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Evolution of copper tolerance in yeast cells

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Abstract

For all living organisms, copper (Cu) is an essential micronutrient taking part, with its redox chemistry, to several metabolic and regulatory cellular events. However, the same redox properties that make Cu essential are responsible for its toxicity. Indeed, Cu participates in reactions that generate Reactive Oxygen Species (ROS). ROS target main cellular macromolecules (proteins, lipids, DNA and RNA), leading to cellular dysfunctions and in the extreme case, to cell death. All living organisms evolved molecular mechanisms for Cu homeostasis. Indeed, uptake, transport and detoxification systems that actively prevent both Cu deficiency and poisoning are well conserved along the phylogenetic tree. Among eukaryotes, these mechanisms have been mainly investigated in the yeast *Saccharomyces cerevisiae* used as a model organism.

Evolutionary engineering is a rational approach that uses the evolutionary principles to direct the selection of organisms with a desired set of phenotypes, allowing for the improvement of microbial properties. This approach can be exploited to obtain Cu-tolerant and Cu-accumulating yeast cells, with potential application in nutraceutics, as nutritional supplements, as well as in bioremediation, for the removal or recovery of metal ions. At the same time, evolutionary engineering is a valuable strategy to gain more insight into the molecular aspects of Cu tolerance in microbial cells.

In the present work is described an evolutionary engineering strategy to improve Cu tolerance of natural yeasts. Strains of *Saccharomyces cerevisiae* and of *Candida humilis* originally endowed with different sensitivity and tolerance toward Cu have been exposed to increasing concentrations of Cu during cell cultivation in liquid medium. This treatment stably improved Cu tolerance of all strains. One evolved

strain for each yeast species was then chosen to analyze in detail the physiological response to Cu. Compared with the original Cu-sensitive strains the two *evolved* strains showed improved cell viability and attenuated production of ROS. A reshaping of the profile of antioxidant enzymes and Cu-binding proteins was observed in both strains as a specific response to copper.

Further investigations carried out on *S. cerevisiae* strains demonstrated a pivotal role of the *CUP1* gene, encoding for a metallothionein. A 7-fold amplification of this gene was found associated with *evolution* of Cu tolerance.

Finally, Cu tolerance in *C. humilis* cells was studied by proteomic analyses. Changes were observed in the levels of several proteins involved in the oxidative stress response (such as glycolytic enzymes), heat shock proteins, proteins involved in protein synthesis and energy production, proteins with a role in phospholipids synthesis. Cu exposure resulted in differential protein expression, in both *non-evolved* and Cu *evolved* cells. In general, changes in protein levels detected in *evolved* cells were smaller. On this basis, it was hypothesized that in the *evolved* cells copper tolerance relies only partly on the molecular mechanisms associated with the oxidative stress response. This work shows once again that evolutionary engineering is a powerful strategy to drive the gain of stable phenotypic traits. The *evolved* strains might found direct application in several biotechnological fields, and provide a kind of "molecular platform" for the investigation on the mechanisms of stress tolerance. The availability of data about the *S. cerevisiae* genome allowed a focused investigation on the molecular actors involved in Cu tolerance. In the case of *C. humilis,* the use of a proteomic approach allowed to compensate for the poor information available on the determinants of Cu tolerance.

Riassunto

Il rame (Cu) è un micronutriente essenziale per tutti gli organismi viventi; questo metallo prende parte a numerose reazioni redox e rappresenta un cofattore essenziale per proteine coinvolte in numerosi *pathways* metabolici e processi di regolazione cellulare. Tuttavia le stesse proprietà redox che rendono il rame essenziale sono responsabili della tossicità di questo metallo. Il rame infatti prende parte a reazioni che generano le cosiddette specie reattive dell'ossigeno (Reactive Oxygen Species, ROS). Queste interagiscono con le principali macromolecole cellulari (proteine, lipidi, DNA ed RNA) causando malfunzionamento e morte cellulare. Tutti gli organismi hanno evoluto sistemi strettamente regolati per l'*uptake*, il trasporto e la detossificazione del rame. Questi sistemi di omeostasi risultano essere altamente conservati lungo tutto l'albero filogenetico e tra le cellule eucariote l'omeostasi del rame è stata principalmente studiata nel lievito *Saccharomyces cerevisiae*.

L'ingegneria evolutiva (*evolutionary engineering)* si basa sui principi dell'evoluzione per selezionare organismi con determinate caratteristiche fenotipiche.

Questo approccio può essere sfruttato per migliorare la *robustness dei ceppi microbici* ed ottenere cellule di lievito resistenti al rame e con migliori capacità di accumulo. Questi ceppi modificati potrebbero trovare applicazione come integratori alimentari e nel settore del biorisanamento. Allo stesso tempo, l'*evolutionary engineering* rappresenta un valido approccio per lo studio della tolleranza al rame, sia negli aspetti fisiologici che molecolari.

In questo lavoro viene descritta una strategia di *evolutionary engineering* mirata al miglioramento della tolleranza al rame di ceppi naturali di *Saccharomyces cerevisiae* e *Candida humilis* caratterizzati

da una diversa sensibilità di base a tale metallo. L'approccio ha previsto l'esposizione delle cellule di lievito a concentrazioni crescenti di rame aggiunto nel terreno di coltura ed ha permesso l'acquisizione di una stabile tolleranza al rame. Le due specie di lievito *evolute* hanno mostrato in generale un miglioramento della vitalità cellulare e diminuita produzione di ROS, oltre che specifici adattamenti nell'attività di alcuni enzimi antiossidanti e nel profilo di proteine che legano il rame.

Studi condotti sul ceppo di *S. cerevisiae* hanno dimostrato che l'amplificazione del gene *CUP1* ha un ruolo centrale nell'evoluzione della tolleranza al rame. Il gene *CUP1* codifica per una metallotioneina. Nel ceppo *evoluto,* l'amplificazione di 7 volte di questo locus genico porta ad un forte aumento dell'espressione genica che è stata messa in relazione con l'attenuazione dello stress ossidativo che generalmente si osserva esponendo le cellule al rame.

Infine, sono presentati risultati riguardanti la tolleranza al rame del ceppo di *C. humilis*. Da analisi di proteomica, realizzate mediante elettroforesi bidimensionale, sono emersi cambiamenti nella regolazione di proteine coinvolte nella risposta da stress ossidativo tra cui enzimi glicolitici, heat shock proteins, proteine coinvolte nella sintesi proteica e nella produzione di energia, proteine coinvolte nella sintesi di fosfolipidi. E' stato osservato che proprio nel ceppo naturale – *non-evoluto* - di *C. humilis* l'esposizione al rame causa cambiamenti più accentuati nel livello di espressione di molte proteine deputate alla difesa da stress ossidativo . Ciò ha fatto ipotizzare che viceversa nel ceppo *evoluto* la risposta all'esposizione al rame sia solo parzialmente affidata ai sistemi di difesa da stress ossidativo.

In generale questo lavoro sottolinea la validità delle strategie di *evolutionary engineering* per ottenere ceppi con proprietà di resistenza al rame stabilmente acquisite. I ceppi *evoluti* potrebbero trovare applicazione diretta in diversi settori delle biotecnologie ed

allo stresso tempo rappresentare una "piattaforma molecolare" per ulteriori indagini sui meccanismi di resistenza allo stress.

Le conoscenze sulla genetica di *S. cerevisiae* hanno permesso di valutare in maniera mirata il coinvolgimento di specifiche proteine nella resistenza al rame. D'altro canto l'approccio di proteomica utilizzato per *C. humilis*, resosi necessario per l'esiguità di informazioni disponibili sulla risposta a stress, ha permesso di individuare specifiche proteine che possano render conto dell'evoluzione della tolleranza al rame in questo lievito.

1. Introduction

1.1 The role of copper in cell biology

It is widely recognized that Copper (Cu), like other transition metals such as Zinc (Zn), Iron (Fe) and Manganese (Mn), is an essential micronutrient required for the survival of all living organisms, from bacteria to humans (1). Cu, cycling between the oxidized (Cu^{2+}) and the reduced (Cu^{1+}) state, is an important catalytic and structural cofactor for several biochemical processes essential for life. Cu has been found as a co-factor of several proteins, where coordination to amino acidic residues occurs with the aid of numerous ligands (such as sulphur, nitrogen and oxygen). The contribution of Cu atoms to structural and functional features/properties of proteins has been already well documented and proved to be involved also in important metabolic and regulatory events (**Table 1**)(2). Its redox chemistry confers to Cu a central role in electron transport in the respiratory chain of biological systems (3). Deficiency or alterations in the activities of Cu-requiring proteins is often associated with diseases and/or with physiological dysfunctions (4). The redox properties of Cu ions are also responsible for their toxicity. Indeed Cu participates in reactions that generate Reactive Oxygen Species (ROS) (5) that are major contributing factors in cancer, diseases of the nervous system and aging (6). An example of the essential and toxic role of Cu is provided by two genetic conditions characterized by the inability to properly distribute Cu to cells and tissues: Menkes and Wilson's diseases (7-9). The first one results in Cu deficiency (10), while the second results in cirrhosis due to Cu-induced oxidative damage in liver and other tissues (11, 12). Moreover, dysfunctions in Cu

metabolism are associated with neurodegenerative diseases as familiar amyotrophic sclerosis (FALS), Alzheimer's disease and prion disease (13) and some studies suggest that a diet low in Cu can contribute to an increased risk of ischemic heart disease (14).

According to the ESSADI (Estimated Safe and Adequate Daily Dietary Intake), the intake of Cu by humans ranges between 0.5-1.18 mg/day (15). The Cu content in the diet is variable and dependent on the nature of the foodstuffs. Cu-rich foods include organ meat (liver), shellfish (oysters), nuts and seeds (16). Drinking water can also be a source of Cu, but its concentration depends on several factors such as natural mineral content, pH and the type of plumbing system (17). **Table 2** lists the relative Cu concentration in foodstuffs and water (18).

Being copper both essential and toxic, maintaining of its balance is critical. Therefore, living organisms evolved complex mechanisms for the uptake, distribution and detoxification of this metal that are finely regulated and conserved through the evolution.

Protein	Function
Amyloid precursor protein (APP)	Protein involved in neuronal development and potentially Cu metabolism; cleavage leads to generation of Aβ peptide that aggregates in plaque associated with Alzheimer's disease
Atox1	Metallochaperone that delivers Cu to ATP7A and ATP7B Cu^{1+} transporters
ATP7A	Cu^{1+}-transporting P-type ATPase expressed in all tissues except liver
ATP7B	Cu^{1+}-transporting P-type ATPase expressed primarily in the liver
Carbon monoxide dehydrogenase to acetyl-CoA synthase	*M. thermoacetica* bifunctional enzyme; reduces CO_2 to CO with subsequent assembly of acetyl-CoA
Ceruloplasmin	Serum ferroxidase that functions in Fe^{3+} loading onto transferrin
CCS	Metallochaperone that delivers Cu to Cu/Zn SOD
CopZ	*A. fulgidus* [2Fe-2S] and Zn^{2+}-containing Cu chaperone
Cox17	Metallochaperone that transfers Cu to Sco1 and Cox11 for cytochrome oxidase Cu loading in mitochondria
Ctr1	High-affinity Cu^{1+}transporter involved in cellular Cu uptake
Cu/Zn SOD (SOD1)	Antioxidant enzyme, catalizes the disproportionation of superoxide to hydrogen peroxide and dioxygen
Cytochrome c oxidase	Terminal enzyme in the mitochondrial respiratory chain, catalyzes the reduction of dioxygen to water
Dopamine β-hydroxylase (DBH)	Oxygenase, converts dopamine to norepinephrine
Ethylene receptor (ETR1)	Member of a plant receptor family that uses a Cu cofactor for ethylene binding and signaling
Hemocyanin	Oxygen transport protein found in the hemolymph of many invertebrates such as arthropods and molluscs
Hephaestin	Transmembrane multi-Cu ferroxidase; involved in iron efflux from enterocytes and macrophages
Glucose oxidase	Pentose phosphate pathway oxidoreductase that catalyzes the oxidation of D-glucose into D-glucono-1,5-lactone and hydrogen peroxide
Laccase	Phenol oxidase involved in melanin production
Lysyl oxidase	Catalyzes formation of aldehydes from lysine in collagen and elastin precursor for connective tissue maturation
Metallothionein	Cystein-rich small-molecular weight metal-binding and detoxification protein
Peptidylglycine-a-amidating mono-oxygenase (PAM)	Catalyzes conversion of peptidylglycine substrate into a-amidated products; neuropeptide maturation
Prion protein (PrP)	Protein whose function is unclear but binds Cu via N-terminal octapeptide repeats
Steap proteins/Fre1/Fre2	Family of metalloreductase involved in Fe^{3+} and Cu^{2+} reduction
Tyrosinase	Monophenol mono-oxygenase; melanin synthesis

Table 1. Cu-requiring proteins and their biological function. From (2).

	Content
µg/g	
Foods	
Muscle meats	0.9-1.0
Fish	
Sea	2-3
Freshwater	0.3-3
Shellfish	12-37
Poultry	0.5-3
Liver	4.6-6.2
Nuts	6-37
Grains and seeds	3-8
Bran	15
Germ	6
White flour	0.6
Legumes	3-7
Vegetables	0.3-3
Fruit	0.4-1.5
Potatoes	2.1
Soils (nutritionally adequate)	
Inorganic	≥4
Organic	20-30
Fresh water	0.0001-0.001
Sea water	
Surface	0.001
-1000 m	0.1
Algae	0.05-0.29
Yeast	8
Animals	1.5-2.5
Humans	1.7

Table 2. Typical Cu content of food, soils, waters and organisms. From (17).

1.2 Yeast as a model organism to study copper metabolism

To maintain Cu homeostasis, all organisms, from prokaryotes to eukaryotes, possess conserved pathways strictly regulated for metal uptake and detoxification (4, 19, 20). Among eukaryotes, Cu homeostasis has been studied in-depth in the yeast *Saccharomyces cerevisiae* (4, 21, 22). The use of yeast as model system is mainly due to broad and exhaustive studies on its genetics and proteomics, that led to the complete sequencing of its genome, and to the availability of wide libraries of single gene deletion and overexpression mutants (23). An overview of Cu metabolism in *S. cerevisiae* is reported in **Figure 1** (24). Extracellular Cu can be transported only in the reduced state, Cu(I). This requires the activity of ferric/cupric reductases Fre1 and Fre2 (25, 26) located at the cell surface. Reduced Cu is imported into yeast cells by the plasma membrane high-affinity transporters Ctr1 and Ctr3(27). Expression of the genes involved in high-affinity transport depends on Cu availability in the medium and is regulated by the transcription factor Mac1. During Cu starvation, transcription is enhanced, enabling Cu uptake. On the contrary, Cu overload results in down-regulation, decreasing Cu uptake (28). Once inside the cell, Cu is routed in three different trafficking pathways that require the action of specific Cu chaperones. These include: Cu delivery to the secretory pathway for the activation of cell surface enzymes; Cu trafficking to Cu/Zn superoxide dismutase (Sod1) in the cytosol; and Cu delivery to the mitochondria where it activates cytochrome oxidase (29).

Cu excess results in the expression of the metallothioneins Cup1 and Crs5, that bind free cytosolic Cu (30, 31), and the Zn/Cu superoxide dismutase Sod1 that is involved in ROS scavenging (32). The expression

of the genes encoding these proteins is regulated by the transcription factor Ace1(33).

More recently, new proteins with a role in Cu metabolism and homeostasis were found: i) Ctr2, a vacuolar low-affinity Cu transporter, deputed to the mobilization of Cu stores to cytosolic chaperones (34); ii) the Heat Shock Protein Hsp12 and the siderophore-iron transporter Arn1 that protect cells against Cu toxicity (24); iii) proteins involved in Cu import or regulation like a protein of the respiratory chain Cyc1 and Ccr1, a putative glycosidase of the cell wall (24); and iv) proteins with vacuolar functions as Pep12 involved in endosome-to-vacuole fusion and the vacuolar H^+-ATPase Vma13 (24).

In yeast, like in other organisms, the metabolism of Cu and Fe are tightly linked. Indeed proteins deputed to Fe uptake contain Cu ions (19, 35). This implies that defects in Cu metabolism have negative consequence on Fe homeostasis too (36).

As previously anticipated, specific proteins and complete pathways of Cu metabolism are conserved from *S. cerevisiae* to humans (37). The elucidation of Cu metabolism in yeast led not only to important insight in mammalian metabolic pathways, but has *per se* applicative revenues such as the design of new strategy of bioremediation of metal contaminated sites, improving dietary availability of Cu and treating metals related human disorders.

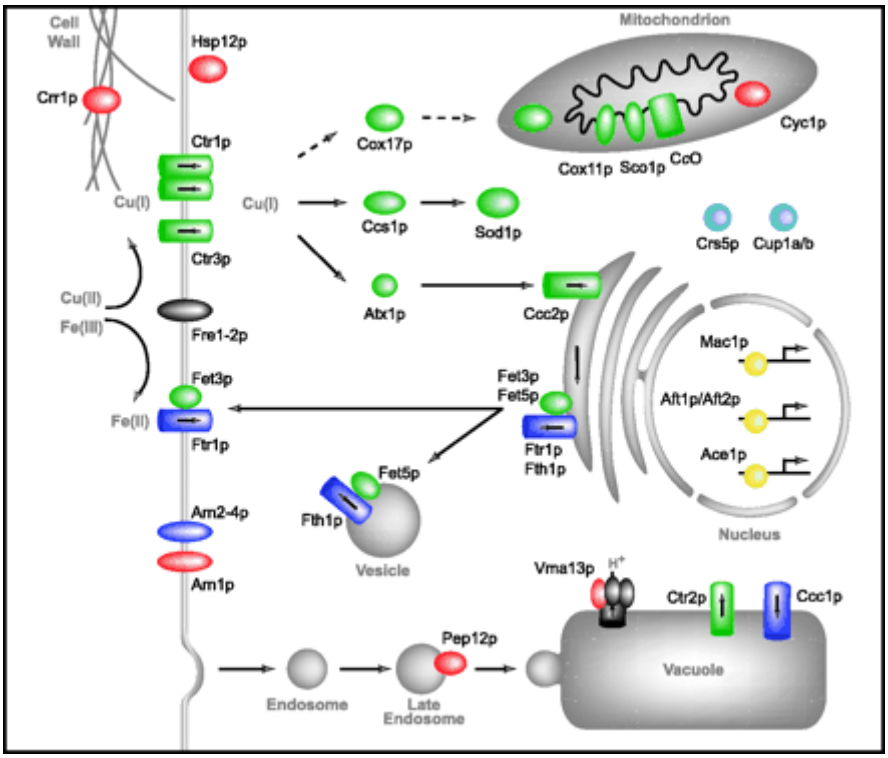

Figure 1. Cu metabolism in *S. cerevisiae*. From (24).

1.3 Antioxidant and prooxidant properties of copper

As already mentioned, one of the functions of Cu ions is the maintenance of protein activity. A classic example of protein whose activity depends on proper coordination of Cu ions in the prosthetic group is the Cu/Zn superoxide dismutase. This enzyme catalyzes the breakdown of the ROS superoxide anions constantly produced during the aerobic metabolism of cells (38). Cu, together with Zn and Se, is involved in cytosolic antioxidant defense as illustrated in **Figure 2** (39): hydroperoxides, including H_2O_2, are reduced to the respective

alcohols and water by the selenoenzyme glutathione peroxidase (GPx) (40, 41).

