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Selection of *Saccharomyces cerevisiae* wine strains improved in glutathione production by an evolution-based strategy and their genome-wide study

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Abstract

Glutathione (L-γ-Glutamyl-L-Cysteinylglycine) is a thiol-containing tripeptide universally distributed in living cells. Due to unique redox and nucleophilic properties, glutathione (GSH) plays central roles in almost all the major biologic processes. For its important contribution in stability and protection of musts and wines, GSH has been the subject of an ever-growing interest in winemaking. In fact, in oenological matrices GSH reduces the *o*-quinones limiting the formation of brown pigments, avoids the formation of atypical aging characters and exerts a protective effect on several aromatic compounds. Nevertheless, GSH content in must and wine as well as the evolution of GSH concentration during alcoholic fermentation is highly variable and depend on multiple factors. Among these, *Saccharomyces cerevisiae* plays a key role since it can utilize or secrete GSH during fermentation altering the final GSH content in wine.

The goals of this research were to select new wine yeast strains able to increase the final amount of GSH in wine and to identify the genetic changes underlying the improved phenotype of the evolved strains at a genome-wide scale. To generate new *S. cerevisiae* strains with enhanced GSH production, we designed an evolution-based strategy that combines the sexual recombination of spores with the application of molybdate Mo(VI) as selective pressure. Specifically, Mo(VI), which is a toxic analogue of sulfate, was effectively used to generate the strains with the desired high-GSH phenotype, probably activating the yeast common metal response that involves GSH.

Our strategy was applied to *S. cerevisiae* UMCC 855, a wine strain from the Unimore Microbial Culture Collection (UMCC), and the selected molybdate-resistant strains were further screened for GSH production in synthetic must and in microvinification assay performed with grape juice. By this non-genetically modified strategy, we obtained two evolved strains, UMCC 2581 and UMCC 2585, able to enhance the GSH content in wine with an increase of 100% and 36%, respectively, compared to the parental strain UMCC 855 and, in particular, the UMCC 2581 strain was successful in increasing the GSH content in almost all the tested media.

Exploiting the potential of next-generation sequence technologies, the genome and transcriptome of parental and selected evolved-strains were determined in order to investigate the changes underlying the high GSH phenotype. We used a three-step process for quantitative trait loci (QTL) mapping to identify genes and pathways

responsible for Mo(VI) resistance trait, and we found three main loci on chromosome four, six and twelve responsible for resistance to Mo(VI). For a comprehensive study of the genetic variation, single nucleotide polymorphisms, insertions and deletions analysis and chromosomal copy number variations were also included. Finally, in order to capture gene expression changes between the parental and selected strains, RNA-seq experiments were performed.

The evolution-based strategy used in this study has proved to be particularly effective and time-saving compared to the common evolutionary approaches. In fact, our strategy does not require multiple rounds of screening and extensive cultivation periods as the evolved strains are rapidly recognized through the selectable molybdate resistance phenotype. Moreover, the genetic information provided in this study could be useful for a further optimization of the evolved strains and for providing an even more rapid approach to identify new strains, with a high GSH production, through a marked-assisted selection strategy.

Selezione di ceppi enologici di *Saccharomyces cerevisiae* migliorati per la produzione di glutatione attraverso una strategia di evoluzione adattativa e studio dei loro genomi

Il glutatione (L-γ-glutamil-L-cisteinilglicina) è un tripeptide ubiquitario caratterizzato dal gruppo funzionale tiolico. Grazie alle sue peculiari proprietà redox e nucleofiliche, il glutatione (GSH) gioca un ruolo centrale in molti processi biologici. L'interesse crescente per il GSH nei processi di vinificazione, è legato al suo importante contributo nella stabilizzazione e protezione di mosti e vini, dove limita l'imbrunimento attraverso la riduzione degli *o*-chinoni, evita la formazione dei caratteri di invecchiamento atipico ed esercita un effetto protettivo verso differenti composti aromatici. Tuttavia, il contenuto di GSH in mosti e vini, così come la sua concentrazione durante la fermentazione alcolica, è estremamente variabile e dipende da molteplici fattori. Tra questi, *Saccharomyces cerevisiae* gioca un ruolo chiave, in quanto può utilizzare o secernere GSH durante la fermentazione alterandone il contenuto finale in vino.

Gli obiettivi di questa ricerca sono stati la selezione di nuovi ceppi enologici di lievito capaci di incrementare il quantitativo finale di GSH nel vino e l'individuazione delle variazioni genetiche alla base del fenotipo migliorato nei ceppi evoluti. Per generare nuovi ceppi di *S. cerevisiae* con un'alta produzione di GSH, abbiamo sviluppato una strategia basata sull'evoluzione adattativa, che combina la ricombinazione sessuale delle spore con l'applicazione del molibdato Mo(VI) come pressione selettiva. Il Mo(VI), analogo tossico del solfato, è stato efficace per la selezione di ceppi con il fenotipo alta produzione di GSH, probabilmente attivando il meccanismo base di resistenza ai metalli pesanti, che nei lieviti coinvolge il GSH.

La nostra strategia è stata applicata al ceppo di *S. cerevisiae* UMCC 855, un ceppo enologico appartenente alla Unimore Microbial Culture Collection (UMCC), e i ceppi selezionati, resistenti al Mo(VI), sono stati valutati per la produzione di GSH in mosto sintetico ed in test di microvinificazione effettuati con mosto d'uva. Grazie a questa strategia, non OGM, abbiamo ottenuto 2 ceppi, UMCC 2581 e UMCC 2585, capaci di incrementare il contenuto di GSH nel vino, rispettivamente del 100% e del 36% rispetto al ceppo parentale UMCC 855: in particolare, il ceppo UMCC 2581 ha mostrato una elevata produzione di GSH in quasi tutti i mezzi testati.

Con lo scopo di studiare le modifiche alla base del fenotipo alta produzione di GSH, sono stati determinati il genoma e il trascrittoma del ceppo parentale e dei ceppi evoluti

selezionati. Per identificare i geni e le vie metaboliche responsabili della resistenza al Mo(VI), abbiamo mappato i loci dei tratti quantitativi (QTL), con un processo in tre passaggi che ci ha permesso di individuare tre loci principali, sui cromosomi quattro, sei e dodici. Per un più completo studio delle variazioni genetiche, sono state incluse anche analisi dei polimorfismi a singolo nucleotide, di inserzioni e delezioni e delle variazioni nel numero di cromosomi. Infine, sono stati effettuati esperimenti di RNA-seq, con l'obiettivo di valutare la variazione dell'espressione genica tra il ceppo parentale e i ceppi evoluti.

La strategia evolutiva utilizzata in questo studio si è mostrata particolarmente efficace, permettendo un considerevole risparmio di tempo rispetto ai comuni approcci evolutivi. Difatti, la nostra strategia non richiede multeplici cicli di selezione e lunghi periodi di coltura, poichè i ceppi evoluti sono rapidamente riconosciuti attraverso il fenotipo di resistenza al Mo(VI). Inoltre, le informazioni genetiche fornite con questo studio potrebbero essere utilizzate per un'ulteriore ottimizzazione dei ceppi evoluti, o per un ancor più rapida identificazione di nuovi ceppi alto produttori di GSH, attraverso strategie di selezione 'marker-assisted'.

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Chapter 1

The importance of glutathione in oenology

1.1 Glutathione: general description

The word "glutathione" (GSH) is composed by gluta- (glutamic acid) + -thi- (thiol, compounds containing a carbon-bonded sulfhydryl group) + -one (ketone, compounds containing a carbonyl group). Consistently, GSH is a tripeptide constituted by L-cysteine, L-glutamate and glycine (Fig. 1.1). It is the most abundant low-molecular-weight

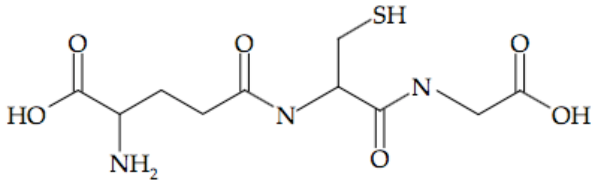

Figure 1.1 – Molecular structure of glutathione (GSH)

intracellular thiol compound (0.2-10 mM) (Anderson 1998), ubiquitously distributed in living organisms, from prokaryotes to eukaryotes (Fahey & Sundquist 1991). The three important chemical properties which make the GSH a fundamental cell peptide are: a very low redox potential (E'_o = -240 mV for thiol disulfide exchange), the unusual γ-glutamyl bond, which confers stability to GSH making it resistant to peptidases and allowing it to exist at high concentrations in the cell (Ganguly et al. 2003), and the reduced state maintained by a NADPH-dependent glutathione reductase (Meister & Anderson 1983). GSH levels as well as the balance between oxidized (glutathione disulfide, GSSG) and reduced forms, need to be carefully maintained in the cell where, generally, the reduced form of GSH occurs with a percentage > of 90%. Indeed, GSH deficiency has been associated with many disease states that include liver diseases, Alzheimer's disease, aging and HIV infections (Wu et al. 2004), however, also higher levels of GSH lead to glutathione toxicity at least in yeast (Srikanth et al. 2005). As a result of these properties, GSH is a strong cellular redox buffer involved in oxidative stress response and detoxification of metals and xenobiotics as well as influences several essential processes such as gene expression, cell proliferation, apoptosis, protein folding and sulfur and nitrogen metabolism (Penninckx & Elskens 1993; Penninckx 2002; Arrigo 1999; Fang et al. 2002; Friedman 1994). Therefore, GSH is considered to be one of the most powerful, versatile and important self-generated defense molecules (Li et al. 2004). For the same characteristics it is widely used as pharmaceutical and cosmetic compound, as food additive, and in sport nutrition, but only recently, its

supplementation is allowed during vinification in musts and bottles (Sies 1999; Beutler 1989; OIV-OENO resolution 446-2015).

1.2 Significance of glutathione in grapes

The first significant source of GSH in winemaking is provided by grape where GSH is synthesized in plant cells in two sequential ATP-dependent reactions (Leustek et al. 2000). As in yeasts, GSH plays similarly important physiological and biochemical roles in plants: it is involved in redox control, detoxification, and sulfur metabolism (Kritzinger et al. 2013). The GSH content in grapes varied from 17 to 114 mg kg^{-1} according to the grape cultivar, but also environmental conditions, location and viticultural practices affect the final GSH concentration (Cheynier et al. 1989). The GSH concentration in *vitis vinifera* berries, increases after the onset of ripening (veraison), regardless to the variety (green, red, seeded and seedless). Because of the positive correlation between GSH and sugar, observed approximately until 16° Brix, it has been proposed that the contribution of phloem components to the berry from sources such as mature leaves, can increase in this stage (Adams & Liyanage 1993; Okuda & Yokotsuka 1999). Since the GSH transport and accumulation occur through the vascular system, the increased GSH in berry is shown accompanied by a decreased GSH content in leaf. Moreover, the GSH amount can vary from different bunches and even from different berries in the same bunch, thus, more studies are required to establish the precise site of GSH synthesis (Liyanage & Adams 1992; Šuklje et al. 2012). However, as mentioned before, also viticultural practices affect the GSH accumulation in grape. Specifically, the GSH content is associated with the nitrogen amount present in the soil during ripening: in particular, when fertilized after bloom, high concentrations of nitrogen in the vineyard result in juice with high yeast assimilable nitrogen content, closely related to the GSH amount in berries. Several studies revealed this direct relationship also in the opposite case, when nitrogen-deficient vine showed a significantly lower GSH concentration (Choné et al. 2006; Lacroux et al. 2008). Nevertheless, further researches should be carried out to define how far GSH levels could be influenced in the vineyard.

1.3 Significance of glutathione in must and wine

The GSH can exert in must and wine various activities, ranging from the improvement of wine aroma by limiting the formation of off-flavor and thiol-related aromas, to antioxidant activity against enzymatic and non-enzymatic wine browning during winemaking and aging, with an efficiency which depends on the final reduced GSH concentration. The detected amount of GSH in must is highly variable (0-100 mg L^{-1}) and it is affected by many factors as grape skin maceration, tyrosinase activity, pressing and exposure to oxygen (Cheynier et al. 1989; Du Toit et al. 2007; Maggu et al. 2007; Patel et al. 2010). However, the amount of GSH and the balance with GSSG, usually much lower (below 1 mg L^{-1}) compared to GSH levels in grape juice, can be modified during winemaking (Kritzinger 2012; Okuda & Yokotsuka 1999). During machine harvesting, oxygen uptake is inevitable but whilst "free-run juice" (must gently extract from the grapes) is characterized by greater GSH concentration, higher press fraction shown almost not detectable amount of GSH (Maggu et al. 2007; Patel et al. 2010). Moreover, it has been shown that reductive treatments during pressing results in a higher GSH levels in the grape juice and the corresponding wine, whereas oxidative handling led to higher GSSG levels (Du Toit et al. 2007). Finally, also skin contact with the grape juice brings to a decrease GSH concentration (Maggu et al. 2007). The GSH present in the grape juice almost disappears at the beginning of the alcoholic fermentation, then increase in concentration as a result of the yeast cell synthesis and lysis and it stabilizes one month after the beginning of the fermentation (Dubourdieu & Lavigne 2004). Due to its metabolism and the interaction with the complex environment, the main factor able to affect the GSH concentration during must fermentation and in wine is the *Saccharomyces cerevisiae* strain. Since the GSH in *S. cerevisiae* is needed in numerous stress response mechanisms (oxidative stress, detoxification of heavy metals/xenobiotics and essential metabolic processes; Penninckx, 2002), the yeast could synthetize GSH, and then release it in fermenting must, as well as assimilate GSH from the must, through GSH transporters (Bourbouloux et al. 2000; Park et al. 2000a; Park et al. 2000b; Dhaoui et al. 2011). Thus, as proposed by several authors (Lavigne et al. 2007; Fracassetti 2010; Kritzinger 2012; Mezzetti et al. 2014), different GSH amount, assimilated or secreted, may result in different GSH contents after alcoholic fermentation, associating the GSH present in wine as depending on the yeast strain. On the other hand, Kritzinger (2012) showed inconsistencies between the GSH production observed when yeast strains were inoculated in synthetic must and when the same strains were inoculated in grape juice.

In the same work, it has been shown that also the time stage and degree of fluctuation of the GSH during fermentation are influenced by the yeast strain. This great variability in results regarding the GSH evolution during alcoholic fermentation is not fully understood, even though it is probably due to the grape juice metabolic complexity (Kritzinger et al. 2013). Indeed, the yeast growth is affected by the interaction of many environmental parameters such as temperature, pH, osmotic pressure, metabolic composition of the juice and bioavailability of nutrients, which change significantly in different musts, with the peculiar genetic background of the yeast strain. Besides yeast strain, also chemical reaction can influence the GSH level in must. GSH may be incorporated into oxidation reaction with phenolic compounds, such as caftaric acid quinones, generating 2-S-glutathionyl caftaric acid (Grape Reaction Product, GRP), leading to a decrease of GSH concentration (Singleton et al. 1985; Cheynier et al. 1986; Sonni et al. 2011a; Sonni et al. 2011b). However, for more definitive clarifications on the fate of GSH during alcoholic fermentation more studies are necessary.

GSH concentration in wine (0-70 mg L^{-1}) is generally lower than in must but it has been observed also equal or higher (Okuda & Yokotsuka 1999; Park et al. 2000b; Du Toit et al. 2007; Fracassetti et al. 2011; Kritzinger 2012). Similarly to what observed during fermentation period, also after fermentation and during wine aging, the GSH content can be affected by technical processes, as the oxygen exposure during bottle aging (Ugliano et al. 2011), and, with contradictory results, by the yeast strain. The effect of yeast lees has been reported both stabilize and decrease the GSH level in wine, underling the dubious contribution of the autolyzed yeast cells (Lavigne et al. 2007; Kritzinger 2012).

In order to achieve high levels of GSH able to efficiently improve the wine quality, several GSH-enriched inactive dry yeast preparations (IDYs) can be added to grape juice. Yeast cells are grown under defined culture conditions with the specific purpose of accumulate elevated concentrations of GSH, that in *S. cerevisiae* can reach the 1% of the dry weight (Penninckx 2000). Then, they are thermal inactivated and added to the must during alcoholic fermentation where GSH is expected to be directly release in the media or where the biosynthesis of GSH can be stimulated allowing to fermenting yeast to assimilate amino acids precursor. Nevertheless, the effect of ethanol, SO_2, the optimal addition time and amount of IDYs required to achieve a positive effect, are still unclear (Ángeles Pozo-Bayón et al. 2009; Andujar-Ortiz et al. 2012).

Due to the important effects of GSH on the improvement of wine quality, its elevated concentration is desired in winemaking. Technical processes and yeast strain influence

the GSH final concentration, but the mainly reasons of the drastically evolution of GSH levels that can be observed during winemaking are yet unknown. Even though the addition of food-grade GSH to wine has recently been permitted (OIV-OENO resolution 446-2015), the use of yeast strain able to excrete, and not assimilate, high amount of GSH remains the cheaper and more effective solution.

1.3.1 Antioxidant activity and interaction with browning pigments

Due to its antioxidant activity, GSH can limit wine browning that is perceived as loss of appearance, aroma, and flavor quality (Li et al. 2008). Browning can occur in must as a

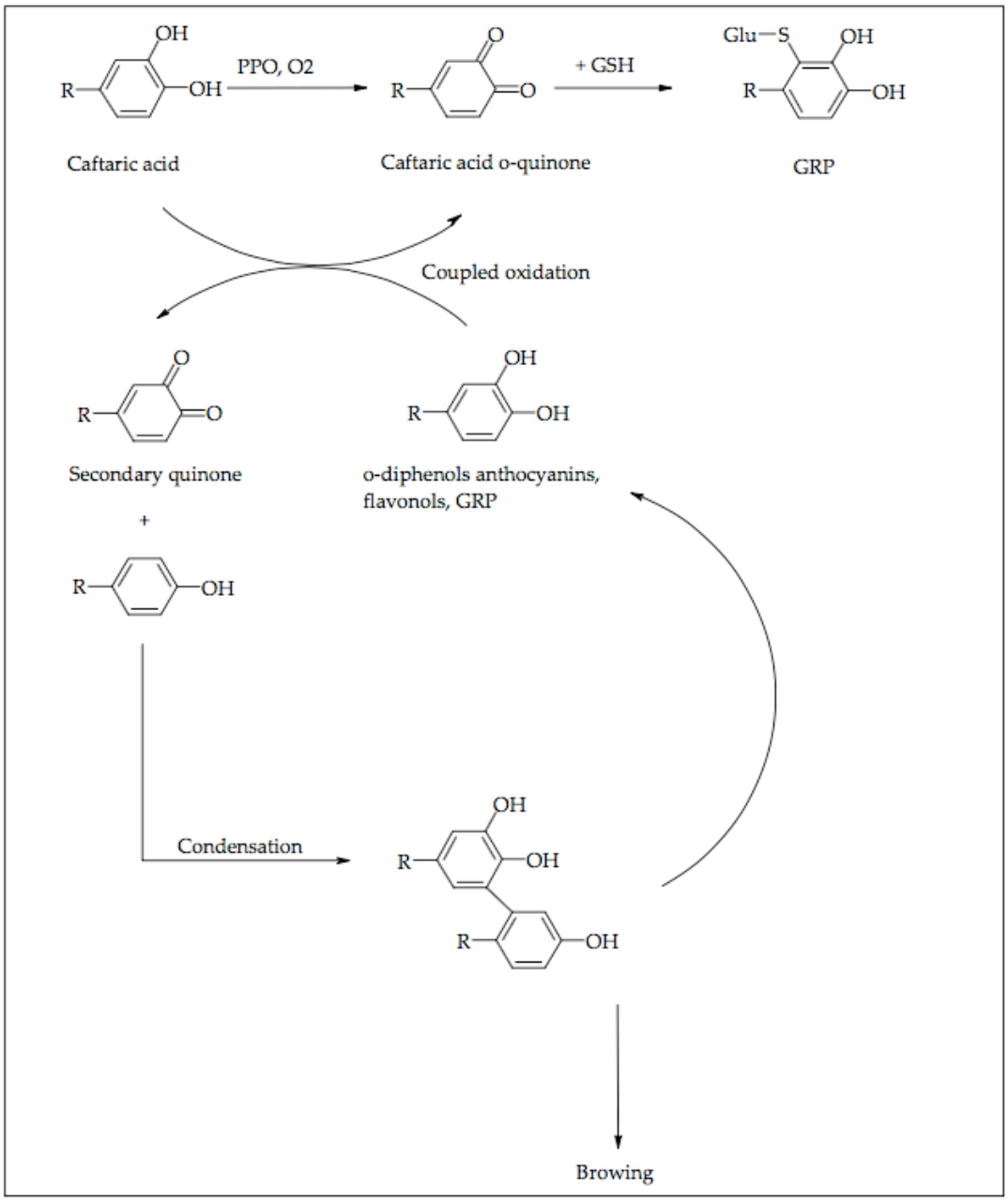

Figure 1.2 - Reaction scheme of enzymatic oxidation with GRP and browning compounds formation in white grape juice (Fracassetti 2010).

result of enzymatic oxidation or during wine aging as effect of non-enzymatic oxidation (Oliveira et al. 2011) but in both cases, the responsible of the oxidative browning in wine are the *o*-diphenols. The main group of phenolic compounds in white grape juice is represented by hydroxycinnamates such as caftaric acid and coutaric acid (Cheynier & van Hulst 1988) that are oxidized by the grape polyphenol oxidase (PPO) to the corresponding electrophilic *o*-quinone. By condensation reactions, *o*-quinone can polymerase with other compounds forming brown pigments (Li et al. 2008; Fig. 1.2). This oxidation takes place only when grapes are crushed because, in intact grapes, hydroxycinnamates are located in vacuole and PPO in the cytoplasm (Singleton et al. 1985). GSH can limit the browning process of white wine by reaction with *o*-quinone to generate 2-(S)-glutathionyl caftaric acid (GRP), a colorless product resistant to further oxidation by PPO (Singleton et al. 1985; Cilliers & Singleton 1990; Fig. 1.2). Moreover, it has been shown that GSH is able to stabilize the wine color also during aging (Vaimakis & Roussis 1996; Dubourdieu & Lavigne 2004), proving its ability to additionally protect phenols to non-enzymatic oxidation. Browning pigments arise during wine aging for the non-enzymatic oxidations of phenols (which in wine are caffeic acid, catechin, epicatechin, and gallic acid) and the subsequent polymerization between phenols or with other wine components as acetaldehyde and glyoxylic acid (Li et al. 2008). The ability of GSH to delay the creation of carboxymethine-bridged (+)-catechin dimers forming GSH addition products with carbonyl compounds, could be responsible of the polymerization reactions inhibition and, therefore, of the unwanted coloration (Sonni et al. 2011a; Sonni et al. 2011b).

1.3.2 GSH influence on aroma

In addition to wine browning, aging leads to a loss of characteristic aromas with the development of new aromas characteristic of older or impaired wines. GSH exerts a protective effect on volatile thiols, esters and terpenes and inhibits the formation of atypical aging characters, as the sotolon, supporting the aroma preservation. Esters, in particular those based on higher alcohols, ethanol, acetic acid and saturated carboxylic acids, bring an significant contribution for the definition of wine aroma by imparting fruity fragrance (Etievant 1991). In several varieties such as Muscat and Gewürztraminer, terpenes represent an important group of aromatic compounds giving floral, coriander and campherous fragrance that characterize the wine (Marais 1983). It has been reported that a GSH concentration higher than 2.5 mg L^{-1} can be inhibitory of

decrease of volatile esters and terpenes (Papadopoulou & Roussis 2008). However, the exact mechanism by which GSH exerts its protective effect is still unknown but is considered to be related to the GSH sulfhydryl (-SH) moiety (Roussis et al. 2009). The same sulfhydryl moiety is proposed to be the way by which GSH provides protection of volatile thiols, in this instance, by a competitive mechanism (Tirelli et al. 2010). Similarly, to esters and terpenes, volatile thiols are important fragrance in the definition of Sauvignon blanc varietal aroma and other cultivars such as Riesling and Merlot (Tominaga et al. 2000). The volatile thiols are highly susceptible to oxidation and during aging *o*-quinone can easily react with them leading to a loss of these varietal aromas. The addition of GSH concentration ranging from 6 to 20 mg L^{-1}, has shown a positive effect on the protection of thiol-related aroma (Dubourdieu & Lavigne 2004; Ugliano et al. 2011). Since GSH is also a thiol, it has been proposed that it can compete with the aromatic thiols to bind to the *o*-quinone, slowing down the decrease of volatile thiols (Tirelli et al. 2010).

With an unknown mechanism, but probably related to its antioxidant properties, GSH is able also to prevent the formation of two compounds responsible for the atypical aging characters: sotolon and aminoacetophenone (Dubourdieu & Lavigne 2004). When their perception threshold is overcome, sotolon (4,5-dimethyl-3-hydroxy-2(5H)-furanone), a volatile furanone, confers dried fig, rancid and curry odor (Cutzach et al. 1999; Lavigne et al. 2008; Silva Ferreira et al. 2003), instead 2-Aminoacetophenone confers wet wool, fusel alcohol and naphthalene aromas (Fan et al. 2007; Schmarr et al. 2007).

Besides the positive effects described above, it has been suggested that, at least in nitrogen- starved situation, GSH may be also a potential source of undesirable H_2S as a result of cysteine degradation (Hallinan et al. 1999; Ugliano et al. 2010).

1.4 Significance of glutathione in yeast

GSH in *Saccharomyces cerevisiae* wild yeasts represents more than the 95% of the non-protein thiol compounds reaching the 1% of the dry weight based on the growth conditions (Elskens et al. 1991). A so high GSH content in yeast cells reveals its fundamental role in several cellular processes. Indeed, GSH is implicated in maintaining housekeeping metabolic function such as mitochondrial, membrane integrity and reproduction as well as in many stress response. It responds to oxidative stress protecting protein thiols against irreversible oxidative modifications and through

glutathione peroxidase detoxification processes (Rauhut 2009). GSH is responsible of the common metal response in yeast by reducing heavy metals and xenobiotics and transporting the derivative compounds into vacuole. Finally during sulfur and nitrogen starvation it can be metabolized supplying the constituent amino acids to satisfy the growth requirement.

1.4.1 GSH metabolic pathway

The GSH biosynthesis, being it an organic sulfur compound, firstly needs sulfate to be taken up and reduced into the cells. In particular, sulfate is transported inside the cell by the two specific plasma membrane permeases Sul1p and Sul2p (Breton & Surdin-Kerjan 1977). Then it is activated by adenylation yielding adenylyl sulfate (APS) and phosphorylated producing phosphoadenylyl sulfate (PAPS) (Mendoza-Cózatl et al. 2005). PAPS is firstly reduced to sulfite and then to sulfide that can be incorporated into the carbon chain of homocysteine (Thomas & Surdin-Kerjan 1997). Subsequently, two transsulfuration reactions allow the interconversion of homocysteine into cysteine via the intermediary formation of cystathionine (Thomas & Surdin-Kerjan 1997). GSH is synthesized in the cytosol by the action of two consecutive enzymes Ɣ-glutamylcysteine synthetase (encoded by GSH1) and GSH synthetase (encoded by GSH2) (Fig. 1.3, Penninckx 2002). In the first reaction, Ɣ-glutamylcysteine is synthesized by condensation of cysteine and glutamate; in the second reaction glycine is added for the synthesis of GSH (Suzuki et al. 2011). Due to its important cellular roles, GSH synthesis is strictly regulated and in particular the Gsh1 protein.

$$\text{Cysteine} + \text{Glutamate} + \text{ATP} \xrightarrow[GSH1]{\gamma\text{-GC synthetase}} \gamma\text{-Glutamylcysteine} + \text{ADP} + \text{Pi}$$

$$\gamma\text{-Glutamylcysteine} + \text{Glycine} + \text{ATP} \xrightarrow[GSH2]{\text{Glutathione synthetase}} \text{GSH} + \text{ADP} + \text{Pi}$$

Figure 1.3 - Enzymatic reaction involved in glutathione synthesis. Synthesis of γ-glutamylcysteine from glutamate and cysteine by γ- glutamylcysteine synthetase (GSH1) and synthesis of GSH from γ-glutamylcysteine by glutathione synthetase (GSH2) (Suzuki et al. 2011).

