulating proteins involved in stress response, methionine metabolism, energy production, protein degradation and novel oxide-reductase enzymes. Moreover, we observed that *Cy. jadinii* M9 and *W. anomalus* M10 displayed ability to activate superoxide dismutase, catalase and chromate reductase activity. Two enzymes from the total cell extracts, type II nitroreductase (Frm2) and flavoprotein wrbA (Ycp4), were identified as possibly responsible for inducing crude chromate-reductase activity in cytoplasm of *W. anomalus* M10 under chromium overload. In *Cy.jadinii* M9, mitochondrial Ferredoxine-NADP reductase (Yah1) and membrane FAD flavoprotein (Lpd1) were identified as probably involved in Cr(VI) reduction. To our knowledge, this is the first study proposing chromate reductase activity of these four enzymes in yeast and reporting a relationship between protein synthesis, enzymatic response and chromium biospeciation in *Cy. jadinii* and *W. anomalus*.

1. Introduction

Although chromium cannot be degraded, in the sixth oxidation state it can be reduced by enzymatic or/and non-enzymatic mechanisms of microorganisms to the less toxic Cr(III). Unlike Cr(III),which cannot pass through cell membranes, Cr(VI) is toxic because of its solubility and high oxidizing power. Solubility of Cr(VI) is due to the chemical and structural similarity of chromate and sulfate. Accordingly, chromate is able to use the sulfate uptake pathway to cross over the cellular membrane and, once inside the cells, Cr(VI) undergoes reduction due to various enzymatic and non-enzymatic activities (Ramirez-Diaz et al., 2008). Furthermore, its high oxidizing power is the result of Cr(VI) reduction, which produces several active intermediates that could directly induce toxic effects on DNA and proteins (Sumner et al., 2005).

In nature, living organisms in contact with toxic substances develop adaptive mechanisms that help them to withstand toxic effects. Microbial remediation using Cr(VI)-resistant species of a variety of genera adapted to the hostile environment has demonstrated to be very useful for neutralizing the negative effects of chromium (Guillen-Jimenez et al., 2008; Machado et al., 2009; Joutey et al., 2015). Among the mechanisms described in tolerant microorganisms, there are two Cr (VI) detoxification mechanisms: efflux of chromate ions from the cell cytoplasm and reduction of Cr(VI) to Cr(III) (Ramirez-Diaz et al., 2008). Enzymes involved in detoxification of free radicals, repair of DNA lesions, and sulfur or iron metabolism were described as critical in Cr(VI) resistance-tolerance mechanisms in microorganisms (Ramirez-Diaz

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et al., 2008; Viti et al., 2014). For example, chromium has been shown to affect mitochondrial function, superoxide dismutase enzyme activity, and production of protein oxidative damage in *S. cerevisiae* (Sumner et al., 2005). The enzymatic reduction of chromium was studied mainly in bacterial cells and was mostly related to soluble NAD(P)H- or NADH-dependent enzymes able to transfer electrons to Cr(VI) (Kwak et al., 2003; Opperman and Heerden, 2008; Paul et al., 2012; Bang et al., 2012; Jin et al., 2012; Zhou et al., 2017)

In a previous work, two indigenous yeasts, *Cyberlindnera jadinii* M9 and *Wickerhamomyces anomalus* M10, were isolated from liquid effluents from a textile-dye effluent channel drainage in the proximity of a textile factory (Famaillá, Tucumán, Argentina) (Fernández et al., 2009). Martorell et al. (2012) and Fernández et al. (2013) have shown that the main resistance mechanism described for theses yeasts are the reduction of Cr(VI) to Cr(III). Although the main detoxification mechanism of these yeasts could be the Cr(VI) biospeciation (Martorell et al., 2012; Fernández et al., 2013, 2017), proteins, enzymes and molecules involved in these capacity are still unknowed.

Despite the great interest in microbial metal bioaccumulation, metabolic pathways involved in chromium-cell interaction in yeasts have still not been fully explored. In a different way both plasmatic membrane and mitochondria are fundamental structures for metal entrance, accumulation and cation exchange, as well as for cell defense against free radicals that are generated during reduction of Cr (VI) to Cr (III). Therefore, the aim of present paper was to evaluate the effect of hexavalent chromium on synthesis of novel and/or specific proteins involved in chromium tolerance and reduction in response to chromium overload in two indigenous yeasts. We expected to find both specific enzymes that interact with Cr (VI) but in turn shared mechanisms, such as those involved in stress response, in the two yeasts studied. This report describes the metabolic pathways and molecules involved in the interaction of Cr(VI) with *Cy. jadinii* M9 and *W. anomalus* M10 yeasts using an enzymatic (superoxide dismutase, catalase and chromate reductase) and proteomic approach of total, membrane and mitochondrial protein. We found novel reductase enzymes in cytoplasm, membrane and mitochondria of *W. anomalus* M10 and *Cy. jadinii*M9, probably involved in Cr(VI) reduction.

2. Materials and methods

2.1. Study organisms and culture conditions

Cyberlindnera jadinii M9 (access number GenBank: FJ865435) and *Wickerhamomyces anomalus* M10 (access number GenBank: FJ865436) were isolated from liquid effluents and wall-attached biofilm from a textile-dye effluent channel drainage located in the proximity of a textile factory (Famaillá, Tucumán, Argentina)(Fernández et al., 2013). Culture medium (YNB') was prepared containing Yeast Nitrogen Base (Difco Laboratories; YNB w/o amino acids and ammonium sulfate, 10% v/v), sucrose (50 gl) and $(NH4)_2SO_4$ (0.6 gl)(Fernández et al., 2009). Cultures were prepared in 250 ml Erlenmeyer flasks containing 50 ml YNB' inoculated at a final concentration of 10^7 cells ml^{-1} with a pre-inoculum previously grown on Czapek Malt (CZM) liquid medium cultivated overnight. Incubation was performed at 25 °C on a rotary shaker at 250 rpm during 8 h for mitochondria extraction (where a better number of mitochondria was obtained) and 18 h for total protein and membrane protein extraction. To analyze the influence of Cr(VI) on the physiology of *Cy.jadinni* M9 and *W. anomalus* M10, the culture medium was supplemented with 1mMof $K_2Cr_2O_7$as Cr(VI) source prior to inoculation. Growth was monitored by optical density at 600 nm.