Notwithstanding Cu is involved in cellular defence against ROS, its redox chemistry makes it also responsible for ROS production. Interaction of Cu ions with thiols such as glutathione – GSH – and oxygen generates superoxide and hydrogen peroxide (see reactions i and ii, where the latter is the sum of reactions iia and iib):

$$2Cu^{2+} + 2R\text{-}SH \rightarrow 2Cu^{+} + R\text{-}SS\text{-}R + 2H^{+} \quad \text{(i)}$$

$$2Cu^{+} + O_2 + 2H^{+} \rightarrow 2Cu^{2+} + H_2O_2 \quad \text{(ii)}$$

$$2Cu^{+} + 2O_2 \rightarrow 2Cu^{2+} + 2O_2^{-\bullet} \quad \text{(iia)}$$

$$2O_2^{-\bullet} + 2H^{+} \rightarrow O_2 + H_2O_2 \quad \text{(iib)}$$

After its generation, Cu^{+} may further interact with hydroperoxides to generate hydroxyl or alkoxyl radicals in Fenton-type reactions (see reaction iii):

$$ROOH + Cu^{+} \rightarrow RO^{\bullet} + OH^{-} + Cu^{2+} \quad \text{(iii)}$$

Cu-induced ROS formation could be the reason why cells evolved defense mechanisms that both depend on Cu and scavenge ROS (39).

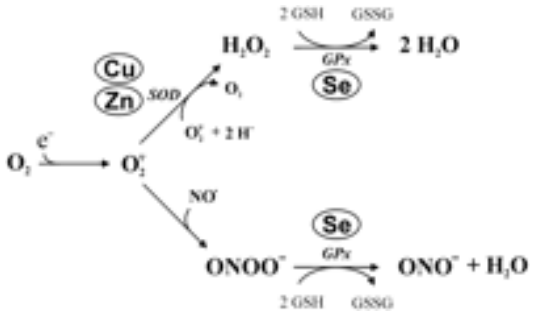

Figure 2. Cu, Zn and Se in the cytosolic defense against ROS. Superoxide is generated by reduction of molecular oxygen. The electrons required may leak out of the mitochondrial respiratory chain or may be derived from oxidation reactions. Cu/Zn superoxide dismutase (CuZn-SOD) catalyzes the dismutation of superoxide; this reaction is competed for by nitrogen monoxide, if generated in sufficient amount, to form peroxynitrite. Both the hydrogen peroxide from superoxide disproportionation and the peroxynitrite may be reduced at the expense of glutathione (GSH) in reactions that are catalyzed by glutathione peroxidase (GPx). From (39).

ROS produced upon Cu exposure target different cell macromolecules causing loss of essential cellular function and/or gain of toxicity (42) (**Figure 3** - (43)). Cu, like other transition metals, can catalyze lipid peroxidation, leading to the conversion of unsaturated lipids to polar lipid hydroperoxides. This results in increased membrane fluidity, efflux of cytosolic solutes and loss of membrane-protein activities (43). Moreover, reactive products of lipid peroxidation, such as malondialdehyde and 4-hydroxynonenal, may attack amino acid side chains (44) and cause fragmentation of DNA (45). DNA is also directly oxidized by ROS such as the hydroxyl radical (46) leading to mutations. Certain proteins are also prone to oxidation depending on their amino acid composition, the presence of metal-binding sites, localization, molecular conformation and rate of degradation (43). During oxidation, amino acid residues like serine, proline, histidine and lysine undergo irreversible carbonylation. Carbonylated proteins have been described to be inactive, not degradable by the proteasome

and to form toxic aggregated species impairing cell viability, as recently reviewed by Avery (43). Beside this direct modification of already synthesized proteins, ROS can affect the translation machinery (43). Finally, intracellular ROS can modulate several signaling pathways triggering apoptotic cell death (43, 47, 48).

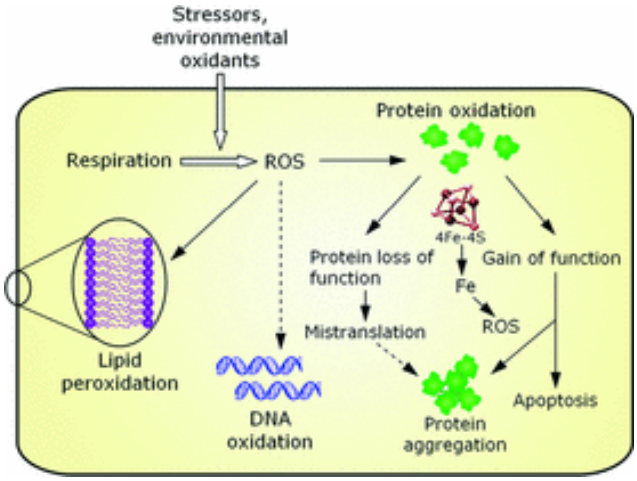

Figure 3. Major routes of ROS action in cells. From (42).

1.4 Protection against copper toxicity

To cope with the toxic effects of Cu and of other heavy metals, cells rely on "protective" proteins such as metallothioneins, that directly bind free cytosolic metal ions and on free radical scavengers.

Metallothioneins (MTs) are a family of cystein-rich, low molecular weight polypeptides (49-51). MTs bind metal ions since they contain multiple sequences motifs like Cys-X-Cys or Cys-X-X-Cys and have a low content of hydrophobic residues (49, 52, 53). Three major features

allow MTs to be effective in metal ions detoxification: (a) the strength of metal ions binding within polymetallic clusters (49, 50, 52, 53); (b) the ease of metal exchange reactions - resulting in the sequestration of potentially toxic ions (54); (c) the metalloregulated gene expression – allowing to modulate MTs concentration in response to the intracellular concentration of a certain metal (55, 56). Many eukaryotic organisms contain a family of MTs genes. At least nine MTs genes are expressed in humans and have been classified in four isoform classes on the basis of their role in metal ion detoxification (57-61).

In *S. cerevisiae* the MTs gene family is composed by two loci: *CUP1* and *CRS5*, encoding two polypeptides unrelated in sequence. Multiple copies of *CUP1* are present in the yeast genome (50, 61-64), whereas only a single copy gene was found for *CRS5* (65). Both genes are transcriptionally activated by Cu ions (56, 65, 66), and their basal and induced expression depends on the transcription factor Ace1 (33, 56, 65-69). The *CRS5* promoter contains only a single recognition sequence for Ace1 while *CUP1* has four recognition sites, resulting in a different induction of the two genes (65, 66, 70, 71). In addition, Cup1 and Crs5 bind a different number of metal ions. In particular, Cup1 binds 7 Cu(I) and 4 Cd(II) ions (71-73), whereas a greater binding capacity was demonstrated for Crs5: 11-12 Cu(I) and 6 Cd(II) (30). However, Cup1-Cu(I) complexes are kinetically more stable than Crs5-Cu(I) complexes (30). Thus, multiple gene copies, greater responsiveness of the promoter to Cu ions and strongest Cu avidity contribute altogether to the dominant role of Cup1 in Cu buffering in *S. cerevisiae*. Indeed, yeast cells defective in *CUP1* gene are hypersensitive to Cu salt (63, 74); on the contrary deletion in *CRS5* gene leads to only reduced sensitivity to Cu (65). Whereas Cup1 expression is established as a primary defense to counteract Cu ions

toxicity, Jensen and collaborators (30) – on the basis of metal-binding studies - proposed for Crs5 a role in Cu bioavailability and utilization.
Besides the promoters of *CUP1* and *CRS5*, the Cu-dependent transcription factor Ace1 recognizes also the promoter of *SOD1*. This gene encodes Sod1, a Cu/Zn superoxide dismutase, and is transcribed upon binding of Ace1 to a single binding site (32). Sod1 protects cells from oxidative damages by the disproportionation of superoxide anions to oxygen and hydrogen peroxide (38). In *S. cerevisiae* – as well as in mammals – Sod1 is mainly located in the cytosol, with a smaller fraction in the intermembrane space of mitochondria (75, 76) The sequence and structure of Sod1 is conserved from prokaryotes to eukaryotes (77). The protein associates to form a dimer (78) stabilized by an intramolecular disulfide bond. Each subunit contains an active site with one Cu ion, acting as the catalyst for superoxide disproportionation, and one Zn ion, that participates in proper folding (79). Insertion of Cu into the active site is facilitated by the metallochaperone Ccs1 that docks with and transfers the metal ion into the disulfide-reduced apo-Sod1 (80, 81).
In *S. cerevisiae* metallothioneins and Cu/Zn superoxide dismutase are directly induced by Cu exposure and protect cells either by buffering Cu ions or by detoxifying Cu-derived ROS. In the cell, among proteins participating in direct ROS detoxification or in redox balance of protein thiols there are also catalases and peroxidases (82). Catalases reduce hydrogen peroxide using the redox properties of a haem group complexed to the polypeptide (83) and peroxidases (glutathione peroxidases and peroxiredoxins) reduce peroxides to the corresponding alcohols using active site cysteine thiols (84, 85).
In addition to enzymatic antioxidant systems, cells possess also non enzymatic defences against metal-induced oxidative stress, the most important of which is the tripeptide glutathione – GSH. In *S. cerevisiae* GSH may account for 1% of the cell dry weight. This small molecule is

a storage form of endogenous sulphur and nitrogen as well as a metal-ion detoxifying agent (86). In the cytosol, GSH has a role of cellular redox buffer, since it is maintained in the reduced state by a NADPH-dependent glutathione reductase (87).

While in *Saccharomyces* cells metallothioneins act as primary defenses against toxic levels of Cu, in other yeasts Cu resistance can be achieved by other mechanisms. For example, in the fission yeast *Schizosaccharomyces pombe* peptides named phytochelatins sequester Cu (88) and in *Candida albicans* a plasma membrane efflux pump can extrude Cu ions by a mechanism described also in some prokaryotic cells (89, 90).

1.5 Yeast as a new food source of essential copper. Is it possible?

Beside its use as a model of eukaryotic cells, yeast is an *ante litteram* biotechnological tool, traditionally used by mankind for thousands years for fermenting food and beverages (91). Starting from ancient times, domestication of naturally occurring yeasts by human selection led to the diffusion of strains optimized for specific fermentation procedures (92). Moreover, yeast can be used in human diet due to its composition in proteins (35-55% dry weight), carbohydrates (25-40%) and fats (up to 3%). Yeast has a high content of total nitrogen (7-9%), 18-20% of which is non-protein nitrogen and is a rich source of soluble vitamins (B group) and calciferol (D group) (93, 94). In addition, yeast biomass contains different trace elements (TE) – specifically bound to the proteic component - such as Cr, Se, I, Fe, Zn, Cu and Mn, even if their amounts are not sufficient to satisfy human requirements (94). However, if properly modified and/or cultured, yeast cells can accumulate significative amounts of TE. Such a

feature makes yeast a valuable food source of TE to compensate deficiencies in specific people categories (95, 96). Another important issue is the chemical form by which TE are supplied: the organic form, present in the majority of foods, is preferred because of its easier uptake and the reduced risk of detrimental side effects compared to inorganic salts and oxides (97). In this context, yeast biomass could be a safe (it is a "GRAS" organism) and good quality source of TE.

A number of literature data and patents report on the preparation and the application of TE-enriched yeasts and, among them, also Cu-enriched yeasts (98-101), for example a patent concerning protocols for the Cu-enrichment of *S. cerevisiae* biomass and its use for preparation of pro-biotic, dietary and nutraceutic products (101). Cu-enriched yeasts are commercially available, for example Lalmin®Cu1000 (Lallemand Health Ingredients, Copenhagen, Denmark) containing 1000-1400 µg Cu/g biomass. Vinson and collaborators (102) compared the bioavailability of different forms of Cu - Lalmin®Cu1000 and Cu-gluconate - as dietary supplement. Cu is present in the body only bound to proteins, but not as a free ion or salt. It has been demonstrated that Cu administered as Cu-enriched yeast, is more bioavailable than Cu-gluconate in rats liver. It has been hypothesized that Cu from yeasts is bound to proteins and/or glutathione and therefore is more easily absorbed into the body from the gastrointestinal tract and then transported to the liver. On the other hand, Cu-gluconate requires proteins as carriers for absorption and, unless associated with a protein-rich diet, this salt form of Cu supplementation is not effective. In a similar study (103), the relative absorption of Cu in animals fed with diets containing Cu-enriched yeast was statistically higher if compared with those fed with diets containing Cu sulphate. However, though these studies support the usefulness of Cu-enriched yeasts, according to the European Food Safety Authority (EFSA) available information for Cu-enriched yeasts is

still not sufficient to assess the safety of their use for nutritional purposes.

1.6 Biotechnological tools to obtain copper-enriched yeast biomass

The use of metal-enriched microbial biomasses, especially *S. cerevisiae* cultures, has been proposed for bioremediation, removal or recovery of metal ions and for analytical purposes (104-107). In this context, based on the understanding of metal uptake mechanisms, genetic technologies have been applied to improve bioaccumulation properties of biomasses (108-110). For example, Kuroda and collaborators (109, 111) obtained a cell-surface modified *S. cerevisiae* strain with an histidine hexa-peptide exposed on the cell surface. This strain displayed improved ability of Cu chelation and self-aggregation properties. Using cell-surface engineering Kuroda and Ueda (112, 113) also constructed yeast cells exposing tandem repeats of metallothioneins leading to an enhanced adsorption of Cu and Cd ions, as well as increased tolerance toward toxic metal concentrations.

Beside the use of engineered cells, the simplest way to obtain metal-enriched and tolerant cells is to exploit the intrinsic capacity of microbial cells to adjust themselves to changing environments. This ability represents the pivotal principle on which experimental designs of *evolutionary engineering* are based on.

1.7 Evolutionary engineering

The term *evolutionary engineering* was introduced for the first time by Butler and collaborators (114) by analogy with *metabolic engineering* (115) to indicate an approach that uses the evolutionary principles to direct the selection of organisms with a desired set of phenotypes. This approach allows improvement of microbial properties by simple random mutation and natural evolution but also by recombination and continuous evolution of large populations over many generations (116).

According to the basic rules of evolution, species evolve through random variations (via mutation, recombination, or other operators); the next step consists in natural selection in which the fittest survive and reproduce themselves, propagating their genetic material to the next generations. In addition, novel metabolic functions can be acquired by mutational activation of *cryptic genes*, which represent a versatile tool to enhance the adaptive potential of a species. These cryptic genes are phenotypically silent DNA sequences, not expressed under normal conditions, that are supposed to have played a role in natural evolution (117). In this context, another important set of genes are the *evolution genes*, that act in DNA repair for the benefit of evolution itself, generating and modulating spontaneous mutations (118, 119).

Evolutionary adaptation of species to changing environments is a process occurring in all simplest cultivation systems. For example the wild-type laboratory strains are the outcome of an evolutionary domestication process (like in the case of *S. cerevisiae*, largely exploited for baking and alcohol production (116)).

A schematic representation of the population dynamics during adaptative evolution of a population is provided in **Figure 4** (116).

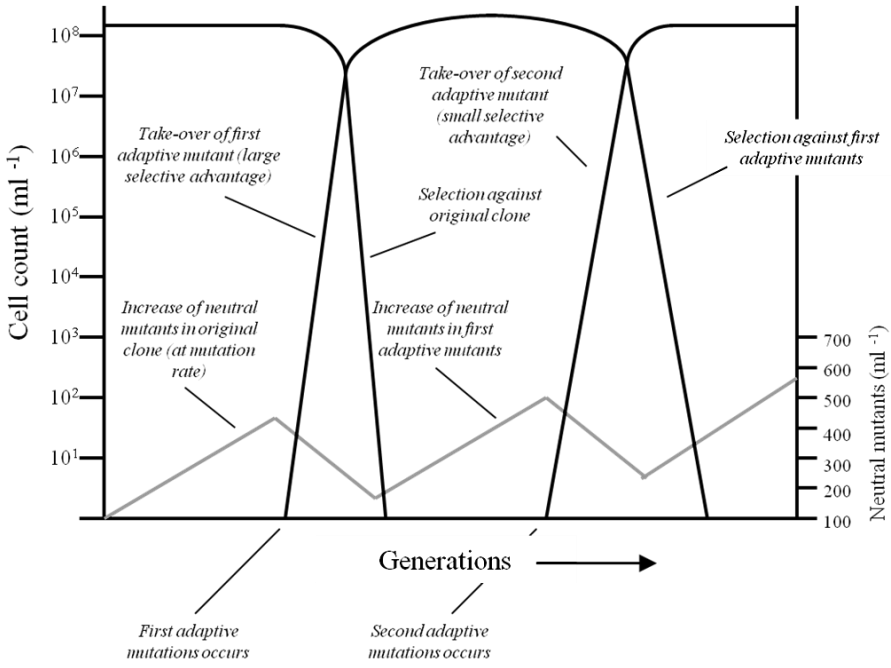

Figure 4. Scheme of the population dynamics during adaptive evolution of an asexual population. The *gray line* at the bottom represents the abundance of neutral mutants (at a linear scale). The other lines indicate periodic selection of two consecutively evolving advantageous mutants (at a logarithmic scale). From (102) inspired by a similar scheme (106).

In a culture inoculated from a single clone, a new advantageous mutation generally occurs in the much larger population that does not carry neutral mutation. Subsequently, the adaptive mutant replaces the existing population (including the fraction of neutral mutants) at the log linear rate of selection. Neutral mutation will continue to arise at the same linear frequency in the adaptive mutant, until another advantageous mutation occurs, again in the still predominant population without the neutral marker phenotype. The abundance of the neutral marker phenotype drops again and the cycle is repeated (116, 120).

Adaptive evolution can be achieved in a number of ways.

Selection on solid media offers the advantage to screen a great number of mutants with a certain phenotype by direct read-out of the progress of the evolutionary adaptation, especially when it is unclear to what extent improvement is possible (121). However this protocol of selection is inefficient for complex phenotypes that require multiple mutations (116).

Adaptation by selection in liquid media allows the evolution of fitter variants over time, with consequent replacement of the parental population. During selection in batch cultures, cells are subjected to changes in environmental conditions and alternate periods of growth and stasis upon serial transfers (116). An exemplar experiment of selection in batch culture was performed by Lensky and coworkers (122-124). They analyzed the fitness of 12 independent *E. coli* populations derived from a single ancestor. These populations were propagated for about 10000 generations by daily serial transfer in shaking flasks containing glucose-supplemented minimal medium. After 10000 generations, the mean fitness of the derived clonal variants was increased by 50% with respect to the common ancestor. Although in the 12 populations the phenotypic changes were consistent, they showed a great genetic diversity. Over time, the evolved genomes became more and more different from the ancestor and from each other. In the evolved populations point mutations occurred rarely, meaning that the accumulated genomic – and phenotypic – changes were mostly a consequence of chromosomal rearrangements. Fundamental mutations shared among all members of a given population represent good candidates for phenotypically relevant mutations. This example illustrates how evolution of adaptative performance is very reproducible, although the same phenotypic adaptation can be achieved by different genotypes (116). Moreover, Lensky and collaborators (123) demonstrated that about half of the phenotypic

improvement occurred within the first 2000 generations, thus the rate of fitness obtained in microbial population assumes an hyperbolic profile, decreasing over time.

In batch culture, cells are subjected to changes in several environmental parameters and the effects of evolutionary adaptation could be attributed to any of the different phases of growth that cells undergo. Conversely, continuous culture systems – in chemostat - providing a constant environment, are a good tool to modulate selective pressure toward a phenotype of interest (120, 125). However, a disadvantage of selection in continuous systems is the strictly sequential appearance and fixation of adaptive mutations. As a consequence, a newly appearing variant may compete only with its immediate one or few predecessor, if the older variants were previously counter-selected. It may happen that new variants exhibit lower fitness compared with the more distant predecessor (126). Therefore, the combination of adaptive mutations during continuous experiments may result in misadapted clones, which may have never directly competed with the later occurring variants (116). Conversely, during selection in batch culture a steady, although hyperbolic improvement in fitness is observed (123).

Evolutionary engineering has been used in biotechnology to improve simple as well as complex cellular subsystems, as illustrated in **Table 3** (116).