Ɣ-glutamylcysteine synthetase is the rate-limiting enzyme in GSH biosynthesis and, to prevent the damaging effects of GSH depletion, it is actively transcribed by Yap1p and

Met4p transcription factors, whilst to prevent over accumulation it is feedback-inhibited by GSH (but not GSSG, Richman & Meister 1975; Penninckx & Elskens 1993). On the other hand, Gsh2p is an unregulated enzyme (Inoue et al. 1998). In addition to its cytoplasmatic biosynthesis, GSH homeostasis is altered by its compartimentalization,

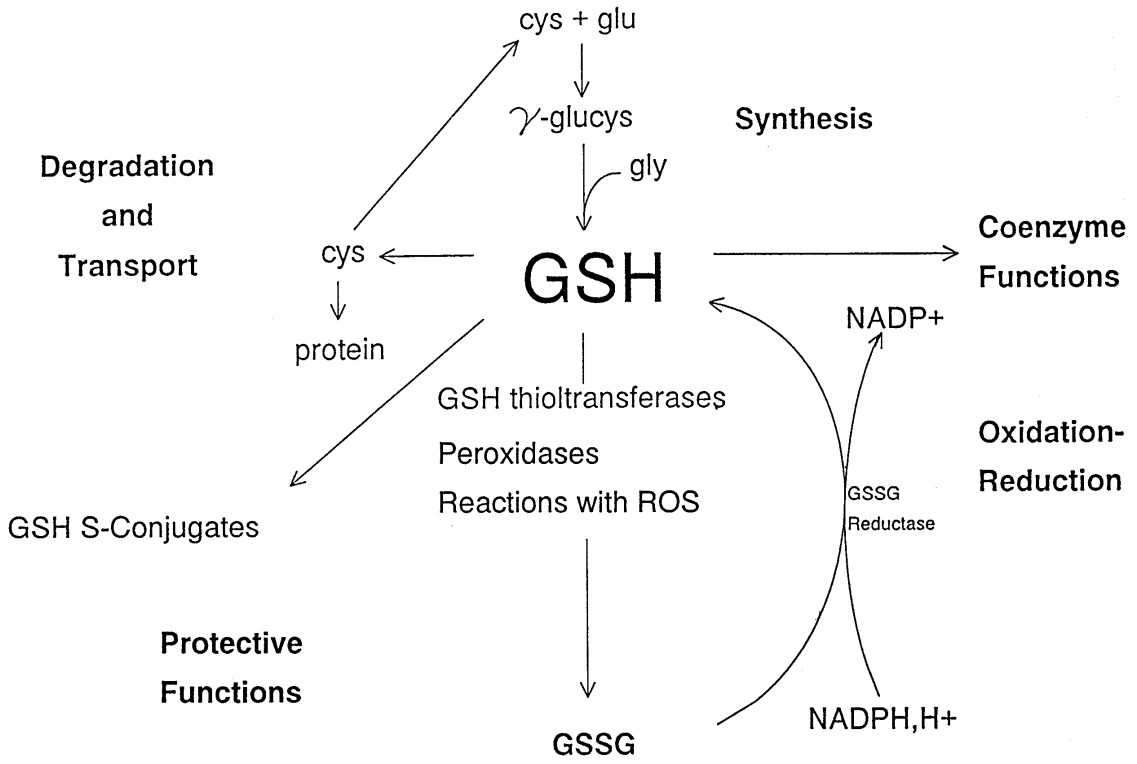

Figure 1.4 - Overview of glutathione (GSH) metabolism (Anderson 1998).

import/export from the cell, degradation and consumption in different processes (Fig. 1.4, Perrone et al. 2005). GSH may be taken up from the extracellular medium through the high affinity GSH transporter Opt1p/Hgt1p (Hauser et al. 2000) able to transport also GSH conjugates into the cell cytoplasm (Bourbouloux et al. 2000). This transporter is actively regulated by sulfur starvation but repressed by cysteine, methionine and partially GSH (Miyake et al. 2002; Srikanth et al. 2005). A new GSH exchanger, Gex1p a glutathione/proton antiporter, was recently described by Dhaoui et al. (2011). GEX1 expression is dependent on the transcription factor Aft2p and its overexpression had shown low intracellular GSH contents with glutathione excreted into the extracellular medium. Aside from the plasma membrane, it was found at the vacuolar membrane like Ycf1p, the vacuolar glutathione S-conjugate transporter in *S. cerevisiae* (Li et al. 1996). The latter however, has as primary function the transport of GSH S-conjugate rather

than GSH (Rebbeor et al. 1998). Thus, these multiple processes combine to maintain a balanced glutathione homeostasis in the cell.

Once in the vacuole, the Ɣ-glutamyltranspeptidase (Ɣ-GT, encoded by ECM38) cleaves the Ɣ-glutamyl moiety releasing glutamate and cysteinylglycine further degraded by L-cysteinyl glycine dipeptidase in the constitutive amino acids cysteine and glycine (Jaspers et al. 1985; Penninckx 1980; Mehdi et al. 2001). Ɣ-GT enzyme is strongly derepressed in case of nitrogen starvation suggesting a role for GSH in the supply of growth requirements in this stress situation (Mehdi & Penninckx 1997). However, a second degrading pathway was proposed by Ganguli et al. (2007). In this Ɣ-GT independent alternative pathway the GSH degradation is mediated by Dug1p/Dug2p/Dug3p proteins forming a specific GSH degradosomal complex.

1.4.2 Role of GSH in oxidative stress

Yeast, as all aerobic organisms, reduces molecular oxygen to H_2O to acquire energy efficiently during respiration or oxidation of nutrients. During reducing processes several reactive oxygen species (ROS) are formed by partially reduced forms of O_2: superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), alkylhydroperoxides (ROOH) and lipid hydroperoxide (LOOH) (Wolff et al. 1986; Jamieson 1998). These compounds are highly reactive and able to attack almost all cellular components from DNA to protein and lipid membrane causing sometime lethal or heavy damage to the cells. GSH performs its defensive role against oxidative damage by means of two ways: by direct interaction with free radicals and indirectly as cofactor for antioxidant enzymes (Fig. 1.4). In the first non-enzymatical way, GSH acts as a radical scavenger thanks to its sulphydryl group: the free radicals are removed from the solution by direct reaction with GSH, which is oxidized to GSSG (Anderson 1998). In the second way, the antioxidant enzymes glutathione peroxidase, glutathione reductase, glutaredoxins and glutathione S-transferases use GSH as equivalents source (Grant 2001). The glutathione peroxidase (GPx) detoxifies the cell from peroxide compounds by catalyzing the reaction in which hydrogen peroxide is reduced to water and the organic peroxide to the corresponding stable alcohols:

$$2GSH + ROOH \longrightarrow GSSG + H_2O + ROH$$

This enzyme, which has a key rule in defense mechanisms, is localized in cytoplasm and mitochondria, where the higher amount of peroxides are produced, and it is induced by oxidative conditions (Galiazzo et al. 1987). GSH is, then, restored by glutathione reductase (GR) in the presence of NADPH (Izawa et al. 1995).

Glutaredoxins (Grx) are GSH using enzymes involved in prevent another effect of oxidative stress: the incorrect protein folding. Their enzyme activity consists of deglutathionylating mixed disulfides between GSH and protein thiols, releasing reduced GSH (Puigpinós et al. 2015). Protein glutathionylation is a reversible mechanism for protecting protein thiols otherwise facing irreversible oxidative modifications, which lead to a proteins degradation (Gallogly & Mieyal 2007).

1.4.3 GSH response to nitrogen and sulfur starvation

Because of the yeast sulfur metabolism that permits the recycling of the by-products, *S. cerevisiae* can grow on either inorganic sulfur sources and organic ones, such as methionine, homocysteine, cysteine or GSH as a sole sulfur source (Miyake et al. 1999). Thus, it is possible that GSH serves as a reservoir for sulfur, nitrogen and precursor amino acids. In fact, it has been reported by Elskens et al. (1991) that GSH might fulfil the role of a sulfur storage compound. During growth on media with sufficient sulfate content, the excess of sulfur is incorporated into GSH; on the contrary in case of sulfate deprivation, the endogenous pool of GSH is used in the synthesis of cysteine, serving as internal sulfur source until reaching the 10% of its normal value. Similarly, when the yeast cells are grown on nitrogen deprived medium the cytoplasmic GSH, that generally represent the 50% of the total content with the remaining 50% occurring in the vacuole, is relocated in the vacuole (Mehdi & Penninckx 1997). Here, following the degrading pathway, the GSH is consumed releasing the constituent amino acids that may serve as an endogenous source for growth and maintenance. Nevertheless, coherently with the sulfur starvation, also during nitrogen starvation about 10% of the intracellular GSH is not degraded, avoiding the complete GSH depletion (Mehdi & Penninckx 1997).

1.4.4 GSH response to heavy metals and xenobiotics

GSH chelation appears to be the most important mechanism for heavy metals such as cadmium, copper, zinc, silver and lead, and xenobiotics resistance in *S. cerevisiae*. These toxic electrophiles can be conjugated to GSH exploiting the cytosolic glutathione S-transferase enzyme, which enable the following reaction:

The resulting GS-X complex is recognized as substrates by transporters for vacuolar sequestration or export outside the cell (Ortiz et al. 1992; Duncan & Jamieson 1996; Mendoza-Cózatl et al. 2005). The Yeast Cadmium Factor (YCF1) represents the main vacuolar glutathione S-conjugate (GS-X) transporter, discovered because of its ability to confer cadmium resistance even though it is able to transport a wide spectrum of GSH-complex (Li et al. 1996). Noteworthy, the Ycf1p and Gsh1p are both activated by the general transcription factors in response to oxidative stress Yap1p, emphasizing the importance of GSH in metals resistance pathway (Wemmie et al. 1994). In this process the transport of the complex into the vacuole is necessary to allow metal tolerance probably because once inside the vacuole, the complex can be decomposed by ϒ-GT (the same enzyme necessary for GSH degradation) and thereafter by other peptidases, restoring the amino acids in the cytoplasm which can be used to the *de novo* synthesis of GSH (Adamis et al. 2007). In this way GSH is recycled ensuring protection against metals, xenobiotics and oxidative stress.

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Aims of the project

In the never-end process of wine improvement, *Saccharomyces cerevisiae* oenological strains play a main role. The yeast wine strains are indeed the principal responsible of the fermentation process and act also as a biocatalyst able to modify flavour and chemical complexity of the final product through the production of functional metabolites. Among them, glutathione (GSH) is the main nonprotein thiol (L-γ-glutamyl-L-cysteinyl-glycine) in yeasts involved in the response to different nutritional and oxidative stresses. Recently GSH has received a growing interest in the winemaking field for its role in limiting the amount of browning pigments by competitive *o*-quinones reduction, avoiding the formation of sotolon and other atypical aging characters and exerting a protective effect on various aromatic compounds. Although it has been postulated that the final GSH amount in wine is dependent of wine strains, few works have been reported regarding the development of new non-genetically modified wine yeast strains, able to produce high amount of GSH.

The first main aim of this PhD project has been the selection of evolved wine yeast strains, high GSH producers, obtained through the application of an evolution-based strategy. Subsequently, metabolic and molecular characterizations have been performed in order to increase knowledge about the complex metabolic processes behind these different phenotypes. Moreover, the genetic information could provide new opportunities for a further future development of optimized oenological strains.

Chapter 2

Evolved *Saccharomyces cerevisiae* wine strains with enhanced glutathione production obtained by an evolution-based strategy

2.1 Introduction

Due to the increasing number of practical applications for GSH, the efficiency of production technologies are increasingly important. Different strategies applied to obtain an enhanced GSH production have been reported (Nisamedtinov et al. 2011). Some of these strategies are based on the modulation of precursor amino acids, in particular cysteine, to overcome substrate limitation (Alfafara et al. 1992; Wen et al. 2006; Wang et al. 2007; Liang et al. 2008). Other strategies consist in the development of genetically engineered strains overexpressing GSH biosynthetic enzymes, or the key enzymes in sulfur assimilation pathways, to increase cysteine biosynthesis (Grant et al. 1997; Hara et al. 2012). In addition, several mutation strategies, based on physical or chemical treatments, have been described in the literature relating to the isolation of GSH over-accumulating variants (Lai et al. 2008; Chen et al. 2012).

Even though recombinant DNA techniques are widely used in biotechnological production of GSH (Li et al. 2004; Hao et al. 2012; Hara et al. 2012), the use of genetically modified (GM) yeast strains for winemaking does not seem to be particularly appreciated by consumers and legal restrictions limit their use in foods and beverages in many parts of the world (Grossmann et al. 2011). On the other hand, wine strains obtained by adaptive evolutionary approaches have high public acceptance (Çakar et al. 2005; Kutyna et al. 2010). Approaches which simply mimic nature by random mutation or genetic recombination of the microorganisms, followed by selection under conditions suitable to the desired phenotype, have been extensively applied to the generation of wine yeast with improved oenological properties (Kuyper et al. 2005; Stanley et al. 2010; Cadière et al. 2011; Çakar et al. 2012; Kutyna et al. 2012). In our evolution-based strategy, recently proposed, we have exploited the resistance to chromate Cr(VI) and molybdate Mo(VI) (toxic sulfate analogues), as selectable phenotypes to rapidly select *S. cerevisiae* strains impaired in the sulfate assimilation pathway (De Vero et al. 2011). These toxic metals enter a yeast cell through Sul1p and Sul2p high-affinity sulfate permeases (Fig. 2.1); mutation in these permeases is one of the most important events, conferring resistance to sulfate toxic analogues (Cherest et al. 1997; Tamás et al. 2006; Wysocki & Tamás 2010). However other mechanisms could be involved, in fact, another important function of the sulfate assimilation pathway is the GSH biosynthesis which has an essential role in the defense against oxidative stress and metal toxicity (Grant 2001; Wysocki & Tamás 2010). In particular, GSH biosynthesis in *S. cerevisiae* takes place in two ATP-dependent steps. In the first, cysteine is linked with glutamate by γ-

glutamylcysteine synthetase (encoded by GSH1) to form γ-glutamylcysteine. In the second step, glycine is added to this intermediate product by glutathione synthetase (encoded by GSH2) to form the final product (Li et al. 2004; Zechmann et al. 2011). In scientific literature it has been reported that GSH is able to chelate heavy metals by forming complexes (metal-GSH complex) that are actively transported into the vacuole or removed from the cell by specific transporters such as Ycf1p and Gex1p (Li et al. 1996; Mendoza-Cózatl et al. 2005; Paumi et al. 2009; Dhaoui et al. 2011).

The aim of this work was to generate evolved *S. cerevisiae* strains with enhanced GSH

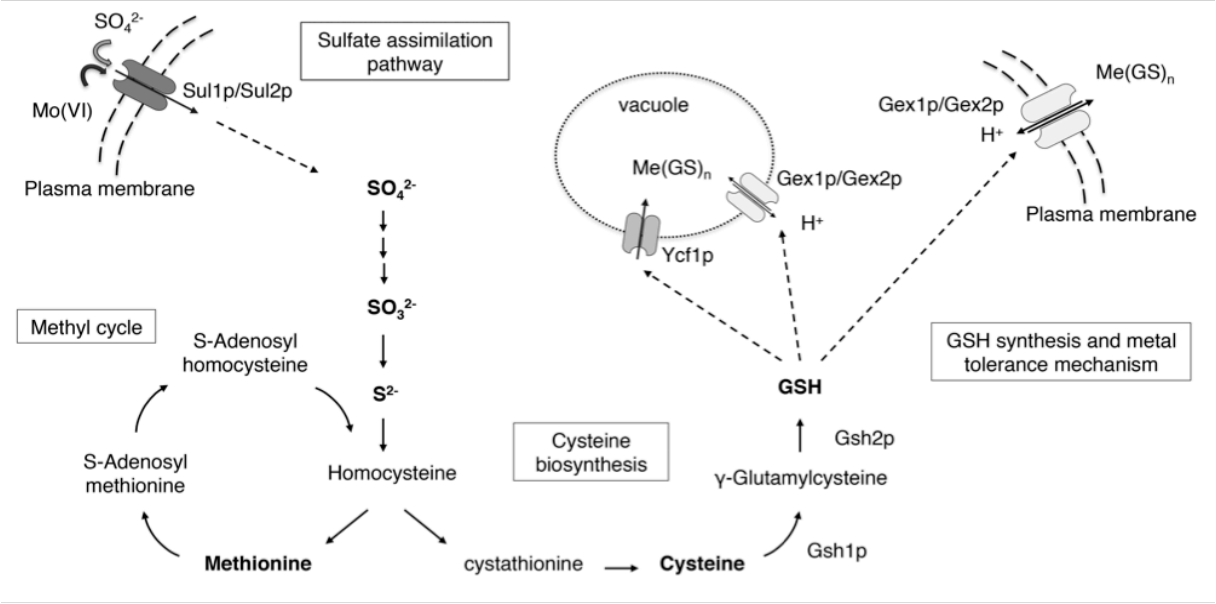

Figure 2.1 - A schematic model for sulfur metabolism, glutathione synthesis and GSH-mediated metal tolerance mechanism in *S. cerevisiae*. The sulfur metabolism can be divided into three parts: the sulfate assimilation pathway, the methyl cycle and the cysteine biosynthesis (Tamás et al. 2006). Glutathione is synthesized in two consecutive reactions and GSH free or conjugate with metal are transported into the vacuole or out of the cell. Sul1p/Sul2p: sulfate transporters; Gsh1p: γ-glutamylcysteine synthetase; Gsh2p: glutathione synthetase; Gex1p/Gex2p: yeast glutathione exchangers; Ycf1p: vacuolar glutathione S-conjugates pump; $Me(GS)_n$: Metal-GSH complex.

production. To achieve this, we applied our evolution-based strategy that combines sexual recombination and the application of molybdate or chromate as specific selective pressure. The resistant strains selected were further screened for GSH production in synthetic grape must and in microvinification assay.

2.2 Materials and Methods

2.2.1 Yeast strains and growth conditions

The *S. cerevisiae* 21T2 parental strain, previously selected in our lab (De Vero & Giudici 2011), was used to obtain the evolved strains in this work. The yeast strains were routinely cultured on YPD agar plates or slants (2% glucose, 2% peptone, 1% yeast extract and 2% agar), incubated at 27 °C for 48 h and stored at 4 °C. For long-term preservation, all the strains were maintained in the University of Modena and Reggio Emilia (Unimore) Microbial Culture Collection (UMCC), Reggio Emilia, Italy, stored at -80 °C in cryovials supplemented with glycerol (final concentration 25%).

2.2.2 Evolution-based strategy and screening of heavy metals resistant strains

To obtain strains improved in GSH production, the evolution-based strategy previously described by De Vero et al. (2011), was applied to the parental strain 21T2 with few modifications. This strain was induced to sporulate on acetate medium (0.5% sodium acetate and 2% agar). After tetrad formation, asci and cells were digested in 0.5 mL distilled water containing 0.5 mg mL^{-1} of Zymoliase 20T (Seikagaku Corp., Tokyo, Japan). The spores, recovered by centrifuging, were re-suspended in 0.5 mL YPD and periodically monitored under a microscope to check the restoration of diploid state by the conjugation of gametes. After that, 10 µL of this suspension was inoculated in 50 tubes containing YPD medium supplemented with 10 mM ammonium molybdate and 2.5 mM potassium chromate (Carlo Erba, Rodano, Italy). The high metals concentration was chosen to lead to significant growth inhibition within 10 days of incubation at 27 °C in YPD. From each tube where cell growth was observed, 100 µL of culture was withdrawn and plated on the YPD agar with Mo(VI) 10 mM or Cr(VI) 2.5 mM, respectively. Then, from each plate, a single colony was re-isolated and purified. The resistant strains selected were subsequently screened on YNB minimal medium plates (0.17% yeast nitrogen base w/o amino acids and ammonium sulfate, DIFCO, Detroit, MI), supplemented with 2% agar, 2% glucose, 100 µM ammonium sulfate (Sigma-Aldrich, St. Louis, MO), and different concentrations of molybdate resulting in a medium (pH 5) containing either 0, 1.0, 2.5 or 5.0 mM Mo(VI) or 0, 0.02, 0.03 or 0.035 mM Cr(VI). Strain pre-cultures were obtained in tubes with 5 mL YNB after 24 h at 27 °C. From each tube, 5 µL of 1 x 10^6 cells mL^{-1} and 5-fold dilution series were spotted on the plates. Colony growth was observed after four days of incubation at 27 °C.

2.2.3 Evaluation of glutathione production in synthetic grape juice

The total GSH (GSH + GSSG) produced by the parental and selected strains was determined with the enzymatic Glutathione Assay Kit (Sigma-Aldrich) following the manufacturer's instructions. The strains were pre-cultured, for 24 h at 27 °C, in 5 mL synthetic grape juice prepared according to Giudici & Kunkee (1994), with complete YNB containing amino acids and ammonium sulfate. The cells were then inoculated at an optical density of 0.05 at 600 nm (OD_{600}) into Erlenmayer flasks, each containing 50 mL synthetic medium, and incubated at 27 °C under shaking conditions (150 r.p.m.). The cells grown until the exponential phase (OD_{600} of 4) were harvested and centrifuged at 1000 g for 5 min at 4 °C. For each sample the supernatant and the pellet were processed, separately, to analyze the extracellular and intracellular GSH respectively. The supernatant was concentrated by freeze-drying equipment (FREEZONE 1L Labconco, Kansas City, MO) before using the Glutathione Assay Kit. The extracellular GSH concentration was expressed as mg GSH L^{-1}.

The pellet was washed three times with ice-cold distilled water and the intracellular GSH was extracted with 100 µL ethanol 25% according to Xiong et al. (2009). After 1 h of incubation at room temperature in a rotary shaker, each sample was centrifuged at 18000 g for 10 min at 4 °C. Then 25 µL 5-sulfosalicylic acid 5% was added to the recovered supernatant, mixed and incubated for 10 min at 4 °C. Finally, after centrifugation at 8000 g for 10 min at 4 °C, the supernatant was recovered and transferred to a fresh tube; GSH was then detected with the enzymatic kit. Intracellular GSH content was expressed as a percentage (%) of GSH concentration (mg L^{-1}) divided by dry cell weight (mg L^{-1} at OD_{600} of 4). Dry cell weight (DCW) was measured after washing the cell pellet twice with distilled water and drying the wet cells at 105 °C for 4 h.

2.2.4 Microvinification assay and analytical methods

Microvinification assay was carried out under static conditions at 25 °C in 250 mL sterile flasks filled with 200 mL pasteurized Trebbiano grape juice (pH 3.44, titratable acidity 8.68 g L^{-1}, reducing sugars 166.59 g L^{-1}) without added sulfites. All yeast strains were pre-cultured in 5 ml synthetic grape juice for 24 h at 27 °C and inoculated in Trebbiano grape juice at a final concentration of 1 x 10^6 cells mL^{-1}. Flasks were sealed with cotton plugs and the fermentation process was evaluated daily by the weight loss due to the evolved CO_2. At the end of the fermentation (constant weight for three consecutive days)

the samples were refrigerated at 4 °C or racked and stored at -20 °C until required for chemical analysis. Titratable acidity and pH were measured using TitroLine easy titrator (Schott Instruments, Mainz, Germany). Reducing sugars were determined by the Fehling method. Enzymatic kits (Megazyme, Bray, Ireland) were used to quantify the concentrations of ethanol (K-ETOH) and total and free SO_2 (K-SULPH) according to the manufacturer's instructions.

The fermentation efficiency was expressed as a percentage (%), calculated from the formula $[(Y_{Eth}/Y_{Eth\ Theor}) \times 100]$, where Y_{Eth} indicates the detected ethanol yield, and $Y_{Eth\ Theor}$ indicates the theoretical ethanol yield as determined by the Gay-Lussac equation.

Glutathione concentration was determined in each wine sample, as previously described on the basis of extracellular GSH analysis.

2.2.5 Evaluation of hydrogen sulfide production

Hydrogen sulfide (H_2S) production by the yeast strains was qualitatively evaluated on bismuth-glucose-glycine-yeast (BiGGY) agar medium (Oxoid Co., Hampshire, UK). Five µL of each strain cultured in YPD medium until OD_{600} of 1, was spotted on BiGGY agar and incubated for 4 days at 27 °C. On this differential medium the colony color turns dark, brown or remains white, depending on the amount of sulfide produced by the yeast. The qualitative amount of H_2S formed during microvinification assay was detected by lead acetate paper strips soaked with 50 µL of 5% lead acetate solution. Throughout the fermentation process, the strips were attached to the top of each flask, away from the liquid. The degree of blackening of the strips is correlated to the amount of H_2S produced during fermentation (Giudici & Kunkee 1994).

2.2.6 Statistical analysis

Statistical analysis was carried out by analysis of variance (ANOVA) using GraphPad Prism v. 6.0 (GraphPad Software Inc., San Diego, CA). Tukey's multiple comparison test was used to identify significant differences between strains ($P < 0.05$). All experiments were performed in triplicate.

2.3 Results

2.3.1 Selection of resistant strains

We used the *S. cerevisiae* 21T2 strain to obtain Mo(VI)/Cr(VI) resistant strains by the evolution-based strategy described above. This parental strain, selected in our lab, was chosen on the basis of its high percentage of asci conversion and its specific oenological aptitude (De Vero & Giudici 2011; Gobbi et al. 2014).

The 21T2 strain was induced to sporulate then, after sexual conjugation of gametes, a cell suspension was inoculated in tubes containing YPD medium with Mo(VI) or Cr(VI) as specific selective pressure. Fifteen evolved strains, codified as Mo21T2 followed by a sequential number (from 1 to 15) and seven Cr21T2 strains (from 1 to 7) were obtained in the preliminary screening. The Mo(VI) resistance of these strains was further assessed on YNB minimal medium plates with a low sulfate concentration (100 µM) and different Mo(VI) concentrations (Fig. 2.2).

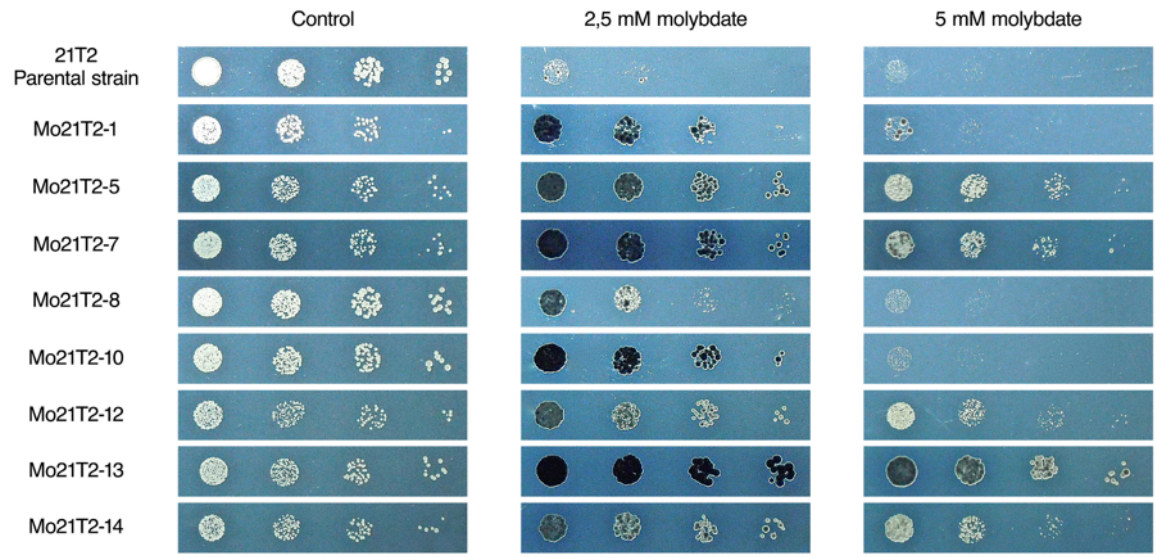

Figure 2.2 - Molybdate resistance screening of the parental and evolved strains. 5-fold dilution series of the strains, starting from 1 x 10^{-6} cell mL^{-1} on the left to the right, were spotted on YNB plates supplemented with 2% glucose, 100 µM of ammonium sulfate and Mo(VI) at the concentrations of 0 (control plates), 2.5 and 5.0 mM as indicated. Plates were photographed after four days of incubation at 27 °C.

The screening on minimal YNB medium allowed for a more stringent selection compared to that made on YPD; in fact, although the sulfate concentration used (100 µM) was not limiting for yeast growth, as observed in the control plates, on YNB there was a marked growth inhibition when Mo(VI) was supplied. This was in alignment with the experiments of Pereira et al. (2008) who investigated chromate toxicity by spotting

yeast cells on YNB plates containing different concentration of Cr(VI) and sulfur source. He found that chromate toxicity increased at low sulfur concentration (100 μM), and that it was strongly dependent on the sulfur source and its concentration. In our screening we observed that the low Mo(VI) concentration (1 mM) was not limiting for either the parental strain or the fifteen evolved strains previously selected on YPD (data not shown). However, in the plates with Mo(VI) 2.5 mM, there was strong inhibition and only eight evolved strains were able to grow (Fig. 2.2).

Strain	Description	UMCC code	Reference
21T2	Laboratory yeast strain	UMCC 855	De Vero & Giudici (2011)
Mo21T2-1*	Molybdate-resistant strain	UMCC 2580	This study
Mo21T2-5*	Molybdate-resistant strain	UMCC 2581	This study
Mo21T2-7*	Molybdate-resistant strain	UMCC 2582	This study
Mo21T2-8*	Molybdate-resistant strain	UMCC 2583	This study
Mo21T2-10*	Molybdate-resistant strain	UMCC 2584	This study
Mo21T2-12*	Molybdate-resistant strain	UMCC 2585	This study
Mo21T2-13*	Molybdate-resistant strain	UMCC 2586	This study
Mo21T2-14*	Molybdate-resistant strain	UMCC 2587	This study

Table 2.1 - *Saccharomyces cerevisiae* parental strain and evolved strains obtained by evolution-based strategy. In bold characters the *S. cerevisiae* parental strain. *Evolved strains generated from 21T2. UMCC - Unimore Microbial Culture Collection, University of Modena and Reggio Emilia, Reggio Emilia, Italy

The Mo(VI) concentration of 5 mM was limiting for almost all the strains, with the exclusion of Mo21T2-13, especially when the lowest cell concentration (8 x 10^3 cells) was spotted on YNB. The eight Mo(VI) resistant strains, selected on the basis of this screening, are listed in Table 2.1.

Differently from what obtained in the resistance screening on minimal YNB medium with Mo(VI) as selective pressure, the screening on the same medium with Cr(VI) of the seven Cr21T2 strains previously obtained did not allow a selection of resistant strains with either, Cr(VI) 0.03 mM, 0.02 mM and 0.035 mM (Fig. 2.3). This has been probably

caused by the extreme high toxicity of the chromate comparing to molybdate, toxicity also stressed by the low sulfur concentration used in a minimal medium as the YNB (Pereira et al. 2008).