2.2. Protein extraction

For total protein extract, after 18 h of yeast cultivation, cells were harvested by centrifugation; pellets were washed twice with distilled water, and then suspended in50 mM Tris-HCl buffer pH: 8.0 with protease inhibitor cocktail I (Calbiochem) in 2-ml Eppendorf tubes plus 0.5 ml of sterilized glass beads. Cells were disrupted using 10 1-min pulses on a vortex mixer, at 1-min intervals on ice between pulses. The homogenates were centrifuged at 1000 $\times g$ at 4 °C for 5 min and the supernatants were recovered. Nucleic acids were removed by treatment with 1 µl of benzonase (Novagen) in the presence of 1 µl of 1 M $MgSO_4$for 30 min at 37 °C. Total protein content in the supernatants was determined via the Bradford method with Bio-Rad 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. Membrane proteins were extracted from 40 mg of frozen yeast pellets using the Proteo Extract™ Native Membrane Protein Extraction Kit (Calbiochem, USA), following the manufacturer's instructions. Protein concentration was determined using the Lowry assay. Preparations of mitochondrial proteins were assayed as described by Mueller (1988). Briefly, after 8 h of incubation, *Cy. jadinni* M9 and *W. anomalus* M10 cultures were centrifuged at 1,000 $\times g$ for 10 min and cell pellets were stored at −20 °C until further analysis. Cell pellets were resuspended in 2 ml of reducing solution (10 mM dithiothreitol (DTT) and 0.1 M Tris-HCl pH: 9.3) and incubated at 30 °C for 30 min. After centrifugation, cell pellets were resuspended in 5.3 ml of Buffer A (25 mM sodium phosphate pH: 7; 1 mM $MgCl_2$; 1 mM EDTA and 2 M sorbitol) and centrifugation step was repeated. Cell pellets were resuspended in Buffer B (25 mM sodium phosphate pH: 7; 1 mM $MgCl_2$; 1 mM EDTA; 0.2 M sorbitol) plus15 mg/ml of lyticase (Sigma L2524) and incubated at 30 °C during 30 min with orbital agitation (250 rpm). After centrifugation (500 $\times g$ 7 min 4 °C) spheroplasts were resupended in 600 µl of Buffer A and 1800 µl of Buffer B and homogenized using Potter in ice bath. The cell debris was removed by centrifugation at 2000 $\times$ g for 10 min and the mitochondrial organelles were isolated by centrifugation at 1000 $\times$ $g$for 10 min. The mitochondrial pellets were suspended in 25 mM sodium phosphate pH: 7 and stored at −20 °C. Mitochondrial protein content in the supernatants was determined via the Bradford method with Bio-Rad reagents using bovine serum albumin as reference protein.

2.3. Gel electrophoresis

One-dimensional (1DE) or two-dimensional (2DE) gel electrophoresis was performed, depending on the sample characteristics. 1DE was carried out for membrane protein extracts. A solution containing sucrose and bromophenol blue was added before re-running the samples on 11% polyacrylamide gels. Total protein (100 µg) was run at 15 mA and 20 mA. Bands were visualized using a modified Coomassie Brilliant Blue staining method (Wang et al., 2007), images were scanned with an Image Scanner III (GE Healthcare Life Sciences) and analyzed with Quantity One (BioRad).

2DE gel was used for total cell extracts and mitochondrial extracts. Cell homogenates were obtained as described above. Total protein extracts were previously precipitated with 9 vol of acetone for 45 min to minimize SDS concentration. Precipitates were washed three times with acetone by centrifugation at 500 $\times$ g for 5 min. After acetone elimination, proteins were resuspended in RHB(200 µl of 8 M urea, 4% (w/v)) CHAPS, 50 mM DTT, 0.5% (v/v) ampholites (pH 3–10; GE Healthcare Life Science) and bromophenol blue. For mitochondrial fraction, the enriched pellet was directly resuspended in RHB. The suspension of both total and mitochondrial proteins was incubated on an orbital shaker for 3 h and centrifuged at 8000 $\times g$ for 5 min to remove insoluble proteins. Proteins were quantified by the Bradford method (BioRad). Isoelectric focusing (IEF) was performed in 11-cm immobilized pH gradient strips (3-11NL, GE healthcare Life Science or/and BioRad). The focused strips were stored at −20 °C until second-dimension electrophoresis was performed. Thawed strips were equilibrated in 370 mM Tris-HCl (pH: 8.8) containing 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol and 130 mM DTT for 15 min, and then equilibrated in a similar buffer, but containing 135 mM iodoacetamide

instead of DTT, for 15 min. Second-dimension SDS-PAGE (2-DE) was performed on 12.5% polyacrylamide gels. Gels used for total proteins were stained using a modified Coomassie Brilliant Blue staining (Wang et al., 2007) and those for mitochondrial proteins were stained with silver nitrate staining. Images were scanned with an Image Scanner III and analyzed with PD Quest (BioRad).

2.4. Proteomic analysis

Three replicates of 2DE gels for total cell extracts and mitochondrial samples and 1DE gels for membrane proteins were obtained for each sample (each replica came from an independent culture). For matching the detected spots in 2DE gels a synthetic master gel was created by matching representative gels with one another (and only maintaining triplicate spots)by using PD Quest software (Bio-Rad). Spots showing increase in protein abundance between cells grown with and without chromium in total and mitochondrial proteomes were digested with trypsin. Mass spectrometric data were obtained using a MALDITOF MS spectrometer, Ultraflex II (Bruker), in the mass spectrometry facility CEQUIBIEM, Argentina. Proteins were identified via peptide mass fingerprinting (PMF) using MASCOT. Fragmentation was carried out with the most intense MS peaks (MS/MS). When possible, MS and MS/MS information was combined for one or more peptide searches. Candidates with a statistically significant score, i.e., without probability ($p < 0.05$) that the protein identified was resulting from a random match, were further compared with *MW* and *pI* experimental values obtained from 2DE gels. The percentage of protein coverage was determined for each spot or band using MASCOT search (Tables 1–3).

2.4.1. Enzyme activity

Cells from cultures prepared as described above were harvested after 18 h of cultivation and washed twice with distilled water for analysis of superoxide dismutase (SOD) and catalase (CAT) activities. Cells were disrupted in 50 mM Tris-HCl buffer, pH 8.0, using glass beads and vortexing for 15 min. Cell-free extracts were centrifuged at 300 $\times g$ for 5 min. Total protein was determined by the Bradford method (Bio-Rad) using bovine serum albumin as a standard. Superoxide dismutase inhibition was achieved by adding Sodium diethyldithiocarbamate (DDC) (Sigma), a specific copper inhibitor, to the total protein at a final concentration of 20 mM. SOD activities in protein extracts were analyzed in native Tris-glycine 10% polyacrylamide gels, as described previously (Irazusta et al., 2006). Equal amounts of proteins were separated by electrophoresis and gels were stained for SOD activity with the tetrazolium salt 3-[4,5-dimethyltiazol-2-yl] − 2,5-diphenyltetrazolium bromide (MTT) and phenazinemethosulfate under light exposure. Densitometry of native gels was carried out on a Gel Doc 2000 analyzer (Bio-Rad) and band densities were determined using the Quantity One software (Bio-Rad). For comparative purposes, SOD activity in protein extracts from control cultures was considered 100%. CAT activities were determined according to Jakubowski et al. (2000). Briefly, extract was added to 60 mM sodium phosphate buffer pH: 7.4 containing 22 mM H_2O_2; then, the decrease in absorbance at 240 nm was monitored with a Beckman DU 640 spectrophotometer at 25 °C. One unit of enzyme activity (U) was defined as the amount of enzyme that converted 1 μmol of substrate per min at 25 °C. Specific activity was expressed as U per mg of protein.

Total protein and membrane protein extracts were used to determine chromate reductase activity. The decrease of chromate concentration by crude chromate reductase (CChR) was assayed at 30 °C using 120 μl of sample preparation in 2 ml reaction mixtures containing (at a final concentration): 50 mM phosphate citrate buffer (pH 5.0), 1 mM K_2CrO_4, 1 mM NADH (Polti et al., 2009). The reaction was started by adding chromate to the mixture and the residual chromate concentration was measured at final point after 30 min. Controls without NADH or CChR were also assayed.

Cr(VI) in solution was determined by colorimetric measurement of the pink-violet colored complex formed after reaction with diphenylcarbazide (DPC) in acid solution (Urone, 1955). Total protein was determined with Bradford assay (Bio-Rad), using bovine serum albumin as standard.

2.5. Statistic analysis

All data are expressed as means ± standard deviation of at least triplicate determinations of independent cultures. Each single measurement came from three replicate cultures belonging to each experimental condition. Data obtained from two paired groups were compared using Student's *t*-test.