Evolved phenotype	Selection system
Novel catabolic activities	
Utilization of carbon substrates (coryneform bacteria)	Plates (with limiting amount of yeast extract)
Utilization of pentoses (*E. coli*)	Plates (non-growing cells)
Novel esterase activities (*P. putida*)	Plates (non-growing cells)
Galactitol dehydrogenase (*Rhodobacter*)	Chemostat (glucose-limited, excess galactitol)
PTS-independent uptake	Chemostat
Improved enzymes properties	
Functionality (*E. coli* mutator strain)	Batch (increasing antibiotic concentrations)
Improved plasmid functions	
Stability (Gram positive, yeast)	Chemostat (antibiotic and auxotrophic marker selection)
Stable host-plasmid combinations (*E. coli*)	Chemostat
Improved stress resistance	
Multiple stress resistance (yeast)	Chemostat and batch (with stress challenges)
Membrane protein overexpression (*E. coli*)	Plates
Improved production properties	
Endo-enzyme overexpression	Chemostat
Protein secretion (*Streptomyces*)	Chemostat (different selection schemes)
Biomass yield (yeast, *E. coli*)	Chemostat (carbon-limited)

Table 3. Recent examples of evolutionary engineering. From (116).

Recent experimental works report on the use of evolutionary engineering to evolve microorganisms resistant to metals, i.e. Cobalt and Boron (127, 128). In these works, selection under continuous exposure to gradually increasing metal concentrations allowed the isolation of hyper-resistant mutant cells. Evolved cells showed acquired cross-resistance to other stress conditions (such as exposure to other metals, heat shock and oxidizing agents). Literature also refers the effect of evolutionary adaptation on different yeast cells

growing in presence of Cu and their properties of bioaccumulation (129, 130).

In conclusion, *in vivo* evolutionary engineering of whole organisms represents a valuable alternative to metabolic engineering, often limited by the great complexity of dynamic interactions in cellular systems (116). In a flow chart taken from Sauer's review (**Figure 5**) (116), it is clearly illustrated how evolutionary engineering well fits in the routes exploited by biotechnology to develop new strains.

Strains obtained with evolutionary engineering 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* (131). In this latter case, characterization of the molecular bases of *evolved* phenotypes is required. In this context *proteomics* and *transcriptomics*, DNA chips and metabolic flux analysis are precious tools for the identification of genes beneficial or detrimental for a specific selected phenotype (132-134). Altogether – and combined with bioinformatics tools for modeling cellular functions and activities (116) – these methodologies contribute to make evolutionary engineering an attractive approach in the field of microbial biotechnologies.

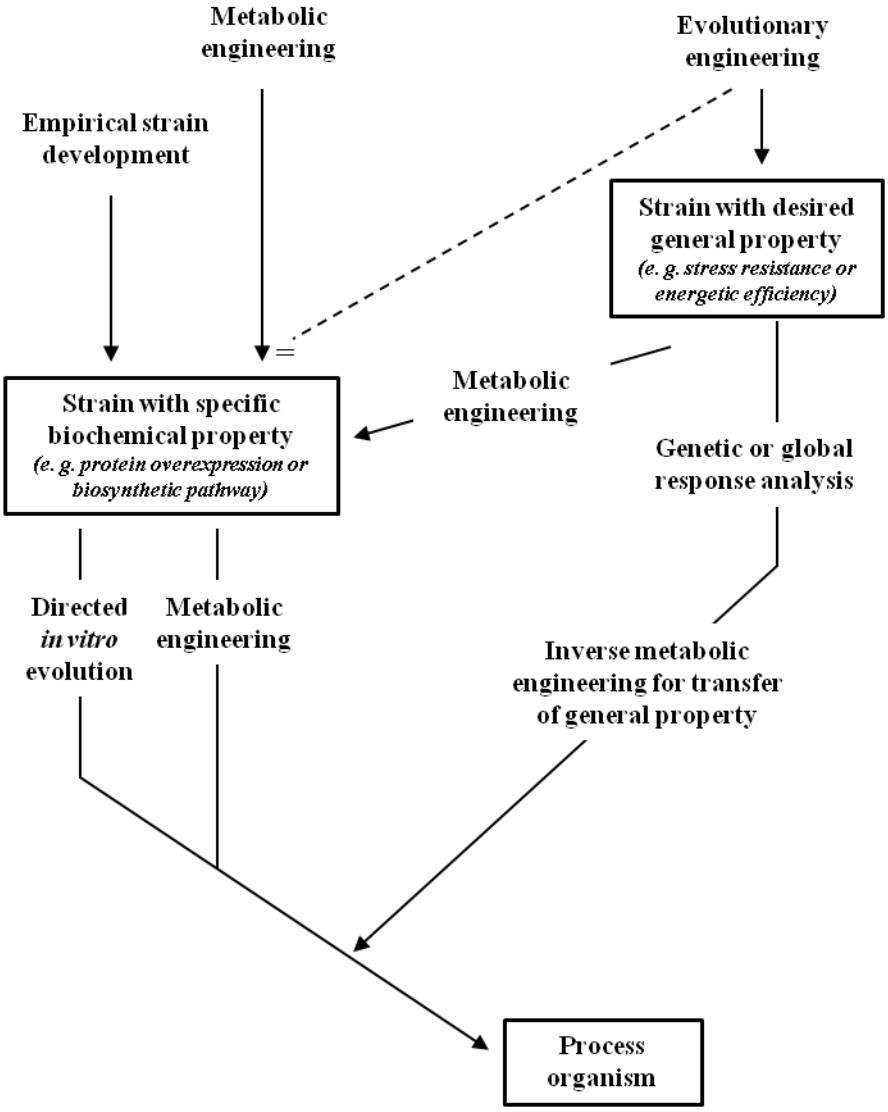

Figure 5. Flow chart for future development of biotechnological strains. From (116).

2. Experimental work

In this section three experimental works are presented:

2.1 Laboratory evolution of copper tolerant yeast strains

GM Adamo, S Brocca, S Passolunghi, B Salvato and M Lotti (*Microbial Cell Factories 2012, **11**:1*)

2.2 Evolution of copper tolerance in *Saccharomyces cerevisiae* relies on amplification of the *CUP1* gene

GM Adamo, M Lotti, MJ Tamás and S Brocca (Manuscript)

2.3 Proteomic analysis of natural and copper-adapted cells of the yeast *Candida humilis*

GM Adamo, S Brocca and M Lotti

(Preliminary results)

As already mentioned in the introduction, evolutionary engineering is a valuable strategy to obtain strains with a desired phenotype. In the first work (2.1), we described an evolutionary engineering strategy to obtain yeast strains endowed with tolerance toward high concentration of Cu ions, supplied as $CuSO_4$. The starting yeast populations were natural strains of *S. cerevisiae* and *C. humilis*, characterized by different basal Cu tolerance, being the former Cu sensitive and the latter Cu tolerant. These strains were step-wise evolved through continuous cultivation in presence of increasing concentration of copper salt. As a result, cells improved their Cu tolerance up to 2.5 g/L $CuSO_4$ and were able to accumulate high amounts of metal ions. The acquired tolerance was permanently maintained, underlying the effectiveness of evolutionary engineering in the acquisition of stably phenotypic traits. A preliminary characterization of Cu-*evolved Saccharomyces* and *Candida* cells revealed common features associated with Cu tolerance: improvement of cell vitality and reduction of ROS production. At the same time, differences in the response of antioxidant enzymes and Cu-binding proteins were found between the two yeasts. An intriguing issue emerging from this work is that, although for both strains evolution of Cu tolerance moves in the same direction (increase in strain robustness), *S. cerevisiae*, naturally more sensitive to Cu, improved its robustness at higher extent than *C. humilis*, characterized by a natural Cu tolerance. This is likely to be associated with different genetic backgrounds of the two strains. This finding prompted us to further investigate the molecular bases of Cu tolerance in *evolved* yeast cells.

In the second work (2.2), we focused our attention on *evolved S. cerevisiae* cells to evaluate the role of the metallothionein Cup1. This work was based on literature data demonstrating the pivotal

role of Cup1 in mediating Cu tolerance of natural *S. cerevisiae* cells (61). Briefly, in the *S. cerevisiae evolved* strain we found out an amplification of *CUP1* gene copy number, triggering a strong increase in gene expression. We ascribed to the over expression of Cup1 the reduction of oxidative stress observed in the *evolved* strain grown in copper medium. At the same time, we found that other factors, such as the metallothionein Crs5 and the radical scavenger Sod1, play a secondary role in protection against Cu-induced oxidative stress. Altogether these results, show the predominant role of *CUP1* amplification in the *evolution* of Cu tolerance.

A different approach was applied to investigate on Cu tolerance of *evolved C. humilis* cells; preliminary results are presented in the last work (2.3). Due to the scarce information available about the *C. humilis* genome, we used a proteomic approach to gain insight into the molecular changes occurring during Cu exposure in *non-evolved* and *evolved* cells (obtained as described in the work 2.1). The differential analysis revealed for both strains a comparable response, in terms of number and type of proteins up- or down- regulated by copper. Identified proteins belong to distinct groups such as glycolytic enzymes, heat shock proteins, proteins involved in protein synthesis and energy production, proteins involved in phospholipids synthesis. All these proteins are involved in the protection against oxidative stress and were previously identified in works performed on *S. cerevisiae* cells (135-138). This is to our knowledge the first proteomic analysis of *Candida* cells during oxidative stress. As a matter of fact, while copper induce changes in the level of proteins of both *evolved* and natural cells, we cannot ascribe the evolution of copper tolerance to the differential expression of one (or few) specific protein. However, any observed change in protein level was less

marked in the *evolved* strain. This observation suggests that the tolerance to copper in *evolved* strain is only partially sustained by molecular mechanisms involved in the oxidative stress response and that other mechanisms can be active in preventing Cu toxicity in the *evolved* strain.

Laboratory evolution of copper tolerant yeast strains

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Background

Yeast strains endowed with robustness towards copper and/or enriched in intracellular Cu might find application in biotechnology processes, among others in the production of functional foods. Moreover, they can contribute to the study of human diseases related to impairments of copper metabolism. In this study, we investigated the molecular and physiological factors that confer copper tolerance to strains of baker's yeasts.

Results

We characterized the effects elicited in natural strains of *Candida humilis* and *Saccharomyces cerevisiae* by the exposure to copper in the culture broth. We observed that, whereas the growth of *Saccharomyces* cells was inhibited already at low Cu concentration, *C. humilis* was naturally robust and tolerated up to 1 $g \cdot L^{-1}$ $CuSO_4$ in the medium. This resistant strain accumulated over 7 mg of Cu per gram of biomass and escaped severe oxidative stress thanks to high constitutive levels of superoxide dismutase and catalase. Both yeasts were then "evolved" to obtain hyper-resistant cells able to proliferate in high copper medium. While in *S. cerevisiae* the evolution of robustness towards Cu was paralleled by the increase of antioxidative enzymes, these same activities decreased in evolved hyper-resistant *Candida* cells. We also characterized in some detail changes in the profile of copper binding proteins, that appeared to be modified by evolution but, again, in a different way in the two yeasts.

Conclusions

Following evolution, both *Candida* and *Saccharomyces* cells were able to proliferate up to 2.5 $g \cdot L^{-1}$ $CuSO_4$ and to accumulate high amounts of intracellular copper. The comparison of yeasts differing in their robustness, allowed highlighting physiological and molecular determinants of natural and acquired copper tolerance. We observed that different mechanisms contribute to confer metal tolerance: the control of copper uptake, changes in the levels of enzymes involved in oxidative stress response and changes in the copper-binding proteome. However, copper elicits different physiological and molecular reactions in yeasts with different backgrounds.

Keywords: yeast, copper, adaptation, evolutionary engineering, oxidative stress response, micronutrients

Background

Metal ions like copper, manganese, zinc and iron are essential micronutrients for living organisms and play a central role in the cell metabolism being the cofactors of a large number of enzymes and electron transport proteins [1]. The metabolism of copper and the mechanisms that control its intracellular concentration are the targets of intense studies since impairments in Cu level, transport and localization have been associated with several human diseases [2, 3]. In fact, while copper deficiency impacts the function of key cell enzymes, Cu overload can generate highly reactive oxygen species (ROS) which produce peroxidation of membrane lipids, displacement of other metal cofactors from their natural ligands in signalling proteins [4], oxidation of proteins and cleavage of DNA and RNA molecules [5] resulting in general cellular damage. Moreover, ROS are thought to play a major role in cancer development and in aging [6]. To cope with such strict constraints, all organisms have developed complex regulatory mechanisms to maintain copper homeostasis.

Yeast cells are a good tool both for the investigation and the manipulation of copper metabolism. Studies on the accumulation of metals in edible microorganisms are of relevance for the production of functional foods enriched in micronutrients (for example the ones about the inclusion of iron, cobalt, copper and manganese in yeast cells [7]) and the industrial production of *Saccharomyces cerevisiae* biomass highly enriched with organic forms of selenium [7, 8]. Yeast cells resistant to and accumulating intracellular copper have been recently patented for cleaning copper from extracellular solutions [9] and use in pro-biotic, cosmetic, dietary and nutraceuticals products [10].

It has been reported that microorganisms can acquire stress tolerance and novel metabolic abilities when exposed to the appropriate selection pressure. This approach is often reported as “evolutionary engineering”, a term introduced by Butler and collaborators in 1996 [11], since it uses evolutionary principles based on the selection of random mutants arising in the microbial population either spontaneously or upon mutagenesis and has been applied for improving complex physiological properties whose genetic and physiological basis is not fully understood [12]. For example, microbial cells were recently *evolved* to improve their

resistance towards multiple stresses [13], cobalt [14], iron- and sulfur-compounds [15], alcohols [16]; and to gain the ability to ferment xylose [17] and lactose [18].

S. cerevisiae is a powerful model organism to investigate copper metabolism and homeostasis in Eukaryotes. As a consequence, a large body of knowledge is available about Cu uptake, intracellular transport and functional role in yeast cells [19], as well as about non-enzymatic and enzymatic mechanisms of protection from ROS and oxidative stress [20].

In this study, yeast strains endowed with different natural robustness towards copper were compared with strains evolved by stepwise adaptation to tolerate high metal concentrations. We report that different and overlapping physiological and molecular responses are elicited in cells with different backgrounds to allow them to tolerate challenging conditions.

Results

Tolerance towards copper of one *Candida* and three *Saccharomyces* strains was first assessed by a drop test on minimal or rich (YPD) solid medium supplemented with increasing concentrations of copper salt ($CuSO_4$) (Fig. 1). In good agreement with results from other laboratories showing that the composition of the culture medium and the growth conditions affect copper sensitivity of yeast cells [21, 22], we observed that on minimal medium, 0.5 $g \cdot L^{-1}$ $CuSO_4$ was sufficient to inhibit the growth of all yeast strains, whereas on YPD plates all of them tolerated up to 1 $g \cdot L^{-1}$ $CuSO_4$. However, above this concentration only *C. humilis* cells still proliferated, suggesting low copper tolerance in all strains assayed but *C. humilis* which showed a higher tolerance.

The tolerance of cells to metals is relevant both for understanding the mechanisms of defence towards stress and for the production of microorganisms enriched in a given micronutrient for biotechnological applications. We applied an evolution-based approach to improve robustness towards copper in all strains, independently from their natural background. The experimental protocol relied on the stepwise cultivation of cells in media supplemented with progressively higher concentrations of copper sulphate. At each step the culture was grown for 72 hours before withdrawing aliquots to be inoculated at a higher $CuSO_4$ concentration. The

starting condition was YPD + 1 g · L^{-1} $CuSO_4$, which is permissive for all strains. The following steps were in YPD + 1.5 g · L^{-1} $CuSO_4$, YPD + 2 g · L^{-1} $CuSO_4$ and YPD + 2.5 g · L^{-1} $CuSO_4$. This last one was the highest concentration applied, since above it copper salts led to acidification of the medium resulting in the precipitation of its protein components. Single colonies isolated after the last step of adaptation displayed relatively high rates of growth when directly re-inoculated in YPD + 2.5 g · L^{-1} $CuSO_4$. These cells are defined in the following as "evolved".

Figure 2 compares the growth kinetics of non-evolved and evolved cells in YPD + 2.5 g ·L^{-1} $CuSO_4$ (for simplicity we will refer to this condition as "copper medium"). Among the natural strains (in the following "non-evolved"), only *C. humilis* AL5 proliferated under this condition, even though growth started after a prolonged lag phase and a very low final cell density was achieved (Fig. 2 a). On the contrary, all evolved strains proliferated in copper medium and reached final biomass densities close to those observed in YPD medium, although with longer duplication times (Additional file 1). Cells subjected to 10 cycles of growth/re-inoculation in YPD without Cu (referred to as "de-adapted"), retained their ability to proliferate if re-inoculated in copper medium, showing only negligible differences when compared with the corresponding evolved strain (Fig. 2 and Additional file 1). This observation suggests that copper tolerance is maintained also in absence of selective pressure. To gain more insight into the behaviour of the copper-sensitive *S. cerevisiae* strains, we compared the kinetics of growth of evolved and non-evolved cells also at 1, 1.5 and 2 g ·L^{-1} $CuSO_4$ (Additional file 2), highlighting a progressive decrease of the proliferation ability of natural cells at increasing copper concentrations. As expected, the same conditions were permissive for evolved *Saccharomyces* cells.

Analysis of the Cu content of *Candida* cell samples from the same cultures shown in Figure 2 revealed relatively high amounts of intracellular copper, i.e. 6.5 and 7.6 mg · g^{-1} biomass in evolved and non-evolved cells, respectively. The kinetics of bioaccumulation was faster in non-evolved cells where Cu measured after 24 hours of growth was three times higher than in the evolved ones (Fig. 3 a). In copper medium non-evolved *Candida* grew poorly and contained high copper concentration from the very beginning of the experiment, while Cu was lower in evolved cells – which proliferated at the same rate as in YPD broth. In both cases,

intracellular copper kept increasing up to 48 hours and then reached a plateau. The amount of copper measured in evolved and de-adapted cells grown in copper medium was always comparable, supporting once more the hypothesis that evolutionary engineering produced stable effects.

The increase of intracellular copper in evolved *S. cerevisiae* was slower and at the end of the experiments we measured 2.1 to 4.2 mg Cu · g^{-1} biomass (Fig. 3 b, c, d). The Cu content of non-evolved and evolved *Saccharomyces* cells was compared also in a milder condition, (1 g ·L^{-1} $CuSO_4$). Whereas both kinds of BL7 cells displayed the same kinetics of copper accumulation (Additional file 3 a), non-evolved EL1 and GL6 showed a faster kinetic of bioaccumulation (Additional file 3 b, c) associated to a growth kinetic slower than in their evolved counterpart (Additional file 2). As in *Candida* samples, also in this case *the* behaviour of evolved and de-adapted cells was similar.

Results obtained up to this point suggested that all *Saccharomyces* strains are rather homogeneous in their response to copper, but different from the *Candida* one. Therefore, subsequent experiments aimed at highlighting possible adaptive changes were focused on a more in-depth comparison between *Candida humilis* and the only *Saccharomyces* cells strain BL7.

Initially, the effect of copper on cell viability was evaluated by citofluorimetry (Fig. 4), that allows to get this information at the single cell level [23]. Samples of *C. humilis* and *S. cerevisiae* cultures were harvested during exponential growth in copper and stained with propidium iodide, an intercalating agent excluded by viable cells, that can instead permeate the surface of seriously injured/dead cells [24]. In both evolved strains the percentage of propidium-positive cells was lower (8.5 % for *C. humilis* and 11 % for *S. cerevisiae*) than in their non-evolved counterpart (28 % for *C. humilis* and 60 % for *S. cerevisiae*). The percentage of propidium-positive cells grown in absence of copper (used as a control) was around 2 % (*data not shown*). Altogether, these results confirm that evolution confers robustness - although not complete insensitivity - to copper. Copper was more detrimental for *S. cerevisiae* than *C. humilis* natural cells, while this difference fainted after adaptive evolution, in good agreement with the kinetics of growth reported in Figure 2.

