INTRODUCTION

Copper (Cu) acts as the cofactor in a variety of enzymes involved in electron transfer and is therefore essential in trace amounts for most living beings. However, similarly to other heavy metals, excess Cu is toxic to cells because of several interconnected effects, e.g. the formation of coordination complexes with cellular components such as phosphates, purines, porphyrins, and cysteinyl and histidyl side chains of proteins (Gadd, 1993). Moreover, Cu ions can elicit oxidative damage either directly through their redox cycling activities that produce the highly reactive OH⁻ radical (Halliwell & Gutteridge, 1984) or indirectly by depleting free radical scavengers such as glutathione and protein-bound thiol groups (Stoehs & Bagchi, 1995). Therefore, a tight regulation of Cu homeostasis is required for cell survival. Accordingly, it was reported that in the yeast Saccharomyces cerevisiae, free Cu inside cells is maintained in a narrow range (Wegner et al., 2011).

Cu homeostasis has been investigated in depth in S. cerevisiae, and complex mechanisms protecting cells from excess Cu have been identified, such as a reduction in Cu uptake (Yamaguchi-Iwai et al., 1997), the induction of metallothioneins (MTs) (Culotta et al., 1994; Gross et al., 2000) and antioxidant enzymes (Romandini et al., 1992), and the activation of vacuolar functions (Szczypka et al., 1997). MTs form a family of cystein-rich, low-molecular-mass polypeptides common in eukaryotic organisms (Kagi, 1991), whose main function is to bind metal ions thereby buffering their intracellular concentration. In S. cerevisiae, MTs are encoded by two genes, CUP1 and CRS5, whose expression is regulated at the transcriptional level, and, in the case of CUP1, also via gene amplification (Butt & Ecker, 1987). A major role for Cup1 has been suggested (Pagani et al., 2007; Peña et al., 1998) and supported by the observation that disruption of the CUP1 locus renders yeast hypersensitive to copper salt (Ecker et al., 1986; Hamer et al., 1985), whereas deletion of CRS5 makes cells...
just slightly sensitive (Culotta et al., 1994). The transcription of both MTs is regulated by Cu through the action of the transcription factor Ace1 (Buchman et al., 1989). Upon Cu exposure, Ace1 also induces the transcription of the SOD1 gene (Gralla et al., 1991) encoding a cytosolic copper–zinc superoxide dismutase that breaks down superoxide ions to dioxygen and hydrogen peroxide (Bannister et al., 1987).

It has been reported that microorganisms can be experimentally endowed with tolerance to different stress conditions through ‘evolutionary engineering’, a term introduced for the first time by Butler et al. (1996) to indicate an experimental approach that uses the principles of adaptive evolution to drive the selection of organisms with desired phenotypic traits. While ‘metabolic engineering’ implies the rational modification of the cellular metabolic pathways (Bailey, 1991), evolutionary engineering relies on the onset of spontaneous or experimentally induced random mutations followed by recombination and continuous evolution of large populations over many generations (Sauer, 2001). For example, strains of S. cerevisiae were recently evolved to gain resistance towards multiple stresses (Çakar et al., 2005) and cobalt (Çakar et al., 2009), and the ability to ferment xylose (van Maris et al., 2007) and lactose (Guimaraes et al., 2008). Evolutionary engineering of whole organisms offers a valuable alternative to metabolic engineering, that is often limited by the great complexity of dynamic interactions in cellular systems (Sauer, 2001). Evolved strains can be used as hosts for further rational improvements by metabolic engineering, or a desired phenotype can be transferred to a production host – an approach known as ‘inverse metabolic engineering’ (Bailey et al., 1996).

In a previous work, we exploited evolutionary engineering to obtain yeast cells tolerant to the presence of high Cu concentrations (up to 2.5 g l⁻¹) in the growth medium (Adamo et al., 2012). Here, we report that in one evolved S. cerevisiae strain, increased CUP1 copy number is the more marked event associated with enhanced Cu tolerance. Increase in the amount of this metallothionein is proposed to be a key factor of protection against Cu-induced oxidative stress.