On the basis of these results, only the molybdate evolved strains were further investigated for their ability to produce GSH and for their oenological aptitude.

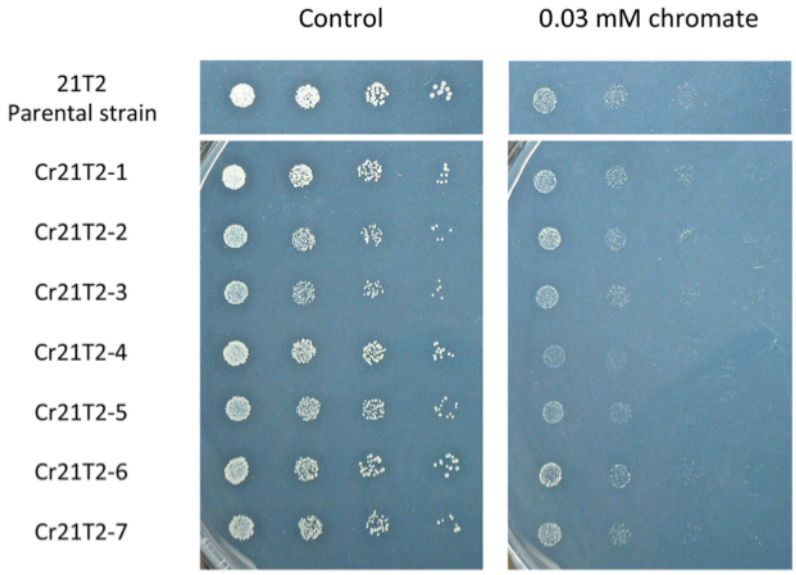

Figure 2.3 - Chromate resistance screening of the parental and strains Cr21T2-1, -7. Fivefold dilution series of the strains, starting on the left to the right. Cr(VI) at the concentrations of 0 (control plates) and 0.03 mM as indicated. Plates were photographed after 4 days of incubation at 27 °C.

2.3.2 Intra- and extracellular GSH production in synthetic grape juice

The native capability of the parental 21T2 and the eight evolved strains to produce GSH was preliminarily evaluated in a defined synthetic grape juice. The enzymatic determination of intra- and extracellular total GSH was assessed in culture samples harvested at the exponential phase.

The highest values of intracellular GSH content in synthetic grape juice were observed for the strains Mo21T2-1 (0.35%) and Mo21T2-12 (0.44%), which were, respectively, 2.1 and 2.6-fold higher than those of the strain 21T2 (0.17%) (Fig. 2.4a). The other evolved strains gave a lower content of GSH ranging from 0.13% to 0.26% with differences not statistically significant compared to the parental strain.

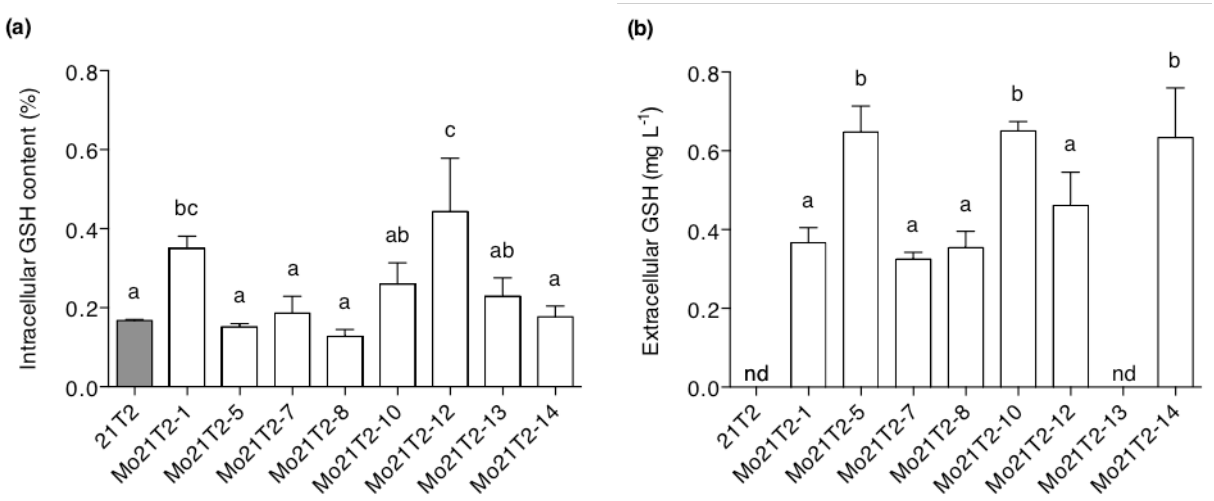

Figure 2.4 - Intracellular and extracellular total glutathione concentration in synthetic grape juice. (a) Intracellular GSH content (%) is expressed as GSH concentration divided by dry cell weight x 100. (b) Extracellular GSH concentration is expressed as mg L^{-1}. *nd* = not detected. Graph shows means with SD (*n* = 3) of 21T2 parental strain (gray column) and evolved strains (white columns). Means with different letters are significantly different from each other as determined by Tukey's test ($P < 0.05$).

Extracellular GSH analysis in synthetic medium made it possible to point out a great difference among almost all the evolved strains and 21T2 (Fig. 2.4b). For the latter, in particular, it was not possible to detect the GSH value as it did not reach the detection limit (0.96 μg mL^{-1}) of the enzymatic kit used. The same condition was observed for Mo21T2-13. The highest extracellular GSH amounts were obtained with the strains Mo21T2-5 (0.65 mg L^{-1}), Mo21T2-10 (0.65 mg L^{-1}) and Mo21T2-14 (0.63 mg L^{-1}). The remaining strains tested showed extracellular GSH concentrations ranging from 0.32 to 0.46 mg L^{-1} with not statistically significant differences.

2.3.3 Microvinification assay: GSH production and analysis of oenological traits

The microvinification assay was performed in Trebbiano white must, inoculated with the parental and the evolved strains, in order to evaluate their ability to produce extracellular GSH in wine, and to assess their oenological aptitude. The must used for the laboratory-scale fermentation showed an initial GSH amount of 2.5 mg L^{-1}. At the end of the fermentation process, the GSH concentration was detected in all the wine samples that had been fermented with the strains tested (Fig. 2.5).

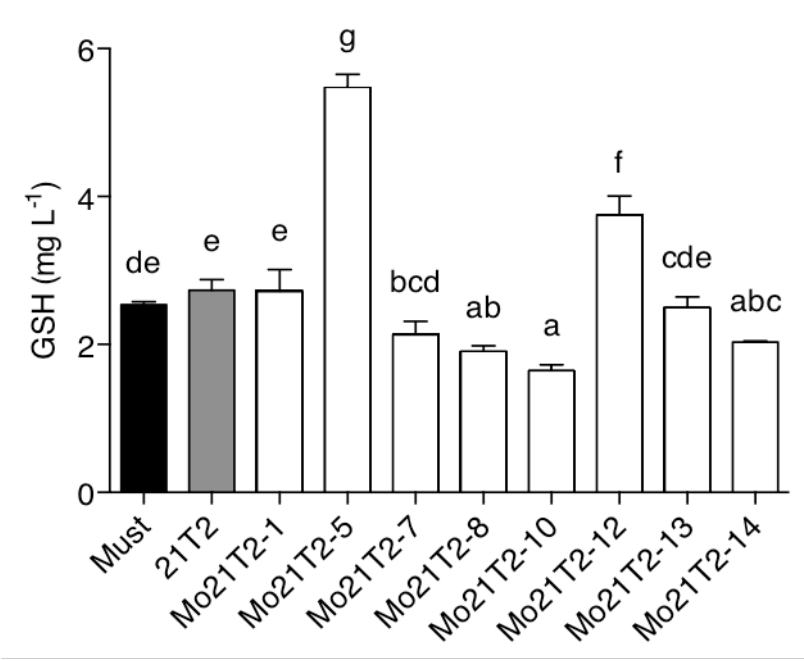

Figure 2.5 - Extracellular total glutathione concentration after microvinification. Graph shows means with SD (n = 3) of must (black column), 21T2 parental strain (gray column) and evolved strains (white columns). Means with different letters are significantly different from each other as determined by Tukey's test ($P < 0.05$).

Compared to the GSH amount of the parental strain (2.75 mg L^{-1}), Mo21T2-5 produced a double amount of GSH (5.5 mg L^{-1}), while Mo21T2-12 showed GSH increasing by 0.36-fold (3.75 mg L^{-1}). On the other hand, Mo21T2-7, -8, -10 and -14 strains displayed a lower GSH production in wine compared to 21T2 and, apart from Mo21T2-7, showed a significantly lower amount of GSH also compared to the initial content in the must. For Mo21T2-10, in particular, the total resultant GSH was 0.35-fold less than the amount in the must.

All the evolved strains detected in the lab-scale fermentation showed notable fermentative aptitudes, comparable to those of the parental strain. After 25 days, all fermentations reached dryness with less than 2 g L^{-1} of reducing sugars (data not shown) indicating the completed fermentation of the must with all the strains used. The oenological traits detected in the microvinification assay, including pH, titratable acidity, ethanol production and fermentation efficiency, are reported in Table 2.2.

The pH analysis displayed a range of values from 3.31 to 3.44 with no significant differences between parental and evolved strains. Titratable acidity analysis (expressed as g L^{-1} of tartaric acid) showed a wider variability with values ranging from 8.87 to 10.21 g L^{-1} even if only the strains Mo21T2-10, -13, -14 were significantly different from

21T2 (9.64 g L^{-1}). The fermentation efficiency observed was close to 90% with no significant differences compared to 21T2, except for Mo21T2-13 (81.81%).

The total SO_2 analysis showed that all strains, parental and evolved, produced almost nil amounts of sulfites, with the exceptions of Mo21T2-5 and -12 that produced 33.3 and 43.1 mg L^{-1} respectively (Fig. 2.6). Interestingly, Mo21T2-5 and -12 were the highest GSH-producing strains in wine. Nevertheless, all strains showed an almost zero amount of free SO_2 (< 2 mg L^{-1}) with no statistically significant difference.

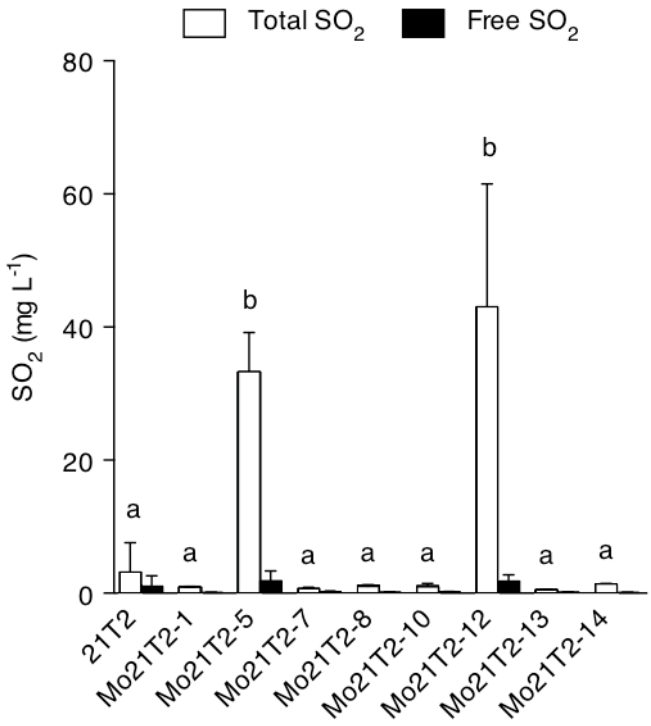

Figure 2.6 - Total and free SO_2 detected after microvinification. Graph shows means with SD (*n* = 3). Means with different letters are significantly different from each other as determined by Tukey's test (P < 0.05). No significant differences were found among all free SO_2 values.

Strains	pH	Titratable acidity (g L^{-1} tartaric acid)	Ethanol Yield (% v/v)	Fermentation efficiency (%)
21T2	3.37^{abc}	9.64^{bcd}	11.18^{bc}	88.34^{bc}
Mo21T2-1	3.31^{a}	9.89^{cde}	11.08^{bc}	87.59^{bc}
Mo21T2-5	3.35^{ab}	9.91^{de}	11.40^{c}	90.09^{c}
Mo21T2-7	3.39^{abc}	9.24^{ab}	11.06^{bc}	87.38^{bc}
Mo21T2-8	3.43^{bc}	9.65^{cd}	10.99^{bc}	86.85^{bc}
Mo21T2-10	3.44^{c}	8.87^{a}	11.27^{c}	89.04^{c}
Mo21T2-12	3.40^{bc}	9.51^{bc}	10.67^{ab}	84.33^{ab}
Mo21T2-13	3.39^{bc}	10.21^{e}	10.35^{a}	81.81^{a}
Mo21T2-14	3.41^{bc}	10.14^{e}	10.61^{ab}	83.86^{ab}

Table 2.2 - Main oenological traits of *Saccharomyces cerevisiae* parental and evolved strains. In bold characters 21T2 parental strain. Data are mean of three independent experiments. Standard Deviation (SD) did not exceed 3% for any of the values. Means with different letters are significantly different from each other as determined by Tukey's test ($P < 0.05$). The titratable acidity was measured at the pH end-point of 7.2. Fermentation efficiency is expressed as $[(Y_{Eth}\ Y_{Eth\ Theor}^{-1}) \times 100]$, where Y_{Eth} indicates the detected ethanol yield and $Y_{Eth\ Theor}$ the theoretical ethanol yield.

2.3.4 Evaluation of H_2S production in BiGGY agar and wine

Hydrogen sulfide formation by parental and evolved strains was qualitatively evaluated on BiGGY agar and on lead acetate strips (Fig. 2.7 a and b). The spotted strains exhibited four different colony color types from white to brown. In particular, we observed white colonies for Mo21T2-5, -8 and -10, light tan colonies for Mo21T2-14, tan colonies for the parental and Mo21T2-7, -12, -13 strains, and brown colonies for Mo21T2-1.

A qualitative measurement of the H_2S produced during fermentation was achieved by the extent of blackening of the lead acetate strips (Fig. 2.7b). In some cases, we encountered a moderate variability within the three replicates performed, therefore, only one representative replicate was considered. The 21T2, Mo21T2-7, -12 and -14 strains appeared to be nil or low producers of H_2S, with a strip blackening ranging from 0 to 1 cm. Four strains, Mo21T2-1, -5, -8, -13, were moderate producers (ranging from 1 to 2 cm). Mo21T2-10 was the highest H_2S-producer, as it blackened the strip completely (3 cm).

Interestingly, it was observed that in some cases there was no correlation between colony color and H_2S formation during fermentation; however, this discrepancy has been already reported by other authors (Spiropoulos et al. 2000; Kumar et al. 2010). In

particular, the Mo21T2-5, -8, -10 strains gave white colonies on BiGGY agar, although they resulted from moderate or high H_2S producers during microvinification. On the other hand, the Mo21T2-7 strain showed tan colonies on the BiGGY medium but was the lowest H_2S-producing strain in wine.

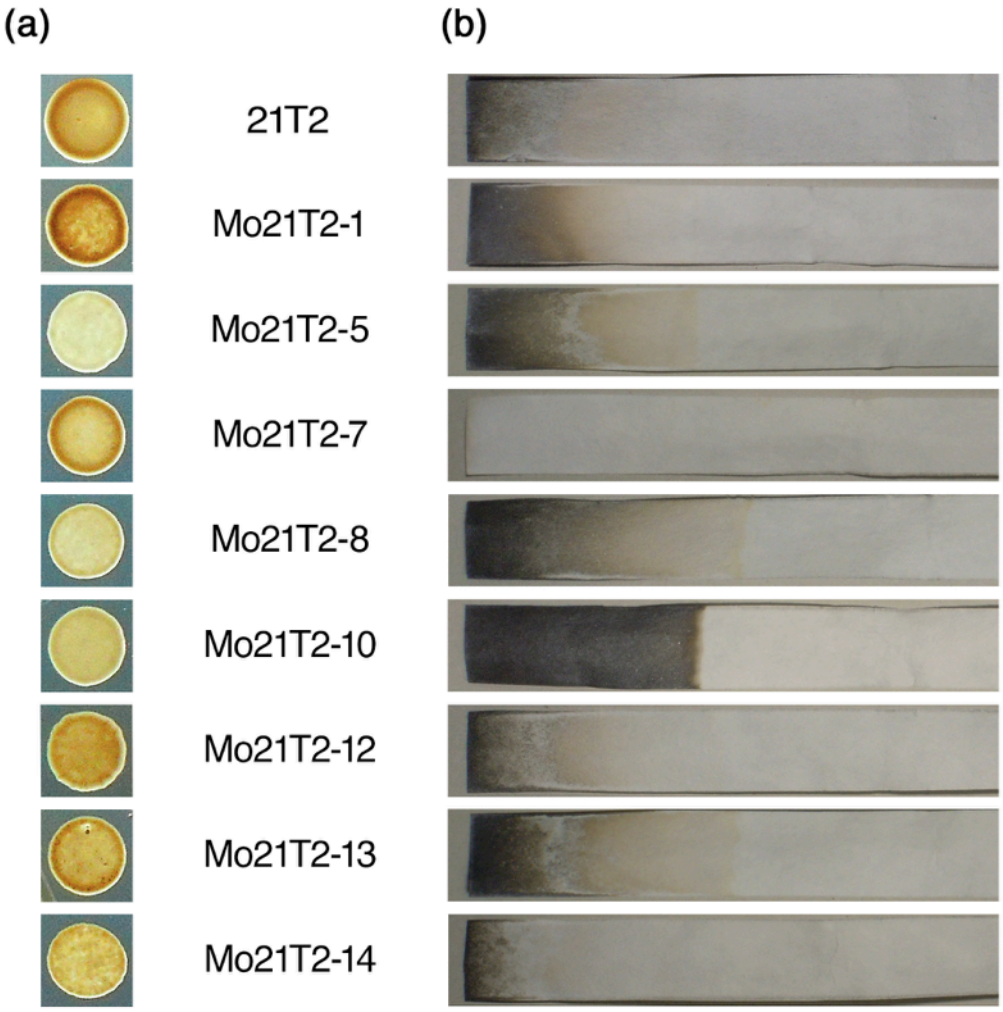

Figure 2.7 - Qualitative evaluation of hydrogen sulfide on BiGGY agar and on lead acetate strips. (a) The colony color of parental and evolved strains were observed after four days incubation. BiGGY agar assay was performed in triplicate. (b) Blackening degree of lead acetate strips after microvinification assay. It is showed one representative replicate of three independent experiments.

2.4 Discussion

In this study we used the *S. cerevisiae* 21T2 strain, previously screened by our laboratory and appreciated for its excellent fermentative and oenological ability, to obtain evolved strains improved for GSH production. To achieve this aim, we applied an

evolution-based strategy that combines the random hybridization of spores and the application of Mo(VI) as specific selective pressure. Particularly, the resistance to this analogue of sulfate, toxic for the cells at high concentrations (Millson et al. 2009), was performed as a rapid and high-throughput screening method for strains with an increased GSH production. In addition, we exploited the genome plasticity of wine yeasts, which are often diploid, heterozygous and homothallic, with a large capacity for genome reorganization through chromosome rearrangements (Sipiczki et al. 2004; Borneman et al. 2011). The eight evolved strains selected showed a wide variability of the phenotype, particularly regarding the production of sulfites, H_2S and GSH. This confirms the effectiveness of meiosis in providing clones with different and frequently better properties than their parental strain, in accordance with the findings of several authors (Pretorius 2000; Marullo et al. 2004; Giudici et al. 2005).

The assessment of intra- and extracellular GSH in synthetic grape juice underlined the variation of the native capability of GSH production in evolved strains. Above all, we obtained two high intracellular GSH-producing strains, Mo21T2-1 and -12, compared to 21T2. Furthermore, all the evolved strains were able to produce extracellular GSH, the only exception being the Mo21T2-13 strain. The highest extra GSH–producing strains were Mo21T2-5, -10 and -14. These data are particularly notable if we consider that, as reported by Perrone et al. (2005), GSH excretion starts at a distinct growth stage, during the end of the exponential phase when the intracellular GSH level has reached a maximum. However, it may be that certain mutations give rise to an altered GSH metabolism with an increased excretion. The genetic characterization of the evolved strains, in particular regarding the expression of genes involved in the GSH metabolism, could be helpful in understanding their different behavior on a molecular level. Even though much work has been done to increase GSH production in *S. cerevisiae* for biotechnological purposes, only one attempt, to our knowledge, has been made with the *S. cerevisiae* wine strain (Hao et al. 2012). Hao et al. constructed a recombinant *S. cerevisiae* strain by self-cloning GSH1 genes and increasing GSH production by 19% in model wine. Approaching from a different direction, we did not use the recombinant DNA technique; instead, we employed an evolution-based strategy more suitable for complex oenological traits. It is remarkable that by this strategy we obtained two evolved strains, Mo21T2-5 and Mo21T2-12, able to enhance GSH content at the end of the fermentation process with an increase of 100% and 36%, respectively, compared to the parental strain, and 120% and 50% compared to initial GSH content in the must.

The reasons for the variation in final GSH levels in different fermentation media remain unexplained but, as also reported by Kritzinger et al. (2013a), they are probably linked to grape juice complexity. However, comparing the GSH assay results obtained in synthetic grape juice and in microvinification, it was remarkable that Mo21T2-5 was the highest GSH-producing strain in wine and one of the best extracellular GSH-producing strains in a synthetic medium. Regarding the Mo21T2-12 strain, which improved the content of GSH in wine by up to 0.36-fold compared to the parental strain, it also showed a high intracellular GSH production in addition to good GSH excretion in a synthetic medium. Overall, our data seem to point to the importance of a homeostasis shifted toward extracellular excretion to obtain wine with an increased GSH content. In the case of Mo21T2-5, the Gex1p glutathione exchanger might play an important role in this shifted homeostasis, according to the behavior of Gex1p-overproducing strains described by Dhaoui et al. (2011). Moreover, overproduction of GSH may be favored by the release of γ-glutamylcysteine synthetase (Gsh1p) from feedback inhibition by GSH at low intracellular concentrations (Richman & Meister 1975; Nisamedtinov et al. 2011).

Regarding sulfite and hydrogen sulfide formation during must fermentation, we also observed that the two highest GSH-producing strains (Mo21T2-5 and Mo21T2-12) were at the same time moderate SO_2-producing strains (SO_2 < 45 mg L^{-1}). These strains were also moderate H_2S producers in must fermentation; therefore we could assume that a fully active sulfate assimilation pathway was involved in the increased GSH metabolism. On the other hand, GSH may also be a source of hydrogen sulfide (Swiegers et al. 2005), following the degradation of cysteine by the cysteine desulfhydrase enzyme to form H_2S. This could be consistent with the Mo21T2-5 screening on the BiGGY medium, where the colony color of the strain was completely white.

In summary, the evolution-based strategy applied in this work was successful for the purpose of generating yeast strains with enhanced GSH production in wine. This strategy, unlike the standard evolutionary approaches, has the advantage of not requiring multiple rounds of screening and extensive cultivation periods because the evolved strains are recognized through a selectable phenotype. In particular, Mo(VI) resistance has proved to be effective for the selection of the desired evolved strains, probably by activating the yeast common metal response that involves sulfur assimilation and GSH biosynthesis.

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Chapter 3

Fermentation trials and glutathione assay with different concentrations of sulfur-containing amino acids

3.1 Introduction

Sulfite is a powerful antioxidant and antimicrobial agent and these properties are exploited in wine and beer industry where it plays an important role in the flavor, stability, quality, and conservation. On the other hand, sulfite is a potentially toxic metabolite in *S. cerevisiae* (Nadai et al. 2016) and is a common human allergen. Moreover it is the metabolic intermediate of the hydrogen sulfide (H_2S), which has an aroma of rotten eggs, and a precursor of other compounds with undesirable sensory characteristics. For these reasons, the sulfur metabolism in *Saccharomyces cerevisiae* is well studied in several works (Giudici & Kunkee 1994; Thomas & Surdin-Kerjan 1997;

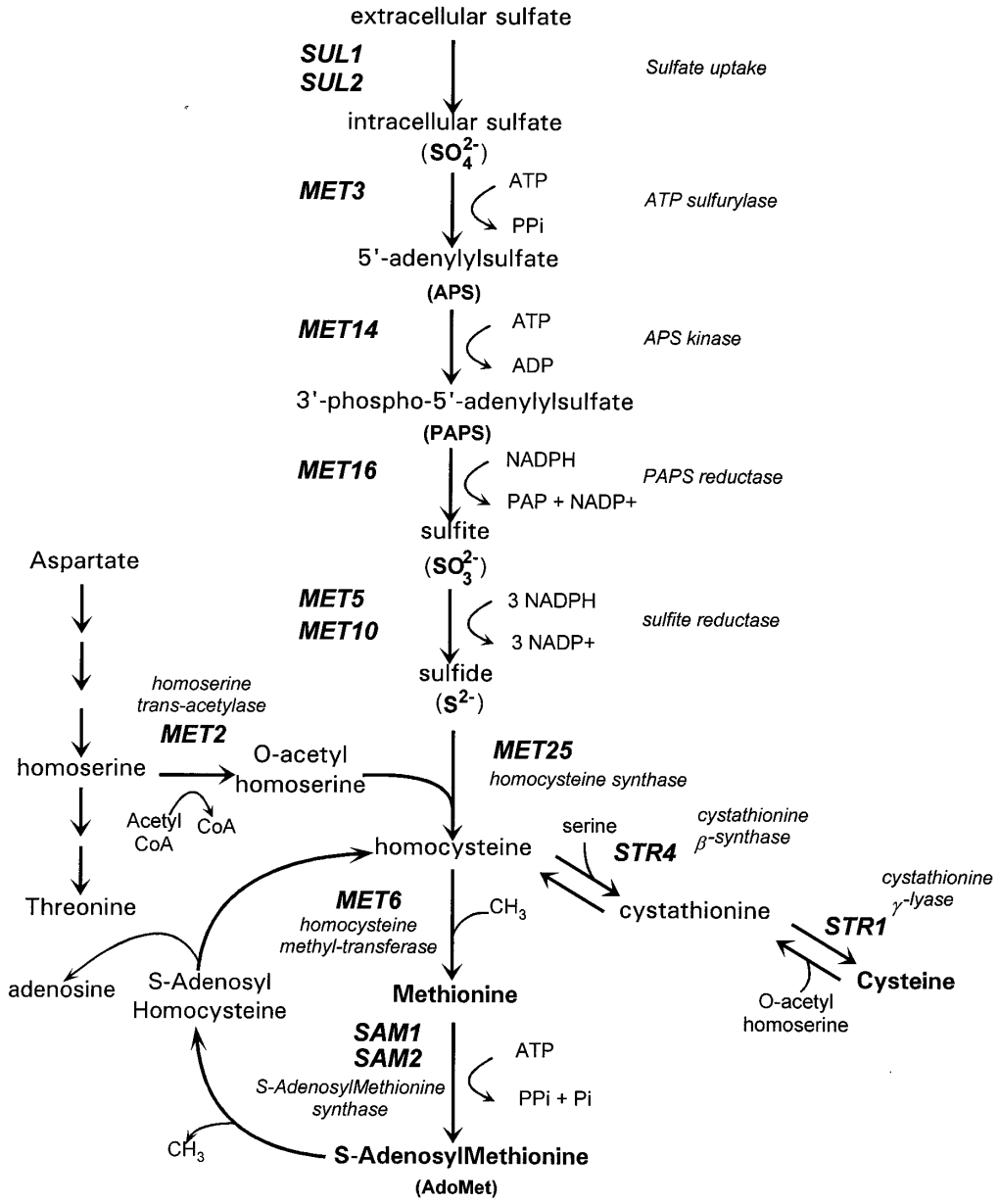

Figure 3.1 - Sulfur amino acid biosynthesis in *S. cerevisiae* (Thomas & Surdin-Kerjan 1997)

Cordente et al. 2009; De Vero et al. 2011). *S. cerevisiae* is able to metabolize almost all inorganic sulfur compounds found in the environment, to reduce them in sulfate and to synthesize organic sulfur metabolites, mostly cysteine and methionine, following the sulfate assimilation pathway (Fig. 3.1). The extracellular sulfate anions are activated into adenylate compounds, subsequently reduced to sulfites, which are further reduced to sulfides by sulfite reductases. Finally, the reduced sulfur atoms can be incorporated into carbon chains by Met17p (Met25p), yielding homocysteine. Homocysteine is the immediate precursor of methionine biosynthesis, through a reaction catalyzed by homocysteine methyltransferase. Regarding the cysteine biosynthesis, it requires two successive steps in the transsulfuration pathway. The cystathionine β-synthase catalyzes the first reaction releasing cystathionine, while cystathionine ϒ-lyase catalyzes the second step, the ϒ-cleavage of cystathionine, giving cysteine. In addition, *S. cerevisiae* contains several enzymatic systems that permit the recycling of the by-products of sulfur metabolism.