Since copper is a strong oxidizing agent, we measured the activities of superoxide dismutase, peroxidase, glutathione peroxidase and catalase - all involved

in the so-called copper-dependent oxidative stress response – in evolved and non-evolved *C. humilis* and in evolved *S. cerevisiae* cells harvested from copper medium in the exponential phase of growth. As a control, basal enzymatic activities were determined in YPD-grown cells (Table 1). In *S. cerevisiae*, exposure to copper resulted in the increase of superoxide dismutase and catalase activities, while peroxidase and glutathione peroxidase activities were only marginally affected. The picture emerging from the analysis of the copper tolerant *C. humilis* strain is different and more exhaustive, since the data set can also include the response of non-evolved cells in copper medium. In all cultivation broths, all enzymatic activities tested in natural *Candida* cells were 2 to 8 fold higher than in *S. cerevisiae*. Interestingly enough, we detected high constitutive superoxide dismutase and catalase activities in non-evolved cells, independently from the composition of the culture broth, while peroxidase and glutathione peroxidase activities were induced only by growth in copper medium. Evolution resulted in lower levels of all tested activities in both YPD and copper medium, with the most remarkable effect on superoxide dismutase. Catalase activity that was high in YPD-grown cells (both kinds of cells), strongly decreased in evolved cells grown in copper medium.

We then evaluated the production of reactive oxygen species (ROS) staining cells with dihydroethidium (Fig. 5). In presence of superoxide anions in the cytosolic space, this probe is oxidized to the fluorescent product ethidium. Therefore, fluorescence intensity reports on oxidative stress. While in YPD medium the amount of ROS was low in both *Candida* and *Saccharomyces* cells, copper exposure clearly triggered oxidative stress, though with milder effects in the evolved cells. Moreover, in agreement with growth and cytofluorimetric data (Fig 2 and 4), the effect on *S. cerevisiae* was stronger than on *Candida* with ROS production three-fold higher. Evaluation of oxidative stress in *S. cerevisiae* at intermediate metal concentration (1, 1.5 and 2 g $\cdot L^{-1}$ $CuSO_4$) showed that while ROS production increases with copper concentration in the non-evolved sample, it remains low in the evolved cells (Additional file 4).

Finally we performed a preliminary analysis of the copper-binding proteins extracted from non-evolved and evolved cells grown either in YPD or in copper medium. Samples were enriched by affinity chromatography as described in Materials and Methods and then analysed by SDS-PAGE. Equal volumes of elution

fractions obtained from the same amount (800 µg) of proteins applied on the column were loaded for the electrophoretic run, therefore assuring that differences detected in the gel reflect changes in composition and content of copper-binding proteins in the starting samples. Figure 6 shows the electrophoretic analysis and Table 2 lists proteins identified by tandem mass spectrometry. The exposure of evolved *S. cerevisiae* cells to copper elicited the induction of several proteins (Fig. 6a, lane 2 and 3) involved in different biochemical and metabolic functions, i.e. the pentose phosphate pathway (band 1), amino acid and sulphur metabolism (bands 2, 3 and 10), glucose metabolism (bands 4 and 7), redox reactions (bands 6 and 8), the translation machinery (bands 5 and 9) and isomerization reactions (bands 1 and 9). The same trend was detected at lower $CuSO_4$ concentration (data not shown). The picture relevant to *Candida* is completely different (Fig. 6 b). Non-evolved *Candida* cells react to copper repressing a number of Cu-binding proteins that would be otherwise expressed during growth in YPD (Fig. 6b, lane 1 and lane 3). Among the down-regulated proteins we could identify ribosomal proteins and components of the protein translation apparatus (band 11, 13 and 16). The profile of Cu-binding proteins extracted from evolved *Candida* cells (Fig. 6, lane 2 and 4) showed a massive enrichment of a protein of ~35 kDa (band 14), identified as glyceraldehyde-3-posphate dehydrogenase 3 (GAPDH). We further observed the increase of a protein of ~ 22 kDa (band 15) identified as peroxiredoxin and of a protein inducible by oxidative stress (band 12).

Discussion

In this work, we obtained copper-enriched and copper-tolerant yeasts through a number of generations smaller than reported in similar recent works [13, 14, 25]. This might be explained on the basis of a different experimental set-up in which relatively few rounds of selection were applied, but with longer cultivation times (72 h) and wider intervals of metal concentration (increases of 0.5 g . L^{-1} at each round). The effectiveness of the method of evolution is substantiated by the preservation of metal tolerance in absence of selective pressure (de-adapted cells), meaning that stable molecular changes occurred. This view is corroborated by the amplification of the CUP1 gene, which encodes for a metallothionein, detected in evolved *S.*

cerevisiae cells (Adamo et al., in preparation). However, it is possible that other adaptive (transient) modifications contribute to the increased resistance.

In the frame of this complex picture, the comparison of yeasts differing in their robustness towards copper allowed us to investigate physiological differences involved in natural and acquired copper tolerance and to obtain some preliminary information about the molecular determinants of this trait. Taken together, our results hint at the concurrence of different mechanisms that we briefly summarize and discuss in the following.

i) Copper uptake. C. humilis cells can grow in copper medium due to their natural tolerance that can be further improved *via* evolutionary engineering. This feature allowed to compare the bioaccumulation of intracellular Cu in non-evolved and evolved cells at the highest copper concentration used (2.5 g $\cdot L^{-1}$ $CuSO_4$), showing that copper is lower in these latter. On this basis we hypothesize that one of the mechanisms of robustness might rely on hindering metal uptake. Consistently, the toxicity of Cu incorporated in the non-evolved strain (mainly in the first hours of growth) might account for the growth impairment and the increase in the rate of propidium positive cells we observed. In this light, the variability in copper sensitivity between *Candida* and *Saccharomyces* cells might depend (at least partly) on a different ability to limit copper uptake and its overload. Such a mechanism has been reported to protect *S. cerevisiae* cells from copper [26] cadmium [27] and cobalt [14] and points to a central role of the plasma membrane [28-30] and of the cell wall [31] in the onset of tolerance to heavy metals.

ii) Antioxidative enzymes and ROS production. Our results indicate that the biochemical bases of copper resistance can be deeply different among yeast species. In *S. cerevisiae,* evolution of copper tolerance is associated with the increase of antioxidative activities, as already documented by others [32], and with a reduced production of ROS. On the contrary, non-evolved cells suffer severe oxidative stress, as showed by the complete inhibition of their growth and the huge percentage of propidium positive cells. Basal activities of most detoxifying enzymes are higher in *C. humilis* than in *S. cerevisiae* cells, fact that could partly explain the natural copper tolerance of *Candida* cells and the reduced ROS production. The response of evolved *Candida* cells to copper is intriguing since our results show a reduction in the production of ROS, a generalised decrease of the antioxidative activities, and

non-responsiveness to copper (compare values reported for evolved cells in YPD and copper medium). Indeed, in evolved cells superoxide dismutase is always lower than in the original strain, independently from the presence of copper. We would assume that the different activity profiles and their re-shaping upon evolution might reflect different and peculiar defence mechanisms responsible for both natural and acquired copper tolerance in *Saccharomyces* and *Candida* strains.

iii) Cu-binding proteins. The hypothesis above is further corroborated by the observation that the amounts of soluble Cu-binding proteins extracted from non-evolved and evolved cells are different too. Over-expression of Cu-binding proteins is consistent with their role in the primary response to copper exposure and results in copper tolerance [33]. We are aware that the enrichment procedure used might lead to overestimation of some proteins that carry modifications such as thiolation [34, 35] or sequence motives that increase their affinity for the chromatographic resin or to underestimation of low-affinity proteins. Nevertheless, the differences detected between the two yeasts are marked and relevant proteins identified are in agreement with literature data reported by others. Among Cu-binding proteins, enzymes involved in sulphur metabolism (i.e. cystathionine-γ-lyase and aspartate-semialdehyde dehydrogenase) were over-expressed by evolved *S. cerevisiae* cells during growth in copper medium, suggesting that copper might redirect the metabolic flux towards the production of GSH to balance the redox equilibrium. The over-expression of glycolytic enzymes such as triose phosphate isomerase and GAPDH3 might be a consequence of the reconfiguration of the glycolytic flux, a mechanism reported to regulate the response to oxidative stress in human [36], plant [37] and yeast [38] cells. Furthermore, the increase of GAPDH3 is consistent with the role of this protein as a sensor of oxidative stress in DNA repair [39] and apoptosis [40, 41]. GAPDH3 increase is also the most remarkable effect triggered by copper in evolved *C. humilis.* Besides, these cells are enriched in the peroxiredoxin Tsa1 [42, 43], known to act as an antioxidant against ROS [44] and to protect actively translating ribosomes from stress conditions [45]. On this basis we propose that in evolved *Candida* cells the inhibition of protein synthesis associated with oxidative stress [46] occurs in an attenuated form in comparison to non-evolved cells, that show a strong decrease of Cu-binding proteins, without any remarkable

down regulation of the ribosomal proteins and other components of the protein translation apparatus.

Conclusion

The comparison between yeast cells naturally resistant or experimentally evolved to tolerate high copper concentration reported in this work supports the view that copper tolerance is due to multiple responses relying on different physiological and macromolecular changes. Yeasts endowed with copper tolerance and able to accumulate metal ions can find application in the biotechnology field for example for bioremediation or as dietary supplement, being these "GRAS organisms" valuable sources of microelements in organic form. Moreover, the comprehension of physiological and molecular responses of microorganisms to metal stress and of the mechanisms triggered during evolution of tolerance could help in the identification of biomarkers for ecotoxicological studies.

Methods

Yeast strains and growth conditions

Yeasts used in this study were isolated from sourdough. Strains were identified by Random Amplification of Polymorphic DNA-PCR (RAPD-PCR) and designated as *Candida humilis* AL5, and *Saccharomyces cerevisiae* BL7, EL1 and GL6 (Veneto Agricoltura - Istituto per la Qualità e le Tecnologie Agroalimentari – VI). Growth was on YPD medium [2% (w/v) glucose, 1% (w/v) yeast extract, 2% (w/v) tryptone] or on minimal medium [2% (w/v) glucose, 0.67% (v/v) yeast nitrogen base]. Solid media contained 2% (w/v) agar.

Copper tolerance was tested on cells grown shaking over night in 3 mL of liquid YPD at 30°C and subjected to serial dilutions in physiological solution [0.9% (w/v) NaCl]. 5 μL aliquots were spotted on either YPD or minimal medium plates containing $CuSO_4$ and incubated at 30° C for 2 days.

Adaptative evolution was performed stepwise starting from cells taken from a fresh culture on agarized YPD and grown overnight at 30°C in 3 mL of liquid YPD + 1 g

· L^{-1} $CuSO_4$. In the subsequent steps, 5×10^5 cells from stationary cultures were inoculated and cultivated for 72 hours in fresh medium containing each time increasing concentrations of copper (1.5 - 2 - 2.5 g · L^{-1}). Single colonies were isolated from the culture on YPD + 2.5 g · L^{-1} $CuSO_4$ by plating on solid YPD and re-inoculated in liquid YPD + 2.5 g · L^{-1} $CuSO_4$. To assess the endurance of metal tolerance, evolved cells were subjected to 10 cycles of inoculation-growth in fresh YPD medium without copper (de-adaptation) prior to be cultivated again in YPD + 2.5 g · L^{-1} $CuSO_4$.

Growth of non-evolved and evolved *S. cerevisiae* cells (BL7, EL1 and GL6) was assayed in liquid YPD medium supplemented with intermediate copper concentrations (1, 1.5, 2 g, · L^{-1} $CuSO_4$).

Growth was determined as the increase of cells number using a cells counter (Particle Count & Size Analyzer, Beckman Coulter).

Copper determination

Cells were harvested from 2 mL culture by centrifugation at 10,000 *g* for 10 min, washed twice with de-ionized water and at least four times with 10 mM citric acid in 0.5% (w/v) NaCl to remove copper ions adsorbed on the cell surface. The biomass was dried by Max-Dry Iyo (Heto) for 30 min, re-suspended in 300 µL of 20% (w/v) trichloroacetic acid (TCA), transferred to a 2 mL screw cap tube containing 100 µL of glass microbeads and subjected to mechanical lysis by three cycles of 20 sec at maximum speed with a Fast Prep® - FP120 (Bio101-Savant). The crude extract was clarified by centrifugation at 10,000 *g* for 10 min and the supernatant transferred to a new tube. Copper was quantified according to Brenner and Harris [47] adapted as follow. The clarified crude extract was diluted in 500 µL of de-ionized water and added to 100 µL of 1% (w/v) ascorbic acid and 400 µL of "BCA reagent" [0.006 % (w/v) 2,2'-biquinoline-4,4'-dicarboxylic acid disodium salt hydrate (Fluka), 3.6% (w/v) NaOH, 15.6% (w/v) Hepes]. After 2 min incubation at room temperature, absorbance was recorded at 354 nm with a spectrophotometer Ultraspec 1000 (Pharmacia Biotec). The amount of copper is referred as mg Cu · g^{-1} dry biomass.

Flow cytometry analysis

Samples containing 10^7 cells were withdrawn during the exponential growth phase. Cells were harvested by centrifugation, washed twice with deionized water and four times with 10 mM citric acid in 0.5% (w/v) NaCl. Cells were washed with 1 mL of PBS (3.3 mM NaH_2PO_4, 6.7 mM Na_2HPO_4, 127 mM NaCl, 0.2 mM EDTA) and stained with 1 mL of the fluorescent probe propidium iodide (5 ng $\cdot$ mL^{-1}). Stained cells were sonicated for 15 sec and then analyzed using a Cell Lab Quanta^{TM} SC flow cytometer (Beckman Coulter) equipped with a diode laser (excitation wavelength 488 nm, laser power 22 mW). The fluorescence emission was measured through a 670 nm long pass filter (FL3 parameter) in logarithmic mode for propidium iodide (PI) signal. Not stained and ethanol-treated samples were used as controls. The sample flow rate during analysis did not exceed 500 cells sec^{-1}. A total of 2×10^4 cells were measured for each sample. Data analysis was performed with WinMDI 2.8 software, build#13 01-19-2000 (Purdue University, Cytometry Laboratories [http://facs.scripps.edu/software.html]).

Preparation of cell-free extracts

Cells from exponential cultures were harvested by centrifugation at 4,900 *g* for 10 min and washed twice with cold deionised water and four times with cold 10 mM citric acid in 0.5% (w/v) NaCl. The cell pellet was finally re-suspended in 0.5 M Tris-Cl pH 8.5, 0.25 M EDTA pH 8.4 added with protease inhibitor cocktail (Sigma) and mechanically disrupted using glass microbeads. Cell debris was removed by centrifugation at 700 *g* for 10 min and the clarified crude extract was used for enzymatic analyses. The protein concentration in cell-free extracts was estimated according to Bradford [48] using bovine serum albumin as the reference.

Enzyme assays

Enzyme activities were measured on cell-free extracts by spectrophotometric assays. Activities were expressed as Units $\cdot$ mg^{-1} proteins. Catalase activity was determined according to Bergmeyer [49] monitoring hydrogen peroxide decrease at 240 nm. Superoxide dismutase activity was measured as the inhibition of the rate of reduction of cytochrome c by the superoxide radical, observed at 550 nm [50]. Peroxidase activity was measured following the oxidation of pyrogallol at 420 nm

[51]. The activity of glutathione peroxidase was determined monitoring NADPH oxidation at 340 nm [52].

Purification of copper-binding proteins

Supernatants obtained by ultracentrifugation (18,000 *g*, 45 min) of cell extracts were heated at 65°C for 10 min to enrich thermostable proteins, since thermostability is common to several copper-binding proteins [53]. Samples were then centrifugated at 10,000 *g* for 10 min and supernatants purified by affinity chromatography on a Sepharose Chelating resin (Sigma) loaded with 0.2 M $CuSO_4$ (copper resin). Samples (ca. 800 µg protein) were incubated for 20 min with 0.5 mL of copper resin previously equilibrated with 1 mL of binding buffer (0.02 M Na_2HPO_4, 0.5 M NaCl, pH 7.2). Unbound proteins were removed by gravity flow and the column washed three times with 0.5 mL of binding buffer. Bound proteins were eluted first with 0.02 M Na_2HPO_4, 0.5 M NaCl, pH 3.5 and then with 0.02 M Na_2HPO_4, 0.5 M NaCl, 0.05 M EDTA, pH 7.2. Proteins from 200 µL of each elution fraction were precipitated with 60 µl of 20% (w/v) TCA, resuspended in 50 µL of SDS-Sample buffer (0.25 M Tris-Cl pH 6.8, 50% (v/v) glycerol, 10% (w/v) sodium dodecyl sulphate, 5% (v/v) β-mercaptoethanol, 0.25% (w/v) bromophenol blue), heated at 99°C for 5 min and applied to 18% (w/v) polyacrylamide gels. Electrophoresis in denaturing conditions (SDS-PAGE) was carried out according to Laemmli [54]. Gels were stained by *GelCode Blue* (Pierce).

Mass spectrometry

Bands were excised from the polyacrylamide gels, cut into small pieces and de-stained by repeated washing cycles alternating 50 mM ammonium hydrogen carbonate and pure acetonitrile. After complete destaining, gel particles were dehydrated by acetonitrile, covered with trypsin solution (12.5 ng/mL in 50 mM ammonium hydrogen carbonate, pH 8.0) and incubated 1 h on ice. Excess liquid was removed and the gel pieces covered with a solution of 50 mM ammonium hydrogen carbonate (pH 8.0) and incubated overnight at 37°C. Tryptic peptides were extracted by alternating incubation in pure acetonitrile and 1% formic acid. Samples were lyophilised, resuspended in 1% formic acid, and desalted by *ZipTip* (Millipore) before ESI-MS analysis.

ESI-MS experiments were performed with a hybrid Quadrupole-Time-of-Flight (q-TOF) mass spectrometer (*QSTAR ELITE*, Applied Biosystems) equipped with a nano-electrospray ionisation sample source. Metal-coated borosilicate capillaries (Proxeon, Denmark) with medium-length emitter tip of 1-□m internal diameter were used for off-line analysis. The instrument was calibrated with standard solution Renin (MH2+ 879.97 Da and its fragment MH+ 110.07 Da, Applied Biosystems). Peptide identification was performed using the *MASCOT* software with the following parameters: 2 missed cleavages, peptide tolerance 0.6 Da, MS/MS tolerance 0.6 Da, peptide charges 2+ and 3+. Only monoisotopic masses were considered as precursor ions.

Spectra of tryptic peptides were acquired in the 400-1,500 *m/z* range, with 1.0 sec accumulation time, ion-spray voltage 1,3000 V, declustering potential 60 V, with active Information Dependent Acquisition (IDA), using rolling collision energy to fragment peptides for MS/MS analysis.

Determination of Reactive Oxygen Species

Samples containing 5×10^6 cells were withdrawn from exponential cultures. Cells were harvested by centrifugation, washed twice with de-ionized water, four times with 10 mM citric acid in 0.5% (w/v) NaCl, once with 1 mL of PBS and finally incubated with 0.5 mL of 5 µM (v/v) dihydroethidium (Sigma, stock solution 30 mM in DMSO) for 30 min in the dark. Stained cells were washed three times with PBS and sonicated for 15 sec. The fluorescence signal was detected using a Cary Eclipse spectrofluorimeter (Varian, CA, USA) at excitation wavelength of 518 nm and emission wavelength of 605 nm. Fluorescence values were normalized against those of not stained cells.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

GMA performed most of the physiological and biochemical characterization of yeasts. SB participated in the design of experiments and in the analysis of results. SP designed and carried out the experiments of cytofluorimetry. BS drew attention on the importance of Cu tolerance in yeast and suggested the experimental strategy of adaptation. ML conceived this study, contributed to the interpretation of results and drafted the paper. All authors read and approved the final manuscript.