**METHODS**

**Strains and growth conditions.** S. cerevisiae cells used in this work derive from a natural Cu-sensitive strain – the BL7 strain described in a previous work (Adamo et al., 2012) – whose growth is inhibited at 1.0 g CuSO₄ l⁻¹ in the culture medium. This parental strain is referred to in the text as non-evolved. Cu tolerance was step-wise evolved in parental cells by exposure to increasing Cu concentrations. The strain tolerant to the highest Cu concentration (2.5 g CuSO₄ l⁻¹) was grown for several generations in non-selective YPD (‘de-evolution’ treatment) maintaining its Cu tolerance (Adamo et al., 2012), and is referred to as evolved; this was used in the present work. The two strains isolated as intermediates of the evolutionary engineering procedures, and tolerant to 1.5 and 2.0 g CuSO₄ l⁻¹ (Adamo et al., 2012) are indicated in the text as int1.5 and int2, respectively. Cells from a fresh culture grown on solid YPD medium [2% (w/v) glucose, 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) agar] were pre-cultured overnight in liquid YPD at 30 °C with shaking at 160 r.p.m. and then inoculated at OD₆₀₀ 0.05 in shaking flasks containing fresh medium. A solution of CuSO₄ was added to cultures exponentially growing in YPD medium. The final concentration of CuSO₄ in the culture broth was 2.5 g l⁻¹ unless stated otherwise.

**RNA extraction and cDNA production.** Total RNA for gene expression analysis was extracted from cells growing in YPD either alone or at different times after Cu addition using the RNeasy Mini kit (Qiagen). The quality of extracted RNA was assessed on a 1.5 % (w/v) agarose gel and RNA was quantified by NanoDrop (ND-1000 v 3.3.2, Saveen Werneve). A dilution of 0.320 μg RNA ml⁻¹ was used to produce the cDNA according to the following protocol: a mix composed of 8.5 μl RNase-free water, 2.5 μl RNA and 1 μl anchored oligo-dT Primer (ABgene) was incubated at 65 °C for 5 min in a thermocycler (T3000, Biometra). After 1 min incubation on ice, 4 μl 5 × first-strand buffer (Invitrogen), 2 μl 0.1 M DTT, 1 μl 10 mM dNTPs was added to the mixture and incubated at 42 °C for 2 min. Reverse transcription was performed by adding 1 μl SuperScript II RT (RetroTranscriptase) followed by incubation at 40 °C for 50 min and 70 °C for 15 min. The obtained cDNA was diluted by adding 80 μl RNase-free water, and 5 μl of the dilution was used for gene expression analysis.

**Genomic DNA extraction.** Genomic DNA was extracted from cells grown overnight at 30 °C in 5 ml YPD medium. Cells were centrifuged at 4900 g for 5 min, resuspended in 200 μl breaking buffer [2% (v/v) Triton X-100, 1% (w/v) SDS], 100 mM NaCl, 10 mM Trizma/HCl pH 8.0, 1 mM EDTA pH 8.0, 200 μl phenol/ chloroform/isoamylalcohol (PCI) at 25:24:1 and transferred to a screw cap tube containing 200 μl acid-washed glass beads (425–600 μm; Sigma). Cells were subjected to mechanical lysis by three cycles of 20 s at maximum speed with a Fast Prep-FP120 (Bio 101-Savant). TE buffer (10 mM Trizma and 10 mM EDTA; 200 μl) pH 8.0 was added to the crude extract and, after centrifugation at 10000 g for 5 min, the aqueous layer was transferred to a new tube. After addition of 1 ml 100% ethanol and centrifugation, the pellet was dried and RNA was removed by incubation for 15 min at 37 °C in 400 μl TE containing 2.5 μl RNase A (20 mg ml⁻¹). Ammonium acetate (4 M; 10 μl) and 1 ml 100% ethanol were added to the sample and, after centrifugation, the DNA pellet was dried and resuspended in 100 μl TE. The DNA was checked on 1.5% (w/v) agarose gel and quantified by NanoDrop (ND-1000 v 3.3.2, Saveen Werneve).