3. Results

3.1. Analysis of Total Proteins of Cy. jadinii M9 and W. anomalus M10

Total cell extracts of exponential cultures of *Cy. jadinii* M9 *and W. anomalus* M10 without metal (control) and with 1 mM of $K_2Cr_2O_7$ as Cr (VI) were analyzed by 2DE (Fig. 1). It is important to clarify that although we observed some down-regulated protein under the conditions studied, we only focus on those proteins that increased their synthesis in the presence of the metal The results of this analysis showed that over-synthesis of at least 9 spots for *Cy. jadinii* M9 and 14 spots for *W. anomalus* M10 were produced in yeasts exposed to 1 mM of K_2CrO_4 as hexavalent chromium (Fig. 1 E and F). Spots that repeatedly showed differences in protein amount in the presence of Cr(VI) were cut from the gel and sent for identification. Proteins were identified using peptide mass fingerprinting or peptide sequencing after MS/MS analysis (Table 1). The exposure of both *Cy. jadinii* M9 and *W. anomalus* M10 cells to chromium triggered the synthesis of several proteins involved in different biochemical and metabolic functions (Fig. 5). Some induced proteins in *Cy.jadinii* M9 and *W. anomalus* M10 shared metabolic pathways, such as proteins involved in methionine biosynthesis (spot 10 of *Cy. jadinii* M9 and spots 1 and 22 of *W. anomalus* M10), and enzymes involved in oxidative stress response (spots 1, 4, 11, 12 and 16, showed homology to molecular chaperones for *Cy. jadinii* M9 and spots 15 to superoxide dismutase Sod1 in). Furthermore, in both yeasts, one protein was found to be involved in protein biodegradation (spots 14 and 10). However, other over-synthesized proteins under chromium overload were specific for only one of the studied yeasts. In *Cy. jadinii* M9, specific proteins were those involved in protein biosynthesis (elongation factor EF-1α) and acidifying vacuolar ATPase (spot 17). In *W. anomalus* M10, were found proteins to be responsible for i) glycolytic metabolism (glyceraldehyde 3-phosphate dehydrogenase, alcohol dehydrogenase, phosphoglycerate kinase and enolase 1); ii) beta 5 subunit of the 20 S proteasome showed relationship with polysaccharide hydrolysis; and iii) the outer membrane porin A was involved in ion transport were recognized as upregulated in chromium stress. Interestingly, type II nitroreductase (Fmr2) and Flavoprotein wrbA (Ycp4) enzymes involved in oxidation-reduction mechanisms were identified in chromium-grown *W. anomalus* M10 cells.

3.2. Analysis of plasma membrane proteins of Cy. jadinii M9 and W. anomalus M10

The plasma membrane performs several important biological functions, including intercellular communication, transport of ions/solutes, and signal transduction. The extracted proteins were separated using SDS-PAGE 1DE. It was not possible to apply 2DE-analysisbecause some substances provided in the Proteo Extract™ Native Membrane Kit cause isoelectric focusing interference. Membrane protein synthesis changed in both *Cy. jadinii* M9 and *W. anomalus* M10 exposed to hexavalent chromium. Nine bands in *Cy. jadinii* M9 and six bands in *W. anomalus* M10 were selected for further identification with peptide mass fingerprinting (Table 2). A total of 12 proteins, 8 for *Cy. jadinii* M9 and 4 for

Table 1
Identification of over-synthesized totals proteins in *Cyberlindnera jadinii* M9 and *Wickerhamomyces anomalus* M10 after incubation with chromium.

Yeast	*Spot*	*Protein*	*gen*	*Specie*	*MW/pI*[a]	*Identification Techniqu*[b]	*MASCOT Score*[c]	*MPN*[d]	*Ratio +Cr/-Cr*[e]
Cyberlindnera jadinii	1	Peptidil-prolilcis-transisomerase	CPR1	*Ajellomyces capsulatus*	17390/7.5	MS/MS	69	5	16.01
M9	4	Heat shock protein	SSA3	*Saccharomyces cerevisiae*	70395/4.7	MS-MS/MS	128	6	2.89
	7	Translational elongation factor EF-1α	TEF2	*Cunninghamellae chinulata*	46803/9.6	MS-MS/MS	206	7	1.66
	10	Methioninesynthase	MET6	*Komagataella pastoris*	86027/6.4	MS-MS/MS	100	8	0.86
	11	Heat shock protein	SSB2	*Meyerozyma guilliermondii*	66421/5.1	MS-MS/MS	344	15	2.35
	12	Heat shock protein	SSB1	*Lachancea thermotolerans*	66106/5.0	MS-MS/MS	211	10	3.38
	14	Cell division control protein	CDC48	*Lodderomyces elongisporus*	92108/4.5	MS-MS/MS	239	29	1.49
	16	Heat shock protein	HSP70	*Candida albicans*	70423/4.7	MS	155	9	4.26
	17	Vacuolar ATPase V1 complex subunit A	VMA1	*Candida albicans*	63592/4.6	MS-MS/MS	208	20	1.42
Wickerhamomyces anomalus	1	Sulfate adenylyltransferase	MET3	*Wickerhamomyces ciferrii*	57654/5.6	MS/MS	77	9	1.30
M10	1	Outer membrane porin A	POR1	*Pseudomonas* sp.	30428/8.1	MS/MS	94	1	1.30
	2	Alcohol dehydrogenase	ADH1	*Wickerhamomyces ciferrii*	36846/6.6	MS-MS/MS	120	6	1.18
	3	Beta-1,3-glucanase	EXG1	*Wickerhamomyces anomalus*	49490/4.3	MS-MS/MS	74	7	3.81
	4	Phosphoglyceratekinase	PGK1	*Meyerozyma guilliermondii*	44738/7.8	MS/MS	34	1	1.19
	6, 7	Transcriptional ADAptor	ADA2	*Lachancea thermotolerans*	50564/6.6	–	*de novo*	1	5.96
	10	Beta 5 subunit of the 20S proteasome	PRE2	*Lodderomyces elongisporus*	31636/6.1	MS-MS/MS	83	5	4.69
	11	Type II nitroreductase	FRM2	*Ogataeapara polymorpha*	21200/7.0	–	*de novo*	1	4.58
	13	Glyceraldehyde 3-phosphate dehydrogenase	TDH1	*Candida tropicalis*	35937/8.5	MS/MS	130	2	2.79
	15	Superoxide dismutase	SOD1	*Ajellomyces capsulatus*	15985/5.9	MS/MS	66	2	2.85
	16	Flavo proteinwrbA	YCP4	*Wickerhamomyces ciferrii*	21238/8.4	MS-MS/MS	182	4	1.72
	17	Glyceraldehyde 3-phosphate dehydrogenase	TDH1	*Komagataella pastoris*	35695/8.5	MS-MS/MS	151	5	3.65
	22	Methionine synthase	MET6	*Wickerhamomyces ciferrii*	86405/6.4	MS-MS/MS	345	10	92.59
	23	Enolase 1	ENO1	*Meyerozyma guilliermondii*	46951/6.6	MS-MS/MS	179	5	28.78

[a] Values for molecular weight (KDa) and isoelectric point.
[b] MS-MS/MS indicates a combination of MS and MS/MS.
[c] 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 ($p < 0.05$). De novo sequencing was inferred from and showed by BLAST homology.
[d] Matched peptides numbers.
[e] Ratio between differential synthesis of proteins in yeast with and without chromium.

Table 2
Identification of over-synthesized membrane proteins in *Cyberlindnera jadinii* M9 and *Wickerhamomyces anomalus* M10 after incubation with chromium.