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Legends

Figure 1. Assay of copper tolerance in yeast strains. Five µl of 1:10 serial dilutions were plated on minimal medium without or with 0.5 g · L^{-1} $CuSO_4$ (**a**) or YPD medium containing 0-2.5 g · L^{-1} $CuSO_4$ (**b**). Plates were incubated two days at 30°C. AL5 is the *C. humilis* strain; BL7, EL1 and GL6 are different *S. cerevisiae* strains.

Figure 2. Growth of yeast cells in YPD + 2.5 g · L^{-1} $CuSO_4$. Evolved (black cirles), non-evolved (black triangles) and de-adapted (black squares) cells of *C. humilis* AL5 (**a**), *S. cerevisiae* BL7 (**b**), *S. cerevisiae* EL1 (**c**), *S. cerevisiae* GL6 (**d**). The values reported are averages of three replicates. Calculated standard deviations are ≤ 0.6, making error bars not appreciable.

Figure 3. Intracellular copper measured during growth in YPD + 2.5 g · L^{-1} $CuSO_4$. *C. humilis* AL5 (**a**); *S. cerevisiae* BL7 (**b**); *S. cerevisiae* EL1 (**c**) and *S. cerevisiae* GL6 (**d**). White bars: evolved cells; black bars: de-adapted cells; grey bars: non-evolved cells. The amount of Cu is reported as mg · g^{-1} biomass. Values are the average of three replicates. Note the change of scale in (**a**).

Figure 4. Cytofluorimetric analysis. Evolved and non-evolved *C. humilis* (**a**) and *S. cerevisiae* (**b**) cells. Cells were collected during exponential growth in YPD + 2.5 g · L^{-1} $CuSO_4$ and stained with propidium iodide. The x- and y-axes of the histogram display the log fluorescence intensity (PI) and the number of collected cells (events) per sample, respectively. Propidium positive cells are on the right side of the distribution whereas viable cells are on the left side. Fluorescence distributions are representative of three replicates obtained in independent experiments. This analysis was carried out with *S. cerevisiae* cells despite their extreme poor growth in copper medium thanks to the very small number of cells required.

Figure 5. Fluorimetric analysis of superoxide anion ($OH^{-\bullet}$) production. Evolved and non-evolved *C. humilis* (**a**) and *S. cerevisiae* (**b**) cells exponentially growing in YPD (white bars) and in YPD + 2.5 g · L^{-1} $CuSO_4$ (grey bars). $OH^{-\bullet}$ formation is expressed as fluorescence intensity of ethidium in arbitrary units. Data presented are the mean of at least three independent analyses.

Figure 6. SDS-PAGE of copper-binding proteins. (**a**) *S. cerevisiae*. Lane 1: proteins from non-evolved cells grown in YPD; lane 2: proteins from evolved cells grown in YPD; lane 3: proteins from evolved cells grown in YPD + $CuSO_4$ 2.5 g · L^{-1}. (**b**) *C. humilis*. Lane 1: proteins from non-evolved cells grown in YPD; lane 2: proteins from evolved cells grown in YPD; lane 3: proteins from non-evolved cells grown in YPD + $CuSO_4$ 2.5 g · L^{-1}; lane 4: proteins from evolved cells grown in YPD + $CuSO_4$ 2.5 g · L^{-1}.

Table 1. **Antioxidant enzyme activities in *S. cerevisiae* and *C. humilis* cells grown in YPD and in YPD + $CuSO_4$ 2.5 g · L^{-1} (Cu).**

Activities measured were superoxide dismutase (SOD), catalase, peroxidase and glutathione peroxidase (GPO). Values, reported as U · mg^{-1} of proteins, are average of three independent measurements. n.d.: not determined due to lack of viable cells.

	S. cerevisiae				*C. humilis*			
	non-evolved		evolved		non-evolved		evolved	
	YPD	Cu	YPD	Cu	YPD	Cu	YPD	Cu
SOD	20.93±0	n.d	39.22±0	64.14±0.92	132.97±17.09	117.2±21.26	32.34±1.08	41.1±8.42
Catalase	3.2±0.45	n.d	3.23±0.45	11.82±1.26	81.5±5.17	73.76±6.74	74.14±11.46	27.7±4.4
Peroxidase	0.001±0	n.d	0.0032±0	0.004±0	0.011±0.001	0.019±0.003	0.014±0	0.008±0
GPO	0.041±0.001	n.d	0.012±0.001	0.013±0.009	0.19±0.024	0.534±0.039	0.235±0.039	0.172±0.07

Table 2. Identification of copper-binding proteins

Band n°	Protein[a]	MW (kDa)	n° peptides[b]
1	Transketolase 1	73.87	4
	Protein disulfide-isomerase	58.53	1
2	NADP glutamate dehydrogenase 1	49.88	14
	NADP glutamate dehydrogenase 2	49.93	6
3	Aspartate-semialdehyde dehydrogenase	40.03	3
	Cystathionine- γ-lyase	42.51	4
4	Glyceraldehyde-3-phosphate dehydrogenase	35.83	4
5	Elongation factor 1-β	22.67	3
6	Uncharacterized oxidoreductase YMR226C	29.19	3
7	Triose phosphate isomerase	26.89	3
8	Cu,Zn-superoxide dismutase	15.95	3
9	Peptidyl-prolyl cis-trans isomerase	17.49	2
	40S ribosomal protein S26-A	13.72	1
10	Hypothetical protein YIL051C (or MMF1)	15.95	3
11	60S ribosomal protein L19	21.69	1
12	Hypothetical protein YDR032C	20.96	1
13	Eucaryotic translation initiation factor 5A-2	17.21	1
14	Glyceraldehyde-3-phosphate dehydrogenase 2	35.93	3
	Glyceraldehyde-3-phosphate dehydrogenase 3	35.83	4
15	Peroxiredoxin TSA1	21.69	1
16	Ubiquitin-40S ribosomal protein S31	17.43	2

[a] ESI-MS indicates that bands 1, 2, 3, 9 and 14 contain more than one protein

[b] number of peptides analyzed by ESI-MS

Additional file 1

Table. Specific growth rate (h^{-1}) and final cell density (cells · mL^{-1}) · 10^7 of non-evolved, evolved and de-adapted cells from *C. humilis* AL5, and *S. cerevisiae* BL7, EL1 and GL6 strains grown on YPD and/or YPD + 2.5 g · L^{-1} $CuSO_4$(Cu). n.d.: not determined.

Additional file 2

Growth of yeast cells in YPD supplemented with 1, 1.5 and 2 g · L^{-1} $CuSO_4$. Evolved (black cirles), non-evolved (black triangles) cells of *S. cerevisiae* BL7 (**a**), *S. cerevisiae* EL1 (**b**), *S. cerevisiae* GL6 (**c**). The values reported are averages of three replicates. For values of standard deviations ≤ 0.6 error bars are not appreciable.

Additional file 3

Intracellular copper measured during growth in YPD + 1 g · L^{-1} $CuSO_4$. *S. cerevisiae* BL7 (**a**); *S. cerevisiae* EL1 (**b**) and *S. cerevisiae* GL6 (**c**). White bars: evolved cells; grey bars: non-evolved cells. The amount of Cu is reported as mg · g^{-1} biomass. Values are the mean of three replicates.

Additional file 4

Fluorimetric analysis of superoxide anion ($OH^{-\bullet}$) production in *S. cerevisiae* BL7 growing at different $CuSO_4$ concentration. Detection of $OH^{-\bullet}$ was carried out after growth in YPD and in YPD supplemented with 1, 1.5 and 2 g · L^{-1} $CuSO_4$. White bars: evolved cells; grey bars: non-evolved cells. $OH^{-\bullet}$ formation is expressed as fluorescence intensity of ethidium in arbitrary units. Data presented are the mean of at least three independent analyses.

Figure 1

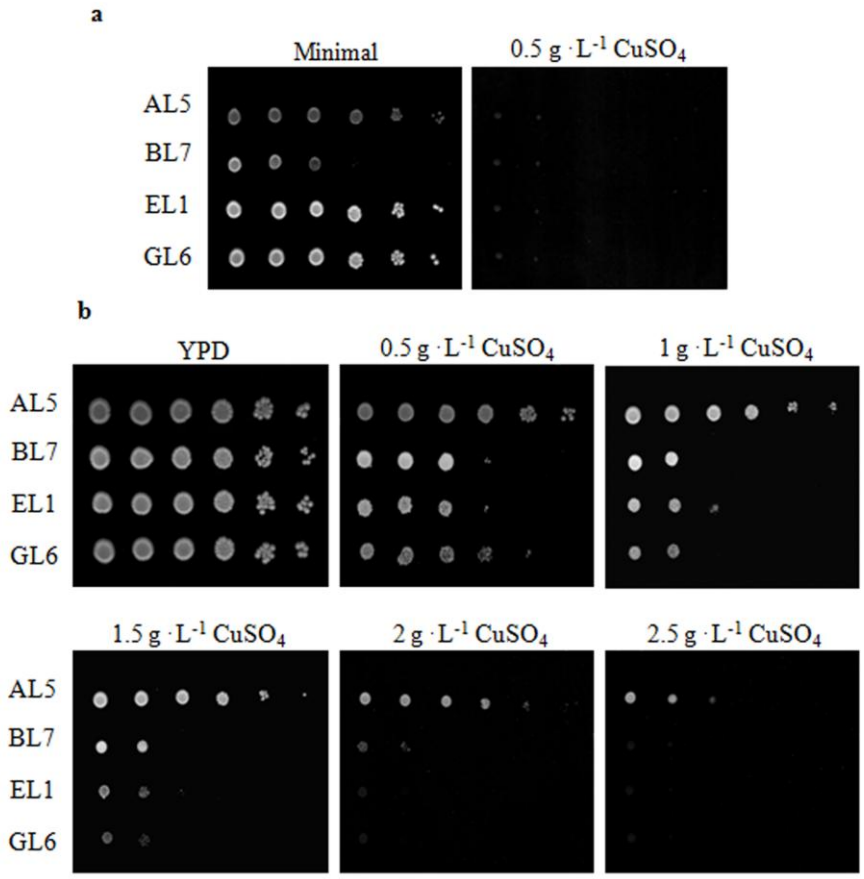
a
Minimal
0.5 g · L-1 CuSO4
AL5
BL7
EL1
GL6
b
YPD
0.5 g · L-1 CuSO4
1 g · L-1 CuSO4
AL5
BL7
EL1
GL6
1.5 g · L-1 CuSO4
2 g · L-1 CuSO4
2.5 g · L-1 CuSO4
AL5
BL7
EL1
GL6

Figure 2

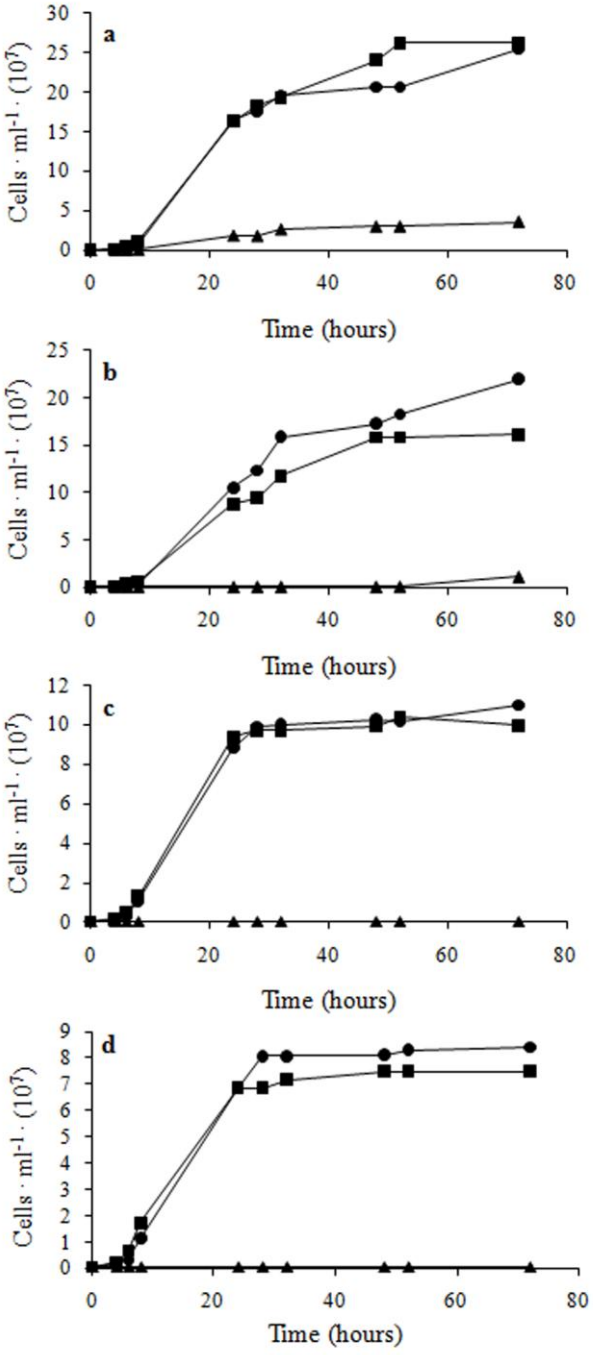

Figure 3

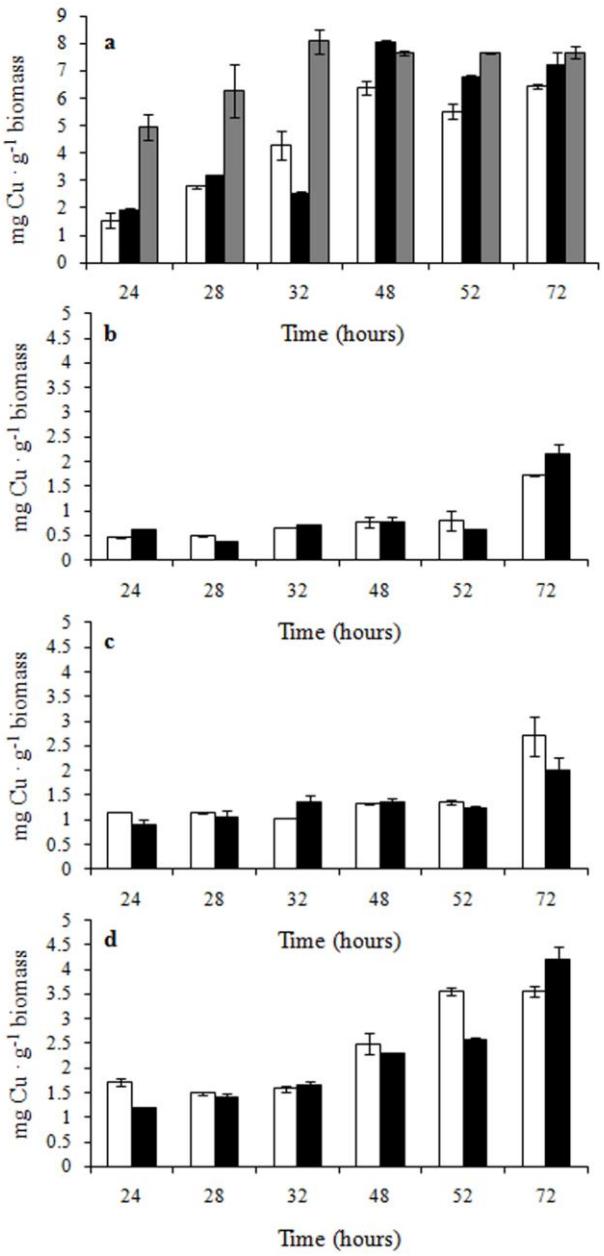

Figure 4

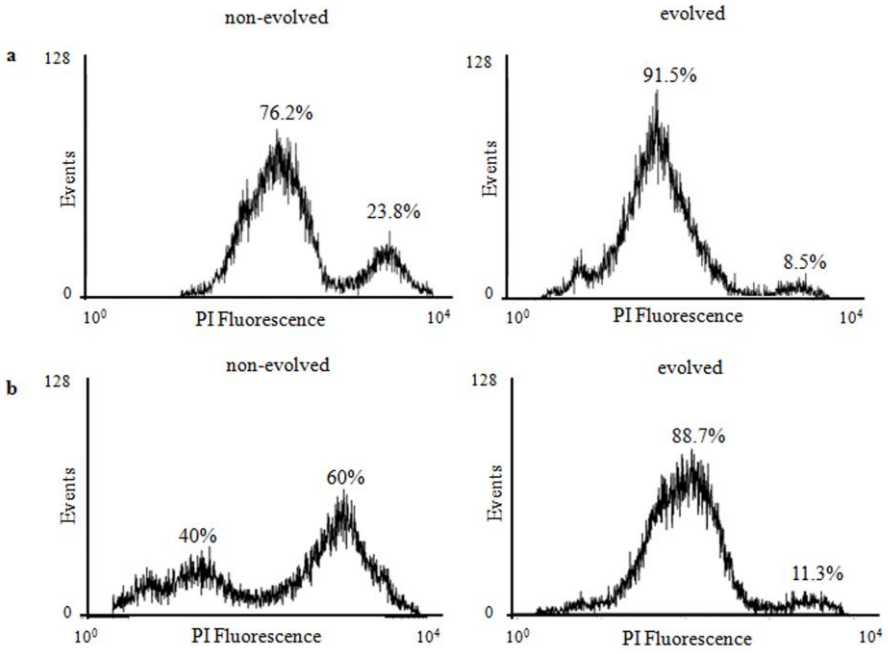
a
non-evolved
128
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Figure 5

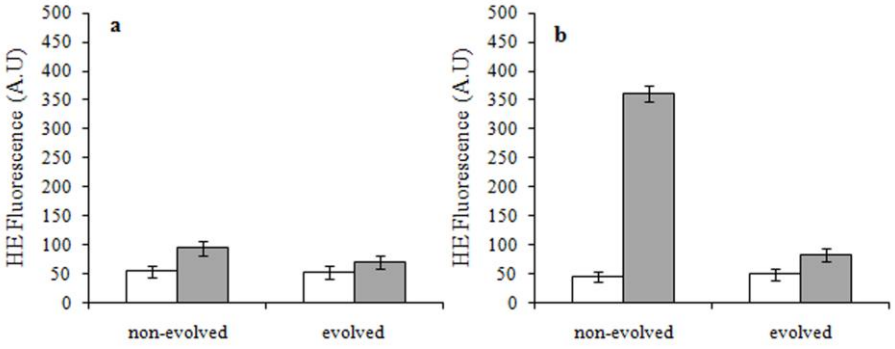
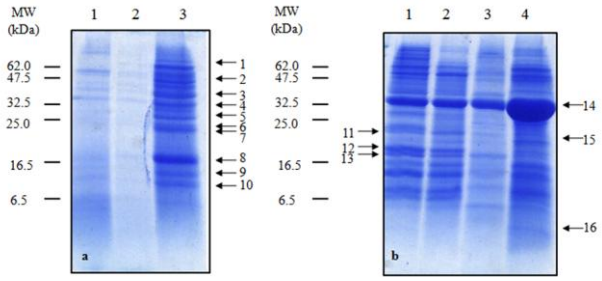