**Gene expression analysis and determination of gene copy number by quantitative real-time PCR (RT-qPCR).** Relative and absolute quantifications were performed by RT-qPCR to determine gene expression and gene copy number, respectively. RT-qPCR was performed using the IQ5 Multicolor real-time PCR detection system (Bio-Rad).

For gene expression analysis, cDNA gene sequences of CUP1, CRSS, SOD1, CTR1, CTR2, CTR3, FET4, CCC2, CCS1, TDH3, TPI1, TKL1 and ACT1 (Table 1) were retrieved from the Saccharomyces Genome Database (www.yeastgenome.org) and the internet-based interface Primer-3 (Rozen & Skaletski, 2000) was used to design PCR oligonucleotides. Individual RT-qPCR mixtures were prepared in a 96-well plate (Bioplastic), each 20 μl reaction containing 5 μl cDNA and 15 μl SYBR green master mix [SYBR Green Supermix (Bio-Rad), 10 μl/m forward and reverse oligonucleotides and water]. Amplification conditions were 3 min at 95 °C followed by 40 cycles at 95 °C for 10 s and at 58 °C for 30 s. Quantification of the target sequence in the samples was estimated generating a standard curve with known
Table 1. Oligonucleotides used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
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| ACT1 | FW CTGGCCGATATTGACCAACT  
       | RV CGGTGATTCCTTGGCATTT |
| CUP1 | FW AAAGTGTAGGTTGAGAATGGC  
       | RV ATTCAGGACGCAGATGAC |
| CRS5 | FW TGACTGTAAGAGCAAGAATGTT  
       | RV TTCCGATGTCCGAGGTTT |
| SOD1 | FW AAGCTGGTTGCCACATCATC  
       | RV CACATTGGTCGTCGTTTCTT |
| CTR1 | FW GTGTTGAGATCTTCAAGGAGA  
       | RV TTGTTCTTCTCCAGGGGAGT |
| CTR2 | FW CCGCTGCTGAGTAGTTTTA  
       | RV GACACTTGTGGCCTGTTT |
| CTR3 | FW GTGCCAAGAGATGGAAGAAGA  
       | RV TGAACGCAACACAGATAAGCA |
| FET4 | FW AACCCCTGTTGGAATATTG  
       | RV TTCTCGGGGTGAATTGAGG |
| CCC2 | FW AGCTCGTGTAGTCTGTCTT  
       | RV CATTAGTCGCCGCTGTGATT |
| CCSI | FW GAAATTACACCGCACGATT  
       | RV GGTTGTTGACTGACGTGAG |
| TDH3 | FW CTGGTGAAAGTTCCTCAGCA  
       | RV TCGTTAAGACCCATGCAAGA |
| TPI1 | FW GTGAGGCTTCTGGTCTT  
       | RV CACAAAGATGACCCGACAAC |
| TKL1 | FW CCGAACACCGCTCATCCT  
       | RV CTCTTGCACAAACACCGTCA |

concentrations of DNA. Values were normalized against the housekeeping gene ACT1.

Genomic DNA (5 µl) was used to calculate the copy number variation in comparison with the non-evolved strain, used as a control. Copy number was calculated according to the 2^-ΔΔCt method (Livak & Schmittgen, 2001). For standardization, the results were expressed as ratios of the amount of the target gene (CUP1 or CRS5) in evolved and non-evolved strains, the reference gene being the housekeeping gene ACT1.