Cysteine (mM)	**Methionine (mM)**	**Reference**
0.25	0.01	Mauricio et al. 2001
From 0 to 0.02	nv	Spayd & Andersen-Bagge 1996
From 0 to 0.029	From 0.0024 to 0.037	Spiropoulos et al. 2000
0	From 0 to 0.35	Park et al. 2000
< 0.08	< 0.07	Landaud et al. 2008
0	0.04	Kumar et al. 2010
From 0 to 0.04	From 0 to 0.17	Henschke & Jiranek 1993
< 0.17	nv	Ugliano & Henschke 2009
Average concentration		
0.05	**0.075**	

Table 3.1 – Amount of cysteine and methionine detected in different musts reported in selected literature. nv: not valuated.

For instance, the methyl cycle consists of reactions that recycle S-adenosylmethionine, a major constituent of intermediary metabolism, transforming into S-adenosylhomocysteine and in homocysteine, which can be again metabolized to

synthesize cysteine and methionine. Besides, closely linked to sulfur metabolism, GSH is biosynthesized through cysteine combined with L-glutamic acid and glycine. The complex physiology of sulfur metabolism in yeast is specifically and negatively regulated and, although all metabolites are interconnected, from the industrial point of view it would be desirable to obtain a diversified expression of specific metabolites. Thus, an increase of SO_2 and decrease of H_2S is preferred in beer (Chen et al. 2012), or a high GSH content together with a low SO_2/H_2S concentration in wine (Chapter 2, De Vero et al. 2011). Moreover, it must be considered the wide variability of the natural environment in which wine fermentations are carried out by oenological yeast strains. Indeed, musts vary considerably year-by-year, in relation to the type of wine, and also to different factors during winemaking (du Toit et al. 2006). For example, on one side the biological content of methionine and cysteine in musts is ranging from 0 to 0.35 mM with average concentrations of 0.075 mM and 0.05 mM, respectively (Table 3.1). On the other hand, the common winery operation called "mutage" (when alcoholic fermentation is stopped by sulfur dioxide addition), alter the final amount of sulfates and the content of biologically active compounds (Giudici et al. 2014).

The objective of this work was to verified the relations between the sulfur amino acids metabolism and the GSH formation, with particular regards to test whether the selected evolved strains were able to store the excess of sulfur compounds into GSH (Elskens et al. 1991) or, conversely, into undesirable sulfur compounds (Eschenbruch 1974).

3.2 Materials and methods

3.2.1 Strains and growth conditions

The *Saccharomyces cerevisiae* strains used in this experiment are deposited in the Unimore Microbial Culture Collection (UMCC - www.umcc.unimore.it) and described in Table 3.2. The strain UMCC 855 was chosen as parental strain from which the selected evolved strains were obtained as described in Chapter 2 (Mezzetti et al. 2014). The strains UMCC 2581 and UMCC 2585 were chosen for their high GSH production in wine (§ 2.4, Table 3.2) while the strain UMCC 2584 was chosen for its low production in wine (§ 2.4, Table 3.2). The yeast strains were routinely cultured on YPD agar plates or slants (2% glucose, 2% peptone, 1% yeast extract, and 2% agar), incubated at 27 °C for 48 h, and stored at 4 °C.

Table 3.2 - *Saccharomyces cerevisiae* strains from Unimore[a] Microbial Culture Collection

UMCC code	Other name	Description	Reference
UMCC 855	21T2	Evolved strain from 20001 (UMCC 845) – parental strain	De Vero et al. 2011; Gobbi et al. 2014; Mezzetti et al. 2014; Solieri et al. 2015; Bonciani et al. 2015
UMCC 2581	Mo21T2-5	Evolved strain from UMCC 855 – high GSH production in wine	Mezzetti et al. 2014
UMCC 2584	Mo21T2-10	Evolved strain from UMCC 855– low GSH production in wine	Mezzetti et al. 2014; Solieri et al. 2015; Bonciani et al. 2015
UMCC 2585	Mo21T2-12	Evolved strain from UMCC 855 – high GSH production in wine	Mezzetti et al. 2014

[a] University of Modena and Reggio Emilia- Reggio Emilia (Italy)

3.2.2 Fermentation trials in different synthetic grape juices

All yeast strains were precultured, for 24 h at 27 °C, in 5 mL synthetic grape juice prepared according to Giudici and Kunkee (1994), with YNB w/o amino acids and ammonium sulfate. The cells were then inoculated at an optical density of 0.1 at 600 nm (OD_{600} nm) into tubes, each containing 10 mL synthetic grape juice with or without the addition of methionine and cysteine at the different concentrations of 0.01, 0.05 and 1 mM. The three different values chosen for methionine and cysteine correspond respectively to low, medium and high concentrations compared to the average concentration of these amino acids found in musts as reported in literature (Table 3.1). The fermentation trials were carried out at 27 °C under static conditions. After 7 days (168h, end of fermentation), cells were harvested and centrifuged at 1000 g for 5 min at 4 °C. For each sample the supernatant and the pellet were separately processed as described in § 2.3 to analyze the extracellular and intracellular GSH, respectively. Supernatant was processed also to analyze the total SO_2 content. The qualitative amount of H_2S formed during fermentation trials was detected by lead acetate paper strips as previously described (§ 2.3).

3.2.3 Analytical methods

The GSH produced by the parental and selected strains was determined as previously described (§ 2.3) with the enzymatic Glutathione Assay Kit (Sigma-Aldrich) following the manufacturer's instructions. Enzymatic kit (Megazyme, Bray, Ireland) was also used to quantify total SO_2 (K-SULPH) according to the manufacturer's instructions.

3.2.4 Statistical analysis

Statistical analysis was carried out by analysis of variance (ANOVA) using GRAPHPAD PRISM v. 6.0 (GraphPad Software Inc., San Diego, CA). Tukey's multiple comparison test was used to identify significant differences between strains ($P < 0.05$). All experiments were performed in triplicate.

3.3 Results

3.3.1 Evaluation of GSH in the fermentation trials

The fermentation trials were performed in synthetic grape juice (SGJ) with or without addition of methionine and cysteine to evaluate whether the evolved strains selected were able to store the excess of these sulfur amino acids in GSH. At the end of the fermentation process, the intracellular, extracellular and total (intra- + extracellular) GSH concentrations, expressed as mg L^{-1} per OD_{600}=1, were detected in all the tested samples (Fig. 3.2a, b and Fig. 3.3). Regarding the intracellular GSH content detected in SGJ without addition of sulfur amino acids, the highest value (0.201 mg L^{-1}) was observed with the parental strain UMCC 855, (Fig. 3.2a). When 1 mM cysteine was added to the SGJ, the best performance was showed by the evolved strain UMCC 2585 (0.255 mg L^{-1}). Almost no statistically significant difference in the intracellular GSH content was observed among all the strains in the media with 0.5 mM methionine while with 1 mM methionine the strains UMCC 855 and 2581, displayed the highest GSH intracellular values (0.288 and 0.278 mg L^{-1}, respectively). A completely different profile was observed for the extracellular GSH concentrations detected (Fig. 3.2b). Compared to all selected evolved strains, UMCC 855 strain produced constantly the lower extracellular GSH concentrations in synthetic grape juice with or without addition of methionine and cysteine. Contrariwise, the UMCC 2581 strain showed the highest GSH values in all tested media with the only exception of 0.05 mM cysteine (the maximum concentration of 0.950 mg L^{-1} was reached with 1 mM methionine). Noteworthy, only

when cysteine was added to SGJ, the UMCC 2584 showed a progressively increase in GSH concentration reaching 0.940 mg L^{-1} with the addition of 1 mM cysteine. Overall, the highest content of total GSH (intra- and extracellular GSH) was obtained with the evolved strain UMCC 2581 (Fig. 3.3). In particular, in SGJ without addition of the sulfur amino acids, a total GSH value of 0.804 mg L^{-1} was achieved with this strain. The resulted

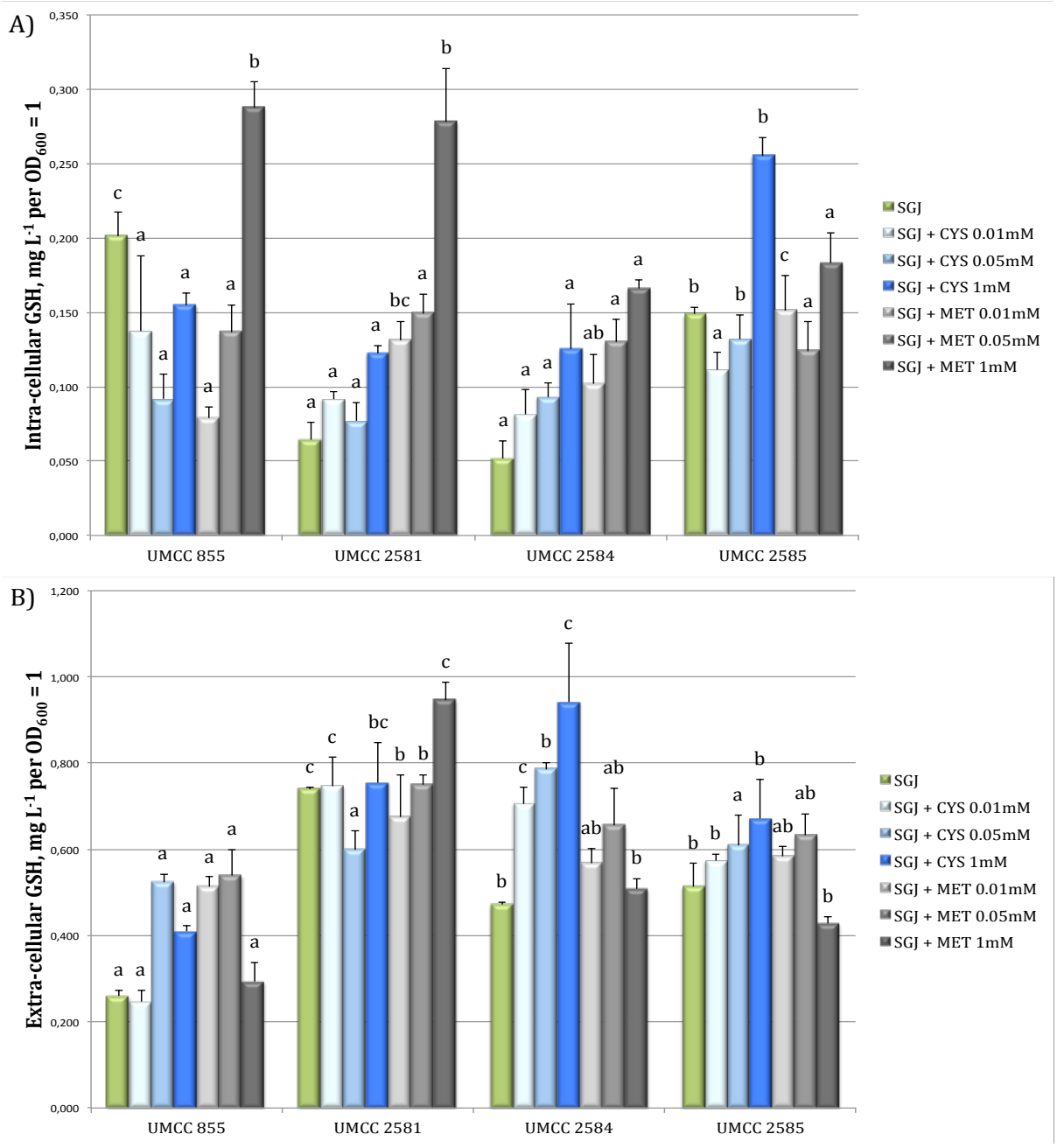

Figure 3.2 – Intracellular and extracellular GSH content after fermentation assay carried out in synthetic grape juice with and without the addition of methionine and cysteine at different concentrations. A) Intracellular GSH concentration is expressed as mg L^{-1} per OD_{600}=1. B) Extracellular GSH concentration is expressed as mg L^{-1} per OD_{600}=1. Graph shows means with SD (n = 3) of UMCC 855 parental strain and selected evolved strains. For each media, means with different letters are significantly different from each other as determined by Tukey's test (P< 0.05).

GSH increment was of 74.3% comparing to parental strain (0.461 mg L^{-1}). With the addition of 0.01 mM cysteine, all the evolved strains showed an increase in total GSH production ranging from 118.6% for UMCC 2581 (0.838 mg L^{-1}) to 78.2% for UMCC 2585 (0.685 mg L^{-1}), compared to UMCC 855 strain (0.383 mg L^{-1}). Instead, in SGJ with the addition of 0.05 mM and 1 mM cysteine, the higher total GSH productions were reached with the evolved strain UMCC 2584 that showed an increase of 39.6% and

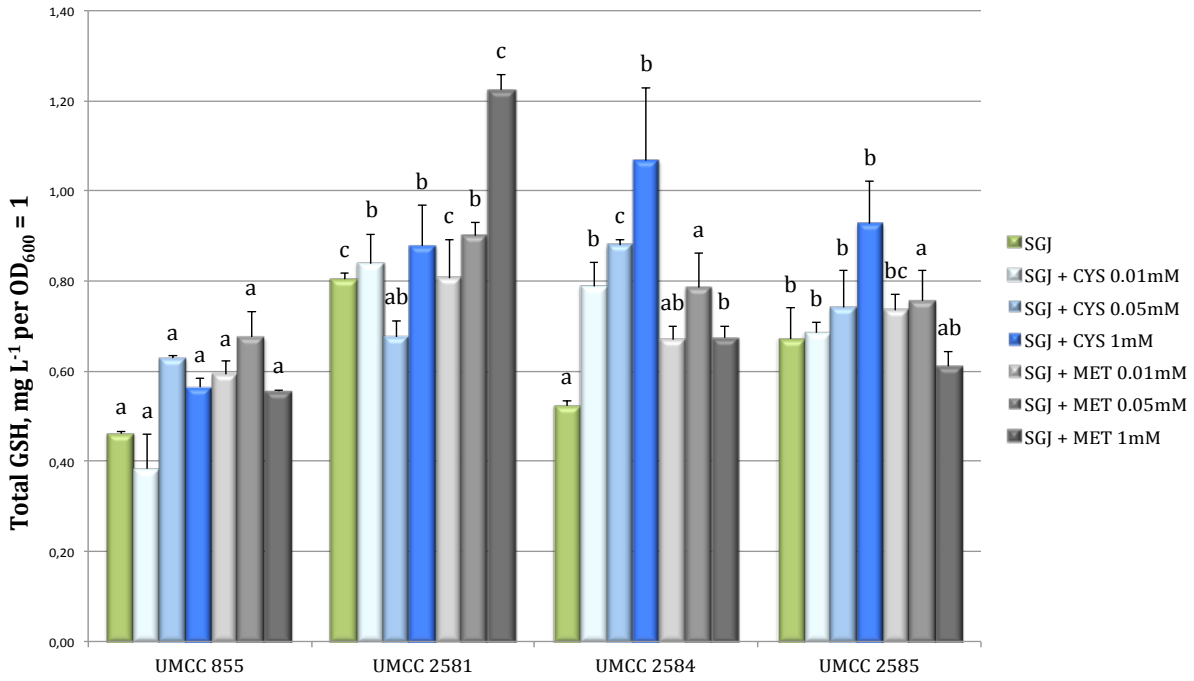

Figure 3.3 - Total (intra- + extracellular) GSH content after fermentation assay carried out in synthetic grape juice with and without the addition of methionine and cysteine at different concentrations. Total GSH concentration is expressed as mg L^{-1}. Graph shows means with SD (n = 3) of UMCC 855 parental strain and selected evolved strains. For each media, means with different letters are significantly different from each other as determined by Tukey's test ($P < 0.05$).

89.0% (0.878 and 1.067 mg L^{-1}) respectively, compared to parental strain (0.629 and 0.565 mg L^{-1}). Finally, the best performances in SGJ supplemented with methionine at all concentrations, were obtained with the strain UMCC 2581. In particular, compared to parental strain, this strain showed an increase of 36.0%, 33.2% and 120.4% respectively in the three different media with the methionine (GSH values with parental strain: 0.593, 0.675, 0.555 mg L^{-1}; GSH values with UMCC 2581: 0.806, 0.899, 1.224 mg L^{-1}). Regarding the strains UMCC 2584/2585, statistically difference in total GSH content, compared to UMCC 855 strain, were observed only in the following conditions: in SGJ containing 1 mM methionine, where the increase was of 21.3% (0.674 mg L^{-1}), and in

SGJ containing 0.01 mM methionine, where the increase was of 24.0% (0.735 mg L^{-1}) with UMCC 2584 and UMCC 2585 respectively.

3.3.2 Evaluation of total SO_2 and H_2S

The total SO_2 analysis showed a final amount profile (Fig. 3.4) that, interestingly, nearly overlapping the extracellular GSH content graph (Fig. 3.2b). The UMCC 2581 evolved strain showed the highest value of total SO_2, expressed as mg L^{-1} per OD_{600}=1, in SGJ without addition of sulfur amino acids reaching the concentration of 9.47 mg L^{-1}. A moderate total SO_2 contents, ranging from 6.25 to 5.13 mg L^{-1}, were detected with the evolved strains UMCC 2584/2585, while the lower concentration (2.84 mg L^{-1}) was reached by UMCC 855 strain. Otherwise, in presence of 0.01 and 0.05 mM cysteine, the UMCC 2581/2584 strains showed the highest sulfites values (9.02, 10.05 and 9.16, 10.33 mg L^{-1} respectively), whereas lower concentrations were obtained with UMCC 855 and UMCC 2585 strains (3.15, 6.61 and 5.18, 6.78 mg L^{-1} respectively). Although the absolute values of total SO_2 concentration were different, the statistical differences between parental and all evolved strains in media supplemented with cysteine 1 mM and all used

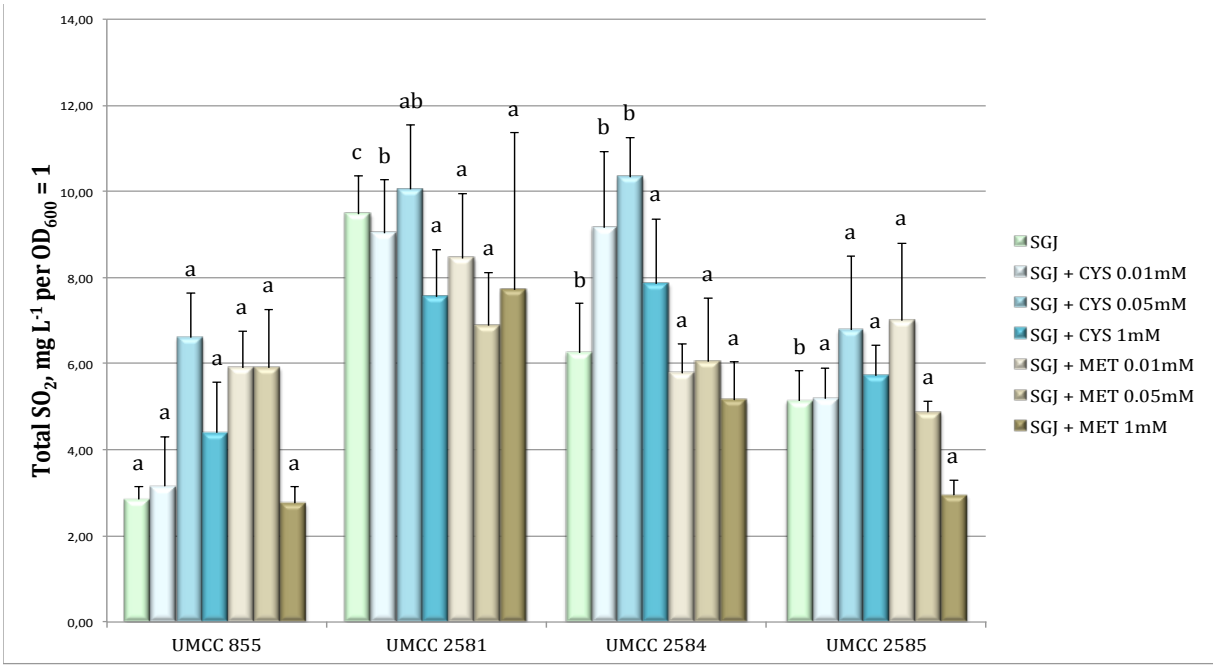

Figure 3.4 - Total SO_2 detected after fermentation assay carried out in synthetic grape juice with and without the addition of methionine and cysteine at different concentrations. Total SO_2 concentration is expressed as mg L^{-1}. Graph shows means with SD (n = 3) of UMCC 855 parental strain and selected evolved strains. For each media, means with different letters are significantly different from each other as determined by Tukey's test ($P< 0.05$).

concentrations of methionine (0.01, 0.05 and 1 mM) were not significant due to high values of standard deviations obtained in this experiment.

A qualitative measurement of the H_2S produced during fermentation assay was achieved by the extent of blackening of the lead acetate strips (data not shown). Remarkably, the H_2S production appear to be nil for all strains in all conditions. In fact, only in few cases we encountered a slight strip blackening, but we never observed a blackening in more than one out of three replicates strips performed.

3.4 Discussion

After the constitution of the new oenological strains able to increase the final GSH amount in wine (Chapter 2), with the fermentation trials carried out in this study we evaluated the relations, in stress situations mimicking the natural must fermentations, between the metabolism of sulfur amino acids and the GSH formation. Specifically, we wanted to test if the evolved strains were able to store the excess sulfur compounds into GSH or if the high value of sulfates led to an increase in the production of undesirable sulfur compounds. The intra- and extracellular graphs (Fig. 3.2a, b) firstly confirmed the important result, from the technological point of view, achieved in the previous experiments (§ 2.3), with the GSH homeostasis shifted to the extracellular media. Furthermore, also the findings with SGJ without addition of sulfur amino acids were nearly comparable with our previous achievements (Chapter 2), even if the data were collected at different fermentations stage. More interesting results, instead, were obtained when sulfur amino acids were added in the media, in particular for UMCC 2581 strain. Indeed, considering all tested media, this evolved strain with respect to UMCC 855 showed an average increase of 63.6% in total GSH content pointing out its great ability to produce GSH almost regardless the media (no statistical difference with parental strain was shown only with SGJ + 0.05 mM of cysteine, Fig. 3.3). Interesting to note also the behaviour of UMCC 2584, which was chosen as low producer in wine (§ 2.3). It displayed a growing increment of intra-, extracellular and therefore total GSH (Fig. 3.2 and 3.3) in media added with cysteine, but the same performances were not achieved adding methionine. This behaviour is probably due to a different metabolic pathway followed by the methionine that not allow to store the excess of this sulfur amino acid into GSH as happened with the cysteine. Finally, the UMCC 2585 strain

confirmed what exhibited in the former experiments in SGJ and wine (§ 2.3), given a high GSH synthesis, but not as intense as detected for UMCC 2581.

Regarding the sulfites and hydrogen sulfide formation, it is noteworthy the nil production of H_2S and, consequently, of the relative undesirable off-flavors. However, the qualitative analysis used for H_2S evaluation (lead acetate strip) and the little volume of the fermentations experiment (10 mL) could affect the final results. The similar profile results observed in the extracellular GSH content and total SO_2 graphs (Fig. 3.2b and Fig.3.4), emphasize the requirement of a fully active sulfate assimilation pathway for an increased GSH metabolism. Marked differences were exhibited only when the synthetic grape juice were supplemented with the highest concentrations of cysteine and methionine (1 mM), but the lower concentrations found in these cases, could be due to the repressive effect of high concentrations of the sulfur amino acids on sulfate assimilation pathway (Hansen & Johannesen 2000; Wheeler et al. 2003). Taking into consideration that our evolved strains were selected on molybdate selective media, these results are consistent with what observed by Thorsen and coworkers (2009). They reported the identification of several metal sensitive mutants having functions in the sulphur assimilation and glutathione biosynthesis pathways as well as transcriptional regulators controlling these pathways, underscoring the importance of the sulfur/glutathione biosynthesis pathways for metal tolerance acquisition.

In this study we tested the ability of selected evolved strains to store the excess of sulfur amino acids in GSH rather than in undesirable compounds. We found that UMCC 2581 was particularly able to produce GSH independently from the fermentative media used and this is an important aspect for a future technological application.

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Chapter 4

Molecular characterization of the evolved strains

4.1 Genetic fingerprinting by interdelta regions, microsatellite and karyotype analyses

Genetic modifications of microorganisms essential in the production of fermented foods such as bread, beer, wine, or cider have always had industrially relevance. Combinatorial approaches can alter the entire cellular environment by strain randomization and subsequent selection/screening for the improved phenotype. An essential step towards the variation between strains of *S. cerevisiae* is the rigorous validation of evolved progeny and different methodologies have been described to discriminate between parents and their progeny, such as karyotyping, microsatellite analysis, PCR-RFLP or sequencing of divergent rRNA gene regions or housekeeping markers (Solieri et al. 2015). Moreover, the discovery of the instability of the yeast karyotype during vegetative growth posed a severe threat for all industries that rely on the propagation and metabolic properties of yeast strains (Carro & Piña 2001). The genetic properties of an unstable strain may vary making it inadequate for the intended process, so another key issue for industrial applications is the genetic and phenotypic stability of the strain, which guarantee the maintenance of its properties in the final products.

In order to obtain a strain typing of the evolved strains, PCR amplification of interdelta and microsatellite regions were performed. Furthermore, the karyotype analysis was carried out in order to assess the effect of sexual recombination and genome reorganization in the evolved strains compared to the parental strain.

4.1.1 Materials and methods

The molecular characterization of the parental and evolved strains was performed through a multi-approach analysis that included PCR amplification of the interdelta and microsatellite regions. Genomic DNA was extracted from 5 mL of YPD (2% glucose, 2% peptone, 1% yeast extract) cultures of the strains using the standard phenol-chloroform extraction method as described by Hoffman & Winston (1987). PCR amplifications were carried out in 25 µL of reaction volumes containing 25 µg of yeast DNA and oligonucleotide primer of the delta family, for the amplification of the interdelta regions, and with primer $(GTG)_5$ for the amplification of the microsatellite regions (Legras and Karst 2003; Baleiras Couto et al. 1996). Amplification products were separated by electrophoresis run on 1.5% agarose gel for 2 h at 80 V. Karyotype analyses of parental

and evolved strains were performed using pulse-field gel electrophoresis (PFGE). Chromosomes were first prepared from overnight cultures in agarose plugs and then separated with a CHEF DRII apparatus (Bio-Rad) as described by Solieri et al. (2015). Agarose gel was then stained with ethidium bromide, washed in TBE buffer and visualized under UV light.