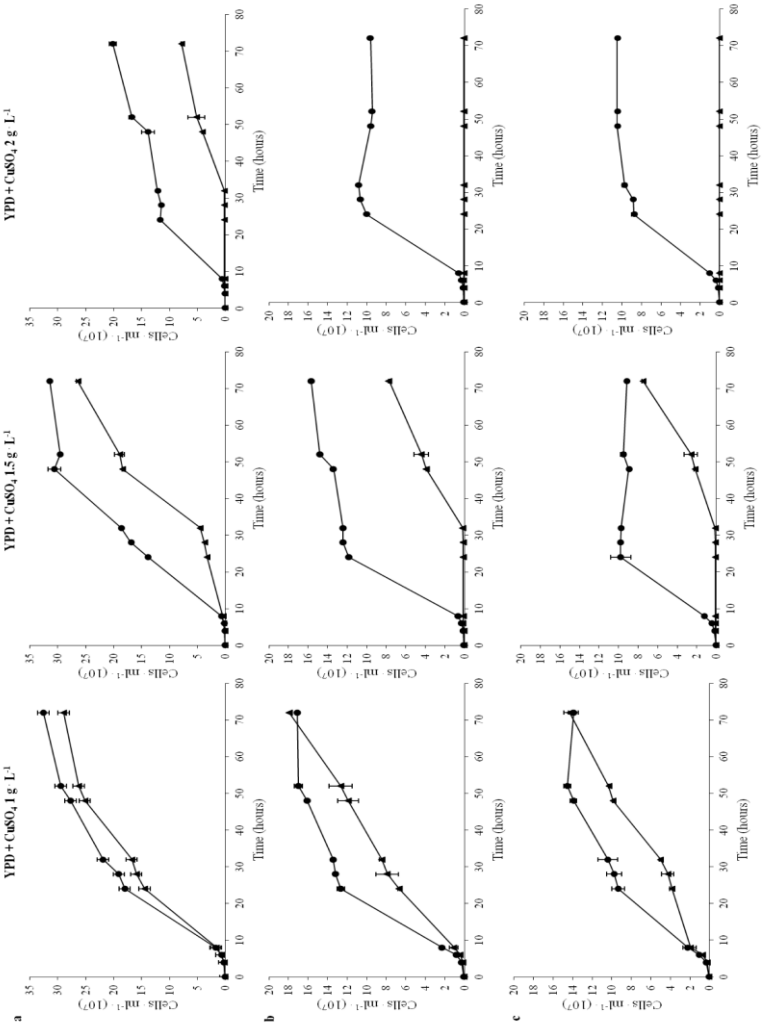
Figure 6



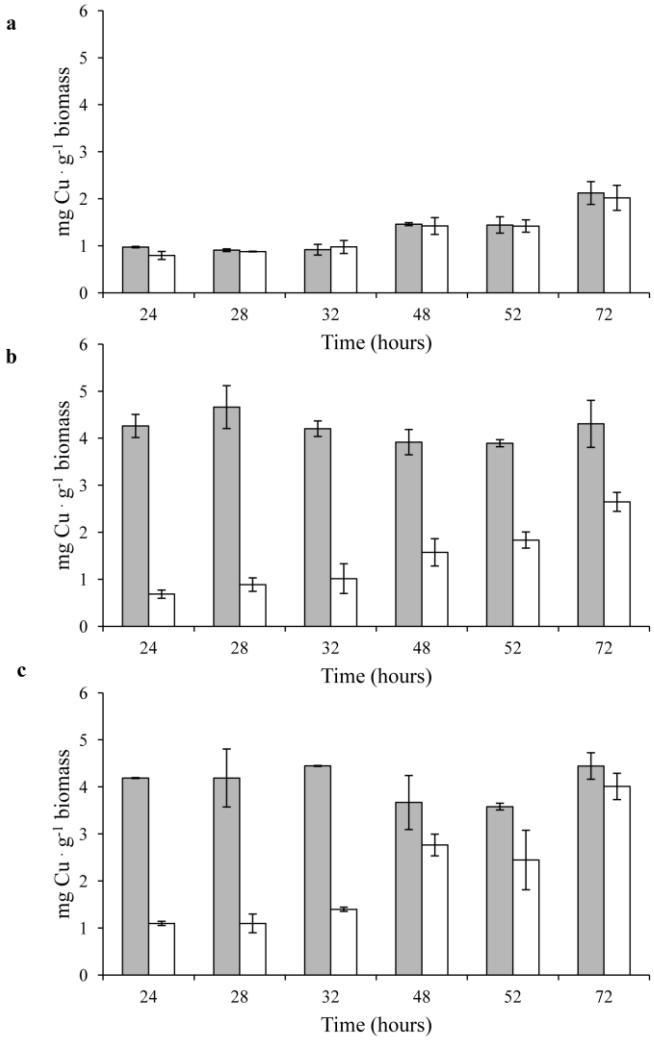
Additional file 1

	Specific growth rate (h^{-1})						Final cell density [(cells · mL^{-1}) · 10^7]					
	non-evolved		evolved		de-adapted		non-evolved		evolved		de-adapted	
	YPD	Cu	YPD	Cu	Cu		YPD	Cu	YPD	Cu	Cu	
AL5	0.50	0.10	0.49	0.33	0.40	AL5	20.75	3.60	21.00	25.57	26.23	
BL7	0.46	n.d.	0.45	0.24	0.31	BL7	24.03	n.d.	24.11	21.97	16.10	
EL1	0.50	n.d.	0.48	0.38	0.40	EL1	13.37	n.d.	13.40	10.98	9.95	
GL6	0.54	n.d.	0.53	0.39	0.43	GL6	10.40	n.d.	10.45	8.40	7.84	

Additional file 2



Additional file 3



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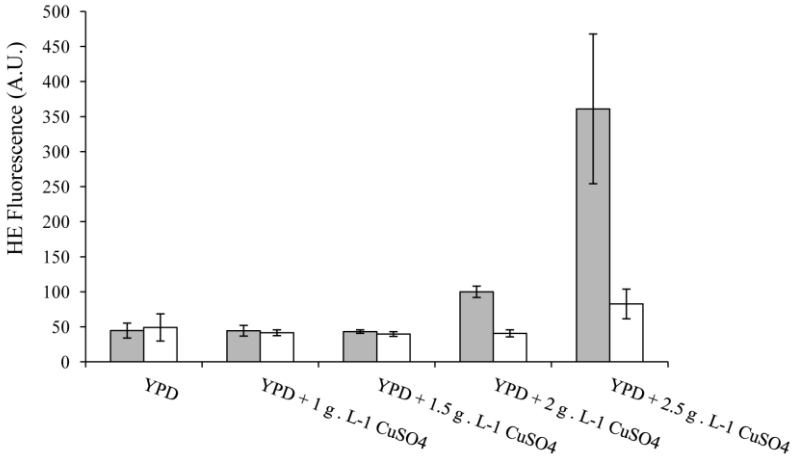

F

Evolution of copper tolerance in *Saccharomyces cerevisiae* relies on amplification of the *CUP1* gene

Manuscript

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Abstract

Background

In living organisms, copper (Cu) contributes to essential functions but may elicit also toxic effects that inhibit the ability of microbial cells to proliferate in the presence of high metal concentrations. Copper-tolerant yeast strains are of relevance both for application in industrial biotechnology and in the study of physiological and molecular mechanisms leading to stress resistance. One way to obtain tolerant strains is to exploit experimental methods that rely on the principles of natural evolution (evolutionary engineering) and allow for the development in microbial cell populations of complex phenotypic traits such as stress resistance and new metabolic abilities. However, in most cases the molecular and physiological bases of the phenotypic changes produced are far to be unraveled.

Results

We investigated the determinants of Cu resistance in a *Saccharomyces cerevisiae* strain *evolved* to tolerate up to 2.5 $g \cdot L^{-1}$ $CuSO_4$ in the culture medium. We found that intracellular copper content and the expression level of *SOD1*, encoding for the superoxide dismutase, were similar in the Cu tolerant (*evolved*) and in the Cu sensitive (*non-evolved*) strain. The major difference detected in the two strains was the copy number of the gene *CUP1* that encodes a metallothionein, with a 7-fold gene amplification observed in the Cu-tolerant cells. Accordingly, the expression of *CUP1* in *evolved* cells exposed to Cu was strongly and steadily increased.

Conclusions

Overall our results highlight a central role of *CUP1* in the protection of the *evolved S. cerevisiae* cells against Cu toxicity. In these cells, robustness towards copper was stably inheritable and could be reproducibly selected by controlling environmental conditions. This finding corroborates the effectiveness of laboratory evolution of whole cells as a tool to develop microbial strains for biotechnological application.

Keywords: Evolutionary engineering, Cu tolerance, gene amplification, oxidative stress, *Saccharomyces cerevisiae*

Background

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, cysteinyl and histidyl side chains of proteins [1]. Moreover, Cu ions can elicit oxidative damage either directly through their redox cycling activities that produce the highly reactive $OH^{\bullet}$ radical [2] or indirectly, by depleting free radical scavengers such as glutathione and protein-bound sulphydryl groups [3]. Therefore, a tight regulation of Cu homeostasis is required for cell survival. Accordingly, it was reported that in yeast free copper is restricted to less than one atom per cell [4].

Copper homeostasis has been investigated in depth in the yeast *Saccharomyces cerevisiae*, and complex mechanisms protecting cells from excess Cu have been identified, such as the induction of metallothioneins [5-7] and of antioxidant enzymes [8], and the activation of vacuolar functions [9]. Metallothioneins (MTs) form a family of cystein-rich, low molecular weight polypeptides common in eukaryotic organisms [10, 11], 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 [12]. The transcription of both MTs is regulated by Cu through the action of the transcription factor Ace1 [6, 13]. Upon Cu exposure, Ace1 induces also the transcription of the *SOD1* gene [14] encoding a cytosolic copper-zinc superoxide dismutase that breaks down superoxide ions to dioxygen and hydrogen peroxide [15, 16].

It has been reported that microorganisms can be experimentally endowed with tolerance to different stress conditions through evolutionary engineering. The term "evolutionary engineering" was introduced for the first time by Butler and collaborators [17] 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 [18], this approach relies on the onset of spontaneous or experimentally induced random mutations followed by recombination and continuous evolution of large populations over many generations [19]. For example, strains of *S. cerevisiae* were recently *evolved* to gain resistance towards multiple stresses [20] and to cobalt [21], and the ability to ferment xylose [22] and lactose [23]. 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 [19]. "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" [24].

In a previous work, we exploited evolutionary engineering to obtain yeast cells tolerant to the presence of high Cu concentrations (up to 2.5 $g \cdot L^{-1}$) in the growth medium [25]. Here we report that in one *evolved S. cerevisiae* strain enhanced tolerance is associated to an increased copy number of the *CUP1* gene resulting in its strong and stable expression. Increase in the amount of this metallothionein is proposed to be the major factor of protection against Cu-induced oxidative stress.

Results

Effects of copper on yeast cells growth

The kinetics of growth of *non-evolved* (Cu sensitive) and *evolved* (Cu tolerant) *S. cerevisiae* cells were compared and are presented in Figure 1. While in YPD medium the growth of the two strains was very similar, the addition of $CuSO_4$ at the final concentration of 2.5 $g \cdot L^{-1}$ to cultures in the exponential phase strongly impaired the growth of *non-evolved* cells, without perturbing the proliferation of the *evolved* ones (Fig. 1a). *Non-evolved* cells exposed to Cu for one hour did not resume multiplicating when plated on YPD (without Cu); on the contrary, in the same conditions *evolved* cells better recovered (Fig. 1b).

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 for metallothioneins. RNA was extracted from cells growing exponentially in absence of Cu or at different hours after the addition of Cu, mRNA was retrotranscribed to the corresponding cDNAs and the expression of the two genes was evaluated by quantitative PCR. In absence of Cu, expression of *CUP1* was hardly detectable in both *non-evolved* and *evolved* cells (Fig. 2 – see 0 h). In response to copper, the level of *CUP1* in the *evolved* strain was rapidly and strongly induced, keeping high along the monitored period of 24 hours (Fig. 2 – white histograms). Copper triggered the expression of *CUP1* also in *non-evolved* cells, although at lower extent (Fig. 2 – grey histograms). Under the same conditions, *CRS5* was expressed at very low level in both *evolved* and *non-evolved* strains (data not shown). Such different responsiveness is well in agreement with the hypothesis of a primary role of Cup1 induction in the response to copper stress [26, 27].

Copy number of CUP1 and CRS5

It has been reported that different yeast strains may bear different copies of the *CUP1* gene [28, 29], whereas *CRS5* is always present in single copy [6]. To investigate whether the copy number of these metallothionein genes could contribute to copper tolerance, we determined by RT-qPCR the number of copies of the *CUP1* and *CRS5* genes in the *evolved* strain and in two "intermediates" of the adaptation process, namely cells tolerant to 1.5 and 2 $g \cdot L^{-1}$ $CuSO_4$ (referred to as *int 1.5* and *int* 2) [25]. As shown in Figure 3, the *CUP1* copy number did not change in *int 1*.5, whereas a sudden 7-fold increase was noticed in *int 2*. No further amplification was detected in cells tolerant to the highest Cu concentration used in this work (2.5 $g \cdot L^{-1}$). No amplification of the *CRS5* copy number was observed in the *evolved* strain (Fig. 3).

Expression level of SOD1

Besides metallothioneins, 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. 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, one hour of Cu exposure resulted in a two-fold 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 just after prolonged cultivation in copper medium.

Copper-induced carbonylation of proteins

ROS produced upon copper stress are known to promote damages to different cell components, including proteins [30] that might undergo carbonylation of amino acids side chain [31]. Therefore, we used an immunoblot assay to detect oxidized proteins, assuming the carbonylation of proteins as indicative of oxidative stress. Total proteins were extracted from cells exponentially growing either in absence of Cu or at different hours 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 copper (Fig. 5 – see 0h in both strains). However, carbonylation increased in samples of *non-evolved* cells at already one hour after copper exposure, and the signal became stronger after prolonged Cu exposure. On the contrary, in *evolved* cells the signal associated to oxidized proteins increased only after several hours of growth in copper medium, although carbonylation was still much lower than in *non-evolved* cells (Fig. 5, compare 24h in both strains).

Quantification of intracellular copper

We next determined the levels of copper accumulated by yeast cells. Within the first hour of Cu exposure, intracellular copper was comparable in both yeast samples (Fig. 6). These data seem to bring into question the previous findings showing that the *evolved* strain has growth advantages and encounters a milder oxidative stress in presence of Cu than the natural strain. However, taking into account that the biochemical assay used for Cu quantification [25] 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 as metallothioneins), 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.

Discussion

Adaptive evolution is a central biological process underlying several phenomena, from the gain of antibiotic resistance in microbial populations to the niche specialization. An intriguing question is what kind of genomic variations are associated with evolution of improved phenotypes and how reproducible the process is [32]. 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 [33-35]. Adaptive evolution can be experimentally attained at the laboratory scale [19]. In this work, we showed that the evolution of increased Cu tolerance in baker's yeasts is mainly mediated by the amplification of the *CUP1* gene, encoding the metallothionein Cup1. These results describe for the first time the bases of Cu tolerance in an industrial strain of *S. cerevisiae* deliberately subjected to a strategy of evolutionary engineering, and are in good agreement with findings by others on genetically modified yeast strains from both laboratory [5, 36, 37] and industrial [28] sources. Compared with its *non-evolved* counterpart, the *evolved* strain encountered a 7-fold amplification of *CUP1* gene, resulting in high expression during growth on Cu medium. Our results suggest that in *evolved* cells the Cup1 dosage provides effective protection against intracellular Cu overload, avoiding toxicity and resulting in growth advantage. On the contrary, a comparable amount of copper internalized by *non-evolved* cells is highly toxic and causes growth impairment.

Looking at the amplification of *CUP1* gene to monitor the whole progress of evolution, we found that a 7-fold amplification arises not only in the hyper-resistant evolved strain, but earlier and suddenly in the *int 2*, an intermediate of the evolutionary engineering procedure. It has been proved that adaptation to selection conditions requires first a small number of mutations that exert large positive effects, then a higher number of mutations with small effects [38-40]. We can conceive that *CUP1* gene amplification found in *int 2* corresponds to a major, primary mutation featuring a cell population that attains a local optimum of the fitness landscape. Other secondary mutations that improve copper tolerance from 2 to 2.5 $g \cdot L^{-1}$ $CuSO_4$ 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 copper tolerance have occurred? According to a neo-Darwinist theory, the overall *CUP1* expression in the natural, *non-evolved S. cerevisiae* cultures might result from stochastic differences in gene dosage among individuals and could average low expression by the large majority of cells, and a high expression by a small subgroup of cells bearing a higher *CUP1* copy number. Indeed, according to the concept of "quasispecies", the result of evolution consists in a distribution of related variants that occupy a distinct region in sequence space [41]. Exposure of the population to high copper 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 [42]. In this scenario, the stresses applied to the cell population might change the genome evolvability by activating mutation mechanisms already well documented in prokaryotic organisms [43], and that appear shared by eukaryotic cells too [44-47].
In the *evolved* strain the expression of *CRS5* was not significantly increased. This can be ascribed both to its lower gene dosage and to its lower responsiveness to Cu mediated by the transcription factor Ace1 [6, 48, 49]. The role of *CUP1* in determining Cu tolerance is dominant also with respect to *SOD1*. Our data, in agreement with results by Gralla and collaborators [14], show that in the *evolved* strain, induction of *SOD1* occurs just in the first hour of Cu exposure. Although the *evolved* and *non-evolved* strains express *SOD1* at similar level and accumulate the same amount of Cu, oxidative stress is stronger in *non-evolved* cells. This finding further corroborates the view that over-expression of Cup1 might allow for the effective buffering of cytosolic Cu. This effect would be sufficient to prevent ROS overproduction and to limit the need for free radical scavengers. On the other hand, upon prolonged Cu exposure, the *non-evolved* strain compensates the overload of dangerous Cu ions with a strong induction of *SOD1* expression. Nevertheless, the late (24h) time point measured (Fig. 4) should be considered with some care due to the low viability of the *non-evolved* strain at that Cu concentration.

Literature data on evolutionary engineering of microbial strains highlight the potential of this approach in selecting strains with specific phenotypes. Moreover, acquisition of cross-resistance to different stresses has been already well documented [50-52]. Recent studies demonstrate that evolution of tolerance to a given metal triggers cross-resistance to other metals and/or other stress conditions [21, 53]. For instance, Çakar and collaborators [21] 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, like iron, manganese, zinc and nickel but not copper and chromium. 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.
Finally, we can observe that amplification of MTs genes cannot be considered a general rule and it has not been encountered in the development of other heavy metal-resistant cells, such as cobalt-resistant *S. cerevisiae* [54]. Therefore, it is likely that metal tolerance may arise from both general and specific molecular mechanisms to be elucidated case by case.

Conclusions

In conclusion, this work demonstrates that the phenotypic traits of a Cu tolerant yeast obtained by evolutionary engineering are mainly associated with the amplification of the locus *CUP1*. Overall, the application of evolutionary engineering emerges as a powerful tool to stably improve the robustness of microbial cells. The evolved strains can find direct applications in several processes of biotechnological interest and provide a starting point for further rational improvements, provided that molecular determinants of *evolved* phenotypes are disclosed.

Methods

Strains and growth conditions

S. cerevisiae cells used in this work derive from a natural Cu-sensitive strain whose growth is inhibited at 1 g · L^{-1} $CuSO_4$ in the culture medium. This parental strain is quoted in the text as "*non-evolved*". In a previous work [25], 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 · L^{-1} $CuSO_4$) was defined as "*evolved*", while two strains isolated as intermediates of the evolutionary engineering procedures, and tolerant to 1.5 and 2 g · L^{-1} $CuSO_4$ [25] are indicated in the text as *int 1.5* 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 under shaking at 160 rpm and then inoculated at optical density (OD_{600}) of 0.05 in shaking flasks containing fresh medium. A solution of $CuSO_4$ was added to cultures exponentially growing in YPD medium. The final concentration of $CuSO_4$ in the culture broth was 2.5 g · L^{-1} if not differently indicated.