Yeast	*Band*	*Protein*	*Gen*	*Specie*	*MW*[a]	*Identification Technique*[b]	*MASCOT Score*[c]	*MPN*[d]	*Ratio +Cr/-Cr*[e]
Cyberlindnera jadinii	3	Methionine synthase	MET6	*Saccharomyces cerevisiae*	85948	MS/MS	35	2	4.19
M9	5	Carboxypeptidase C	PRC1	*Wickerhamomyces ciferrii*	59322	MS/MS	68	2	2.47
	5	FAD flavoprotein	LPD1	*Millerozyma farinosa*	53665	MS/MS	64	3	2.47
	7	Enolose 1	ENO1	*ZygoSaccharomyces bailii*	46708	MS-MS/MS	157	10	1.91
	8	Methionine adenosyl transferase	SAM1	*Candida dubliniensis*	42535	MS-MS/MS	352	13	1.75
	9	Glyceraldehyde 3-phosphate dehydrogenase	TDH1	*Cyberlindnera jadinii*	35969	MS/MS	98	4	2.23
	9	Urate oxidase	UOX	*Cyberlindnera jadinii*	34382	MS/MS	55	6	2.23
	10	Phosphoglycerate mutase	GPM1	*Candida maltosa*	27411	MS/MS	78	6	2.70
Wickerhamomyces anomalus	13	Maltase alfa glucosidase	MAL7	*Wickerhamomyces ciferrii*	67127	MS-MS/MS	127	11	4.34
M10	14	Myoinositol phosphate synthase	INO1	*Ascochyta rabiei*	59642	MS/MS	104	1	1.82
	15	Methionine adenosyl transferase	SAM2	*Candida tenuis*	42295	MS-MS/MS	165	10	2.64
	16	Eisosome	LSP1	*Saccharomyces cerevisiae*	38048	MS/MS	36	1	2.24

[a] Values for molecular weight (KDa) and isoelectric point.
[b] MS-MS/MS indicates a combination of MS and MS/MS.
[c] MS:proteinscores > 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 ($p < 0.05$). De novo sequencing was inferred from and showed by BLAST homology.
[d] Matched peptides numbers.
[e] Ratio between differential synthesis of proteins in yeast with and without chromium.

W. anomalus M10, were identified as upregulated under Cr(VI) overload (Table 2). Bands 3, 8 and15 were recognized as part of methionine biosynthesis; bands7, 9 an10 were involved in glycolytic metabolism, and band 13 was related to polysaccharide hydrolysis (Fig. 5). Two proteins involved in membrane biosynthesis, eiosome component and myoinositol phosphate synthase, were found to be upregulated in membranes exposed to hexavalent chromium in *W. anomalus* M10. FAD flavoprotein enzyme involved in oxido-reduction mechanisms was identified in chromium-grown *Cy. jadinii* M9.

3.3. Analysis of mitochondrial proteins of Cy. jadinii M9 and W. anomalus M10

Mitochondrial enrichment was performed to determine if mitochondrial proteins were involved in chromium stress resistance and/or chromium reduction. A proteomic study using 2DE-analysis was carried out with 8 h of cultivation in order to obtain exponentially grown yeast with metabolically active mitochondria (Fig. 2). Eleven and seven spots were selected for identification in *Cy. jadinii* M9 and *W. anomalus* M10, respectively. Only seven proteins of a total of 18 selected could be identified (Table 3), probably due to the low concentration of these proteins and technical inconveniences, such as staining with silver nitrate. We identified four proteins, heat shock protein, alfa tubulin, translational elongation factor EFT2α and ferredoxine-NADP reductase for *Cy. jadinii* M9, and three proteins, ATPasa D mitochondrial, 6 phosphoglucanate dehydrogenase and alcohol dehydrogenase, for *W. anomalus* M10.

3.4. Antioxidant enzyme activities

Catalase (CAT) and superoxide dismutase (SOD) activities were assayed in order to recognize the oxidative status of the yeasts exposed to hexavalent chromium. As shown in Fig. 3 F, CAT activity increased in the presence of Cr (VI) in *Cy. jadinii* M9 and *W. anomalus*M10 strains. Interestingly, basal enzyme levels in the absence of metal in *Cy. jadinii* M9 were statistically lower than those in *W. anomalus*M10 (*p* value 0.017).

To reveal whether SOD activity was altered in chromium presence, we analyzed the activity of both Sod isoenzymes using native gels stained for SOD activity. As shown in Fig. 3E, enzyme patterns - depicted as white bands on a dark background- differed for each strain. In order to discriminate between Sod1 and Sod2 isoenzymes, different inhibitors were explored. The specific copper inhibitor sodium diethyldithiocarbamate (DDC) was able to avoid cytosolic copper-dependent CuZn-SOD (Sod1) activity (Fig. 3). Addition of DDC allowed us to differentiate between Sod isoenzymes in the studied yeasts. Sod1 is represented by the first band on the left of gel (1) in *Cy. jadinii* M9 and by the last band on the right of gel (4) in *W. anomalus* M10. Accordingly, we can infer that bands 2 and 3 correspond to mitochondrial manganese-dependent isoenzyme (Mn-SOD: Sod2) (Fig. 3E). Native gels were analyzed using Quantity One software (Bio-Rad). As shown in Fig. 3A and B, Cr (VI) increased CuZn-SOD activity in both *Cy. jadinii* M9 and *W. anomalus* M10 cells. We also investigated the effects of Cr (VI) on Mn-SOD activity and found that *Cy. jadinii* M9 increased Sod2 activity by 80% compared with cells growing in the absence of chromium (Fig. 3C), whereas in *W. anomalus* M10, cells under Cr(VI) overload exhibited an non-significant increase of isoenzyme activity of about 25% (Fig. 3 D).

3.5. Total and membrane chromate reductase activity

A previous study analyzed Cr(VI) reduction activity in *Cy. jadinii* M9 and *W. anamalus* M10 cells (Martorell et al., 2012). The authors tested cell free extracts of *Cy. jadinii* M9 and *W. anomalus* M10, and found that a soluble enzyme was possibly responsible for Cr(VI) reduction.

To further investigate the contribution of plasma membrane extract to the reduction of hexavalent chromium, we performed an enzymatic study of total and membrane extracts. Both protein extracts exhibited an increased capacity of *Cy. jadinii* M9 and *W. anomalus* M10 to reduce Cr(VI) in the presence of hexavalent chromium (Fig. 4). Crude chromate reductase activity (CChR) obtained from *Cy. jadinii* M9 cells grown in the absence or presence of 1 mM Cr(VI) increased from 4.13 ± 0.17 to 6.05 ± 0.28 (mmoL Cr(VI) min^{-1}mg $prot^{-1}$). Furthermore, the reduction capacity of *W. anomalus* M10 increased in total extracts in the presence of chromium, from a basal activity of 2.13 ± 0.87 to 7.22 ± 0.099 (mmoL Cr(VI) min^{-1} mg $prot^{-1}$) in the presence of metal.

Similar results in terms of effects of responses to chromium were observed in extracts of plasma membrane: an increase in the specific activity was observed in the presence of metal, indicating an induction

Table 3
Identification of over-synthesized mitochondrial proteins in *Cyberlindnera jadinii* M9 and *Wickerhamomyces anomalus* M10 after incubation with chromium.