4.1.2 Results and discussion

Discrimination of strains within the species *Saccharomyces cerevisiae* is achievable only combining results obtained with different techniques, since there is no single PCR-mediated typing technique that, taken by itself, allows a good discrimination between strains (Baleiras Couto et al. 1996). In the genome of S288c *S. cerevisiae* strain, about 300 delta elements are described and are therefore good candidate targets for identification of polymorphisms. For the amplification of interdelta regions, we chosen the optimized primers proposed by Legras & Karst (2003). On the other hand, how described by Baleiras Couto et al. (1996), another PCR approach able to generate a high degree of diversity in *S. cerevisiae* is the PCR fingerprinting with primers $(GTG)_5$. The strain typing of parental and all evolved strains was therefore performed by combining interdelta regions analysis and $(GTG)_5$-fingerprinting. The higher degree of diversity was obtained with amplification of delta elements (Fig. 4.1a), which gave rise to six different profiles comparing to parental strain UMCC 855: UMCC 2580/2581, UMCC 2582, UMCC 2583/2584, UMCC 2585, UMCC 2586, UMCC 2587. However, as mentioned above, a single PCR-mediated typing technique does not allow a good discrimination between strains, thus a $(GTG)_5$-fingerprinting was performed as well (Fig. 4.1b). Differently, the $(GTG)_5$ amplification showed only three different profiles: UMCC 2580/2582/2584/2587, UMCC 2581 and UMCC 2583/2585/2586. Combining the results of both amplification experiments, we obtained a different profile for each evolved strain since the strains UMCC 2580/2581 and UMCC 2583/2584, that showed an identical profile after amplification of delta elements, showed a different profile after amplification of $(GTG)_5$. As might be expected after a sexual recombination of spores, forced during the evolution-based strategy, each evolved strain has shown a different electrophoretic profile compared to the parental strain and to the other evolved strains, proving that parental and all evolved were different strains. In addition, the karyotyping experiments, performed by PFGE, underlined the effect of sexual recombination and genome reorganization. Indeed, CHEF analysis showed multiple polymorphic bands

between parental and evolved strains corresponding in particular to chromosomes IV, XVI, V/VIII and IX (indicated by white star in Fig. 4.2). Chromosome IV, apparently lost in parental strain, missed also in UMCC 2584/2587 strains but not in the other evolved strains. Similar situation was found for the chromosome XVI, present only in UMCC 2584/2585 and for chromosomes V/VIII present in UMCC 2582/2587 but not in the

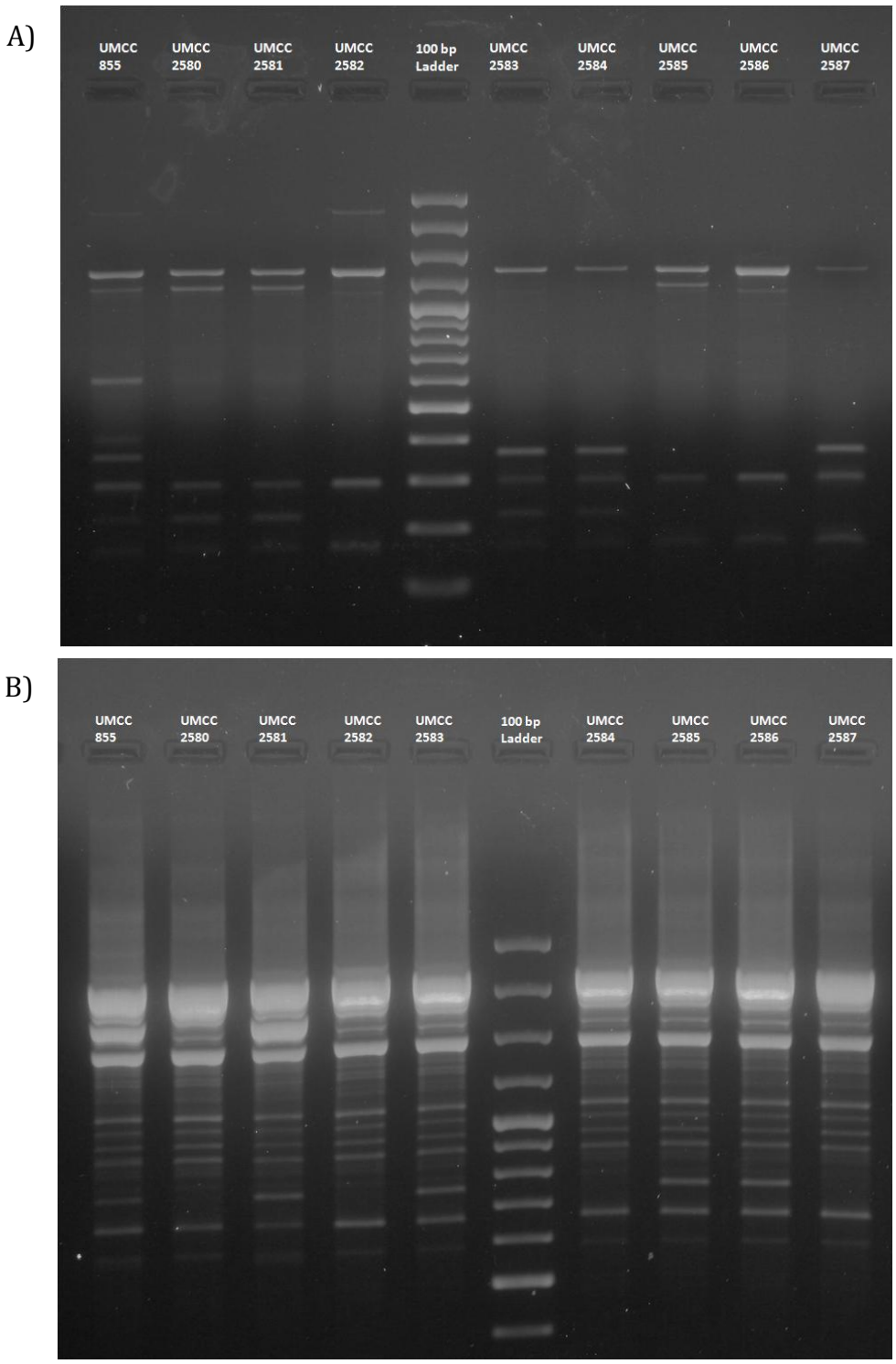

Figure 4.1 – PCR amplification profiles of parental strain (left line) and evolved strains. A) Interdelta profiles. B) $(GTG)_5$-PCR profiles. Marker: GeneRuler 100bp DNA Ladder (Fermentas, Thermo Fisher).

other tested strains. Instead, the chromosome IX showed probably an altered mobility since the gel showed two bands in UMCC 855 parental strain rather than one as in line M (marker) and in all other lines. Although our experiment showed a so high chromosome variability, it is known that yeast strains isolated from nature may show high rates of changes in their karyotypes (Carro & Piña 2001). Noteworthy however, changes used to occur primarily in the higher-mobility chromosomal bands, which would correspond to chromosomes I, VI, and III, while in our experiment the bands with altered mobility correspond to chromosome IV, XVI, V/VIII and IX.

Overall, our data seem to point out to the effectiveness of evolution-based strategy to exploit the genome plasticity with a large capacity for genome reorganization through chromosome rearrangements of wine yeasts (Borneman et al. 2011).

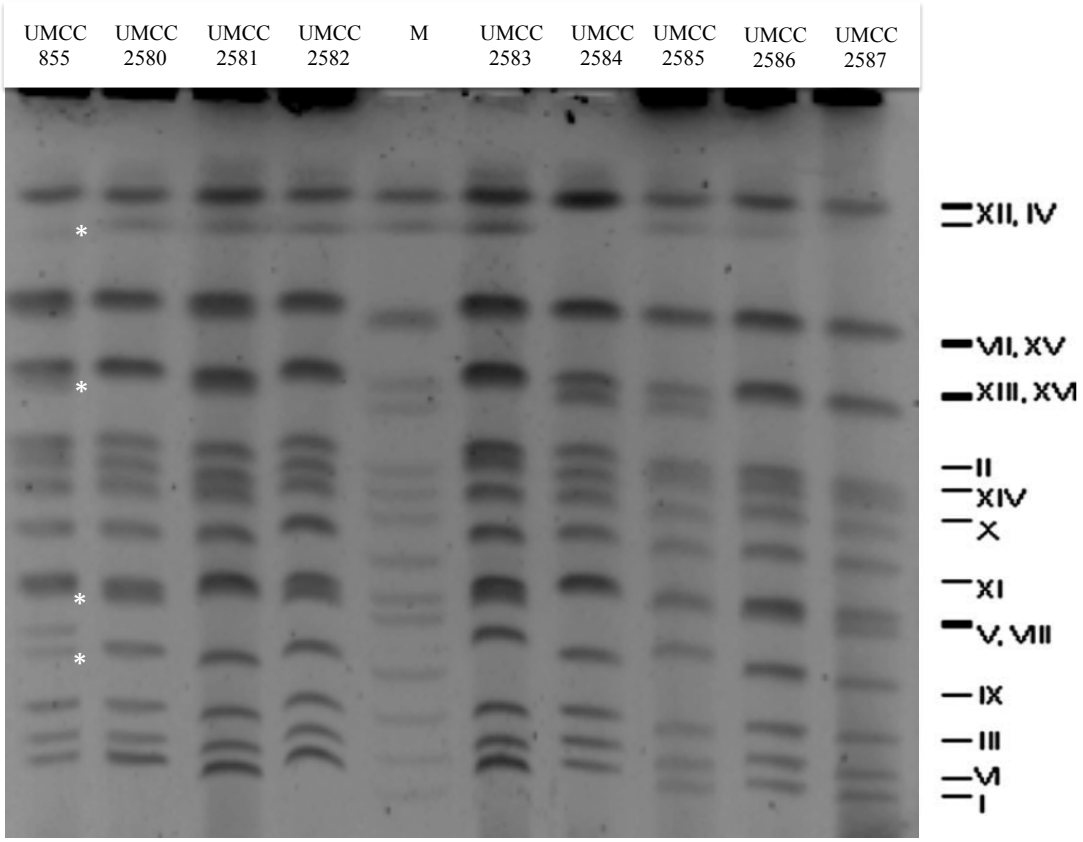

Figure 4.2 - Karyotype profiles of UMCC 855 and evolved strains. *S. cerevisiae* YNN 295 (Bio-Rad Laboratories) was used as reference strain (lane M). Polymorphic bands are marked with white stars.

4.2 Genome-wide analysis

The genetic variability found in wine yeast wild strains as well as in evolved strains obtained from hybrids or different techniques, is correlated with metabolic variations (Giudici & Zambonelli 1992; Ambroset et al. 2011; Engle & Fay 2012; Treu et al. 2014). The induction of recessive mono-, or at most, polygenic mutations has been the most used analyzing method to dissect metabolic pathways in *S. cerevisiae* (Inoue et al. 1999; Linderholm et al. 2008; Puigpinós et al. 2015), although the silenced enzymes were not often able to fully explain the variations among individuals. In a complex living organism, indeed, the metabolic dynamics vary in a multi-factors, continuous way, and not as all-or-nothing switches, following a typical polygenic determinism (Pretorius 2000; Marullo et al. 2004; Giudici et al. 2005). More recently, the quantitative trait loci (QTL) mapping analysis, has allowed to detect the multitude of genes that affect the value of a single quantitative trait, also overcoming the necessity of a priori hypothesis on gene function typical of rational or "classic" metabolic strategy (Cakar et al. 2012). In addition, the current high-throughput DNA and RNA sequencing technologies permit to produce gigabases of sequence information in a single experiment, allowing a rapid identification of genetic variations and facilitating, therefore, an efficient genetic mapping. In this work, we have combined the whole-genome sequencing, which reveals the repertoire of point mutations and copy number polymorphisms, with the gene expression analysis carried out by RNA-sequencing (RNA-seq), that allows an accurate measurement of gene expression and an effective comparison between the transcriptional profiles of parental and evolved strains. The RNA-seq has proved to be a better method for the study of industrial wine strains comparing to microarray. Indeed, it is more sensitive in detecting genes with very low expression and more accurate in the quantification of highly expressed genes, due to its wider dynamic range (Nagalakshmi et al. 2008; Treu et al. 2014). Our aim was to analyze the molybdate resistance phenotype as a specific quantitative trait, in order to understand the relations with and the reasons of the higher GSH production showed by UMCC 2581 evolved strain in comparison to UMCC 855 parental strain (§ 2.3, § 3.3). In fact, as previously shown (Chapter 2 and 3), we successfully applied our evolution-based strategy to *S. cerevisiae* UMCC 855 strain, obtaining evolved strains improved for GSH production. This strategy, which combines random hybridization of spores and the application of Mo(VI) as specific selective pressure, resulted in the generation of yeast strains with enhanced GSH by activation of the yeast common metal response that involves GSH biosynthesis,

as we assumed. Thus, the identification and the characterization of the *S. cerevisiae* UMCC 855/2581 strains genomic diversity were fundamental to understand their phenotypic potential and to provide genetic tools for a further optimization of new oenological yeast strains.

4.2.1 Materials and methods

4.2.1.1 Strains and growth conditions

The *Saccharomyces cerevisiae* strains used in these experiments are the same described in Table 3.2 (§ 3.2.1) with the addition of UMCC 2580 that showed a high GSH production in synthetic grape juice but not in wine (Chapter 2). The parental strain UMCC 855, from which were obtained the selected evolved strains described by Mezzetti et al. (2014, Chapter 2), was used to generate the monosporic clones (MSCs) throughout this study.

Yeast cells were grown at 28 °C on YPD complete medium (1% yeast extract, 2% peptone and 2% glucose) with the addition of 2% agar when necessary.

4.2.1.2 Sporulation and screening of the monosporic cultures

An overnight culture of UMCC 855 grown at 28 °C on YPDA, was resuspended in 3 mL of 1% Potassium Acetate (Fisher Scientific) and incubated overnight at 30 °C and 300 r.p.m. to induce sporulation. The asci were diluted 2-fold with the same volume of 10 mg mL^{-1} Zymolyase (20T, Fisher Scientific), spotted in YPDA plate and incubated for 1 h at 30 °C. Asci dissection was performed using a micromanipulator (Singer Instruments MSM System 200) and the obtained monosporic cultures were subsequently plated on

UMCC code	Description	Color phenotype on 2.5 mM	Growth phenotype on 2.5 mM
UMCC 855	Parental strain	White/Light Blue	Low/Intermediate
UMCC 2580	Evolved strain	Dark Blue	Resistant
UMCC 2581	Evolved strain	Dark Blue	Resistant
UMCC 2584	Evolved strain	Dark Blue	Resistant
UMCC 2585	Evolved strain	Dark Blue	Resistant
Cluster Sensitive	pool of 37 segregants of 21T2	///	Sensitive
Cluster Resistant-Parental	pool of 9 segregants of 21T2	White/Light Blue	Low/Intermediate
Cluster Resistant-Evolved	pool of 9 segregants of 21T2	Dark Blue	Resistant
Cluster Resistant-Other	pool of 14 segregants of 21T2	Miscellaneous	Various but able to grow at least at 1 mM

Table 4.1 - Schematic indication of the clusters features

Yeast Nitrogen Base (YNB) without amino acids and ammonium sulfate (DIFCO) supplemented with different molybdate Mo(VI) concentrations (1 mM, 2.5 mM and 5 mM) to evaluate their resistance phenotype according to Mezzetti et al. (2014).

On the basis of the phenotype showed, the monosporic cultures were clustered in four different groups: Sensitive, Resistant strain-like Parental (Resistant-Parental), Resistant strain-like Evolved (Resistant-Evolved), Resistant strain-like Other (Resistant-Other) (Table 4.1). In the Sensitive group we clustered all clones that were not able to grown on Mo(VI) (at all concentrations). On the other hand in the resistant groups, we clustered all clones that were able to grown at least on 1 mM Mo(VI) but they have been subsequently separate in three different groups taking into account the color of the colonies in addition to the molybdate resistance grade. In the Resistant-Parental group we gathered all clones that have shown dark blue colonies on the media with 1 mM Mo(VI), white colonies on the media with 2.5 mM Mo(VI) and no growth on 5 mM Mo(VI). In the Resistant-Evolved group we clustered all clones that have shown dark blue colonies on the media with concentrations of 1 mM and 2.5 mM Mo(VI), while clones that have shown all other phenotypes have been collected in the Resistant-Other group.

4.2.1.3 Genomic DNA extraction, sequencing and data handling

Genomic DNA (gDNA) was extracted using the ZR fungal/bacterial DNA miniprep kit (Zymo Research) following the manufacturer's instructions. The gDNA of the monosporic clones were pooled in equimolar amount in the respective cluster and the whole-cluster gDNA was sequenced along with gDNA of the parental and evolved strains.

Genome sequencing was performed with the Ion Torrent ProtonTM platform (Life Technologies) by the Genomics Core Facility at Saint Louis University School of Medicine. Sequence reads were mapped to the *S. cerevisiae* reference genome, S288c (Cherry et al. 2012), using BWA (Burrows-Wheeler Aligner, Li & Durbin 2009). Sequencing were then manually edited using Picard 1.114 (http://broadinstitute.github.io/picard) and the Genome Analysis Toolkit (GATK v3.4-46, DePristo et al. 2011) was used for the variant discovery (single nucleotide polymorphisms, SNPs; insertions and deletions, InDels). The information was finally saved in ".vcf" format (variant call frequency).

In order to map the Quantitative Trait Loci (QTL) putatively involved in the evolved phenotype, we selected the heterozygous sites of the parental strain UMCC 855 using a self-written perl script on the list in "vcf" format. The Alleles Frequencies (AF) were calculated and only sites with an AF ranging from 0.25 – 0.75 and coverage reads greater than 20 were filtered out and used in the subsequent comparison. In Clusters Resistant-Parental/Resistant-Evolved comparison a p-value was assigned to each sites according to the Fisher's Exact Test. A $-\log_{10}$(p-value) greater than 20 log unit was chosen as a cutoff to call QTL peak and the width of each peak was determined dropping 5 log unit.

The SNPs and InDels specific of parental strain UMCC 855 and evolved strain UMCC 2581 were identified comparing the list in "vcf" format. The discovered variants were analyzed using the snpEff software (Cingolani et al. 2012, http://snpeff.sourceforge.net) to classify them according to their effect on protein-coding genes. Reads alignments and subsequent comparison of the "vfc" files underwent a strict quality and coverage verification that led to the exclusion of SNPs and InDels with coverage lower than 20x and frequency lower than 25% of the reads.

To discover regions of chromosomal copy-number variations (CNVs), the average sequencing coverage over a 1000 bp window size were calculated using IGVtools (Thorvaldsdottir et al. 2013).

4.2.1.4 Differential gene expression analysis

To capture gene expression changes between the parental and evolved strains, we performed RNA-seq experiments. Selected strains were inoculated in Erlenmeyer flasks filled with 100 mL of chemically defined synthetic must (SM) prepared according to Giudici & Kunkee (1994). Cell growth was monitored by measuring the optical density at 600 nm hourly until reaching the end of exponential phase. At each time point, the cells were harvested and centrifuged, then supernatant was removed and the pellet was immediately frozen in liquid nitrogen and stored at -80 °C until sample analysis. For each sample, the time point corresponding to the ¾ of exponential phase was chosen and the total RNA was extracted with hot acidic phenol (Ausubel et al. 2002). mRNA purification, RNA-Seq library preparation and sequencing were performed by the Genomics Core Facility at Saint Louis University School of Medicine using Ambion Dynabeads mRNA Direct Micro Kit (Life Technologies), Ion Total RNA-seq kit v2 (Life Technologies) and the Ion Torrent ProtonTM platform (Life Technologies) respectively. Bowtie 2 (Langmead & Salzberg 2012) was used to align reads for each sample to the reference genome S288c, Picard 1.114 to edit the reads while DESeq (Anders & Huber 2010) was used to identified genes differentially expressed between parental and evolved strains. Only genes with a false discovery rate (FDR) lower than 0.05 were considered differentially expressed. The gene ontology analysis was performed by using the on-line tool GO Term Finder (http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl) within the SGD database. The statistical analysis of the GO term "molecular function" was performed using the BiNGO plug-in (Maere et al. 2005) in Cytoscape (http://www.cytoscape.org).

4.2.1.5 Note

The analysis of the mapped QTL peaks with the identification of SNPs and InDels in the candidate genes as well as the analysis of RNA-seq data, have been carried out only on the parental strain and the evolved strain UMCC 2581 which showed the best performance in GSH production. The selection of just one evolved strain to deep analyze, has been a forced choice since the huge amount of data collected with genome and transcriptome sequencing has been done in the last year of my PhD thesis project.

4.2.2 Results

4.2.2.1 Chromosomal variations

In order to catalogue copy-number variation (CNV) in the UMCC 855 and 2581 yeast genomes, the depth of sequencing coverage determined for each genomes were calculated (Fig. 4.3). Whether the parental strain exhibited a normal chromosomal asset

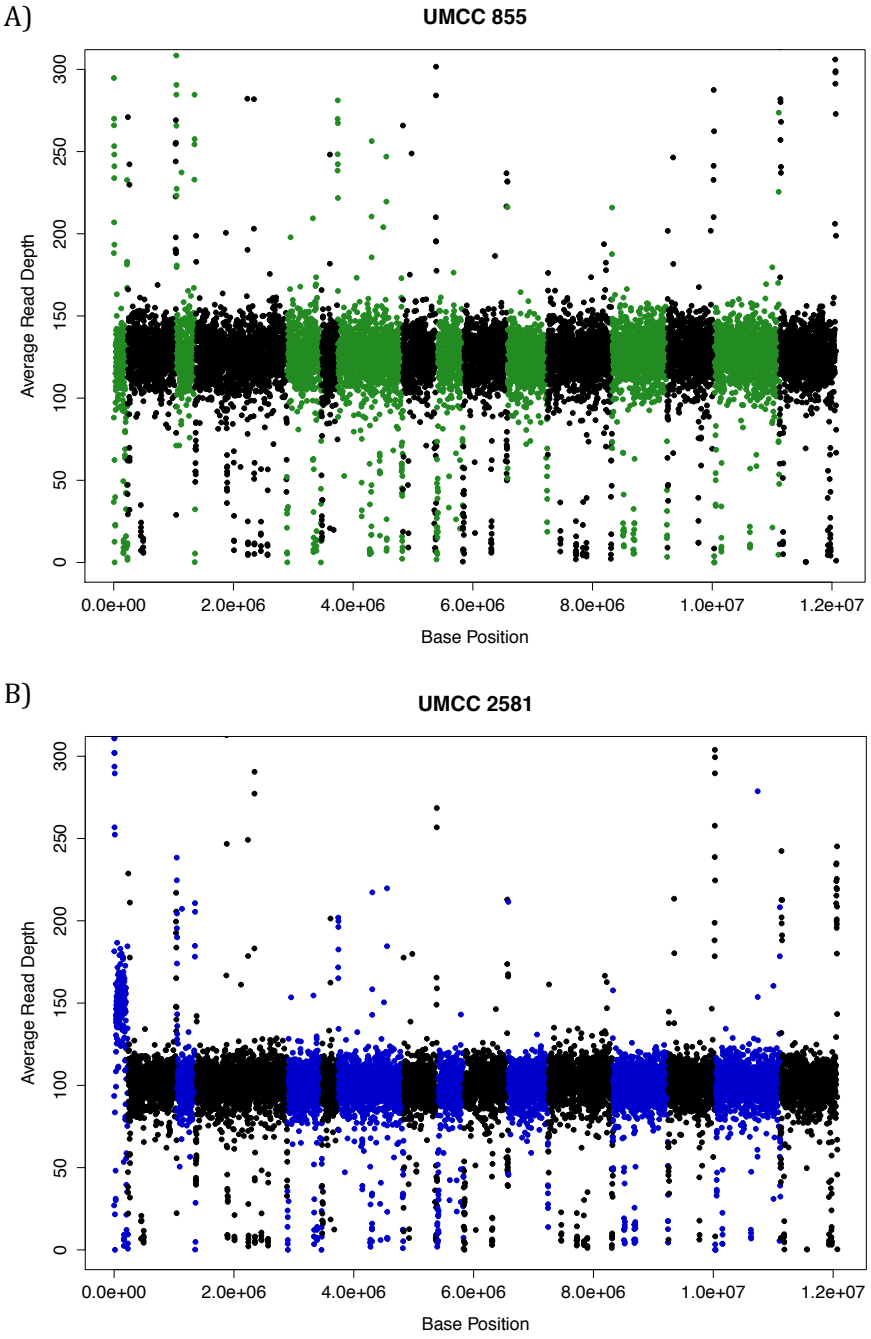

Figure 4.3 - Chromosomal aneuploidy determined by whole-genome sequencing coverage. The average sequencing coverage was determined using a sliding window of 1000 bp and plotted in chromosomal order (each represented by a colored dot). The dots colors (green or blue and black) alternate when change the chromosome of reference, starting from the green or blue chromosome 1 on the left. A) Coverage plot of UMCC 855 parental strain. B) Coverage plot of UMCC 2581 evolved strain.

(Fig. 4.3a), the evolved strain revealed a whole-chromosome amplification (Fig. 4.3b). The read depth of chromosome 1 was indeed 1.5-fold greater than the median of the strain, pointing out the presence of an extra-copy of this chromosome in UMCC 2581.

4.2.2.2 QTL mapping

To study the genetic basis of variations that produce a greater resistance to Mo(VI) and production of GSH in evolved phenotype, we used a three step process for QTL mapping (Fig. 4.4) similar to that proposed by Parts et al. (2011).

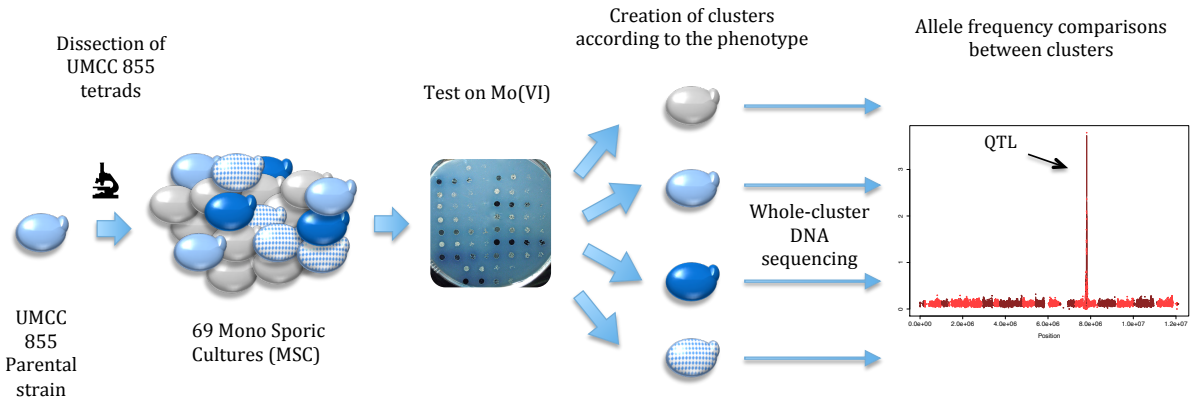

Figure 4.4 – QTL mapping strategy. Creation of pool of spores by dissection of UMCC 855, test on selective media, formation of cluster according to the phenotype and detection of fixed alleles via sequencing total DNA from the cluster.

First, we generated a large pool of mono sporic cultures (MSC) (69) starting from parental strain UMCC 855. We then tested them on the same selective media, with addition of different molybdate Mo(VI) concentrations (1 mM, 2.5 mM and 5 mM), used in the evolution-based strategy (§ 2.2). According to the phenotype showed, each MSC was clustered in the respective group: Sensitive, Resistant-Parental, Resistant-Evolved, Resistant-Other. Finally, we sequenced all clusters to assess the changes in allele frequencies throughout the genome in Resistant-Parental / Resistant-Evolved clusters.

The genomic DNA of clusters, as well as of the parental and selected evolved strains, was extracted and submitted to sequence analysis (§ 4.3, Table S5 in Supplementary materials). The sequence reads were then aligned with the S288c standard sequence and only the sites heterozygous in the UMCC 855 parental strain (filtered as described in § 4.3) were selected and used in the subsequent analysis. The allele frequency ratio p-

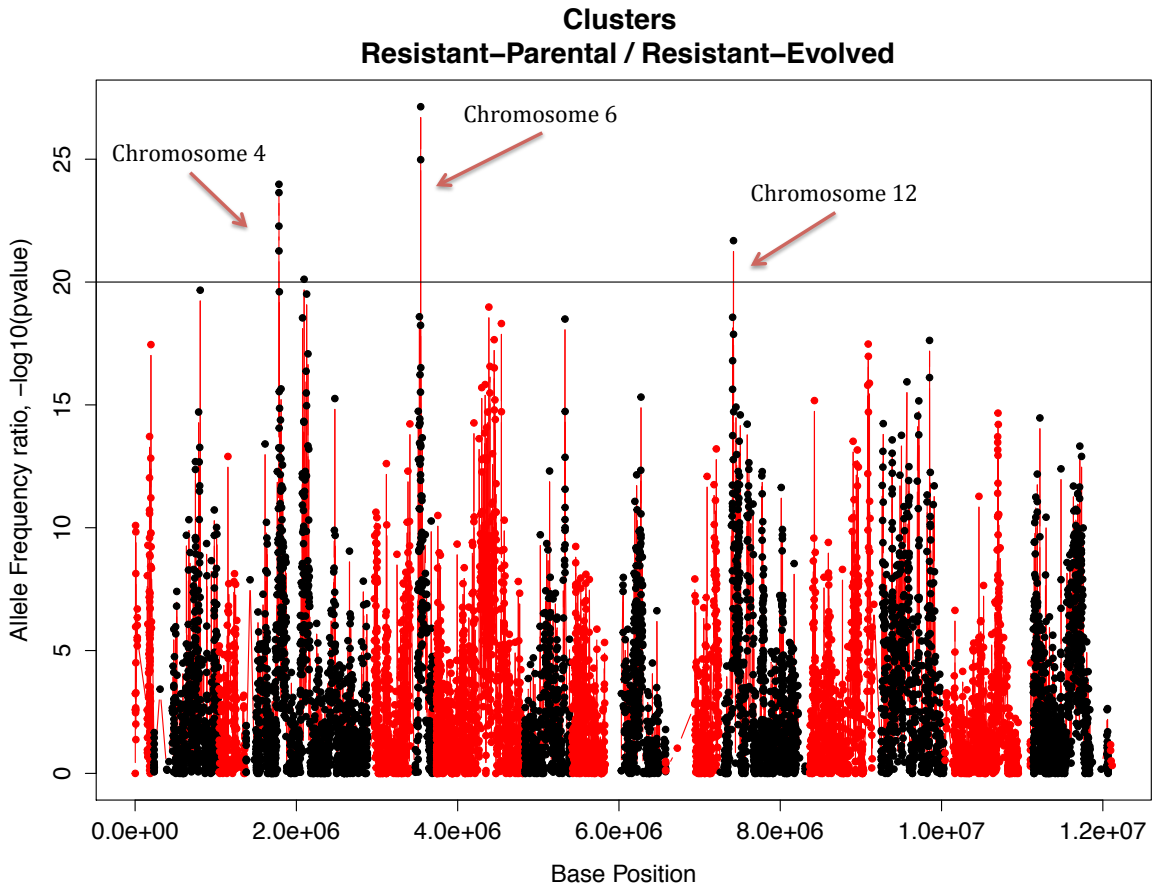

Figure 4.5 - Allele frequency ratio plot. The variants $-\log_{10}$ p-values for Fisher's Exact Test (each represented by one dot) comparing clusters Resistant-Parental and –Evolved are shown. The dots colors (red and black) alternate when change the chromosome of reference, starting from the red chromosome 1 on the left. The QTL cutoff threshold was chosen at 20 log unit (black line). Candidate QTLs were found on chromosomes 4, 6 and 12.

value of the selected variants in the DNA of the clusters Resistant-Parental and Resistant-Evolved was then plotted against the variant position on the chromosome. The result is shown in Fig. 4.5. The cutoff threshold chosen at 20 log unit allowed the identification of four QTLs: two peaks were present on chromosome 4, one on chromosome 6 and one on chromosome 12. The width of each peak was determined dropping 5 log unit from the top of the peak. The QTLs present on chromosome 4 (detailed in Fig. 4.6a) presented the larger peaks with a width of about 26 Kbp (between 423000 and 449000 bp) and 67 Kbp (between 717000 and 784000 bp) respectively for peak 1 and 2. Consequently, also the numbers of genes in these intervals were high, with 19 genes identified in peak 1 and 34 genes in peak 2 (Table S6). Contrariwise, the narrowest peak was found on chromosome 12 (Fig. 4.6c) with a width of about 11 Kbp (between 164000 and 175000 bp) and 9 genes detected (Table S8). Instead, the peak on

chromosome 6 (Fig. 4.6b) showed a width of about 17 Kbp (between 57000 and 74000 bp) but the minimum number of genes (6) found (Table S7).