**Southern blotting.** For Southern blotting analysis, genomic DNA was digested with EcoRI. The DNA restriction fragments obtained were separated by electrophoresis on 0.8 % (w/v) agarose gel. The fragments were visualized under UV light after ethidium bromide staining and were transferred by capillary on a Hybond-N+ membrane (Amersham Biosciences) as described by Sambrook et al. (2001). A CUP1 DNA probe was obtained by amplification of genomic DNA with the primers used also for RT-qPCR. Labelling was done with the DIG DNA Labelling kit (Roche) and hybridization was carried out as suggested by the manufacturer. Nucleic acids were detected using the DIG luminescent detection kit (Boehringer Mannheim), according to the manufacturer’s instructions.

**Protein extraction and electrophoresis in denaturing conditions.** Culture samples (50 µl) were withdrawn at different times after Cu addition and processed for protein extraction. The biomass was resuspended in 2 ml 2 M NaOH, incubated for 2 min at room temperature and then 2 ml 50 % (w/v) TCA were added. After centrifugation for 3 min at 10000 g, pellets were resuspended in 1 ml 1 M Tris/HCl pH 8.0 and centrifuged again. Proteins were resuspended in 250 µl 1 x SDS sample buffer [0.0625 M Tris/HCl pH 6.8, 10 % (v/v) glycerol, 3 % (w/v) SDS, 5 % (v/v) β-mercaptoethanol, 0.004 % (w/v) bromophenol blue] and heated at 99 °C for 5 min. The protein concentration in cell-free extracts was estimated by the RC-DC protein assay (Bio-Rad) using BSA as the reference. Proteins (20 µg; derivatized to detect carbonyl groups, see below) were applied to 12 % (w/v) polyacrylamide gel. SDS-PAGE was carried out according to Laemmli (1970).

**Detection of carbonyl groups in proteins.** Analyses of carbonylated proteins were performed using the chemical and immunological reagents of an OxyBlot oxidized protein detection kit (Millipore). The carbonyl groups in the protein side chains were derivatized to DNP-hydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNP-derivatized proteins were analysed immunochromically by Western blot using rabbit anti-DNP as primary antibody (Sigma-Aldrich). A goat anti-Hog1 antibody (Santa Cruz Biotechnology) was used to detect Hog1 as a control of protein quantity. Donkey anti-rabbit and donkey anti-goat antibodies ODyssey (LI-COR Bioscience) were used as secondary antibodies. Visualization of carbonylated proteins was performed using Odyssey Infrared Imaging System 2.1 (LI-COR Bioscience). Densitometric analysis was carried out on three different biological replicates of the experiment to calculate the percentage of carbonylated proteins at different hours after Cu addition.

**Cu determination.** Intracellular Cu in non-evolved and evolved cells was measured within the first hour of Cu exposure as described previously (Adamo et al., 2012). The amount of Cu is referred as mg Cu (g dry biomass)^-1.

**Determination of reduced glutathione (GSH) content.** Reduced GSH was quantified on TCA-treated protein samples. Thiol content was quantified following the conversion of 5,5-dithiobis-2-nitrobenzolic acid (DTNB) in 5-thio-2-nitrobenzoic acid (TNB) in a spectrophotometric assay at 412 nm (Sedlak & Lindsay, 1968). Analyses were carried out on non-evolved and evolved cells before and after 1 h Cu exposure and results were expressed as nmol reduced GSH (µg protein)^-1.

**RESULTS**

**Effects of Cu on yeast cell growth**

The growth kinetics of non-evolved (Cu-sensitive) and evolved (Cu-tolerant) S. cerevisiae cells were compared and are presented in Fig. 1. Growth of the two strains was very similar (Fig. 1a) in YPD medium. Addition of CuSO4 to a final concentration of 2.5 g l^-1 to cultures in exponential phase (indicated by an arrow in Fig. 1b) affects cells growth (compare Fig. 1a and b). It was also evident that Cu exerted stronger growth impairment on non-evolved cells than on the evolved ones (Fig. 1b). Fig. 1(c) illustrates a drop test on YPD plate of non-evolved and evolved cells harvested before and after 1 and 24 h of Cu addition. After 1 h of Cu exposure, non-evolved cells did not fully resume multiplying; in contrast evolved cells recovered better, although this difference tends to be attenuated after prolonged Cu exposure (24 h Cu).