RNA extraction and cDNA production

Total RNA for gene expression analysis was extracted from cells either growing in YPD or at different hours after Cu addition using the RNeasy Mini Kit (Quiagen). The quality of extracted RNA was assessed on 1.5 % (w/v) agarose gel and RNA was quantified by NanoDrop® (ND-1000 v 3.3.2 – Saveen Wernev). A dilution of 0.320 µg· μL^{-1} of RNA was used to produce the corresponding cDNA according to the following protocol: a mix composed of 8.5 µL RNase-free water, 2.5 µL RNA and 1 µL of anchored oligo-dT Primer (ABgene) was incubated at 65°C for 5 min in a thermocycler (T3000 - Biometra®). After 1 min of incubation on ice, the mix was added of 4 µL 5× first-strand buffer (Invitrogen), 2 µl 0.1 M DTT, 1 µL 10 mM dNTPs and incubated at 42°C for 2 min. Retrotranscription was performed adding 1 µL SuperScript™ II RT (Retro Transcriptase) 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 were used for gene expression analysis.

Genomic DNA extraction

Genomic DNA was extracted from cells grown overnight at 30°C in 5 mL of YPD medium. Cells were centrifuged at 4,900 × g for 5 min, re-suspended in 200 µL of breaking buffer [2% (v/v) Triton X-100, 1% (w/v) sodium dodecyl sulphate (SDS)], 100 mM NaCl, 10 mM Trizma-HCl pH 8.0, 1 mM ethylene-diamine tetra acetic acid (EDTA) pH 8.0, 200 µL PCI (phenol/chlorophorm/isoamylalchool at 25/24/1) and transferred to a screw cap tube containing 200 µL of acid-washed glass beads (425-600 µm – Sigma). Cells were subjected to mechanical lysis by three cycles of 20 sec at maximum speed with a Fast Prep® - FP120 (Bio101-Savant). Two hundred µL of 10 mM TE (Trizma and EDTA) pH 8.0 were added to the crude extract and, after centrifugation at 10,000 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 $mg \cdot mL^{-1}$). Ten µL of 4 M ammonium acetate and 1 mL 100% ethanol were added to the sample and, after centrifugation, the DNA pellet was dried and re-suspended in 100 µL TE. The obtained DNA was checked on 1.5 % (w/v) agarose gel and quantified by NanoDrop® (ND-1000 v 3.3.2 – Saveen Wernev).

Gene expression analysis and determination of gene copy number by RT-qPCR

Relative and absolute quantifications were performed by quantitative real time PCR (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*, *CRS5*, *SOD1* and *ACT1* (Table 1) were retrieved from the *Saccharomyces Genome Database* (www.yeastgenome.org) and the internet-based interface Primer-3 [55] 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 µM forward and reverse oligonucleotides and water]. Amplification conditions were: 3 min at 95°C followed by 40 cycles at 95°C for 10 sec and at 58°C for 30 sec and 81 cycles at 55°C for 8 sec. Quantification of the target sequence in the samples was estimated

generating a standard curve with known concentrations of DNA. Values were normalized against the housekeeping gene *ACT1*.

Five µL of genomic DNA were 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 $2^{\Delta\Delta Ct}$ method [56, 57]. 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*.

Protein extraction and electrophoresis in denaturing conditions

Fifty mL samples were withdrawn from cultures at different times after Cu addition and processed for protein extraction. Biomass was re-suspended in 2 mL of 2 M NaOH, incubated for 2 min at room temperature and then 2 mL of 50% (w/v) trichloroacetic acid (TCA) were added. After centrifugation for 3 min at 10,000 × *g*, pellets were re-suspended in 1 mL of 1 M Tris-HCl pH 8.0 and centrifuged again. Proteins were re-suspended in 250 µL 1× SDS sample buffer (0.0625 M Tris-Cl 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 bovine serum albumin as the reference. Twenty µg of proteins (derivatized to detect carbonyl groups – see below) were applied to 12% (w/v) polyacrylamide gel. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to Laemmli [58].

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 2,4-dinitrophenyl-hydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenyl hydrazine (DNPH). The DNP-derivatized proteins were analyzed immunochemically by Western blot using rabbit anti-DNP as primary antibody (Sigma-Aldrich). A goat anti-Hog1 antibody (Santa Cruz – Biotechnology) was used as a control. Donkey anti-Rabbit and donkey anti-Goat antibodies Odyssey® (Li-Cor – Bioscience) were used as

secondary antibody. Visualization of carbonylated proteins was performed using Odyssey®- Infrared Imaging System 2.1 (Li-COR – Bioscience).

Copper determination

Intracellular Cu in *non-evolved* and *evolved* cells was measured as previously described [25], within the first hour of Cu exposure. The amount of Cu is referred as mg $Cu \cdot g^{-1}$dry biomass.

Acknowledgements

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Table 1. Oligonucleotides used for quantitative RT-PCR

Gene	Sequence (5'→3')
ACT1	FW 5'-CTGCCGGTATTGACCAAACT-3'
	RV 5'-CGGTGATTTCCTTTTGCATT-3'
CUP1	FW 5'-AAGGTCATGAGTGCCAATGC-3'
	RV 5'-ATTTCCCAGAGCAGCATGAC-3'
CRS5	FW 5'-TGACTGTTAAGGCGAATGTTG-3'
	RV 5'-TTCACAAGTGCACGTGGTTT-3'
SOD1	FW 5'-AACGTGGGTTCCACATTCAT-3'
	RV 5'-CACCATTTTCGTCCGTCTTT-3'

Legends

Figure 1. Effect of Cu on cells growth. **a)** Growth kinetics of *non-evolved* (black circles) and *evolved* (black squares) *S. cerevisiae* cells. Addition of Cu (supplied as $CuSO_4$ to a final concentration of 2.5 $g \cdot L^{-1}$) to cells exponentially growing on YPD medium is indicated by an arrow. Values are the mean of three biological replica. **b)** Drop test of *non-evolved* and *evolved* strains harvested before (0h) and after one hour (1h) of copper exposure. Five µl of 1:10 serial dilutions were plated on YPD and plates were incubated for two days at 30°C.

Figure 2. Relative expression of *CUP1*. *Non-evolved* (grey bars) and *evolved* (white bars) cells were harvested at different hours after the addition of copper (0h-24h). Gene expression was quantified by RT-qPCR and normalized for *ACT1* expression. Values are the mean of two biological replica performed in triplicate.

Figure 3. Determination of the gene copy number in *evolved* cells. Copy number of the *CRS5* gene was determined on the *evolved* strain tolerant to 2.5 $g \cdot L^{-1}$ of $CuSO_4$ (*CRS5 evolved*); for *CUP1* gene analysis was carried out on different "intermediates of evolution"-strains evolved to tolerate 1.5 $g \cdot L^{-1}$ of $CuSO_4$(*CUP1 int1.5*), 2 $g \cdot L^{-1}$ of $CuSO_4$ (*CUP1 int2*) - and on the *evolved* strain tolerant to 2.5 $g \cdot L^{-1}$ of $CuSO_4$(*CUP1 evolved*). Estimations were made according to the $2^{\Delta\Delta Ct}$ method. Values are the mean of two biological replica performed in triplicate.

Figure 4. Relative expression of *SOD1*. *Non-evolved* (grey bars) and *evolved* (white bars) cells were harvested at different hours after Cu addition (0h-24h). Gene expression was quantified by RT-qPCR and normalized for *ACT1* expression. Values are the mean of two biological replica performed in triplicate.

Figure 5. Protein carbonylation upon Cu exposure. *Non-evolved* and *evolved* cells were harvested at different hours after Cu addition (0h-24h). Equal amounts of proteins were assayed after derivatization with DNPH and western blotting with an anti-DNP-hydrazone antibody. Cntr-: not-derivatized protein samples. The expression level of Hog1 is included as a control of protein amount. The picture represents one of three independent experiments.

Figure 6. Measurement of intracellular Cu. Intracellular Cu was measured in *non-evolved* (grey bars) and *evolved* (white bars) cells at 0, 5, 10, 15, 20, 60 min after Cu addition. The amount of Cu is reported as $mg \cdot g^{-1}$dry biomass. Values are the mean of three biological replica.

Figure 1

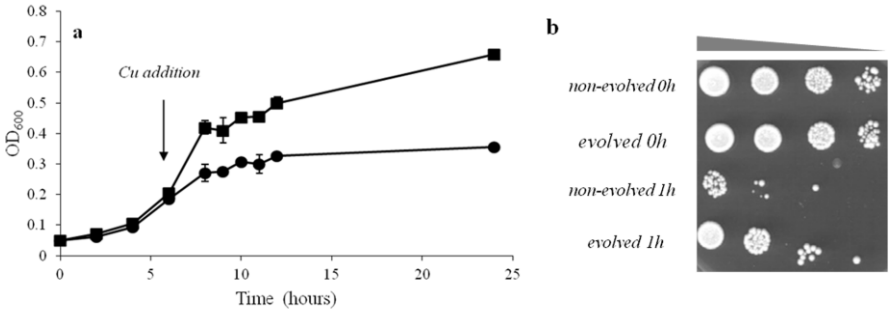


Figure 2

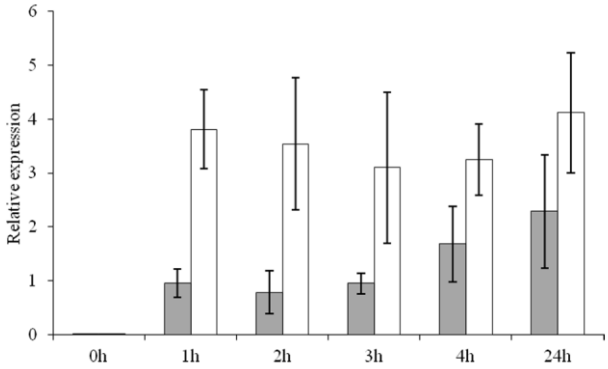


Figure 3

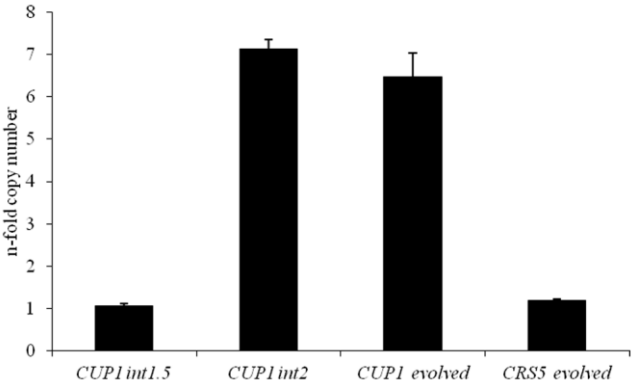


Figure 4

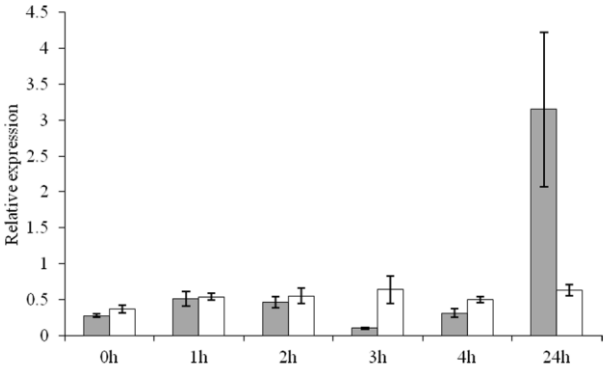


Figure 5

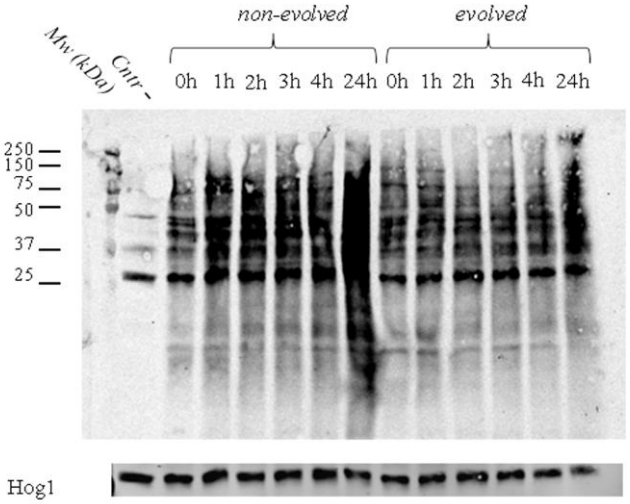
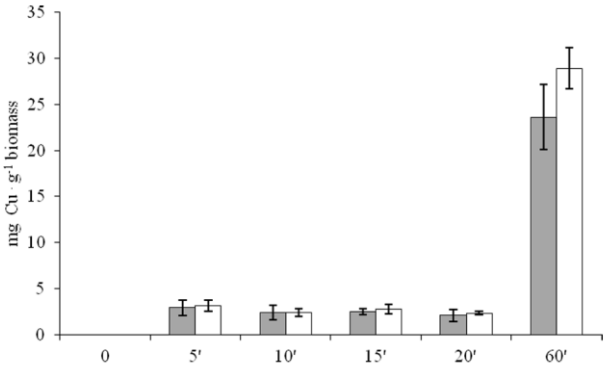


Figure 6



Proteomic analysis of natural and copper-adapted cells of the yeast *Candida humilis*

Preliminary results

Giusy Manuela Adamo, Stefania Brocca and Marina Lotti

Background

Copper (Cu), like other transition metals, is an essential micronutrient required for the survival of all living organisms (1). Indeed, cycling between the oxidized (Cu^{2+}) and the reduced (Cu^{1+}) state, copper acts as an important catalytic and structural cofactor for several biochemical processes essential for life (2). However, the redox properties of Cu are also responsible for its toxicity, since Cu participates in reactions that generate Reactive Oxygen Species (ROS) (3). ROS target different cell macromolecules, such as proteins, lipids, DNA and RNA, causing loss of essential cellular functions and/or gain of toxicity (4). In microbial cells, tolerance toward toxic concentrations of copper, as well as toward other stress conditions, can be experimentally improved by "evolutionary engineering" that is the application of the principles of natural evolution to drive the selection of organisms with a desired set of phenotypes (5). These *evolved* strains may find a direct application in several areas, from the industrial sector to bioremediation and allow gaining insight in the processes of adaptive evolution at the basis of the specific properties acquired by the evolved strains.

Beside *Saccharomyces cerevisiae*, extensively studied in its Cu metabolism (6, 7), other yeasts are attractive for their marked natural tolerance to high copper, but still poorly investigated.

In a previous work, starting from a strain of *Candida humilis* endowed with some natural resistance to the presence of copper, we exploited a strategy based on step-wise adaptation obtaining cells able to proliferate at high metal concentration

(8). In copper medium, the *evolved* strain featured growth advantage and decreased production of ROS in comparison with the natural strain (*non-evolved*). In the frame of a broader research program aimed at the characterization of the molecular determinants of copper robustness in yeasts, we performed a differential proteomic analysis on *non-evolved* and *evolved C. humilis* cells grown in high Cu concentration.

Methods

Strains and growth conditions

In this work were used *evolved* and *non-evolved C. humilis* strains respectively tolerant and partly sensitive to copper sulphate ($CuSO_4$). The *evolved* strain was obtained from the *non-evolved* one through evolutionary engineering as previously described (8). Cells from a fresh culture on agarized YPD medium were pre-cultured overnight in liquid YPD medium [2% (w/v) glucose, 1% (w/v) yeast extract, 2% (w/v) peptone] at 30°C under orbital shaking (140 rpm) and then inoculated at initial OD of 0.05 in shaking flasks containing YPD or YPD + $CuSO_4$ 2.5 g/L.

Extraction and quantification of total proteins

Two grams of cells from exponential cultures were harvested by centrifugation at 4,900 *g* for 10 min at 4°C. The cell pellet was washed twice in cold deionised water and re-suspended in a breaking buffer containing 8 ml of deionised water, 200 µl of 0.5 M Tris-Cl pH 8.5, 200 µl of 0.25 M ethylenediaminetetraacetic acid pH 8.4 added of protease inhibitor cocktail (Sigma). Cells were subjected to mechanical lysis with glass microbeads by four cycles of 20 sec at maximum speed with a Fast Prep® - FP120 (Bio101-Savant). Each lysis cycle was followed by two minutes of incubation on ice. Cell debris was removed by centrifugation at 700 *g* for 10 min at 4°C. Total proteins were precipitated from the crude extract with an excess of acetone maintaining samples for two hours at -20°C. Samples were centrifuged again for 20 min, acetone was discarded and when the pellet of precipitated proteins was completely dried, proteins were re-suspended in a solubilization buffer containing 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-

Cholamidopropyl)dimethylammonium]-1-propanesulfonate (CHAPS), 60 mM dithiothritol (DTT), 20 mM iodacetamide (IAA).

Protein concentration was estimated according to Bradford (9) using protein bovine serum albumin as the reference.

Two-dimensional gel electrophoresis

Six hundred µg of proteins were diluted in 300 µL of sample buffer containing 60 mM DTT, 60 mM IAA, 1% (v/v) carrier ampholytes and traces of bromophenol blue. For separation in the first dimension, samples were applied on a 17-cm pH 4-7 Immobilized pH Gradient (IPG) strip (BioRad) and isoelectrofocusing was performed with the use of a isoelectrofocusing apparatus (BioRad PROTEAN IEF Cell) under the following conditions: passive rehydratation with sample (1h), 200 V (1 h), 3500 V (2.5 h), 3500 V (3 h), 8000 V (4 h), 8000 V (6 h) and 500 V h (hold). The focused IGP strips were first equilibrated in equilibration buffer (6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS) in 0.05 M Tris-HCl pH 6.8) containing 2% (v/v) DTT for 20 min and then in equilibration buffer containing 2.5 % (w/v) IAA for 20 min. Gel strips were adjusted on the top of a 12% SDS-polyacrylamide gel and incorporated with 0.5% (w/v) agarose. Electrophoresis was carried out in a refrigerated system (EttanDALTsix electrophoresis unit, Amersham Biosciences) at 15-30 mA/gel. Gels were finally stained with GelCode Blue Stain Reagent (Pierce).

Spot matching

Images of the stained gels were analyzed using DECODON Delta 2D software (http://www.decodon.com). The differential analysis was performed for pairs of image: in each pair a reference image was warped with an image of interest first using automatic warping and later setting specific landmarks manually. The software provides the creation of a fused image were spots can be detected and transferred back to all analyzed gels. The intensity of each protein spot is normalized to the total intensity of all valid spots detected on each gel.

Proteins were considered to be differentially expressed if there was at least 1.5-fold absolute difference in the intensity of corresponding spots between the reference and the sample of interest. Expression changes were considered significant if the *p* value

of the corresponding spot provided by the program (running an Anova test) was lower or close to 0.05.