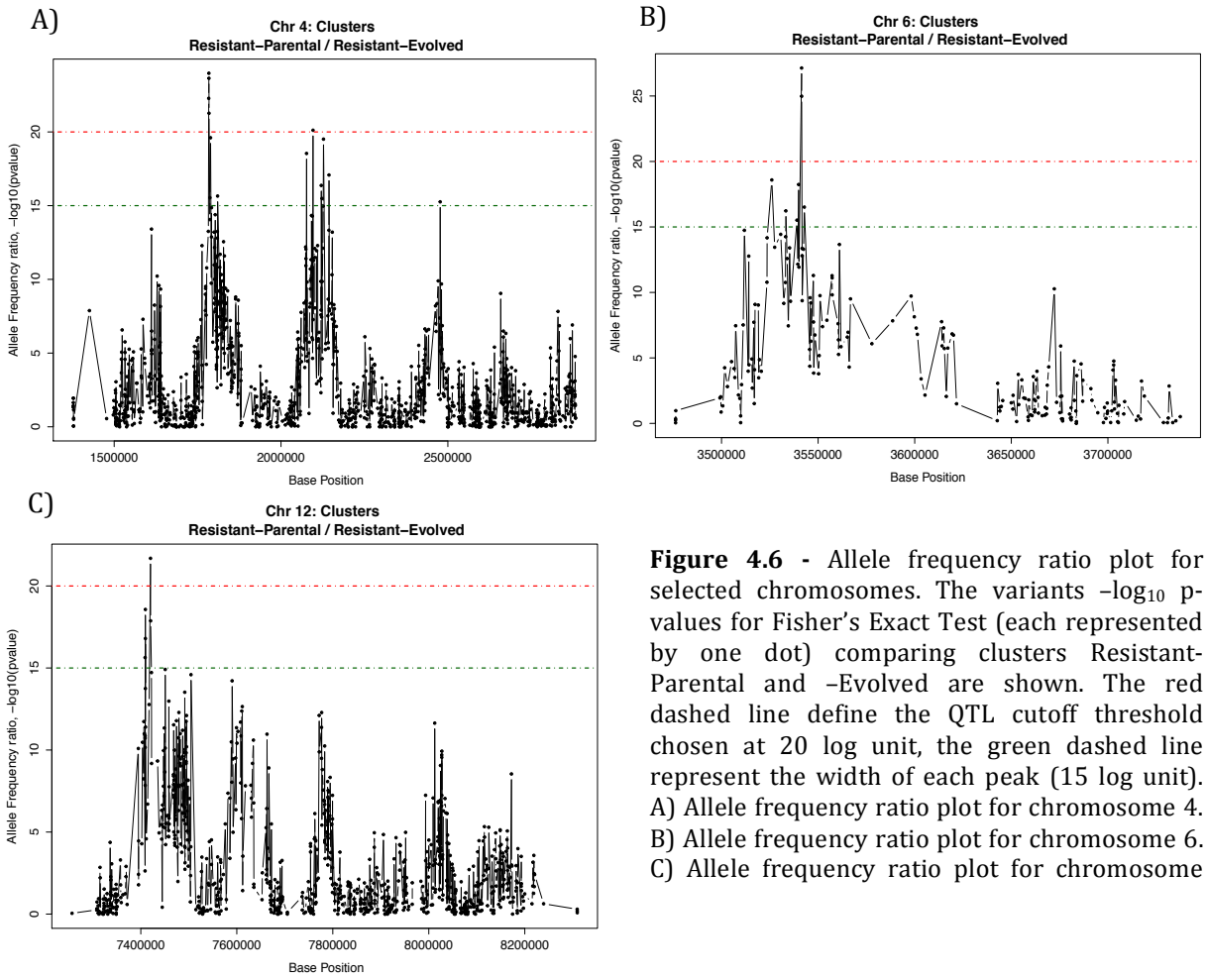

Figure 4.6 - Allele frequency ratio plot for selected chromosomes. The variants $-\log_{10}$ p-values for Fisher's Exact Test (each represented by one dot) comparing clusters Resistant-Parental and –Evolved are shown. The red dashed line define the QTL cutoff threshold chosen at 20 log unit, the green dashed line represent the width of each peak (15 log unit). A) Allele frequency ratio plot for chromosome 4. B) Allele frequency ratio plot for chromosome 6. C) Allele frequency ratio plot for chromosome

4.2.2.3 Identification of candidate genes

In order to identify the genes underlying the QTL, we initially examined the *S. cerevisiae* genome database (http://www.yeastgenome.org). Considering all peaks together, in the 121 Kbp mapped region 68 genes were annotated. Among these seven were dubious ORFs (YDL016C, YDL011C, YDR133C, YDR136C/VPS61, YDR149C, YDR154C and YDR157W) and seven were proteins of unknown function (YDL009C, YDL007C-A, YDL012C, YDR132C, YDR161W, YLR012C and YFL034W) that we did not consider any further. Besides dubious ORFs and unknown function protein, seven genes were attributed to cellular process involved in reproduction (CDC7, APC11, MCD1, RMD1, SWI5, CPR1 and NSE1), two to fatty acid/phospholipid metabolism (TSC13 and EKI1), five to transport function (ERP3, DOP1, PEX7, ENT5 and SEC1), nine to

transcription/translation process (NOP1, MTQ2, TAF12, CTH1, GIR2, RPA14, CWC15, RPL22B and PPR1), three to mitochondrial function (ATP16, KGD2 and PAM18), five to ubiquitin/proteasome process (SLX5, RPT2, YDR131C, RUB1 and SAN1) and nine to cell integrity (NHP10, FIN1, MKC7, NBP2, TUB2, MOB2, TEN1, GAT3 and BRE2). None of these genes had any obvious relationship to GSH production, resistance to metals or to oxidative stress, so we did not consider them as good candidates. In contrast, PTC1 and NUM1 had roles in metals resistance mechanisms (Ruiz et al. 2006; Paumi et al. 2007) and SSY1 in regulation of GSH precursor amino acids permeases (Forsberg et al. 2001), but they did not show any genomic variations between parental and evolved strain (on NUM1 sequence we faced reads alignment bias that did not allow any speculation for this gene). Also these genes were not considered as good candidates. On the other hand, GRX6 and MED2 on chromosome 4 peak 1, YCF1, RGP1, HPR1, HOM2 and SAC3 on chromosome 4 peak 2, RPO41 and RIM15 on chromosome 6 and RLP24 and LOT6 on chromosome 12 were considered to be worth further investigation. In particular, comparing the UMCC 855 and UMCC 2581 coding regions, GRX6 that encodes for a glutaredoxin involved in oxidative stress response, showed a single nucleotide polymorphism (SNP), whereas MED2 gene, that encodes for a subunit of the RNA polymerase II mediator complex, involved as well in oxidative stress response, showed one SNP and two insertions (Table S6). Instead, on chromosome 4 peak 2, YCF1 (Yeast Cadmium Factor: vacuolar glutathione S-conjugate transporter), RGP1 (gene implicated in retrograde transport from endosome to Golgi), HPR1 (components of conserved THO nuclear complex) and SAC3 (mRNA export factor), were all related to metals detoxification. The YCF1 gene differed in UMCC 2581 respect to UMCC 855 sequence, only for a single SNP in the coding region, two in case of HPR1 and three in both, RGP1 and SAC3 (Table S6). HOM2, the last gene annotated on chromosome 4 peak 2, which catalyzes the second step for methionine biosynthesis, displayed a single variant in the genetic sequence. Regarding the genes annotated on peaks on chromosomes 6 and 12, they were all related to oxidative stress response. On chromosome 6, the mitochondrial RNA polymerase RPO41 showed one SNP in the coding region, instead on the protein kinase RIM15, we detected the higher number of genomic variations in a single gene with ten SNPs and one insertion (Table S7). Finally, both genes annotated on chromosome 12, displayed one SNP comparing parental and evolved DNA sequences (Table S8).

4.2.2.4 YAP1 transcription factor

The function of yeast AP-1 (YAP1) transcription factor in response to oxidative stress and in exposure to a variety of cytotoxic agents (Toone & Jones 1999; Rodrigues-Pousada et al. 2010) as well as its role in activation of glutathione biosynthetic pathway (by activation of the target gene GSH1, Wu & Moye-Rowley 1994), are well known. For these reasons, the YAP1 gene was investigated although not annotated in QTL peaks (Table S9). The two sequences, UMCC 855 and 2581, presented one nucleotide polymorphism but, more interesting, both revealed one allele with a disruptive insertion at amino acid position 399.

4.2.2.5 Transcriptome profile comparison of the UMCC 2581 and UMCC 855 strains

To assess the gene expression level of the entire genome of the evolved and parental strains, we performed a transcriptome analysis. The three quarter of the exponential phase was chosen for analyzing gene expression. This point was chosen in order to precede the major transcriptional reprogramming event during fermentation of a

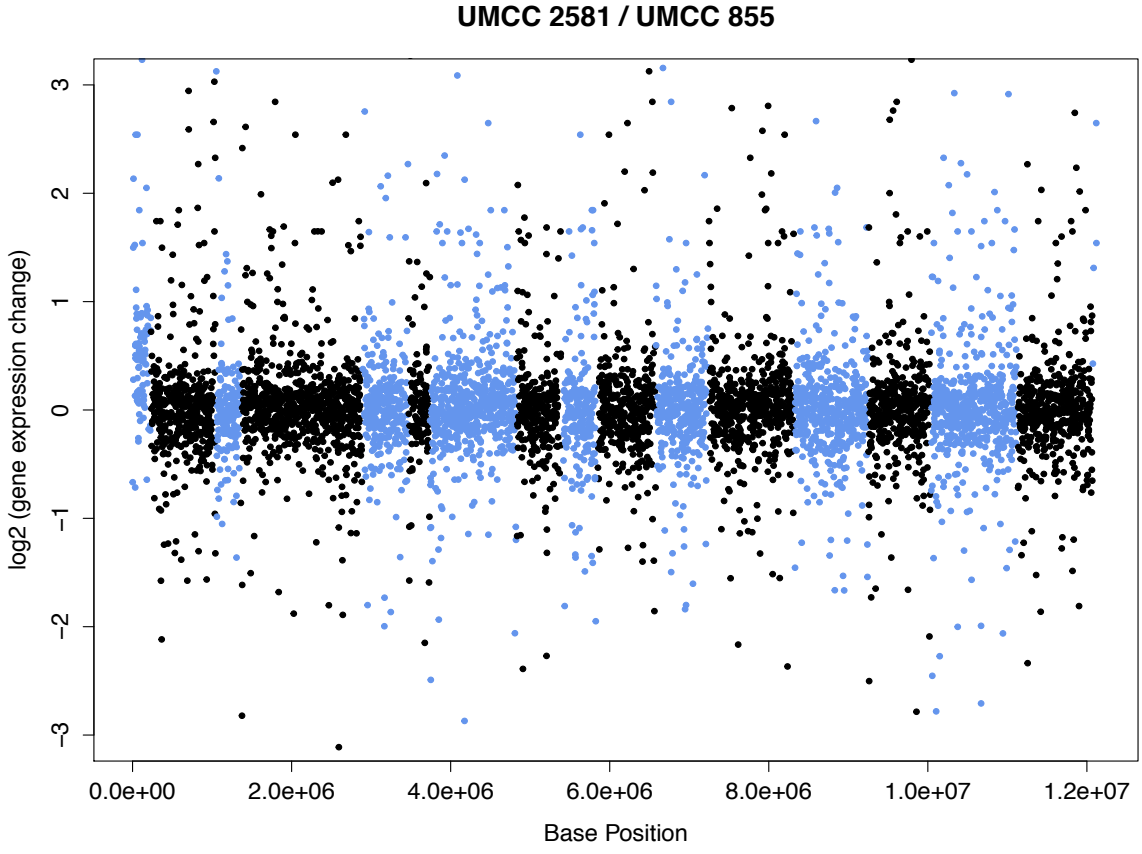

Figure 4.7 – Expression profile plot comparing the UMCC 2581 to UMCC 855. Each dot represents the $\log_2$ of the gene expression fold-change comparing the two strains. The dots colors (light blue and black) alternate when change the chromosome of reference, starting from the light blue chromosome 1 on the left.

synthetic grape juice that is triggered entering into the stationary phase (Rossignol et al. 2003; Treu et al. 2014). Under the chosen condition, the transcriptome is stable and expected to provide a relevant picture regarding the different strains capacity to produce GSH in mimicking natural must condition.

The transcriptomes of UMCC 2581 evolved strain and UMCC 855 parental strain were firstly compared in the expression profile plot (Fig. 4.7). The graph shows an average high expression of the genes present in the chromosome 1 of the evolved strains 2581 (0.57) comparing to the average expression of all other chromosomes taking together, that results 0.04. Since the average expression in a comparison between two strains with a normal chromosomal asset is expected to be 0, this result confirm, as previously described (Fig. 4.3), the presence of an extra copy of chromosome 1 in UMCC 2581

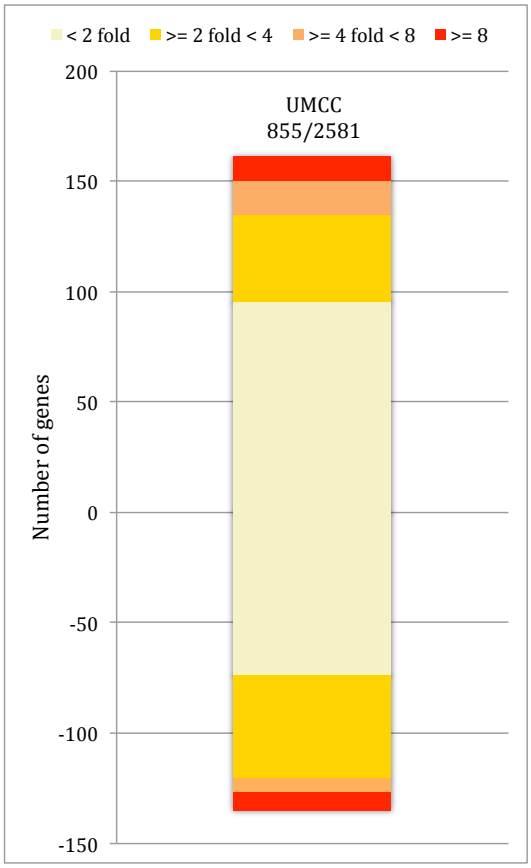

Figure 4.8 - Genes differentially expressed in the comparison between the two strains (UMCC 855/2581). The positive values of the vertical axis represent the number of genes more expressed in UMCC 2581 in comparison to UMCC 855, while the negative values reports the number of genes down expressed. Four different thresholds were considered, the genes with a low expression difference (light green and orange, differentially expressed between 2- and 4-fold), those with a medium expression difference (dark orange) (between 4- and 8-fold) and those with high expression difference (red) (more than 8-fold) (FDR<0.05).

strain. Accordingly with the demonstrated aneuploidy, all UMCC 2581 differentially expressed genes presents in chromosome 1 (47 genes) were not considered in the subsequent analysis. Without counting the chromosome 1, we observed that 296 genes were differentially expressed between the two strains at FDR < 0.05 (Fig. 4.8 and Table S1). Of the 161 genes up-regulated, 66 genes modified their expression more than two-fold as well as 61 genes out of 135 genes down-regulated. Our results indicate that a small fraction of the genes in the genome are differentially expressed but with quite a large subset (almost half) displaying strong variations.

Among the top 10% of genes that were strongly over-expressed (16 genes, Table S1), UMCC 2581 exhibited a small but significant set of permease genes including two amino acids permeases (DIP5 and GNP1) involved in transportation of GSH precursor amino acids (cysteine, methionine, glutamate and glycine) and SUL1 gene involved in sulfate assimilation pathway. On the other hand, the top 10% of genes strongly under-expressed (14 genes), was characterized by the null production of two genes (MAL11 and MAL13) that could impair the strain ability to utilize maltose.

4.2.2.6 Gene Ontology (GO) analysis

To have a more accurate comparison between strains, enriched functional classes of genes were obtained using "GO Term Finder" within the SGD database. GO term annotation analysis was used to detect enriched biology process (Table S2), function (Table S3 and Table 4.2) and component (Table S4) in UMCC 2581 compared to UMCC 855. All the GO analysis performed displayed consistent results that revealed, for the up-regulated genes, the term related to the transport activity striking enriched. Conversely, the same analysis considering the down-regulated genes, exhibited enriched term only for four genes (THI2, THI4, THI20, THI21) related to metabolism of thiamine in GO term "Process". The graphical analysis of the GO term "molecular function" (Fig. 4.9), showed that within the dataset of transporter activity, the terms associated with the amino acids transporter were particularly extent. Coherently, as observed in Table 4.2, many of the genes that strongly characterize all the enriched function classes, were genes involved in amino acids transporter. Noteworthy, 7 out of 9 genes in the GO class "amino acid transmembrane transporter activity" (genes in bold in Table 4.2) were genes related to GSH precursor amino acids. In particular, Dip5p (more than 20 fold-change) mediates high-affinity transport of L-glutamate but it is also a transporter for glycine, YCT1 and MUP3 encode, respectively, for high-affinity cysteine transporter and low affinity

methionine permease, Gnp1p transports both as well as Agp1p together with glycine and Gap1p is a general amino acid permease. Finally, even though not a transporter for amino acid, the high affinity sulfate permease, encoded by SUL1, is involved in methionine and cysteine biosynthetic process by the sulfate assimilation pathway.

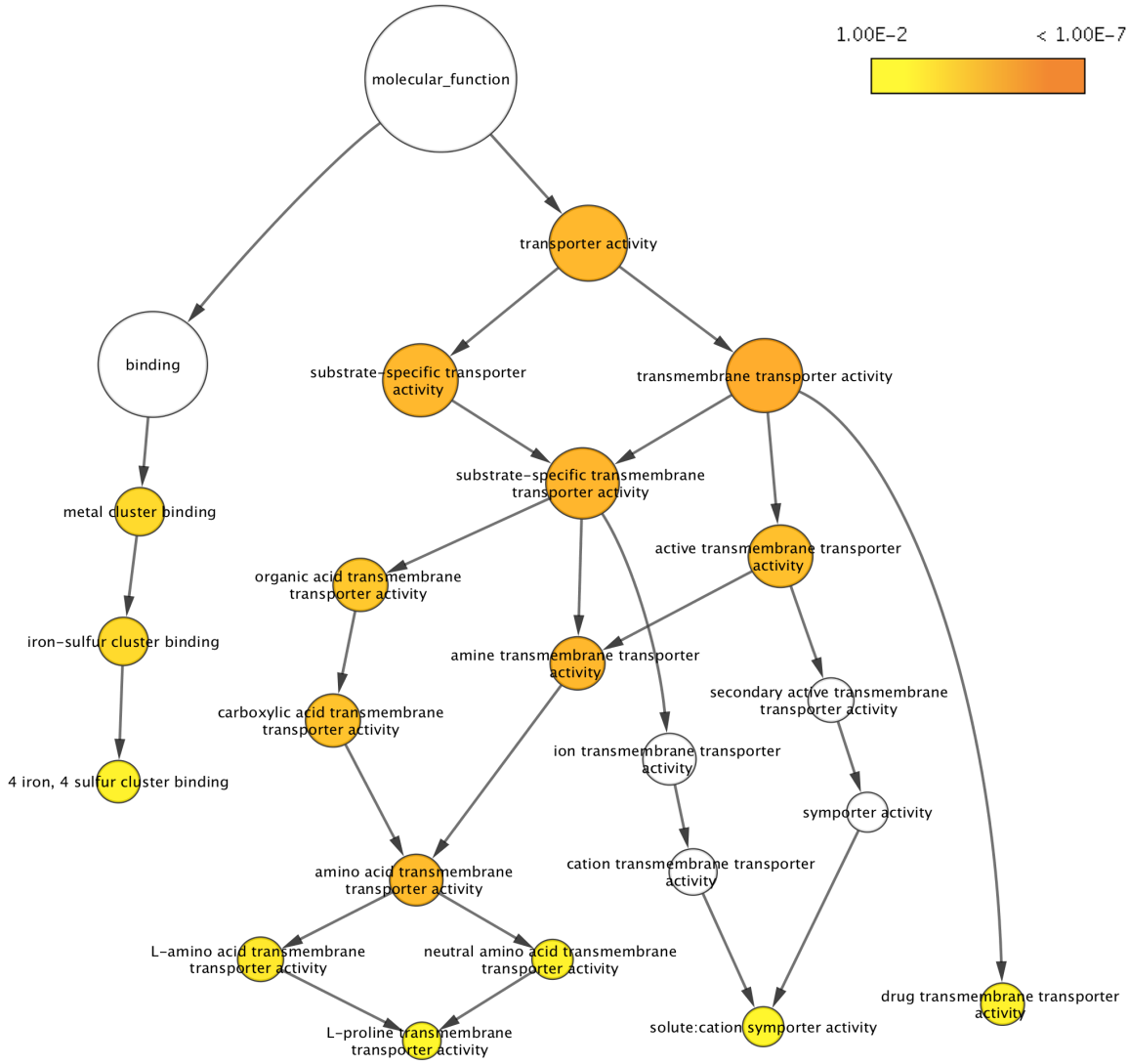

Figure 4.9 – BiNGO result for the over-expressed genes in UMCC 2581 considering the GO term "Molecular function". The node size corresponds to the number of proteins that are assigned with the individual term. The terms with a p-value below 0.01 were defined as significant (yellow), and a darker color represents a lower p-value (orange < 1.00E-7). White nodes are not significantly over-represented, they are included to show the colored nodes in the context of the GO hierarchy.

Furthermore, although not present in enriched GO classes, also MET5, GLT1 and SER3 genes were found over-expressed in UMCC 2581. These genes were involved in biosynthesis of methionine and cysteine (MET5, sulfite reductase beta subunit),

glutamate from glutamine and alpha-ketoglutarate (GLT1, NAD(+)-dependent glutamate synthase) and glycine (SER3, 3-phosphoglycerate dehydrogenase).

Gene Ontology term	Number of genes	p-value	Genes annotated to the term
anion transmembrane transporter activity	16	3.68E-08	CTP1, VBA2, **SUL1**, PHO89, **AGP1**, YCF1, **GNP1**, HNM1, MPC3, **MUP3**, **GAP1**, **YCT1**, **MMP1**, ATR1, TAT2, **DIP5**
carboxylic acid transmembrane transporter activity	13	1.38E-07	CTP1, VBA2, **AGP1**, YCF1, **GNP1**, HNM1, MPC3, **MUP3**, **GAP1**, **YCT1**, **MMP1**, TAT2, **DIP5**
substrate-specific transmembrane transporter activity	27	1.72E-06	FUR4, CTP1, VBA2, **SUL1**, PHO89, **AGP1**, YCF1, HXT7, **GNP1**, STL1, FCY2, HNM1, MEP1, MPC3, DUR3, **MUP3**, HXT5, QDR2, **GAP1**, **YCT1**, **MMP1**, ATR1, HXT2, FET3, TAT2, ENB1, **DIP5**
transmembrane transporter activity	28	2.81E-06	FUR4, CTP1, VBA2, **SUL1**, PHO89, **AGP1**, SNQ2, YCF1, HXT7, **GNP1**, STL1, FCY2, HNM1, MEP1, MPC3, DUR3, **MUP3**, HXT5, QDR2, **GAP1**, **YCT1**, **MMP1**, ATR1, HXT2, FET3, TAT2, ENB1, **DIP5**
amino acid transmembrane transporter activity	9	2.78E-05	VBA2, **AGP1**, **GNP1**, **MUP3**, **GAP1**, **YCT1**, **MMP1**, TAT2, **DIP5**
cation transmembrane transporter activity	15	0.00255	FUR4, PHO89, **AGP1**, **GNP1**, STL1, HNM1, MEP1, DUR3, **MUP3**, QDR2, **GAP1**, **YCT1**, FET3, TAT2, ENB1

Table 4.2 - Comparison between gene expression levels of the UMCC 2581 and UMCC 855 strains. Selected Gene Ontology (GO) function enriched for over-expressed genes in UMCC 2581 are reported. In bold permeases genes related to GSH precursor amino acids.

4.2.2.7 Genes related to GSH metabolism

As regard to GSH, we did not found any genes up- or down-regulated directly related to the GSH biosynthesis (GSH1, GSH2). Nevertheless, two genes involved in GSH metabolism and in metals detoxification, YCF1 and ECM38, were found over-expressed. YCF1 encodes for a vacuolar glutathione S-conjugate transporter and its role in detoxification of heavy metals is widely reported in literature (Li et al. 1996; Paumi et al. 2009; Wysocki & Tamás 2010). Gamma-glutamyltranspeptidase (Ecm38p), also a vacuolar protein, is the major GSH degrading enzyme involved in the recycle of GSH with a role in the protection against metals, xenobiotics and oxidative stress (Adamis et al. 2007; Ubiyvovk et al. 2006).

The GPX2 gene, that encodes for an enzyme that protect cells from phospholipid hydroperoxides during oxidative stress, was initially named phospholipid

hydroperoxide glutathione peroxidase because of homology with other GPx amino acids sequence and described as using GSH as electron donor (Inoue et al. 1999). However subsequent investigations, indicated that GPX2 encodes an atypical 2-Cys peroxiredoxin which uses thioredoxin, and not GSH, as electron donor (Tanaka et al. 2005). Therefore, albeit over-expressed gene in UMCC 2581, it was not further considered as GSH related.
Lastly, in UMCC 2581 transcriptome, the ZAP1 gene was observed slightly down-regulated. This could be interesting because Perrone et al. (2005), showed that *zap1* (Zn^{2+} responsive transcription activator) mutant cells had a higher level of extracellular GSH comparing to the relative parent.

4.2.3 Discussion

We previously showed that the evolved strains, obtained applying the evolution-based strategy to UMCC 855 parental strain, were able to a greater resistance to Mo(VI) (§2.3) and in some cases to increase the final GSH content in must and synthetic grape juice (§2.3, §3.3). Thus, evolved strains revealed to differ in two, but putatively related, aspects by UMCC 855 strain. In order to catalogue the precise genetic changes that underpin these diverse characteristics, we applied whole-genome and transcriptome sequencing of UMCC 855 parental and UMCC 2581 evolved *Saccharomyces cerevisiae* strains. We were able to identify several genetic differences among these strains including single nucleotide polymorphisms, chromosome copy number variation and differentially expressed genes that probably provide the basis for the phenotypic differences observed.

4.2.3.1 Chromosomal variations

Saccharomyces cerevisiae wine yeasts are characterized by the complexity of their nuclear genome and, rather than being strictly diploid, many strains display chromosomal copy number variation (polyploidy, aneuploidy) or rearranged chromosomes (Bidenne et al. 1992; Mortimer 2000; Borneman et al. 2011). In this work, we observed that the median read depth of UMCC 2581 chromosome 1 was greater by 1.5-fold (3:2 ratio represent at least one extra genomic copy in a diploid strain) than the median of the strain, pointing out the presence of an extra copy of this chromosome (Fig. 4.3b) and confirming the high level of polymorphism in wine yeasts. Therefore, this

large-scale genomic reorganization provided an average high expression of all genes present in chromosome 1 of the evolved strains UMCC 2581 (Fig. 4.7). However, how the increased expression of these genes can affect the different phenotype is unknown and hard to define because of the large number of genes simultaneously involved (117 ORF).
Interestingly, the genome coverage analysis revealed almost none overlapping results with the karyotyping experiment performed by PFGE (Fig. 4.2). In fact, the only chromosomal polymorphism found in strains UMCC 855/2581 by whole-genome sequencing was the UMCC 2581 aneuploidy on chromosome 1. Contrariwise, by PFGE, chromosomal polymorphisms were observed on chromosome 4, 16, 8 and 9, while on chromosome 1, as predictable, a more marked band was seen. A so evident discrepancy between different genotypic data results was not expected even though the high frequency of chromosomal polymorphisms and unexplained abnormal migration behavior (Bidenne et al. 1992; Ibeas & Jimenez 1996) could justify our observation.

4.2.3.2 Identification of candidate genes in QTL

In order to map trait loci we used a three steps approach: a pool of spores was initially generated from parental strain and then tested on selective media added with molybdate (same media used in evolution-based strategy). We applied selective pressure to group together individuals with a phenotype similar to parental or evolved phenotype. Lastly, through the analysis of the two clusters of interest (Resistant-Parental/Resistant-Evolved), it was possible to map the QTL that characterize the evolved phenotype. The allele frequency plot revealed four major loci on chromosome 4, 6 and 12 where 68 genes were annotated. Among these, eleven genes (GRX6, MED2, YCF1, RGP1, HPR1, HOM2, SAC3, RPO41, RIM15, RLP24 and LOT6) presented genomic variations comparing parental and evolved strains sequences and were functional related to GSH production, resistance to metals or to oxidative stress, so we considered them as good candidates.
The HOM2 gene is the only candidate gene that could be related to GSH production by its amino acid precursor biosynthesis. Indeed, the gene product, aspartic β-semialdehyde dehydrogenase, catalyzes the second step of the threonine and methionine metabolic pathway starting from aspartic acid (Thomas & Surdin-Kerjan 1989). The HOM2 sequence displayed only one single nucleotide polymorphism, but it occurs in the NAD binding domain. Therefore, we can speculate that the resulting replacement of histidine by asparagine (His29Asn, Table S6), both uncharged amino acids but with different 3D-

structure, could affect the efficiency of binding NAD and, consequently, the catalytic efficiency of the protein.