**Expression levels of CUP1 and CRS5**

To gain insight into the determinants of Cu tolerance in evolved cells, we evaluated the expression levels of the genes
CUP1 and CRS5, both encoding MTs. RNA was extracted from cells growing exponentially in absence of Cu or at different hours after the addition of Cu, mRNA was reverse transcribed to cDNA and the expression of the two genes was evaluated by quantitative PCR. In the absence of Cu, expression of CUP1 was hardly detectable in either non-evolved or evolved cells (Fig. 2a, see 0 h). In response to Cu, the level of CUP1 in the evolved strain was rapidly and strongly induced, and remained high during the monitored period of 24 h (Fig. 2a). Cu also triggered the expression of CUP1 in non-evolved cells, although to a lower extent (Fig. 2a).

In absence of Cu, the expression level of CRS5 was very low in both strains (Fig. 2b, see 0 h). Cu exposure triggered a stronger induction of CRS5 in the evolved strain than in the non-evolved one (Fig. 2b). Although after 24 h of Cu exposure a fivefold induction of CRS5 was detected, this increase was moderate compared with that observed for CUP1 (Fig. 2).

Copy number of CUP1 and CRS5

It has been reported that different yeast strains may bear several copies of the CUP1 gene (Liti et al., 2009; Stroobants et al., 2009; Welch et al., 1983), whereas CRS5 is always present in single copy (Culotta et al., 1994). We applied RT-qPCR to investigate CUP1 gene amplification in the evolved strain and in two ‘intermediates’ of the evolution process, namely cells tolerant to 1.5 and 2.0 g CuSO4 l⁻¹ (referred to as int 1.5 and int 2) (Adamo et al., 2012). As shown in Fig. 3, the CUP1 copy number did not change in int 1.5, whereas a sudden sevenfold increase was observed in int 2. No further amplification was detected in cells tolerant to the highest Cu concentration used in this work (2.5 g l⁻¹). Southern blot analysis (Fig. S1, available with the online version of this paper) confirmed the
amplification of the CUP1 locus in int 2 and in the evolved strains, whose hybridization profiles show a defined, extra band of higher length with respect to non-evolved and int 1.5. This observation sustains the hypothesis that a local genetic rearrangement, i.e. tandem gene duplication, has occurred to produce a cluster of linked gene copies (Zhang, 2003). The CRS5 copy number was evaluated just for the evolved strain and we found that CRS5 was not amplified (Fig. 3). We hypothesize that the CUP1 amplification is an important contributor to Cu resistance of the evolved strain.

Expression level of SOD1

Other than an increase in CUP1 gene number, several genetic or either metabolic changes are likely to contribute to Cu tolerance of the evolved strain. Besides MTs, yeast cells respond to excess of Cu by increasing the level of Sod1, a cytosolic Cu,Zn-superoxide dismutase that participates in the scavenging of superoxide anions resulting from Cu redox metabolism (Bannister et al., 1987). Therefore, we evaluated the expression of SOD1 in evolved and non-evolved cells by quantifying its mRNA in the same conditions described above (Fig. 4). For both strains, 1 h Cu exposure resulted in a twofold increase of SOD1 expression level. In the evolved cells, this value did not further increase with time, whereas in the non-evolved strain, a marked increase was appreciable only after prolonged cultivation in Cu medium. This increase should be considered with some care; indeed it might represent a secondary effect of Cu exposure, due to cell death and lost of mitochondrial integrity, with consequent leaking of H₂O₂ (Park & Choi, 2012) and rise in of oxidative damage (see below).