Yeast	*Spot*	*Protein*	*Gen*	*Specie*	*MW/pI*[a]	*Identification Technique*[b]	*MASCOT Score*[c]	*MPN*[d]	*Ratio +Cr/-Cr*[e]
Cyberlindnera jadinii	8	Heat shock protein	HSP70	*Dothistroma septosporum NZE10*	71377/4.7	MS/MS	72	10	1.8
M9	9	Alfa tubulin	TUB1	*Komagataella pastoris GS115*	50407/4.8	MS-MS/MS	62	3	1.5
	10	Translational elongation factor EFT2α	EFT2	*Cyberlindnera jadinii*	22232/6.2	MS-MS/MS	170	5	1.6
	11	Ferredoxine-NADP reductase	YAH1	*Saccharomyces cerevisiae S288c*	18932/5.3	–	*de novo*	1	1.5
Wickerhamomyces anomalus	4	ATPasa D mitochondrial	ATP7	*Zygosaccharomyces rouxii*	19809/9.7	–	*de novo*	1	4.1
M10	5	6 Phosphoglucanate dehydrogenase	GND1	*Saccharomyces cerevisiae S288c*	42840/6.6	–	*de novo*	1	58
	6	Alcohol dehydrogenase	ADH	*Saccharomyces cerevisiae S288c*	36849/6.6	MS/MS	69	5	2.9

[a] Matched peptides numbers.
[b] Values for molecular weight (kDa) and isoelectric point.
[c] MS-MS/MS indicates a combination of MS and MS/MS.
[d] 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 ($p < 0.05$). De novo sequencing was inferred from and showed by BLAST homology.
[e] Ratio between differential synthesis of proteins in yeast with and without chromium.

of CChR activity due to the presence of Cr(VI). CChR activity was almost tripled in *Cy. jadinii* M9 yeast grown in presence of chromium, showing in this condition the lowest value for such activity (Fig. 4 B).

Moreover and due to the low protein obtained in mitochondrial purifications we were unable to evaluate the chromate reductase activity in these extracts.

4. Discussion

We performed a proteomic and enzymatic study to elucidate the mechanisms employed by two different yeast strains in bioremediation of Cr(VI). We found that in *Cy. jadinii* M9 and *W. anomalus* M10 exposed to high concentrations of Cr(VI), some proteins associated with either cytoplasm, membrane and/ or mitochondria were over-synthesized. Below we discuss the implications of the over-expression of these proteins and their correlation with enzymatic activities and Cr(VI) reduction.

4.1. Stress proteins

Proteins involved in stress responses, such as heat shock proteins and molecular chaperones, and specific enzymes such as SOD and CAT, are readily induced in microorganisms in response to a wide range of environmental stress conditions, including heavy metals. Heat shock proteins (Hsps) are a family of proteins that were shown to be upregulated in the presence of arsenic, cadmium, copper, iron and chromium in heavy metal-resistant yeasts (Tamás et al., 2014). Since the ATP-dependent Hsp70 chaperone system significantly attenuates the folding-inhibitory effect of metal ions (Tamás et al., 2014), the oversynthesis of chaperones would be an adaptive response to chromium exposure in *Cy. jadinii* M9. Furthermore, this response was observed in total and mitochondrial protein analyses, demonstrating the mitochondrial localization of Hsp70 (Tables 1 and 3). Interestingly, we previously found that chaperones helped to protect other indigenous yeasts, such as *Rhodotorula mucilaginosa* RCL-11 and *Candida fukuyamaensis* RCL-3, against copper-induced oxidative stress (Irazusta et al., 2012, 2016). Moreover, chaperone protein synthesis was previously found to increase in other microorganisms exposed to Cr(VI) ions, such as *Euglena gracilis* and *Pseudomonas aeruginosa* (Kiliç et al., 2010). Cyclophilin (spot 1of *Cy. jadinii* M9, Table 1), which was identified as over-expressed, is part of a conserved cis–trans peptidylprolylisomerase that is involved in protein folding and acts as a molecular chaperone. Perez and Weis (2008) found that high levels of cyclophilin were synthesized in response to stress in plant tissues. Since it has been demonstrated that Cpr1 is essential for oxidative defense against ROS in yeast (Kim et al., 2011), we propose that this protein would be another molecular chaperone implicated in defense capabilities in *Cy. jadinii* M9.

We identified SOD enzymes in chromium overload in *W. anomalus*M10. These enzymes have an antioxidant function by catalyzing the disproportionation of superoxide anion to hydrogen peroxide (Baron et al., 2015). Yeast cells possess two SODs: the CuZn-depending cytosolic Sod1 and the Mn-depending Sod2 in the mitochondrial matrix (Sturtz et al., 2001). In this work we found that Sod1 were over-synthesized in the presence of chromium in *W. anomalus* M10 yeast. These results are in agreement with previous observations in prokaryote and eukaryote organisms, in which over-synthesis and over-activity of SODs proteins were observed under stress conditions (Ackerley et al., 2006; Irazusta et al., 2006). Ackerley et al. (2006) observed the activation of superoxide dismutase and catalase in *E. coli*, indicating the presence of chromate protective systems. Accordingly, Sumner et al. (2005) demonstrated that the antioxidant function of SOD proteins is specifically protecting *S. cerevisiae* from Cr toxicity. There are many examples of over-synthesis and activity of these enzymes; for example, in filamentous fungi *Trichosporon* the presence of heavy metals like copper, cadmium and chromium stimulated SOD and CAT enzyme (Lazarova