Mass spectrometry (MS)

Spots of interest were excised from the polyacrylamide gels, cut into small pieces and destained by repeated washing cycles alternating 50 mM ammonium hydrogen carbonate and pure acetonitrile. After complete destaining, gel particles were dehydrated by acetonitrile, covered with trypsin solution (12.5 ng/mL in 50 mM ammonium hydrogen carbonate, pH 8.0) and incubated 1 h on ice. Excess liquid was removed and the gel pieces covered with a solution of 50 mM ammonium hydrogen carbonate (pH 8.0) and incubated overnight at 37°C. Tryptic peptides were extracted by alternating incubation in pure acetonitrile and 1% (v/v) formic acid. Samples were lyophilised, re-suspended in 1% (v/v) formic acid, and desalted by *ZipTip* (Millipore) before Electro Spray Ionization-Mass Spectrometry (ESI-MS) analysis. ESI-MS experiments were performed with a hybrid Quadrupole-Time-of-Flight (q-TOF) mass spectrometer (*QSTAR ELITE*, Applied Biosystems) equipped with a nano-electrospray ionisation sample source. Metal-coated borosilicate capillaries (Proxeon, Denmark) with medium-length emitter tip of 1-µm internal diameter were used for off-line analysis. The instrument was calibrated with standard solution Renin (MH2+ 879.97 Da and its fragment MH+ 110.07 Da, Applied Biosystems). Peptide identification was performed using the *MASCOT* software with following parameters: 2 missed cleavages, peptide tolerance 0.6 Da, MS/MS tolerance 0.6 Da, peptide charges 2+ and 3+. Only monoisotopic masses were considered as precursor ions. Spectra of tryptic peptides were acquired in the 400-1,500 *m/z* range, with 1.0 sec accumulation time, ion-spray voltage 1,300 V, declustering potential 60 V, with active Information Dependent Acquisition (IDA) using rolling collision energy to fragment peptides for MS/MS analysis.

Determination of thiobarbituric acid reactive substances (TBARS)

Determination of TBARS to evaluate lipids peroxidation was carried out following the procedure previously described by (10). Cells exponentially growing on either YPD or YPD + 2.5 g/L $CuSO_4$ were harvested, mechanically disrupted and the crude extract mixed with a solution containing 15% (w/v) TCA, 0.375% (w/v)

Thiobarbituric acid and 0.25 (v/v) HCl. After boiling and cooling, samples were centrifuged and the presence in the supernatants of malondialdehide (MDA) – a product of lipids peroxidation - was monitored spectrophotometrically at 532 nm. Results were expressed as mM MDA/g biomass.

Results

In a previous work (8) through prolonged cultivation of *C. humilis* cells in presence of increasing amounts of copper sulphate, we obtained cells (hereafter referred to as *evolved*) that, compared with the natural strain (hereafter *non-evolved*) exhibited an increased robustness toward copper toxicity. Such a robustness lied in marked growth advantage and diminished rate of mortality during cultivation in copper medium and was accompanied by a negligible production of ROS. These features of copper tolerance acquired by the *evolved* strain resulted stably maintained.

Proteomic analysis

To characterize more in-depth changes induced by adaptation, we compared the proteomes of *non-evolved* and *evolved* cells cultured in both YPD and YPD + 2.5 g/L $CuSO_4$. Total proteins were extracted from cells collected at the exponential phase of growth and resolved in the pI and the molecular mass ranges of 4-7 and 11-245 kDa, respectively. With the aim to detect both intra- and inter- strain differences, we performed the differential analysis comparing the proteomic profile of the two strains in different conditions in the following "comparison couples"

(i) *non-evolved* and *evolved* strain grown in YPD

(ii) *non-evolved* strain grown in YPD and in YPD + 2.5 g/L $CuSO_4$

(iii) *evolved* strain grown in YPD and in YPD + 2.5 g/L $CuSO_4$

(iv) *non-evolved* and *evolved* strain grown in YPD + 2.5 g/L $CuSO_4$

For the couples (i) and (iv) the protein pattern of the *non-evolved* strain was used as the reference, whereas for the couples (ii) and (iii) the reference was the protein pattern obtained during growth in YPD. Figure 1 shows the proteomic profiles in four of the above-described conditions, whereas Figure 2 presents one representative gel in which relevant spots are indicated.

No statistically relevant differences were found between proteins of the couple (i), hence we have no indication that *non-evolved* and *evolved* cells may feature different physiology during growth in permissive conditions. Instead, several spots differed in the couple (ii), that considers changes induced by copper in the natural strain. In this case, we found that 9 proteins were up-regulated and 4 down-regulated by the presence of the metal. Out of these proteins, 4 were differentially represented also in the *evolved* cells challenged by copper (couple iii). The direction of regulation –either up or down – was the same detected in the *non evolved* population. Finally, no major changes were highlighted in the comparison couple (iv), suggesting that the improved Cu tolerance in the *evolved* strain cannot be ascribed to a sharp up- or down-regulation of a few group of proteins. Taken together these data suggest that the major variations in the cells proteome are associated with copper exposure rather than with the evolution of Cu tolerance.

The differentially regulated proteins were successfully identified by ESI-MS analysis followed by MASCOT database searches. Table 1 lists the proteins identified in comparison couples (ii) and (iii) and the corresponding statistically significative fold change. In table 1 proteins are classified according to their biochemical functions in: heat shock response (spot n° 6, 7, 9, 11, 13), protein translation (spot n° 8, 10), energy production (spot n° 16), phospholipids synthesis and sulphur aminoacids metabolism (spot n° 14), glycolysis (spot n° 2, 4) and alcoholic fermentation enzymes (spot n° 1-3). Out of them, all heat shock proteins, S-adenosylmethionine synthase (a protein participating in phospholipids synthesis and sulphur aminoacid metabolism), ATP synthase and proteins involved in protein synthesis were up-regulated during copper exposure. The glycolytic enzymes pyruvate kinase and glyceraldehydes-3-phosphate dehydrogenase as well as alcohol dehydrogenase were found to be down-regulated On Two-dimensional gels this last protein was identified in two different spots (n°1 and n°3 – see Figure 2) with different isoelectric points, suggesting the occurrence of post translational modifications.

Lipids peroxidation

As already mentioned, one of the targets of ROS are membrane lipids that can be converted into polar lipids hydroperoxides, causing increase in membrane fluidity,

efflux of cytosolic solutes and loss of membrane-protein activities (4). Moreover, reactive products of lipids peroxidation may attack amino acid side chains (11) and cause fragmentation of DNA (12). Determination of TBARS revealed a reduced extent of lipids peroxidation in the *evolved* than in the natural strain grown in the presence of copper (Figure 3).

Discussion

This work is intended as the first step in the molecular characterization of evolved tolerance to copper in *Candida humilis* cells. Cu is a strong oxidizing agent that promotes the formation of ROS (13). To cope with the effects triggered by ROS, cells have evolved several defence and repair mechanisms highly conserved from unicellular organisms to multicellular eukaryotes. In bacteria and yeasts the mechanisms of protection against oxidation damages partly depend on changes in gene expression (14, 15). However, a recent study demonstrated that, under certain stress condition, changes in the microbial transcriptome and proteome are surprisingly small (16). A similar conclusion can be drawn from the data reported in this work. Indeed, the protein expression profiles of *evolved* and *non-evolved* cells are very similar, with only a few proteins produced at different amount in response to copper. In the following, these results are commented with some more detail for the different groups of proteins

Heat shock proteins

Changes in the expression of heat shock proteins (HSPs) were elicited by copper both in *non-evolved* and in *evolved* cells. Both cytosolic and mitochondrial-localized HSPs were up-regulated in response to Cu. The activation of Heat shock genes in response to oxidizing agents (17, 18) as well as to other stress conditions has been already reported (19-23). The biological meaning of this response can be appreciated in the frame of a general homeostasis strategy to cope with oxidative stress injuries acting through the protein quality control (24), the resolubilization of protein aggregates (24, 25), the control of mitochondrial membrane integrity and of the cellular redox state (26). We have observed that *C. humilis* strains respond to Cu-induced oxidative stress increasing the level of HSPs, with a more pronounced

up-regulation in the *non-evolved* strain, which suggests a more stringent need to activate the defence systems for the sensitive strain.

ATP synthase

ATP synthase is a mitochondrial protein involved in the energy production (27). A greater amount of the β subunit of this protein was found to in the *non-evolved* strain during growth in copper medium. This observation is in agreement with literature data demonstrating the induction of ATP synthase in response to osmotic and oxidative stress conditions in plants (28, 29) and in yeast cells (30).

S-adenosylmethionine synthase

S-adenosylmethioninesynthetase (Sam) amount was reduced in *non-evolved* cells exposed to copper. This enzyme catalyzes the production of S-adenosylmethionine, AdoMet (31-33), a cell metabolite with antistress properties. Malakar and co-workers (34, 35) demonstrated the protective role of AdoMet in *S. cerevisiae* cells exposed to acids. The authors proposed that AdoMet promotes the production of phosphatidilcoline, required to repair the damages induced in the cell membrane by the exposure to toxic inorganic acids, maintaining plasma membrane integrity and thus preserving the activity of integral proteins, such as H^+-ATPase, that counteract acidic stress (36). Since plasma membrane lipids are one of the targets of oxidative stress as well, up-regulation of Sam in *C. humilis* could reflect the activation of a cell surface repair system. Being AdoMet also involved in glutathione production (37), the increased amount of Cu-dependent Sam could indicate the involvement of sulphur amino acid metabolism in protecting cells from Cu-induced ROS (38).

Glycolytic and fermentative enzymes

Recent studies carried out on *S. cerevisiae* demonstrated that glycolytic enzymes are among the major targets of oxidative stress (39), and metabolic reconfiguration of the carbohydrate flux is a key strategy to counteract oxidative stress (40-42). According to our data, in *C. humilis* exposure to Cu down-regulates pyruvate kinase and glyceraldheyde-3-phosphate dehydrogenase, with a stronger effect in *non-evolved* cells. Glyceraldehyde-3-phosphate dehydrogenase catalyzes the conversion of glyceraldehydes-3-phosphate to 1,3-biphosphoglycerate and pyruvate kinase catalyzes the conversion of phosphoenolpyruvate to pyruvate in one of the check-

point reactions of the glycolytic pathway (43). A recent work demonstrated that in yeast cells, the reduction of pyruvate kinase activity triggers a metabolic feedback loop that reduces ROS production (40) re-routing the carbohydrate source to the pentose phosphate pathway with production of NADPH. This latter provides the redox power for known antioxidant systems (44, 45). Under oxidative stress, down-regulation of the glyceraldehyde-3-phosphate dehydrogenase also contributes to balance the intracellular redox equilibrium In response to various oxidants, this enzyme is inactivated and transported into the nucleus. Moreover, it can undergo post-translational modifications (such as S-nitrosylation, S-thionylation, S-glutathionylation, carbonylation) that alter its activity (46-50). In summary, we can conclude that the observed down-regulation of the pyruvate kinase and glyceraldheyde-3-phosphate dehydrogenase in *C. humilis* cells is well in agreement with the need to produce reducing equivalents to cope with the formation of ROS triggered by copper. Down-regulation of alcohol dehydrogenase in both *non-evolved* and *evolved* cells exposed to Cu is consistent with the hypothesis that the alteration of metabolites homeostasis contributes to balance the redox equilibrium in cells subjected to oxidative stress. Since alcohol dehydrogenase is involved in ethanol production (51), one could also hypothesize that the reduced activity of pyruvate kinase results in a decreased availability of the substrates for alcoholic fermentation, with consequent decrease of the related enzymes.

In general, our results indicate a major effect of Cu on proteins involved in the oxidative stress response. This effect is more prominent in the *non-evolved* strain, suggesting that in the *evolved* yeast are active additional defence mechanisms to counteract copper toxicity and that reduce therefore the need to trigger the oxidative stress response. The hypothesis that different defence mechanisms could have been implemented by adaptation is supported also by the lower degree of lipids peroxidation detected in the *evolved* strain. Moreover, a preliminary characterization (8) of the *evolved* strain showed a lower ROS production, a non-conventional responsiveness of antioxidant enzymes and lower intracellular level of Cu. However, the determinant of Cu tolerance in the *evolved* strain are still largely unknown and further experiments aimed to their disclosure are required.

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Legends

Figure 1. Electrophoretic separation of total proteins from the *non-evolved* and *evolved C. humilis* cells grown either in YPD or YPD + 2.5 g/L $CuSO_4$. Proteins were resolved in the pI range of 4-7 and the molecular mass range of 11-245 kDa on a 12% polyacrilamide gel.

Figure 2. Total proteins of *non-evolved C. humilis* cells grown in copper medium with relevant spots circled. Numbers of spots refer to proteins in Table 1.

Figure 3. TBARS production in *non-evolved* and *evolved C. humilis* cells during growth in YPD (white bars) and YPD + 2.5 g/L $CuSO_4$ (grey bars). Data represent the average of three biological replica and are expressed as mM MDA/g biomass.

Table 1. Proteins indentified as significantly up- or down- regulated in *non-evolved* and *evolved C. humilis* cells upon Cu exposure.

					Fold change[c]	
Spot n°	**Protein**	**Function**	**MW[a]**	**pI[b]**	**(ii)[d]**	**(iii)[e]**
6	SSE1	Heat Shock proteins	78,818	5.20	+1.5	
7	SSB1		66,732	5.32	+1.55	
9	SSC3		70,042	5.93	+1.61	
11	HSP71		70,452	5.06	+1.69	+1.89
13	HSP60		60,374	5.22	+1.73	+1.92
8	Guanine nucleotide-binding protein subunit β	Translation machinery	34,898	5.80	+1.57	
10	Elongation factor 1 α		50,426	9.09	+1.68	
16	ATP synthase subunit β	Energy production	54,035	5.16	+1.85	
14	S-adenosylmethionine synthase 2	Sulphur amino acids and phospholipids synthesis	42,515	5.18	+1.73	
2	Pyruvate kinase 1-2	Glycolysis	55,905	6.25	-2.41	-1.72
4	Glyceraldehyde-3-phosphate dehydrogenase 1-3		36,067	5.25	-1.72	
1	Alcohol dehydrogenase 1-2	Alcoholic fermetnation	37,183	6.25	-2.91	
3	Alcohol dehydrogenase 1-2		37,183	6.25	-1.97	-1.5

[a] Nominal MW (in Dalton)
[b] Calculated pI
[c] Absolute differences in the intensity of corresponding spots between the reference and the sample of interest
[d] Fold change of proteins of the *non-evolved* strain expressed in YPD+$CuSO_4$ 2.5 g/L compared to the expression in YPD (reference)
[e] Fold change of proteins of the *evolved* strain expressed in YPD+$CuSO_4$ 2.5 g/L compared to the expression in YPD (reference)

Figure 1

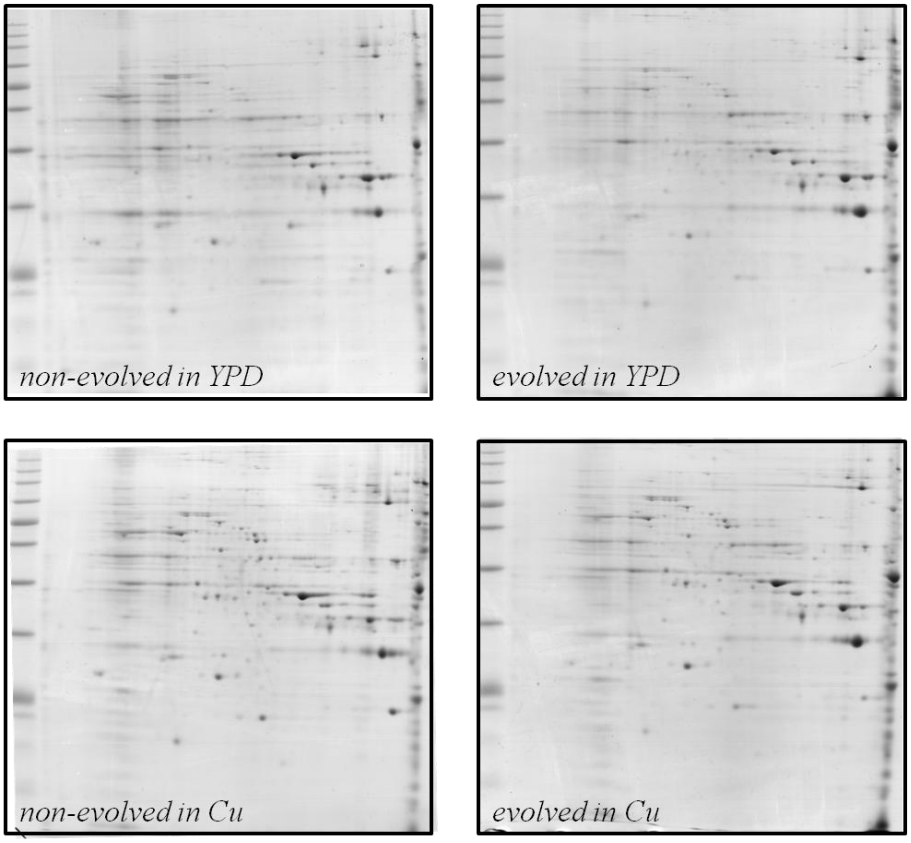
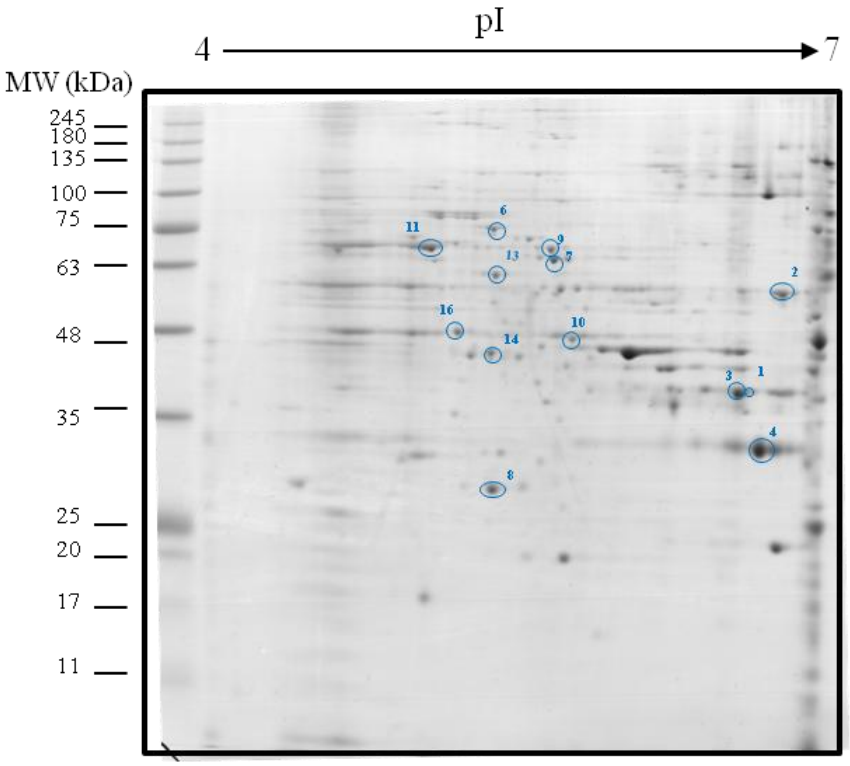

non-evolved in YPD
evolved in YPD
non-evolved in Cu
evolved in Cu

Figure 2


pI
4
7
MW (kDa)
245
180
135
100
75
63
48
35
25
20
17
11
6
11
9
7
13
2
16
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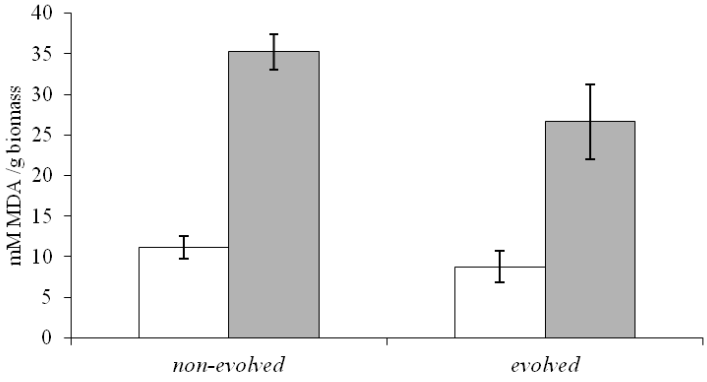
3
1
4
8

Figure 3



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