Expression levels of genes involved in Cu transport and genes responding to oxidative stress

We analysed the expression of genes encoding plasma membrane Cu transporters (CTR1, CTR3 and FET4), intracellular importers (CTR2 and CCC2) and a chaperone (CCS1) involved in Cu distribution in the cell. Changes in the expression levels were monitored by RT-qPCR after 1 h Cu exposure (Fig. 5). In both evolved and non-evolved cells, Cu inhibited the expression of genes for the high-affinity Cu transporters CTR1 and CTR3 (Pena et al., 2000; Wu et al., 2009) and of low affinity transporter FET4 (Hassett et al., 2000) (Fig. 5a–c). Expression of CCS1 (Fig. 5d), encoding a cytosolic chaperone that delivers Cu to Sod1 (Brown et al., 2004), was enhanced by Cu in both strains. This result fits well with the increase in SOD1 expression observed at the same time point and reported in Fig. 4. CTR2 encodes a vacuolar low affinity Cu importer (Rees et al., 2004) and CCC2 encodes a P-type ATPase that transports Cu across the vesicular membranes (Solioz & Vulpe, 1996). Cu exposure did not affect their expression levels (Fig. 5e, f), although we observed that in the evolved strain basal expression is lower.

In yeast cells, glycolytic enzymes are among the major targets of oxidative stress (Shanmuganathan et al., 2004) and metabolic reconfiguration of the carbohydrate flux toward the pentose phosphate pathway is a key strategy to counteract oxidative stress (Grant, 2008; Ralser et al., 2007). We previously reported that the levels of some glycolytic enzymes and enzymes of the pentose phosphate
pathway are highly enhanced in evolved cells grown in the presence of Cu (Adamo et al., 2012). We therefore measured the extent to which Cu exposure affects the expression of the genes TPI1, TDH3 and TKL1 (encoding the enzymes triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and transketolase, respectively). The expression of glycolytic genes (Fig. 5g, h) was unaffected or only marginally modified by Cu exposure in both kinds of cell, whereas a small decrease was observed for the gene TKL1 (Fig. 5i).

**Cu-induced carbonylation of proteins**

Reactive oxygen species (ROS) produced upon Cu stress are known to promote damage to different cell components, including proteins (Davies, 1987) that might undergo carbonylation of amino acid side chains (Stadtman & Levine, 2006). Therefore, we used an immunoblot assay to detect oxidized proteins, assuming the carbonylation of proteins is indicative of oxidative stress. Total proteins were extracted from cells exponentially growing either in absence of Cu or

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**Fig. 5.** Relative expression of CTR1 (a), CTR3 (b), FET4 (c), CCS1 (d), CTR2 (e), CCC2 (f), TPI1 (g), TDH3 (h) and TKL1 (i). Non-evolved (grey bars) and evolved (white bars) cells were harvested before (0) and 1 h after (1) Cu addition. Gene expression was quantified by RT-qPCR and normalized for ACT1 expression. Values are the mean ± SD of two biological replicates performed in triplicate.
at different times after Cu addition. Extracted proteins were derivatized with DNPH that specifically reacts with carbonyl groups. We observed a comparable amount of carbonylated proteins in the non-evolved and the evolved populations cultivated without Cu (Fig. 6a, see 0 h in both strains). However, carbonylation increased rapidly and reached high levels in samples of non-evolved cells exposed to Cu, while in evolved cells, a lower increase in the signal associated with oxidized proteins was observed at any time of growth in Cu medium (Fig. 6). As mentioned above, the marked increase in protein carbonylation observed for non-evolved strain following 24 h Cu exposure might be also ascribed to cell death.