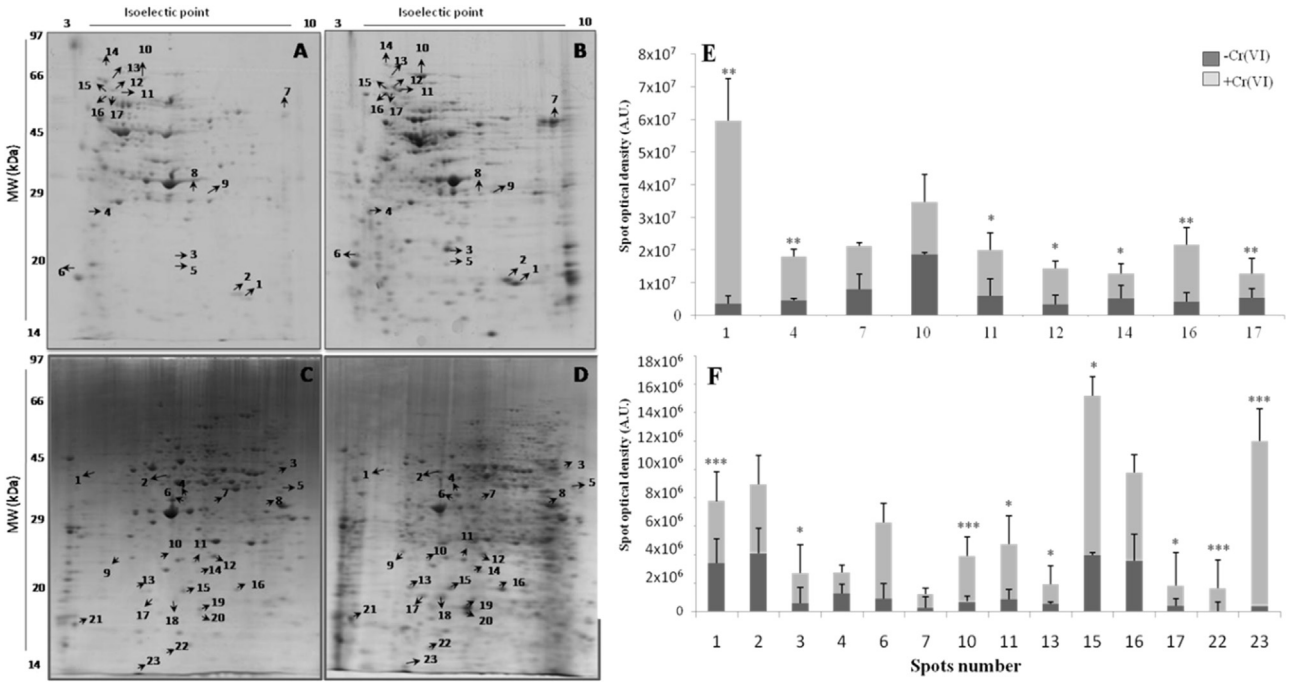

Fig. 1. Analysis of the proteomes of *Cy. jadinii* M9 and *W. anomalus* M10 in the presence and absence of 1 mM Cr(VI). Cells were grown in minimal medium in the presence or absence of chromium and then 200 μg of total cell lysate were separated by 2DE-gel electrophoresis. A) *Cy.jadinii* M9 without chromium; B) *Cy.jadinii* M9 supplemented with Cr(VI); C) *W. anomalus* M10 without chromium; D) *W. anomalus* M10 supplemented with Cr(VI); E) *Cy.jadinii* M9 analysis of identified spots optical density with and without chromium; F) *W. anomalus* M10 analysis of identified spots optical density with and without chromium. Gels were stained with Coomassie Brilliant Blue R-250, digitalized by Image Scanner III (GE Healthcare Life Sciences) and analyzed with PD Quest (BioRad). Spots showing difference in protein abundance between presence and absence of metal in *jadinii* M9 and *W. anomalus* M10 proteomes are indicated with arrows in gels and represented in graphics (E, F). Data are expressed as means ± standard deviation of at least triplicate determinations of independent cultures and were analyzed using Student's *t*-test.*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$.

et al., 2014).

In this work we observed that both *Cy. jadinii* M9 and *W. anomalus* M10displayed ability to activate SOD and CAT enzymes, although only over-synthesis of Sod1 in M10 was observed (Fig. 3). Interestingly, the over-synthesis of proteins involved in defense under oxidative stress implied a great stress situation in *Cy. jadinii* M9 and *W. anomalus* M10 yeasts (Fig. 5).

4.2. Proteins involved in energy production

An elevated energy demand in yeast under heavy metal stress may explain the over-synthesis of four enzymes involved in the glycolytic pathway: enolase 1, phosphoglycerate kinase, alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase (Tables 1–3 and Fig. 5). An increased expression of proteins involved in the glycolytic pathway has been observed in other organisms under stress conditions, such as sorbic acid-stress, copper overload and, interestingly, in the presence of chromium overload (Kiliç et al., 2010; Irazusta et al., 2016). Increased expression was also reported in transcriptomic studies of genes related to carbohydrate metabolism in bacteria exposed to chromate (Monsieurs et al., 2011).

The protein involved in energy production 6 phosphoglucanate dehydrogenase -part of pentose phosphate pathways- was over-synthesized under Cr(VI) overload in *W. anomalus*M10. Remarkably, this enzymes is involved in biosynthesis of NADPH, an essential molecule and cofactor for enzymes involved in the reduction of Cr(VI) to Cr(III) (Viti et al., 2014). Interestingly, 6-phosphogluconate dehydrogenase catalyzes the second oxidative reduction of $NADP^+$ to NADPH and is required for adaptation to oxidative stress, because it is an essential cofactor of many antioxidant enzymes (Maaheimo et al., 2001). It is

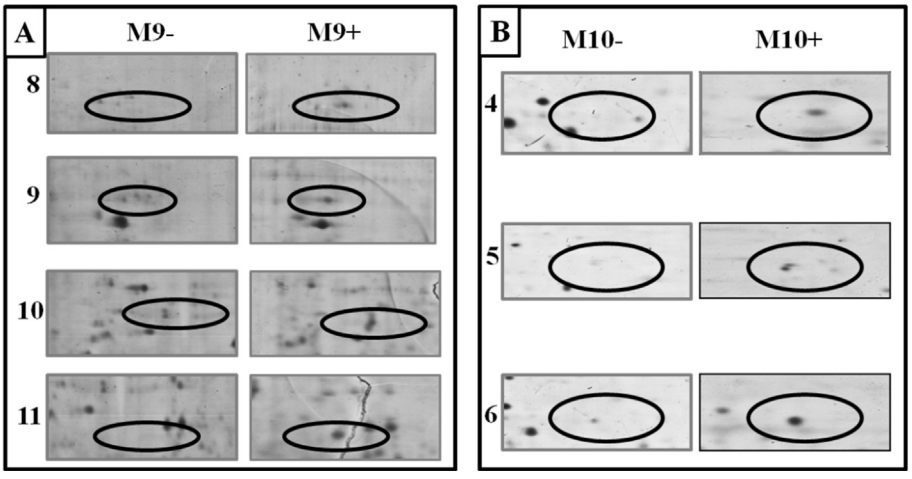

Fig. 2. Analysis of mitochondrial proteomes of *Cy. jadinii* M9 and *W. anomalus*M10 in the presence and absence of 1 mM Cr(VI). A) *Cy. jadinni* M9 without (-) and with (+) chromium in the culture medium. B) *W. anomalus* M10 without (-) and with (+) chromium in the culture medium.