On behalf of its function and its close relation with GSH, the most convincing candidate gene in relation with the metals resistant phenotype, seemed to be YCF1. The Yeast Cadmium Factor (YCF1) is a well-studied ATP-binding cassette (ABC) protein localized in vacuolar membrane as glutathione S-conjugate (GS-X) transporter (Li et al. 1996). It was discovered by Szczypka and co-workers (1994) who described its ability to confer cadmium resistance. Indeed, the YCF1 is also able to transport into the vacuole a broad range of heavy metals as well as xenobiotic substrates providing resistance to cells (Gueldry et al. 2003; Mendoza-Cózatl et al. 2005; Prévéral et al. 2006; Paumi et al. 2009; Thorsen et al. 2009). The SNP found as heterozygous in UMCC 855 sequence, and fixed as homozygous alternative allele in UMCC 2581, changed the glutamine amino acid in position 899 with a histidine (Table S6). This variation occurred in regulatory domain and in particular next to K890, an ubiquitination site (Eraso et al. 2004; Swaney et al. 2013). Taking into account that no DNA variations were exhibited on the consensus element YRE (binds by Yap1p, the transcriptional activator) (Fernandes et al. 1997), and that RNA-seq experiments showed a slight increased YCF1 expression in UMCC 2581, we propose that the presence of His, possibly for steric hindrance, reduced or prevent the ubiquitination of lysine 890 leading to a lower degradation of the protein.

Although the vacuole emerges as a major hot-spot for metal detoxification, a number of pathways that play a more general, less direct role in promoting cell survival under stress conditions as mRNA processing and transport, can be identified. In this context, besides YCF1, also RGP1, HPR1 and SAC3 were found to be involved in the metal detoxification. In particular, Rgp1p is a subunit of a Golgi membrane GTP exchange factor (Ric1p-Rgp1p) that activates the GTPase Ypt6p by nucleotide exchange (Siniossoglou et al. 2000). These genes work together in the retrograde transport from endosome to Golgi, which is required for vacuolar protein sorting. Together with all the different steps in vesicle-mediated transport, they proved to be important in metals detoxification (Kakimoto et al. 2005; Chesi et al. 2012). In RGP1 sequence three non-synonymous mutations were detected (Table S6): in position 61 and 71 (valine > isoleucine and valine > alanine, respectively) the alternative allele was fixed in evolved strain, whereas in position 167 (asparagine > histidine) was restored the reference allele. However, very few is known about the protein structure and more study are necessary to infer any hypothesis.

Hpr1p, along with Mft1p, is a component of the evolutionarily conserved THO nuclear complex, functionally involved in mRNP (messenger ribonucleoprotein) biogenesis and transport, transcriptional elongation, and is a key player in the coupling of transcription and RNA export (Zenklusen et al. 2002; Rondon et al. 2003). In the work of Arita et al. (2009), deletion strain of Hrp1 was identified as sensitive strain to nickel, but the exact role that the nucleocytoplasmic transport process, more specifically the THO complex, plays in metals toxicity still needs to be studied. Of the two polymorphisms displayed in this sequence, the most significant was the deletion of the nucleotide A in ORF position 1427 (CA > C, Table S6) in one allele of parental strain (mutation not observed in evolved strain) that produce a frameshift variation and probably caused a nonfunctional protein.

The nuclear pore complex-associated protein Sac3p, was the last candidate gene involved in metals tolerance. It functions in mRNA export, transcription elongation and biogenesis of small ribosomal subunit (Lei et al. 2003). Ruotolo et al. (2008) reported mutations affecting nuclear pore complex subunits and mRNA export factors (included Sac3p) as cadmium/nickel-sensitive, pointed to trafficking (particularly nuclear export) as a novel hot spot of metal toxicity. Peculiarly, a polypeptide sequence identical to SAC3, except for N-terminal truncation, was reported also for LEP-1, a gene that augments the transcription of the leucine permease activity in *Saccharomyces* organisms (Stella et al. 1999). Interestingly, all the three genomic variations that differ between UMCC 855 and 2581 (from position 734 to 792, Table S6) appeared in the leucine permease transcriptional regulator helical domain. Although the SAC3/LEP-1 sequence did not show motifs homologous to transcription factors, the biologic functions observed suggest its regulatory activity of various target genes (Kuwahara et al. 2000), nevertheless our knowledge are insufficient to explain the effect of the observed mutations and further study are required.

Metal toxicity may be caused by impaired DNA repair, inhibition or disturbing of enzyme function but also by oxidative stress that originates from toxic levels of oxygen-derived reactive species (ROS) stimulated directly or indirectly by metals (Sumner et al. 2005; Ruotolo et al. 2008; Thorsen et al. 2009). ROS attack and damage all cellular macromolecules, leading to protein oxidation, lipid peroxidation and DNA damage (Wysocki & Tamás 2010). For this reason proteins related to oxidative stress were considered as candidate genes in our analysis and even, they have been the major represented function (6 out of 11 genes). Among these genes, GRX6, RIM15 and LOT6

have proven to be directly involved in the oxidative stress response (Izquierdo et al. 2008; Mesecke et al. 2008). The Grx6p, cis-golgi localized monothiol glutaredoxin, enzyme activity consists of deglutathionylating mixed disulfides between glutathione and protein thiols, releasing reduced glutathione. Protein glutathionylation is a reversible mechanism for protecting protein thiols against irreversible oxidative modifications (Puigpinós et al. 2015). However, the missense mutation detected in this protein (serine > threonine, Table S6) in position 93 is far from the active domain and, for the close similarity between the two switched amino acids, probably is also of little effect.

Rim15p is a protein kinase by which *Saccharomyces cerevisiae* regulates the post diauxic shift, entry into meiosis and stationary phase and life-span (Reinders et al. 1998; Fabrizio et al. 2001). The reduced life-span observed in *rim15* deletion cells was probably due to their deficiency in oxidative damage prevention (Cameroni et al. 2004), indeed the Rim15p regulon comprises genes implicated in oxidative stress. In our observation, the RIM15 gene was a hot-spot of mutations, gathering eleven polymorphism between the two sequences. Noteworthy, none of them were in protein kinase domain even though a frameshift mutation (GA > G) in position 1066 (Table S7) observed in one allele of evolved strain, arose between the two protein kinase domain, probably decreasing or deleting the protein functionality.

The candidate gene LOT6, encoded for a soluble quinone reductase (Sollner et al. 2007). This enzyme affords protection against the cytotoxic effects of electrophilic quinones by catalyzing a strict two-electron reduction. However, the metabolism of quinones within a cell has a direct effect on the cell's ability to cope with oxidative stress (Soballe & Poole 1999) and Lot6p plays a role in protecting yeast cells against quinone and semiquinone potential in generation of reactive oxygen species (Sollner et al. 2007). The SNP detected in this gene was a C > T change at position 120, resulting in a stop codon in parental strain (Table S8), sequence restored with the reference allele in evolved strain.

As mentioned before regarding metals detoxification, some housekeeping processes appear to play a significant role also in case of hyperoxia resistance. Consistent with these observations, in our QTL genes involved in controlling the activity of general transcription factors and RNA polymerase II (Myers et al. 1998; Sakurai & Fukasawa 2000) as Med2p, in the production of 60S ribosomal subunits (Harnpicharnchai et al. 2001) as Rlp24p, or encoding for a mitochondrial RNA (mtRNA) polymerase (Greenleaf et al. 1986) as Rpo41 were found. The specific function carried out in response to

oxidative stress by these genes is not clearly understood, however their involvement in the response against oxidative stress was reported by several authors (Higgins et al. 2002; Outten et al. 2005; Bonawitz et al. 2006; Okada et al. 2014). The most severe mutation revealed in MED2 sequence was a protein disruptive deletion GTTATTA > G in ORF position 998 (Table S6) fixed in UMCC 2581 strain, which predicted effect is to cut the carboxy-terminal domain. On the other hand, the effects of the SNP T > A in RPO41, that lead to change serine to threonine in position 259 of evolved strain sequence (Table S7), and the point mutation G > A arose in a UMCC 855 allele, which cause a non-synonymous mutation on amino acid position 39 (alanine > threonine, Table S8) on RLP24 gene, are difficult to predict.

The deeper analysis of the sequences revealed that in four genes, HPR1 and HOM2 on chromosome 4 and the two candidate genes on chromosome 12 RLP24 and LOT6, the UMCC 2581 evolved strain presented the restored reference allele, suggesting that in these cases the alternative alleles arose in parental strain lead to lost, rather than a gain, of function. The corresponding fully functional proteins, in particular Hpr1p and Lot6p where the alternative alleles brought a frameshift and a stop codon UMCC 855, probably provide a contribution in the resistant phenotype. On the contrary, in some other cases the mutations observed as heterozygous on UMCC 855 sequence and fixed in the evolved strain, as in MED2 and RIM15 seems to result in a corrupted protein. This unexpected protein dysfunction, reported only in UMCC 2581, could nevertheless comply the mechanisms of GSH overproduction recently proposed by Zhu et al. (2015). In this work the authors proposed that, first mutant cells accumulated high ROS levels because of deficient mutated protein, and then the accumulated endogenous ROS subsequently led to chronic oxidative stress and triggered the oxidative stress response, resulting in overproduction of GSH. On the other hand it is also possible that the partially impaired regulation of the main transcription factor involved in oxidative stress response, Yap1p (described below), may require to be balanced. However, the suggested hypothesis and the mutations effects proposed requires further study to be confirmed.

Our findings on the QTL that characterize the evolved phenotype, demonstrated also the great importance of the tested media used for phenotype definition. In fact, in the identification of the candidate genes, none of them related to GSH production with the

only exception of HOM2, was clear the limit of using resistance to molybdate as discriminant phenotype instead of directly the high glutathione production.

4.2.3.3 Analysis of YAP1 gene

Of particular interest was the analysis of YAP1 gene. Although not annotated in QTL peaks it was investigated for its critical role as transcription factors in response to oxidative stress and multidrug resistance as well as in activation of GSH1, encoding the

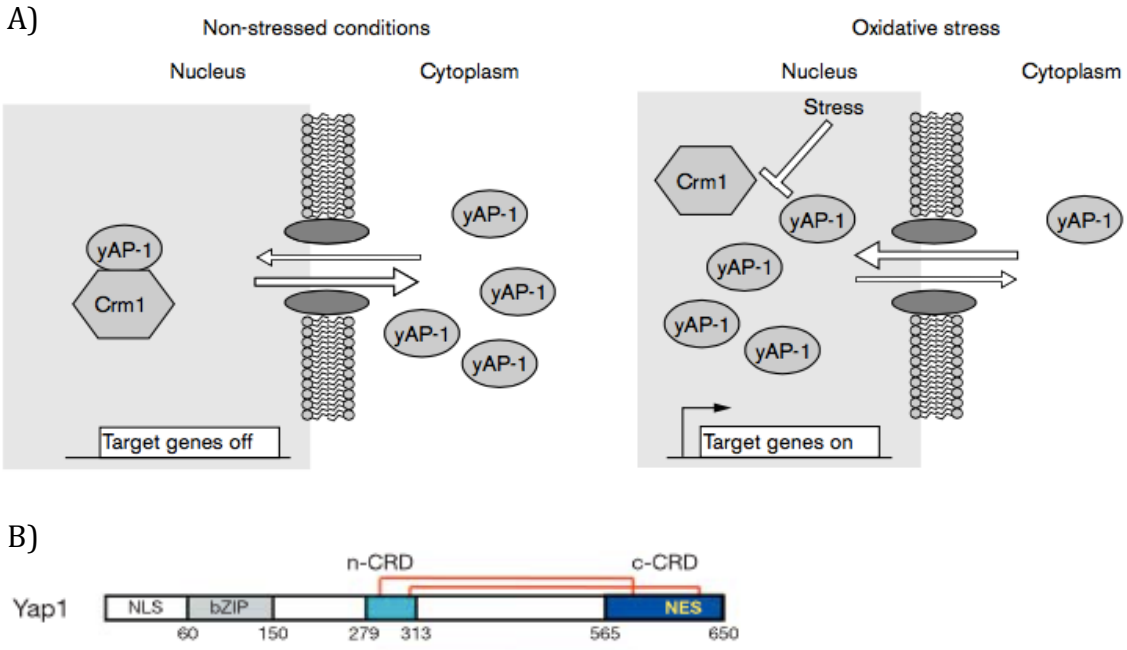

Figure 4.10 - Regulation of intracellular localisation of Yap1p transcription factor and schematic structure. A) Under normal growth conditions the Yap1p protein is cytoplasmic and is maintained in the cytoplasm through the Crm1p transporter that binds the nuclear export signal. Under oxidative stress conditions, this export pathway is blocked and the Yap1 protein accumulates in the nucleus resulting in increased Yap1p-dependent gene expression. (Toone & Jones 1999). B) Yap1 contains three conserved regions: a basic leucine zipper DNA binding domain (bZIP), an N-terminal cysteine-rich domain (n-CRD, Asn279 to Arg313) and a C-terminal-CRD, (Asn565 to Asn650). The nuclear localization signal (NLS) and nuclear export signal (NES) are located at the N and C termini, respectively. The Cys303–Cys598 and Cys310–Cys629 disulfide bonds are shown with red lines (Wood et al., 2004).

rate-limiting enzyme in glutathione biosynthesis (Sugiyama et al. 2000; Wheeler et al. 2003; Jun et al. 2012). The regulation of YAP1 is redox-dependent, and more than affects the protein level, which remains constant, it is mainly accomplished through its subcellular localization. Following activation by increased levels of reactive oxygen species, Yap1p is rapidly redistributed to the nucleus where it regulates the expression of up to 70 genes (Kuge et al. 1997; Fig. 4.10). The mechanism of regulation involved a reversible intramolecular disulfide bond formation between a conserved α-helix and the

nuclear export signal (NES) in the carboxy-terminal cysteine-rich domain (Cys303–Cys598 and Cys310–Cys629). In the active oxidized form, the NES is masked by the disulfide bond while upon reduction of the disulfide bonds, Yap1p undergoes a change to an unstructured conformation that exposes the NES and allows redistribution to the cytoplasm by the nuclear exporter Crm1 (Wood et al. 2004). Moreover, Kuge and coworkers (1997) reported that *yap1* (1-373), mutant lacking the C-terminal of the protein, showed high levels of Yap1p basal activity, up to ~10-fold more than in wild-type cells, and a slightly increased expression levels of GSH1. Considerably, we observed a disruptive insertion (G > GTAGCACTGA, Table S8) corresponding to amino acid position 399 in one allele of both, parental and evolved strains sequences. We speculate that in our strains the nuclear export of Yap1p is (at least partially since heterozygous strains) arrested because it can no longer interact with the conserved nuclear exporter Crm1p leading, as previously reported for similar mutant strain, to an higher basal activity of the enzyme and a consequent increased expression of GSH1. This hypothesis could also explain the almost total absence of genes related to GSH production annotated in the QTL (with the only exception of HOM2 where the protein function was recovered in evolved strain). Indeed our parental strain already showed an enhanced GSH pathway that has been conserved in the evolved strain, and mutations were accumulated in other genes, in particular involved in a better response to oxidative stress.

4.2.3.4 Transcriptome profiles comparison

With the aim to find relevant evidences supporting the different strains capacity to produce GSH, the variations in UMCC 855/2581 genes expression level was assess (§ 4.4). The synthetic grape juice was used in order to mimicking natural must condition and the sampling point was chosen to avoid both, the most critical phase at the beginning of the fermentation and the major transcriptional reprogramming event that triggered entering into the stationary phase. The most abundant overexpressed GO classes in UMCC 2581 were all involved in transport activity strongly underling how the major differences between the two strains were situated in this process. Important to notice, the terms associated with the amino acids transporter were particularly extended in the graphical representation (Fig. 4.3), but also highly characterizing the other enriched function classes (Table 4.1). A deeper analysis of the gene annotated in 'amino acid transmembrane transporter activity' revealed, remarkably, that 7 out of 9

were genes related to all GSH precursor amino acids. Cysteine and methionine are transported by Yct1p, Mup3p, Gnp1p and Agp1p, glutamate and glycine by Dip5p (the most differentially higher expressed gene) and Agp1, all are transported by Gap1p, a general amino acid permease (Hinnebusch 1992; Düring-Olsen et al. 1999; Regenberg et al. 1999). Moreover, among the differentially over-expressed genes, other genes potentially related to GSH production were found (Table S1). SUL1, present together with the above-mentioned amino acids permeases in numerous GO enriched classes, encodes for high affinity sulfate permease (Smith et al. 1995) and along with MET5, that encode for the β-subunit of the *S. cerevisiae* sulfite reductase (Masselot & De Robichon-Szulmajster 1975), are involved in the sulfate assimilation pathway that precede the synthesis of sulfur-containing amino acids cysteine and methionine (Thomas & Surdin-Kerjan 1997). GLT1, which encodes for GOGAT (glutamate synthase), synthesizes two molecules of glutamate out of one molecule of glutamine and one molecule of α-ketoglutarate (Filetici et al. 1996). Finally, Ser3p, phosphoglycerate dehydrogenase, catalyze the first reaction of serine and glycine biosynthesis from the glycolytic metabolite 3-phosphoglycerate (Albers et al. 2003). So, our results evidenced that all the GSH precursor amino acids biosynthetic pathway (sulfur-containing amino acids, glutamate and glycine) were over-expressed, from permeases to synthetic enzymes. Thus, it is suggested that this aptitude to collect the precursor amino acids from the media, considering also the partially constitutive expression of the GSH1 transcriptor regulator Yap1p (described above) and the over-represented biosynthetic steps in each amino acid pathway, might lead to an overproduction of glutathione by providing large amount of precursors. This despite the fact that we did not find any difference in the expression of the two GSH specific biosynthetic reactions. The gene expression pattern here observed is of particular importance for the technological point of view: although genomic regulation may differ in natural musts with a different nutritional status, the ability to gather precursor amino acids from the media is probably relevant for a constantly high GSH production in real fermentations where must are different year by year (see also § 3.3).

Albeit not reported in the enriched GO classes, the analysis of genes related to GSH metabolism was carried out. Notable, we found two over-expressed GSH-related genes that are involved in metals detoxification: YCF1 and ECM38. The YCF1 gene was the only one detected in both analyses, whole-genome and transcriptome sequencing, and its important role in providing resistance to heavy metals and xenobiotics as GSH S-

conjugate transporter was previously described. The Ɣ-glutamyltranspeptidase (Ɣ-GT), in *S. cerevisiae* encoding by ECM38, is the major GSH degrading enzyme. Once is transported into the vacuole, GSH is degraded by the vacuolar membrane-bound Ɣ-GT and L-cysteinyl glycine dipeptidase by the cleavage of the Ɣ-glutamyl moiety and the release of cysteinylglycine, further degraded to its constitutive amino acids (Jaspers et al. 1985). Analogue mechanism might be responsible for the recycle of xenobiotics/metal-GSH complex stored in the vacuole, which can be excreted from cells (Adamis et al. 2007; Ubiyvovk et al. 2006) suggesting a possible mechanism for molybdate resistance in evolved strain.

We can conclude that the analysis of the transcriptional profile revealed two very important aspects. The first is the global over-expression of the amino acids permeases, noteworthy especially for the final purpose of the evolved strain: high GSH production in oenological applications. The second aspect is the remarkable role of transport processes in the definition of the phenotype. This character appear as extremely significant in particular in transcriptome analysis where was the only enriched process, but also in the QTL analysis we detected key genes related to transport activity. Since the selective pressure applied to obtain evolved strains, YCF1, detected in both analyses, emerged as a major hot spot for metal detoxification. Besides the YCF1, a number of genes associated with a more general transport pathways (for example vesicle and nucleocytoplasmic transport), probably play also a role in promoting cell survival under metal/oxidative stress conditions and in the GSH production and homeostasis. Nevertheless, the link between the differences in genes expression and genomic variations is not understood and further studies are needed.

4.2.3.5 Conclusion

In this work, we applied quantitative genetics to study the genetic changes underlying the high GSH production showed by the wine *S. cerevisiae* strains UMCC 2581 selected in a molybdate enriched environment after sexual recombination. We identified four peaks within 11 candidate genes in QTL analysis and 296 genes differentially expressed between parental and evolved strain. The complex genetic traits and the wide variations produced by sexual recombination resulted in a presumed additive phenotype effects. The high GSH production phenotype included over-expression of amino acids permeases and biosynthetic enzymes in addition to impaired Yap1p transcription factor, whereas GSH production and metabolism, transporter activity, vacuolar detoxification

and oxidative stress response enzymes were probably added resulting in the molybdate resistance phenotype. However to address the exact relationships between evolved phenotypes and candidate genes variations (expression and sequence polymorphisms), correlation experiments are to be carried out. Thorough understanding of the genes variations effects and the scope of the aneuploidy consequence on chromosome 1 are necessary prerequisites for the development of the next generation of oenological industrial yeasts.

4.2.4 References

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4.3 Supplementary materials

4.3.1 RNA-seq

Table S1 – Differentially expressed genes in evolved strain UMCC 2581 comparing to UMCC 855 as determined by RNA-seq. Since aneuploidy on chromosome 1, all genes in this chromosome are not reported. Only genes with a p-adjusted < 0.05 (FDR, Benjamini & Hochberg) are reported.

Systematic gene name	Standard gene name	Gene description	Log_2(Fold change)	p-adjusted
YPL265W	DIP5	Dicarboxylic amino acid permease	4,35	7,31E-15
YDR536W	STL1	Glycerol proton symporter of the plasma membrane	4,23	1,40E-06
YMR011W	HXT2	High-affinity glucose transporter of the major facilitator superfamily	4,02	6,06E-14
YHR033W		Putative protein of unknown function	3,58	1,76E-25
YHR096C	HXT5	Hexose transporter with moderate affinity for glucose	3,45	9,69E-06
YLR413W	INA1	Putative protein of unknown function	3,42	7,89E-27
YDR508C	GNP1	High-affinity glutamine permease	3,38	1,93E-12
YFL053W	DAK2	Dihydroxyacetone kinase	3,27	4,13E-02
YKL187C	FAT3	Protein required for fatty acid uptake	3,16	1,68E-02
YGL089C	MF(ALPHA)2	Mating pheromone alpha-factor, made by alpha cells	3,09	4,51E-02
YBR296C	PHO89	Plasma membrane Na+/Pi cotransporter	3,03	1,55E-02
YOL013W-A		Putative protein of unknown function	2,92	2,23E-04
YEL065W	SIT1	Ferrioxamine B transporter	2,75	1,76E-25
YNL194C		Integral membrane protein	2,68	2,40E-06
YBR294W	SUL1	High affinity sulfate permease of the SulP anion transporter family	2,66	4,94E-14
YDL222C	FMP45	Integral membrane protein localized to mitochondria	2,61	3,99E-14
YBR117C	TKL2	Transketolase	2,59	1,00E-04
YLR265C	NEJ1	Protein involved in regulation of nonhomologous end joining	2,58	9,33E-04
YDL241W		Putative protein of unknown function	2,42	1,73E-02
YOR028C	CIN5	Basic leucine zipper (bZIP) transcription factor of the yAP-1 family	2,28	4,98E-07
YPL223C	GRE1	Hydrophilin essential in desiccation-rehydration process	2,27	5,07E-15
YKR093W	PTR2	Integral membrane peptide transporter	2,17	3,17E-03
YDR342C	HXT7	High-affinity glucose transporter	2,10	4,89E-02
YFR032C	RRT5	Putative protein of unknown function	2,09	9,73E-03
YOL052C-A	DDR2	Multi-stress response protein	2,07	6,83E-07
YNL195C		Protein of unknown function	2,00	9,78E-09
YNL145W	MFA2	Mating pheromone a-factor	1,80	1,73E-02
YJL089W	SIP4	C6 zinc cluster transcriptional activator	1,72	3,41E-03
YEL039C	CYC7	Cytochrome c isoform 2, expressed under hypoxic conditions	1,64	1,71E-02
YMR085W		Putative protein of unknown function	1,63	2,35E-06
YGR043C	NQM1	Transaldolase of unknown function	1,62	1,48E-03

YHR022C		Putative protein of unknown function	1,61	1,22E-02
YDL039C	PRM7	Pheromone-regulated protein	1,61	1,49E-05
YNR014W		Putative protein of unknown function	1,60	1,34E-06
YPR002W	PDH1	Putative 2-methylcitrate dehydratase	1,60	2,27E-17
YHL026C		Putative protein of unknown function	1,57	2,61E-05
YDR534C	FIT1	Mannoprotein that is incorporated into the cell wall	1,52	3,52E-03
YOR161C	PNS1	Protein of unknown function	1,51	3,99E-07
YML116W	ATR1	Multidrug efflux pump of the major facilitator superfamily	1,44	3,55E-21
YIL121W	QDR2	Plasma membrane transporter of the major facilitator superfamily	1,42	8,79E-13
YLR174W	IDP2	Cytosolic NADP-specific isocitrate dehydrogenase	1,42	3,57E-09
YOL047C	LDS2	Protein Involved in spore wall assembly	1,40	2,44E-02
YNL279W	PRM1	Pheromone-regulated multispanning membrane protein	1,36	5,41E-04
YLL061W	MMP1	High-affinity S-methylmethionine permease	1,35	2,61E-05
YGR248W	SOL4	6-phosphogluconolactonase	1,32	5,32E-03
YDL214C	PRR2	Serine/threonine protein kinase	1,31	1,80E-02
YGR121C	MEP1	Ammonium permease	1,25	1,22E-09
YDL223C	HBT1	Shmoo tip protein, substrate of Hub1p ubiquitin-like protein	1,24	2,23E-04
YOL158C	ENB1	Endosomal ferric enterobactin transporter	1,23	8,98E-08
YOL155C	HPF1	Haze-protective mannoprotein	1,23	2,90E-09
YBR244W	GPX2	Phospholipid hydroperoxide glutathione peroxidase	1,23	6,51E-04
YDL049C	KNH1	Protein with similarity to Kre9p	1,22	1,09E-04
YBR008C	FLR1	Plasma membrane transporter of the major facilitator superfamily	1,20	2,40E-11
YCR021C	HSP30	Negative regulator of the H(+)-ATPase Pma1p	1,15	2,06E-07
YLL057C	JLP1	Fe(II)-dependent sulfonate/alpha-ketoglutarate dioxygenase	1,14	1,19E-02
YJL108C	PRM10	Pheromone-regulated protein	1,13	1,41E-09
YGR197C	SNG1	Protein involved in resistance to nitrosoguanidine and 6-azauracil	1,13	2,09E-02
YOL020W	TAT2	High affinity tryptophan and tyrosine permease	1,13	2,48E-03
YDR011W	SNQ2	Plasma membrane ATP-binding cassette (ABC) transporter	1,11	2,61E-05
YJL191W	RPS14B	Protein component of the small (40S) ribosomal subunit	1,10	1,21E-06
YIR032C	DAL3	Ureidoglycolate lyase	1,09	1,57E-03
YHL016C	DUR3	Plasma membrane transporter for both urea and polyamines	1,09	6,87E-03
YNL058C		Putative protein of unknown function	1,07	2,61E-03
YFL026W	STE2	Receptor for alpha-factor pheromone	1,04	6,33E-03
YCL025C	AGP1	Low-affinity amino acid permease with broad substrate range	1,03	1,24E-04
YDR216W	ADR1	Carbon source-responsive zinc-finger transcription factor	1,02	1,61E-02