Quantification of intracellular Cu

We next determined the levels of Cu accumulated in yeast cells. Within the first hour of exposure, intracellular Cu was comparable in both yeast samples (Fig. 7). At first sight, these data seem to disagree with the findings reported above showing that in the presence of Cu the evolved strain has growth advantages and suffers a milder oxidative stress than the natural strain. However, taking into account that the biochemical assay used for Cu quantification (Adamo et al., 2012) does not discriminate between toxic Cu ions (e.g. those participating in reactions generating ROS or improperly bound to cellular proteins) and non-toxic Cu ions (e.g. those bound to buffering molecules such as MTs), it is reasonable to hypothesize that although we detected a similar quantity of Cu, the toxicity of Cu ions might vary in the two strains. Note that in the present work we quantified intracellular Cu in exponentially growing cells, whilst intracellular Cu was previously quantified in stationary phase cells (Adamo et al., 2012). This latter condition appears more restrictive since it causes the death of non-evolved cells and hence means that the two quantifications are not directly comparable.

Intracellular content of GSH

To investigate the contribution of small molecules to Cu tolerance we determined the amount of reduced GSH in non-evolved and evolved cells. Cu exposure for 1 h resulted in an equal, strong depletion of GSH in both evolved and non-evolved cells (Fig. S2).

Fig. 7. Measurement of intracellular Cu measured in non-evolved (grey bars) and evolved (white bars) cells at different times after Cu addition. The amount of Cu is reported as mg (g dry biomass)^{-1}. Values are the mean±SD of three biological replicas.
DISCUSSION

Adaptive evolution is a central biological process underlying several phenomena, from the gain of antibiotic resistance in microbial populations to niche specialization. An intriguing question is what kind of genomic variations are associated with evolution of improved phenotypes and how reproducible the process is (Elena & Lenski, 2003; Gresham et al., 2008; Hendrickson et al., 2002). Adaptive evolution in microbial populations can typically occur through relatively few mutations that confer large benefits, rather than through countless mutations with minor benefits. The most extreme beneficial mutations are greatly overrepresented owing to selection (Rozen et al., 2002). Adaptive evolution can be experimentally attained at the laboratory scale (Sauer, 2001). We report here the characterization of a natural yeast strain used in an industrial process and deliberately subjected to a strategy of evolutionary engineering for improving its Cu tolerance. Among the investigated aspects, amplification of the CUP1 gene was most prominent event associated with the phenotypic traits of Cu tolerance achieved by evolutionary engineering. We therefore propose that CUP1 gene amplification plays an important role in protecting cells from Cu toxicity. Our results and the proposed mechanism are in agreement with findings by others on natural and genetically modified yeast strains from both laboratory and industrial sources (Bi et al., 2007; Fogel & Welch, 1982; Koller et al., 2000; Stroobants et al., 2009).

Looking at the amplification of the CUP1 gene to monitor the whole progress of evolution, we found that a sevenfold amplification arises not only in the hyper-resistant evolved strain but also earlier and more abruptly in int 2, an intermediate of the evolutionary engineering procedure. The localized amplification of the gene gave rise to a cluster of copies in a single locus, as occurs upon tandem duplication (Zhang, 2003). We should consider that the amplification of CUP1 might not be the unique beneficial mutation leading to the evolution of our Cu-tolerant yeast strains and the possible scenario might be more complex. Indeed, we can conceive that CUP1 gene amplification found in int 2 corresponds to one of the genetic alterations featuring a cell population that attains a local optimum of the fitness landscape. Other mutations might contribute to the final adaptation of the evolved strain, presumably placing it closer to a global fitness optimum in the conditions of selection.