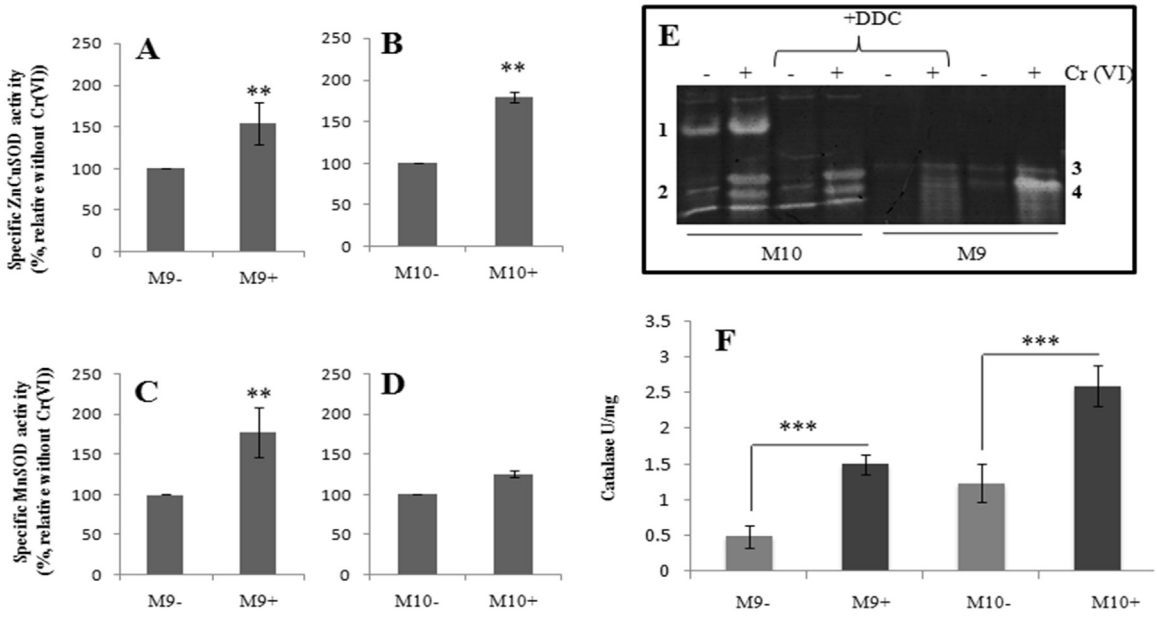

Fig. 3. Increase of superoxide dismutase and catalase activity in *Cy. jadinii* M9 and *W. anomalus* M10 under chromium overload. A) Specific CuZnSOD activity in *Cy. jadinii* M9 with and without chromium supplementation. B) Specific CuZnSOD activity in *W. anomalus* M10 with and without chromium supplementation. C) Specific MnSOD activity in *Cy. jadinii* M9 with and without chromium supplementation. D) Specific MnSOD activity in *W. anomalus* M10 with and without chromium supplementation. E) Cultures of *Cy. jadinii* M9 and *W. anomalus* M10 with and without Cr(VI) supplementation and cultures amended with or without DCC were grown in minimal medium, and cell lysates (20 μg of protein) were analyzed using native gel electrophoresis; SOD activity staining showed the activity and amounts of both isoenzymes (bands 1 and 4 for CuZnSOD, and 2 and 3 for MnSOD). F) Catalase activity in *Cy. jadinii* M9 and *W. anomalus* M10 in cells cultured with and without Cr(VI) supplementation. Data are expressed as means ± standard deviation of at least triplicate determinations of independent cultures and were analyzed using Student's *t*-test.*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$.

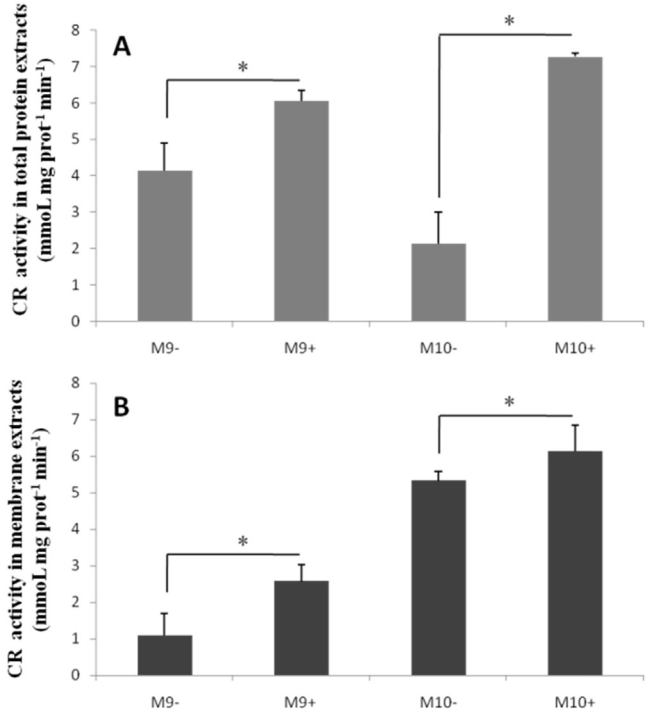

Fig. 4. Specific Chromate reductase activity of A) *Cy. jadinii* M9 and *W. anomalus* M10 total protein extracts obtained from cultures amended with and without Cr(VI), and B) *Cy. jadinii* M9 and *W. anomalus* M10 membrane extracts obtained from cultures amended with and without Cr(VI). Data are mean of three independent assays and error bars indicate standard deviation. Data were analyzed using Student's *t*-test.*$p \leq 0.05$.

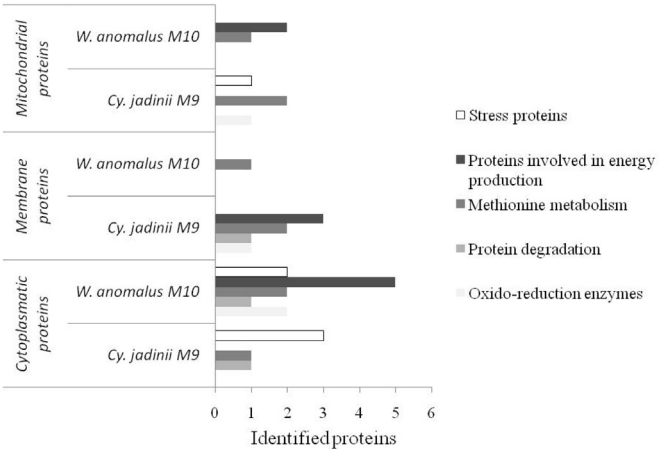

Fig. 5. Clustering of cytoplasm, membrane and mitochondria differentially synthesized proteins in *Cy. jadinii* M9 and *W. anomalus* M10 after incubation with chromium. Five groups were chosen as the most representative to determine the main and shared biochemical and metabolic functions: stress protein, proteins involved in energy production, methionine metabolism, protein degradation and oxide-reduction enzymes. Each bar of the graph represents the number of identified proteins belonging to: the biochemical group, the protein fraction and the yeast under study.

well known that NADH/NADPH generated in the pentose phosphate pathways is necessary to prevent oxidative stress in the cell. The induction of Gnd1 in *W. anomalus* M10 may be related to the need to generate NADH/NADPH molecules, which are cofactor enzyme systems, such as chromate reductase.

ATP synthase (Atp7), encoding ATPase d-subunit of the stator stalk of mitochondrial F_1F_0, was observed as upregulated in mitochondrial fraction of *W. anomalus* M10 cells. Increased demand for ATP production by induction of ATPasa proteins like F_1F_0 ATPase subunits in cells under different stresses was also reported (Yin et al., 2009; Irazusta et al., 2016).

4.3. Methionine metabolism

Cy. jadinii M9 and *W. anomalus* M10 responded to Cr(VI) stress by stimulation of methionine metabolism. Three enzymes, methionine

synthase (MET6), sulfate adenylyltransferase (MET3) and methionine adenosyltransferase (SAM1 and SAM2),were found in total and membrane proteomic analyses (Tables 1 and 2 and Fig. 5). It is interesting to note that in both total proteins extract in *W. anomalus* M10 and in membrane extract in *Cy. jadinii* M9, methionine synthase (MET6) was the protein with the highest radius of over-synthesis in presence of the metal (Table 1 and Table 2). Studies using proteomic and transcriptomic tools have demonstrated that bacteria respond to chromate stress by up-regulating the genes/proteins involved in methionine/cysteine biosynthesis (Kiliç et al., 2010). Furthermore, the absence of glutathione (GSH) was found to increase Cr(VI) toxicity in bacteria (Helbig et al., 2008). Methionine biosynthesis was correlated with GSH system. Bothe systems work in the detoxification of free radicals generated during exposure to Cr (VI) these yeasts. The regulation of the sulfur metabolic pathway in bacteria stressed by Cr(VI) demonstrate the nexus between methionine biosynthesis and GSH system (Viti et al., 2014). It was demonstrated that the lack of GSH free radical-preventing enzymes causes heavy metal sensitivity in yeast. Although the role of GSH in response to chromate in fungi is controversial, the importance of sulfur metabolism appears to be clear in yeast. Pepi and Baldi (1992) demonstrated that supplementation of *Candida* sp. with sulfur-containing compounds such as methionine and cysteine makes strains more resistant to chromate than when grown in the presence of sulfate. We observed that *Cy. jadinii* M9 and *W. anomalus* M10 yeasts under chromium overload stimulated a higher production of enzymes involved in methionine molecules than that in GSH molecules. These results are in agreement with the need of Cr(VI)-sensitive yeasts for methionine to tolerate Cr(VI) toxicity (Pepi and Baldi, 1992).

4.4. Protein degradation

While protein damage takes place continuously in cells, it is exacerbated by adverse intrinsic and environmental conditions, such as unbalanced protein synthesis, and metabolic, oxidative and heavy metals stress. Nevertheless, induction of defense proteins was observed, also demonstrating that Cr(VI) induces protein damage (Viti et al., 2014). Pre2 protein, an essential component of the proteosome, was over-synthesized in *W. anomalus* M10 exposed to chromium. In *W. anomalous* M10 cells, some proteins may undergo oxidation and/or inactivation in the presence of chromium. Another protein involved in proteolysis-connected pathways, the cell division control protein (Cdc48), was identified in *Cy. jadinii* M9. This cytosolic protein "escorted" ubiquitylated proteins to the 26S proteasome for degradation (Buchberger et al., 2010). In *S. cerevisiae*, Cdc48 was shown to be hypersensitive to cell wall perturbing and stress agents (Hsieh and Chen, 2011). In addition, a vacuolar carboxypeptidase (Prc1) involved in non-specific protein degradation in the vacuole was found to be induced in *Cy. jadinii* M9 membranes proteins. Hence, a differential expression of over-expressed proteins of Pre2, Cdc48 and Prc1 involved in protein degradation in cells under chromium stress is not surprising.

4.5. Oxido-reduction enzymes (crude chromate reductase activity)

Martorell et al. (2012) revealed that CChR activity was involved in remove toxic hexavalent chromium by *Cy. jadinii* M9 and *W. anomalus* M10. These strains were resistant to chromium and showed Cr(VI) reduction to undetectable levels at a maximum of 96 h of incubation (Fernández et al., 2009, 2010). Experiments with cell-free extracts of *Cy. jadini* M9 and *W. anomalus* M10 indicated that a soluble intracellular type of enzymes was responsible for Cr(VI) reduction (Martorell et al., 2012). In this study, we observed that both type II nitroreductase (Frm2) and flavoprotein wrbA (Ycp4), which were probably involved in the reduction of Cr(VI), were over-synthesized in the presence of chromium in *W. anomalus* M10.

Bacterial Cr(VI) reductases, some of which confer resistance to chromate, have been previously studied and characterized (Ramirez-Diaz et al., 2008; Paul et al., 2012); however, little is known about yeast chromate reductases. These enzymes usually show a NADH flavin oxidoreductase activity and have the ability to reduce chromate as a secondary function. For example, the primary activity of intracellular nitroreductases NfsA/NfsB from *Vibrio Harveyi* is nitrofurazone reduction and a secondary function is Cr(VI) reduction (Kwak et al., 2003). Two putative nitroreductase-like proteins, Frm2 and Hbn1, have been identified in *S. cerevisiae* (de Oliveira et al., 2007). Interestingly, Frm2 can protect microorganisms against oxidative stress exerted by nitro aromatic compounds and copper (Bang et al., 2012). However, to date there is no evidence of chromate reductase activity by Frm2 in yeast. This is the first work proposing that this enzyme plays a role in detoxification of Cr(VI). Studies in order to characterize this enzyme are being carried out in our laboratory.

WrbA, a member of multimeric flavodoxin-like family, binds to flavin mononucleotide (FMN) coenzyme, and is able to transfer electrons at low oxidation–reduction potential (Patridge and Ferry, 2006). This enzyme was found to be involved in reduction of Fe(III), U(VI), quinines and Cr(VI) (Jin et al., 2012). The function of both *E. coli* WrbA protein and eukaryotic NAD(P)H:quinone oxido reductases is to catalyze quinine (or chromate) reduction via simultaneous two-electron transfer by the cellular one-electron reducers and generation of quinols that promote tolerance to ROS (Ackerley et al., 2004; Cardona et al., 2011; Eswaramoorthy et al., 2012). Interestingly, flavodoxin is located in the cytoplasm in *Gluconacetobacter hansenii*. Little is known about intracellular chromate reductases (CR); further studies should be conducted to explore these two candidates identified in this work. Reduction of Cr(VI) may be associated with the cell membrane or with the soluble fraction (Ramirez-Diaz et al., 2008). Regarding the CChR activity measured both in total protein extracts and in the membrane protein fraction, *W. anomalus* M10 showed greater Cr(VI) reducing power (Fig. 4). In addition, a higher increase of CR activity in total protein extract including cytoplasmic enzymes was observed in Cr(VI) presence in *W. anomalus* M10 than in *Cy. jadinii* M9. This reducing capability can be explained by the presence of both intracellular enzymes (Frm2 and Ycp4), which were found to be stimulated in the presence of metal in the strain *W. anomalus* M10.

In contrast to the quinone reductases and the nitroreductases, the dihydrolipoamide dehydrogenase (Lpd1) has a non-covalently bound FAD instead of FMN as well as a redox active disulfide. In *Thermuss cotoductus* SA01 this enzyme is located in peripheral membrane and was able to reduce Cr(VI) to Cr(III) (Opperman and Heerden, 2008). *Cy. jadinii* M9 under chromium treatment showed Lpd1 FAD flavoprotein over-synthesis, which indicates the reducing ability of the membranes of these yeasts. This finding is in agreement with the high stimulation of CChR activity in *Cy. jadinii* M9 membrane extract in the presence of chromium (Fig. 4).

Mitochondrial Ferredoxine-NADP reductase Yah1 was found to be over-synthesized under chromium exposure in *Cy. jadinii* M9. The capacity of Cr(VI) reduction can be attributed to NAD(P)H-dependent flavoenzymes, including the Ferredoxin-NADP oxidoreductases (Shi and Dalal, 1990). However, there are no evidences of Yah1 Cr(VI) reduction in yeast. Further studies of these novels CR enzymes will be necessary to characterize chromium reducing activity.

To the best of our knowledge, this is the first report showing the relationship among protein synthesis, enzymatic response and chromium biospeciation in both *Cy. jadinii* and *W. anomalus*. The ability of these yeasts to adapt to diverse stress conditions by means of an overproduction of industrially significant enzymes could be of particular interest. Understanding the molecular basis of this phenomenon could lead to enhanced bioremediation of contaminated environments.

5. Conclusion

What happens in yeast cell sunder Cr(VI) overload? Here, we observed that the major responses against acute chromium stress in both

*Cy. jadinii*M9 and *W anomalus* M10 yeasts were related to: stress response proteins, methionine synthase, energy and degradation proteins, and oxide-reduction proteins. The analysis of catalase and superoxide dismutase activities indicated that yeasts increased both activities under Cr(VI) overload. This fact was in agreement with a stress situation in *Cy. jadinii* M9 and *W. anomalus* M10 cells exposed to Cr(VI). As expected stress response was a shared mechanism under metal overload.

However specific enzymes related to Cr(VI) detoxification were found in each of the yeast studies. Four novel reductase enzymes probably involved in the Cr(VI) reduction were found to be over-synthesized in *Cy. jadinii* M9 and *W. anomalus* M10. Interestingly, in total protein extracts, two enzymes: type II nitroreductase (Frm2) and flavoprotein wrbA (Ycp4) in *W anomalus* M10 and two -a mitochondrial enzyme, Ferredoxine-NADP reductase (Yah1) and a membrane FAD flavoprotein (Lpd1) - in *Cy jadinii* M9, were identified as probably involved in Cr(VI) reduction. Further studies will be needed to confirm these enzyme activities and their implications. To the best of our knowledge, this is the first report showing the relationship between protein synthesis, enzymatic response and chromium biospeciation in both *Cy. jadinii* and *W. anomalus*.

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Conflict of interest

None declared.

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