How could the evolution of Cu tolerance have occurred? According to a neo-Darwinist theory, the overall CUP1 expression in cultures of natural non-evolved S. cerevisiae might result from stochastic differences in gene dosage among individuals. The average expression could result from low expression by the large majority of cells, and high expression by a small subgroup of cells bearing a higher CUP1 copy number. Indeed, according to the concept of 'quasi species', the result of evolution consists of a distribution of related variants that occupy a distinct region in sequence space (Biebricher & Eigen, 2006). Exposure of the population to high Cu levels might reduce the original heterogeneity of the culture (number of variants) by selecting amplification mutants of the CUP1 locus since this beneficial mutation contributes to their fitness. On the other hand, we cannot exclude that adaptive mutations, and specifically amplification of the CUP1 locus, could have occurred during the prolonged cultivation in the presence of Cu (Hersh et al., 2004). In this scenario, stresses applied to the cell population might change the genome evolvability by activating mutation mechanisms, as already well documented in prokaryotic organisms (Hastings et al., 2009) and that appear to be shared by eukaryotic cells too (Finch & Goodman, 1997; Hall, 1995; Harris et al., 1994; Strauss, 1992). Putting together all the results obtained in this study and in previous work, we hypothesize that the first line of defence of yeast cells towards Cu relies mainly on increased activities of detoxifying enzymes. This early but transient metabolic response could give cells the time to develop stable tolerance.

In the evolved strain, Cu also induced the expression of CUP5, though to a lower extent than that of CUP1. This can be ascribed to both its lower gene dosage and its lower responsiveness to Cu mediated by the transcription factor Ace1 (Culotta et al., 1994; Jensen et al., 1996).

Our data, in agreement with results by Gralla et al. (1991), show that in the evolved strain, induction of SOD1 occurs within the first hour of Cu exposure. Although the evolved and non-evolved strains express SOD1 at similar levels and accumulate the same amount of Cu, non-evolved cells experience more oxidative stress as shown by protein carbonylation assays (Fig. 6). According to our view, buffering of cytosolic Cu by overexpression of Cup1 would be sufficient to prevent ROS overproduction, limiting the need for free radical scavengers.

GSH is a small molecule with a key role in the defence against oxidative stress and metal toxicity (Grant, 2001). Our yeast strains exposed to Cu encountered the depletion of GSH, an effect known to be associated with Cu toxicity (Stohs & Bagchi, 1995). Depletion occurred at the same extent in evolved and non-evolved yeast strains; hence we cannot hypothesize about the contribution of GSH in the gain of Cu tolerance. Moreover, other experimental data show that Cu tolerance is independent of GSH activity (Bi et al., 2007; Gharieb & Gadd, 2004).

Besides MTs and radical scavengers, Cu homeostasis also depends on plasma membrane transporters and intracellular Cu importers and chaperones. Moreover, Cu-induced oxidative stress might impact on carbon metabolism. Overall, in the considered time slot, the non-evolved and evolved strains showed a similar and expected response of Cu homeostasis genes and almost unchanged expression of carbon metabolism enzymes. Although hard to interpret at this stage, we observed that...
expression of CTR2 and CCC2 genes appeared constitutively lower in the evolved strain.

To conclude, published data on evolutionary engineering of microbial strains highlight the potential of this approach in selecting strains with specific phenotypes. Recent studies demonstrate that evolution of tolerance to a given metal triggers cross-resistance to other metals and/or other stress conditions (Çakar et al., 2009; Şen et al., 2011). For instance, Çakar et al. (2009) found that cobalt-resistant S. cerevisiae cells can also evolve tolerance to metals that are in the proximity of cobalt in the Periodic Table of the Elements, such as Fe, Mn, Zn and Ni but not Cu and Cr. However, cross-resistance to other metals was not detected in evolved Cu-tolerant S. cerevisiae cells used in this work (data not shown), underscoring the specificity of the Cu tolerance acquired during evolution. Moreover, the observation that cup1Δ cells are sensitive to Cu but not to other metals/metalloids (Ecker et al., 1986; R. Wysocki & M. J. Tamas, unpublished data) sustains the hypothesis of a physiological role of Cup1 specifically associated with Cu detoxification (Wysocki & Tamas, 2010). Thus, metal tolerance may arise from both general and specific molecular mechanisms to be elucidated case by case. Although the evolved strains can find direct applications in several biotechnological processes of interest, the understanding of molecular determinants of evolved phenotypes will provide a starting point for further rational improvements.

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