YLL055W	YCT1	High-affinity cysteine-specific transporter	1,00	1,65E-04
YML091C	RPM2	Protein subunit of mitochondrial RNase P	0,99	3,73E-07
YJL107C		Putative protein of unknown function	0,99	1,26E-02
YMR169C	ALD3	Cytoplasmic aldehyde dehydrogenase	0,98	7,98E-05
YIR031C	DAL7	Malate synthase	0,98	1,58E-04
YOR374W	ALD4	Mitochondrial aldehyde dehydrogenase	0,97	7,12E-05
YKL161C	KDX1	Protein kinase	0,96	6,85E-08
YDL171C	GLT1	NAD(+)-dependent glutamate synthase (GOGAT)	0,96	6,35E-08
YIL101C	XBP1	Transcriptional repressor	0,95	3,08E-02
YGR243W	MPC3	Highly conserved subunit of mitochondrial pyruvate carrier	0,95	7,25E-03
YOR346W	REV1	Deoxycytidyl transferase	0,94	4,94E-03
YDR259C	YAP6	Basic leucine zipper (bZIP) transcription factor	0,94	6,87E-03
YOR356W	CIR2	Putative ortholog of human ETF-dH	0,93	5,73E-05
YEL040W	UTR2	Chitin transglycosylase	0,93	2,84E-06
YMR058W	FET3	Ferro-O2-oxidoreductase	0,93	1,83E-06
YHR023W	MYO1	Type II myosin heavy chain	0,90	3,11E-03
YIR027C	DAL1	Allantoinase	0,89	2,73E-02
YBR021W	FUR4	Plasma membrane localized uracil permease	0,89	2,31E-02
YGR052W	FMP48	Putative protein of unknown function	0,87	3,36E-06
YMR032W	HOF1	Protein that regulates actin cytoskeleton organization	0,87	3,92E-02
YNR044W	AGA1	Anchorage subunit of a-agglutinin of a-cells	0,86	3,24E-02
YLR297W		Protein of unknown function	0,86	1,19E-02
YOL084W	PHM7	Protein of unknown function	0,85	3,44E-05
YLR304C	ACO1	Aconitase	0,84	6,05E-10
YPR149W	NCE102	Protein of unknown function	0,84	8,67E-05
YIL024C		Putative protein of unknown function	0,84	4,51E-02
YGR088W	CTT1	Cytosolic catalase T	0,82	1,46E-02
YJR124C		Putative protein of unknown function	0,81	5,32E-03
YBR150C	TBS1	Putative protein of unknown function	0,80	1,73E-02
YOL126C	MDH2	Cytoplasmic malate dehydrogenase	0,79	1,07E-03
YNL129W	NRK1	Nicotinamide riboside kinase	0,78	3,59E-03
YMR246W	FAA4	Long chain fatty acyl-CoA synthetase	0,75	1,34E-05
YBR293W	VBA2	Permease of basic amino acids in the vacuolar membrane	0,75	2,44E-02
YML076C	WAR1	Homodimeric Zn2Cys6 zinc finger transcription factor	0,72	3,05E-03
YPL017C	IRC15	Microtubule associated protein	0,72	8,88E-03
YER024W	YAT2	Carnitine acetyltransferase	0,70	1,08E-02
YLR108C		Protein of unknown function	0,68	3,88E-04
YJL172W	CPS1	Vacuolar carboxypeptidase S	0,68	1,61E-04
YHR097C		Putative protein of unknown function	0,68	2,01E-02
YBR005W	RCR1	Protein of the ER membrane involved in cell wall chitin deposition	0,68	1,25E-02
YIR029W	DAL2	Allantoicase	0,67	3,82E-02
YLR346C		Putative protein of unknown function found	0,67	4,58E-02

		in mitochondria		
YJL078C	PRY3	Cell wall-associated protein involved in export of acetylated sterols	0,67	4,99E-03
YGR023W	MTL1	Putative plasma membrane sensor	0,67	3,60E-02
YER081W	SER3	3-phosphoglycerate dehydrogenase	0,67	3,31E-05
YDL025C	RTK1	Putative protein kinase, potentially phosphorylated by Cdc28p	0,67	4,45E-04
YGL114W		Putative protein of unknown function	0,66	3,24E-03
YPR160W	GPH1	Glycogen phosphorylase required for the mobilization of glycogen	0,66	6,87E-03
YIL155C	GUT2	Mitochondrial glycerol-3-phosphate dehydrogenase	0,65	4,51E-02
YOR382W	FIT2	Mannoprotein that is incorporated into the cell wall	0,65	1,73E-02
YKR039W	GAP1	General amino acid permease	0,65	4,94E-02
YLR401C	DUS3	Dihydrouridine synthase	0,64	3,79E-02
YJL035C	TAD2	Subunit of tRNA-specific adenosine-34 deaminase	0,64	4,06E-02
YLL027W	ISA1	Protein required for maturation of mitochondrial [4Fe-4S] proteins	0,64	1,70E-02
YNL068C	FKH2	Forkhead family transcription factor	0,64	4,80E-02
YKL051W	SFK1	Plasma membrane protein that may act to generate normal levels of PI4P	0,64	4,80E-02
YGR092W	DBF2	Ser/Thr kinase involved in transcription and stress response	0,63	2,54E-02
YBR183W	YPC1	Alkaline ceramidase	0,63	4,56E-02
YDR334W	SWR1	Swi2/Snf2-related ATPase	0,62	1,69E-02
YGR238C	KEL2	Protein that negatively regulates mitotic exit	0,62	3,12E-02
YLR299W	ECM38	Gamma-glutamyltranspeptidase	0,62	1,71E-03
YPL014W		Putative protein of unknown function	0,62	1,88E-02
YGR239C	PEX21	Peroxin required for peroxisomal matrix protein targeting	0,61	1,36E-02
YLR168C	UPS2	Mitochondrial intermembrane space protein	0,61	3,03E-03
YBR291C	CTP1	Mitochondrial inner membrane citrate transporter	0,61	2,73E-02
YHR146W	CRP1	Protein that binds to cruciform DNA structures	0,61	6,25E-03
YDR034C	LYS14	Transcriptional activator involved in regulating lysine biosynthesis	0,61	1,51E-03
YMR189W	GCV2	P subunit of the mitochondrial glycine decarboxylase complex	0,59	3,24E-03
YNL074C	MLF3	Serine-rich protein of unknown function	0,58	9,65E-03
YNL311C	SKP2	F-box protein of unknown function	0,57	4,51E-02
YLR414C	PUN1	Plasma membrane protein with a role in cell wall integrity	0,57	5,57E-03
YJL088W	ARG3	Ornithine carbamoyltransferase	0,56	6,69E-03
YGR090W	UTP22	Component of the small-subunit processome	0,56	4,33E-02
YMR318C	ADH6	NADPH-dependent medium chain alcohol dehydrogenase	0,55	4,46E-03
YMR300C	ADE4	Phosphoribosylpyrophosphate amidotransferase (PRPPAT)	0,54	2,61E-03
YHL036W	MUP3	Low affinity methionine permease	0,54	4,51E-02

YOR120W	GCY1	Glycerol dehydrogenase	0,54	2,93E-02
YHR216W	IMD2	Inosine monophosphate dehydrogenase	0,52	1,46E-02
YMR016C	SOK2	Nuclear protein that negatively regulates pseudohyphal differentiation	0,51	4,13E-02
YER164W	CHD1	Chromatin remodeler that regulates various aspects of transcription	0,51	4,51E-02
YER056C	FCY2	Purine-cytosine permease	0,50	1,80E-02
YOR136W	IDH2	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase	0,49	1,17E-02
YNL037C	IDH1	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase	0,47	1,48E-02
YJL200C	ACO2	Putative mitochondrial aconitase isozyme	0,47	9,84E-03
YJL141C	YAK1	Serine-threonine protein kinase	0,46	4,86E-02
YMR070W	MOT3	Transcriptional repressor and activator with two C2-H2 zinc fingers	0,45	4,51E-02
YGR061C	ADE6	Formylglycinamidine-ribonucleotide (FGAM)-synthetase	0,45	1,46E-02
YNR016C	ACC1	Acetyl-CoA carboxylase, biotin containing enzyme	0,45	5,09E-03
YER069W	ARG5,6	Acetylglutamate kinase and N-acetyl-gamma-glutamyl-phosphate reductase	0,44	1,73E-02
YDL140C	RPO21	RNA polymerase II largest subunit B220	0,44	1,80E-02
YDR135C	YCF1	Vacuolar glutathione S-conjugate transporter	0,44	3,08E-02
YGL009C	LEU1	Isopropylmalate isomerase	0,43	1,33E-02
YGL077C	HNM1	Plasma membrane transporter for choline, ethanolamine, and carnitine	0,43	3,79E-02
YDR074W	TPS2	Phosphatase subunit of the trehalose-6-P synthase/phosphatase complex	0,43	1,66E-02
YJR137C	MET5	Sulfite reductase beta subunit	0,38	4,00E-02
YBR214W	SDS24	Protein involved in cell separation during budding	-0,41	4,85E-02
YNL160W	YGP1	Cell wall-related secretory glycoprotein	-0,44	4,80E-02
YER152C		Protein with 2-aminoadipate transaminase activity	-0,48	3,44E-02
YDL048C	STP4	Protein containing a Kruppel-type zinc-finger domain	-0,49	3,16E-02
YJL056C	ZAP1	Zinc-regulated transcription factor	-0,50	2,73E-02
YOR188W	MSB1	Protein of unknown function	-0,50	4,51E-02
YER088C	DOT6	Protein involved in rRNA and ribosome biogenesis	-0,51	4,48E-03
YLR136C	TIS11	mRNA-binding protein expressed during iron starvation	-0,51	4,51E-02
YHR188C	GPI16	Subunit of the glycosylphosphatidylinositol transamidase complex	-0,51	1,28E-02
YNL200C		NADHX epimerase	-0,52	4,80E-02
YCR098C	GIT1	Plasma membrane permease	-0,54	4,28E-02
YFL042C	LAM5	Putative sterol transfer protein	-0,54	4,50E-02
YGL156W	AMS1	Vacuolar alpha mannosidase	-0,55	3,21E-03
YOR344C	TYE7	Serine-rich protein that contains a bHLH DNA binding motif	-0,55	1,73E-02
YNR039C	ZRG17	Endoplasmic reticulum zinc transporter	-0,55	1,01E-02
YBR169C	SSE2	Member of the heat shock protein 70	-0,56	1,14E-02

		(HSP70) family		
YBR201W	DER1	ER membrane protein that promotes export of misfolded polypeptides	-0,56	4,80E-02
YPL026C	SKS1	Putative serine/threonine protein kinase	-0,57	4,91E-02
YJL082W	IML2	Protein of unknown function	-0,57	5,54E-03
YOR321W	PMT3	Protein O-mannosyltransferase	-0,57	5,77E-03
YJL020C	BBC1	Protein possibly involved in assembly of actin patches	-0,57	2,50E-03
YLR045C	STU2	Microtubule-associated protein (MAP) of the XMAP215/Dis1 family	-0,59	2,40E-02
YKL029C	MAE1	Mitochondrial malic enzyme	-0,59	3,24E-03
YOL007C	CSI2	Protein of unknown function	-0,60	2,12E-02
YNR026C	SEC12	Guanine nucleotide exchange factor (GEF)	-0,60	2,51E-02
YNL289W	PCL1	Cyclin, interacts with cyclin-dependent kinase Pho85p	-0,60	6,69E-03
YOL055C	THI20	Trifunctional enzyme of thiamine biosynthesis, degradation and salvage	-0,61	7,80E-04
YJR061W		Putative protein of unknown function	-0,62	3,05E-03
YPR091C	NVJ2	Lipid-binding ER protein, enriched at nucleus-vacuolar junctions (NVJ)	-0,62	4,44E-02
YNL168C	FMP41	Putative protein of unknown function	-0,63	2,42E-02
YIR035C		Putative cytoplasmic short-chain dehydrogenase/reductase	-0,64	7,93E-03
YNR040W		Putative protein of unknown function	-0,64	4,85E-02
YDR320C	SWA2	Auxilin-like protein involved in vesicular transport	-0,64	1,76E-03
YOR274W	MOD5	Delta 2-isopentenyl pyrophosphate:tRNA isopentenyl transferase	-0,65	4,32E-03
YIL124W	AYR1	Bifunctional triacylglycerol lipase and 1-acyl DHAP reductase	-0,66	1,14E-02
YPL134C	ODC1	Mitochondrial inner membrane transporter	-0,66	2,55E-02
YER175C	TMT1	Trans-aconitate methyltransferase	-0,66	3,34E-02
YGL256W	ADH4	Alcohol dehydrogenase isoenzyme type IV	-0,66	1,25E-02
YOL082W	ATG19	Receptor protein for the cytoplasm-to-vacuole targeting (Cvt) pathway	-0,66	3,01E-03
YLR126C		Putative glutamine amidotransferase	-0,67	4,81E-03
YKR003W	OSH6	Member of an oxysterol-binding protein family	-0,69	1,43E-02
YCL028W	RNQ1	[PIN(+)] prion	-0,69	1,51E-03
YPL277C		Putative protein of unknown function	-0,70	1,44E-03
YOL083W	ATG34	Receptor protein involved in selective autophagy during starvation	-0,71	4,86E-02
YMR170C	ALD2	Cytoplasmic aldehyde dehydrogenase	-0,72	2,52E-02
YIL119C	RPI1	Transcription factor, allelic differences between S288C and Sigma1278b	-0,73	4,45E-02
YOR003W	YSP3	Putative precursor to the subtilisin-like protease III	-0,73	4,35E-02
YER158C		Protein of unknown function	-0,74	3,05E-03
YLR059C	REX2	3'-5' RNA exonuclease	-0,75	1,25E-02
YPR196W		Putative maltose-responsive transcription factor	-0,76	4,42E-04

YBR045C	GIP1	Meiosis-specific regulatory subunit of the Glc7p protein phosphatase	-0,76	4,32E-03
YDR205W	MSC2	Endoplasmic reticulum zinc transporter	-0,77	4,53E-03
YOR125C	CAT5	Protein required for ubiquinone (Coenzyme Q) biosynthesis	-0,78	1,70E-02
YMR096W	SNZ1	Protein involved in vitamin B6 biosynthesis	-0,78	1,80E-04
YDR400W	URH1	Uridine nucleosidase (uridine-cytidine N-ribohydrolase)	-0,79	3,51E-04
YCL049C		Protein of unknown function	-0,82	2,01E-04
YJL157C	FAR1	CDK inhibitor and nuclear anchor	-0,83	1,77E-04
YGR144W	THI4	Thiazole synthase	-0,84	3,01E-03
YOL128C	YGK3	Protein kinase related to mammalian GSK-3 glycogen synthase kinases	-0,85	1,97E-03
YDR506C	GMC1	Protein involved in meiotic progression	-0,85	3,79E-02
YER015W	FAA2	Medium chain fatty acyl-CoA synthetase	-0,85	4,50E-06
YMR020W	FMS1	Polyamine oxidase	-0,85	1,04E-02
YML054C	CYB2	Cytochrome b2 (L-lactate cytochrome-c oxidoreductase)	-0,85	6,29E-03
YLR231C	BNA5	Kynureninase	-0,88	5,13E-06
YKL049C	CSE4	Centromere protein that resembles histone H3	-0,89	3,63E-02
YGL185C		Putative protein with sequence similar to hydroxyacid dehydrogenases	-0,91	3,47E-03
YNR075W	COS10	Protein of unknown function	-0,92	1,61E-04
YBL049W	MOH1	Protein of unknown function, essential for stationary phase survival	-0,93	1,13E-02
YOR092W	ECM3	Non-essential protein of unknown function	-0,93	7,10E-04
YJR025C	BNA1	3-hydroxyanthranilic acid dioxygenase	-0,93	4,14E-04
YOR071C	NRT1	High-affinity nicotinamide riboside transporter	-0,94	2,14E-02
YFR055W	IRC7	Beta-lyase involved in the production of thiols	-0,99	3,72E-06
YGL121C	GPG1	Proposed gamma subunit of the heterotrimeric G protein	-0,99	1,07E-03
YER130C	COM2	Transcription factor that binds IME1 Upstream Activation Signal (UAS)ru	-0,99	3,35E-06
YLR237W	THI7	Plasma membrane transporter responsible for the uptake of thiamine	-1,00	8,73E-10
YJR149W		Putative protein of unknown function	-1,01	1,15E-02
YLR154C	RNH203	Ribonuclease H2 subunit	-1,03	1,68E-02
YOR301W	RAX1	Protein involved in bud site selection during bipolar budding	-1,05	1,61E-04
YFL050C	ALR2	Probable Mg(2+) transporter	-1,07	9,41E-08
YGL257C	MNT2	Mannosyltransferase	-1,08	8,65E-07
YDR379C-A	SDH6	Mitochondrial protein involved in assembly of succinate dehydrogenase	-1,08	4,03E-02
YGL262W		Putative protein of unknown function	-1,09	9,84E-03
YIL072W	HOP1	Meiosis-specific protein required for chromosome synapsis	-1,09	3,17E-03
YLR004C	THI73	Putative plasma membrane permease	-1,10	1,34E-09
YPL191C		Putative protein of unknown function	-1,12	5,64E-03

YIL099W	SGA1	Intracellular sporulation-specific glucoamylase	-1,13	8,08E-03
YDR461C-A		Putative protein of unknown function	-1,14	2,72E-03
YDR503C	LPP1	Lipid phosphate phosphatase	-1,14	1,44E-04
YNL254C	RTC4	Protein of unknown function	-1,15	6,80E-07
YHL028W	WSC4	Endoplasmic reticulum (ER) membrane protein	-1,16	1,80E-04
YHL048W	COS8	Nuclear membrane protein	-1,16	4,92E-05
YPR006C	ICL2	2-methylisocitrate lyase of the mitochondrial matrix	-1,17	2,24E-02
YMR095C	SNO1	Protein of unconfirmed function	-1,20	9,73E-03
YMR199W	CLN1	G1 cyclin involved in regulation of the cell cycle	-1,21	4,05E-10
YOR386W	PHR1	DNA photolyase involved in photoreactivation	-1,21	3,96E-02
YDR243C	PRP28	RNA binding protein	-1,22	3,03E-04
YPL248C	GAL4	DNA-binding transcription factor required for activating GAL genes	-1,23	1,13E-04
YBL029W		Non-essential protein of unknown function	-1,24	6,28E-08
YKL043W	PHD1	Transcriptional activator that enhances pseudohyphal growth	-1,26	4,98E-12
YGL205W	POX1	Fatty-acyl coenzyme A oxidase	-1,29	2,86E-08
YOR049C	RSB1	Suppressor of sphingoid LCB sensitivity of an LCB-lyase mutation	-1,30	4,98E-03
YHR140W		Putative integral membrane protein of unknown function	-1,32	7,25E-03
YBR033W	EDS1	Putative zinc cluster protein, predicted to be a transcription factor	-1,32	2,97E-02
YPL258C	THI21	Hydroxymethylpyrimidine (HMP) and HMP-phosphate kinase	-1,34	1,21E-12
YBR072W	HSP26	Small heat shock protein (sHSP) with chaperone activity	-1,38	3,06E-09
YDR406W	PDR15	Plasma membrane ATP binding cassette (ABC) transporter	-1,39	4,47E-17
YJR079W		Putative protein of unknown function	-1,40	5,69E-03
YKL132C	RMA1	Putative dihydrofolate synthetase	-1,50	4,19E-05
YLR070C	XYL2	Xylitol dehydrogenase	-1,55	4,34E-03
YBR240C	THI2	Transcriptional activator of thiamine biosynthetic genes	-1,56	1,42E-14
YBL048W	RRT1	Protein of unknown function	-1,58	1,83E-02
YNL065W	AQR1	Plasma membrane transporter of the major facilitator superfamily	-1,66	1,80E-15
YMR182C	RGM1	Putative zinc finger DNA binding transcription factor	-1,67	4,81E-02
YER060W-A	FCY22	Putative purine-cytosine permease	-1,73	1,52E-02
YEL049W	PAU2	Member of the seripauperin multigene family	-1,80	3,79E-02
YIL160C	POT1	3-ketoacyl-CoA thiolase with broad chain length specificity	-1,81	7,72E-06
YPR121W	THI22	Protein with similarity to hydroxymethylpyrimidine phosphate kinases	-1,81	1,99E-10
YJR156C	THI11	Protein involved in synthesis of the thiamine precursor HMP	-1,86	3,53E-07

YIR039C	YPS6	Putative GPI-anchored aspartic protease	-1,95	3,43E-04
YER060W	FCY21	Putative purine-cytosine permease	-2,00	6,94E-11
YGR287C	IMA1	Major isomaltase (alpha-1,6-glucosidase/alpha-methylglucosidase)	-2,06	9,68E-10
YFR026C	ULI1	Putative protein of unknown function	-2,15	4,82E-07
YGL258W	VEL1	Protein of unknown function	-2,49	5,41E-09
YOR161W-B		Protein of unknown function	-2,71	3,24E-03
YOL131W		Putative protein of unknown function	-2,78	3,17E-05
YNL012W	SPO1	Meiosis-specific prospore protein	-2,78	7,30E-03
YDL244W	THI13	Protein involved in synthesis of the thiamine precursor HMP	-2,82	2,51E-16
YDR380W	ARO10	Phenylpyruvate decarboxylase	-3,11	1,80E-02
YOR387C		Putative protein of unknown function	-3,40	6,50E-30
YMR319C	FET4	Low-affinity Fe(II) transporter of the plasma membrane	-3,88	3,64E-32
YGR108W	CLB1	B-type cyclin involved in cell cycle progression	NULL	7,49E-22
YGR109C	CLB6	B-type cyclin involved in DNA replication during S phase	NULL	5,59E-03
YGR288W	MAL13	MAL-activator protein	NULL	8,99E-17
YGR289C	MAL11	High-affinity maltose transporter (alpha-glucoside transporter)	NULL	2,17E-06
YIR042C		Putative protein of unknown function	NULL	5,17E-14

Table S2 - Comparison between gene expression levels of the UMCC 2581 and UMCC 855 strains. Gene Ontology (GO) process enriched for over-expressed and under-expressed genes in UMCC 2581 are reported.

Process Ontology

Gene Ontology Enrichment, up-regulated genes

Gene Ontology ID	Gene Ontology term	Cluster frequency	Background frequency	p-value	FDR	Expected False Positives	Genes annotated to the term
6820	anion transport	19 out of 140 genes, 13.6%	119 out of 4737 background genes, 2.5%	5.42e-07	0.00	0.00	CTP1/ YBR291C, VBA2/ YBR293W, SUL1/ YBR294W, PHO89/ YBR296C, AGP1/ YCL025C, YCF1/ YDR135C, GNP1/ YDR508C, HNM1/ YGL077C, MPC3/ YGR243W, MUP3/ YHL036W, FAT3/ YKL187C, GAP1/ YKR039W, YCT1/ YLL055W, ATR1/ YML116W, FET3/ YMR058W, FAA4/ YMR246W, TAT2/ YOL020W, ENB1/ YOL158C, DIP5/ YPL265W
46942	carboxylic acid transport	14 out of 140 genes, 10.0%	67 out of 4737 background genes, 1.4%	2.71e-06	0.00	0.00	CTP1/ YBR291C, VBA2/ YBR293W, AGP1/ YCL025C, YCF1/ YDR135C, GNP1/ YDR508C, HNM1/ YGL077C, MPC3/ YGR243W, MUP3/ YHL036W, FAT3/ YKL187C, GAP1/ YKR039W, YCT1/ YLL055W, FAA4/ YMR246W, TAT2/ YOL020W, DIP5/ YPL265W
15849	organic acid transport	14 out of 140 genes, 10.0%	68 out of 4737 background genes, 1.4%	3.33e-06	0.00	0.00	CTP1/ YBR291C, VBA2/ YBR293W, AGP1/ YCL025C, YCF1/ YDR135C, GNP1/ YDR508C, HNM1/ YGL077C, MPC3/ YGR243W, MUP3/ YHL036W, FAT3/ YKL187C, GAP1/ YKR039W, YCT1/ YLL055W, FAA4/ YMR246W, TAT2/ YOL020W, DIP5/ YPL265W
15711	organic anion transport	15 out of 140 genes, 10.7%	100 out of 4737 background genes, 2.1%	8.37e-05	0.00	0.00	CTP1/ YBR291C, VBA2/ YBR293W, AGP1/ YCL025C, YCF1/ YDR135C, GNP1/ YDR508C, HNM1/ YGL077C, MPC3/ YGR243W, MUP3/ YHL036W, FAT3/ YKL187C, GAP1/ YKR039W, YCT1/ YLL055W, FAA4/ YMR246W, TAT2/ YOL020W, ENB1/ YOL158C, DIP5/ YPL265W
6811	ion transport	22 out of 140 genes,	215 out of 4737 background	0.00011	0.00	0.00	CTP1/ YBR291C, VBA2/ YBR293W, SUL1/ YBR294W, PHO89/ YBR296C, AGP1/ YCL025C, YCF1/ YDR135C,

		15.7%	genes, 4.5%				GNP1/ YDR508C, HNM1/ YGL077C, MEP1/ YGR121C, MPC3/ YGR243W, DUR3/ YHL016C, MUP3/ YHL036W, QDR2/ YIL121W, FAT3/ YKL187C, GAP1/ YKR039W, YCT1/ YLL055W, ATR1/ YML116W, FET3/ YMR058W, FAA4/ YMR246W, TAT2/ YOL020W, ENB1/ YOL158C, DIP5/ YPL265W
43605	cellular amide catabolic process	5 out of 140 genes, 3.6%	9 out of 4737 background genes, 0.2%	0.00126	0.00	0.00	DUR3/ YHL016C, DAL1/ YIR027C, DAL2/ YIR029W, DAL7/ YIR031C, DAL3/ YIR032C
71705	nitrogen compound transport	18 out of 140 genes, 12.9%	192 out of 4737 background genes, 4.1%	0.00564	0.00	0.02	FUR4/ YBR021W, VBA2/ YBR293W, AGP1/ YCL025C, YCF1/ YDR135C, GNP1/ YDR508C, FCY2/ YER056C, HNM1/ YGL077C, YGL114W, UTP22/ YGR090W, MEP1/ YGR121C, DUR3/ YHL016C, MUP3/ YHL036W, GAP1/ YKR039W, PTR2/ YKR093W, YCT1/ YLL055W, MMP1/ YLL061W, TAT2/ YOL020W, DIP5/ YPL265W
6865	amino acid transport	8 out of 140 genes, 5.7%	39 out of 4737 background genes, 0.8%	0.00708	0.00	0.02	VBA2/ YBR293W, AGP1/ YCL025C, GNP1/ YDR508C, MUP3/ YHL036W, GAP1/ YKR039W, YCT1/ YLL055W, TAT2/ YOL020W, DIP5/ YPL265W

Gene Ontology Enrichment, down-regulated genes

Gene Ontology ID	Gene Ontology term	Cluster frequency	Background frequency	p-value	FDR	Expected False Positives	Genes annotated to the term
42723	thiamine-containing compound metabolic process	4 out of 109 genes, 3.7%	5 out of 4737 background genes, 0.1%	0.00055	0.02	0.02	THI2/ YBR240C, THI4/ YGR144W, THI20/ YOL055C, THI21/ YPL258C
42724	thiamine-containing compound biosynthetic process	4 out of 109 genes, 3.7%	5 out of 4737 background genes, 0.1%	0.00055	0.01	0.02	THI2/ YBR240C, THI4/ YGR144W, THI20/ YOL055C, THI21/ YPL258C
6772	thiamine	4 out of	5 out of 4737	0.00055	0.01	0.02	THI2/ YBR240C, THI4/ YGR144W, THI20/ YOL055C,

	metabolic process	109 genes, 3.7%	background genes, 0.1%				THI21/ YPL258C
9228	thiamine biosynthetic process	4 out of 109 genes, 3.7%	5 out of 4737 background genes, 0.1%	0.00055	0.01	0.02	THI2/ YBR240C, THI4/ YGR144W, THI20/ YOL055C, THI21/ YPL258C

Table S3 - Comparison between gene expression levels of the UMCC 2581 and UMCC 855 strains. Gene Ontology (GO) function enriched for over-expressed and under-expressed genes in UMCC 2581 are reported.

Function Ontology

Gene Ontology Enrichment, up-regulated genes

Gene Ontology ID	Gene Ontology term	Cluster frequency	Background frequency	p-value	FDR	Expected False Positives	Genes annotated to the term
8509	anion transmembrane transporter activity	16 out of 140 genes, 11.4%	75 out of 4737 background genes, 1.6%	3.68e-08	0.00	0.00	CTP1/YBR291C, VBA2/YBR293W, SUL1/YBR294W, PHO89/YBR296C, AGP1/YCL025C, YCF1/YDR135C, GNP1/YDR508C, HNM1/YGL077C, MPC3/YGR243W, MUP3/YHL036W, GAP1/YKR039W, YCT1/YLL055W, MMP1/YLL061W, ATR1/YML116W, TAT2/YOL020W, DIP5/YPL265W
46943	carboxylic acid transmembrane transporter activity	13 out of 140 genes, 9.3%	50 out of 4737 background genes, 1.1%	1.38e-07	0.00	0.00	CTP1/YBR291C, VBA2/YBR293W, AGP1/YCL025C, YCF1/YDR135C, GNP1/YDR508C, HNM1/YGL077C, MPC3/YGR243W, MUP3/YHL036W, GAP1/YKR039W, YCT1/YLL055W, MMP1/YLL061W, TAT2/YOL020W, DIP5/YPL265W
5342	organic acid transmembrane transporter activity	13 out of 140 genes, 9.3%	53 out of 4737 background genes, 1.1%	3.05e-07	0.00	0.00	CTP1/YBR291C, VBA2/YBR293W, AGP1/YCL025C, YCF1/YDR135C, GNP1/YDR508C, HNM1/YGL077C, MPC3/YGR243W, MUP3/YHL036W, GAP1/YKR039W, YCT1/YLL055W, MMP1/YLL061W, TAT2/YOL020W, DIP5/YPL265W
8514	organic anion transmembrane transporter activity	13 out of 140 genes, 9.3%	60 out of 4737 background genes, 1.3%	1.57e-06	0.00	0.00	CTP1/YBR291C, VBA2/YBR293W, AGP1/YCL025C, YCF1/YDR135C, GNP1/YDR508C, HNM1/YGL077C, MPC3/YGR243W, MUP3/YHL036W, GAP1/YKR039W, YCT1/YLL055W, MMP1/YLL061W, TAT2/YOL020W, DIP5/YPL265W
22891	substrate-specific	27 out of 140 genes, 19.3%	271 out of 4737	1.72e-06	0.00	0.00	FUR4/YBR021W, CTP1/YBR291C, VBA2/YBR293W, SUL1/YBR294W, PHO89/YBR296C, AGP1/YCL025C,

	transmembrane transporter activity		background genes, 5.7%				YCF1/YDR135C, HXT7/YDR342C, GNP1/YDR508C, STL1/YDR536W, FCY2/YER056C, HNM1/YGL077C, MEP1/YGR121C, MPC3/YGR243W, DUR3/YHL016C, MUP3/YHL036W, HXT5/YHR096C, QDR2/YIL121W, GAP1/YKR039W, YCT1/YLL055W, MMP1/YLL061W, ATR1/YML116W, HXT2/YMR011W, FET3/YMR058W, TAT2/YOL020W, ENB1/YOL158C, DIP5/YPL265W
15075	ion transmembrane transporter activity	23 out of 140 genes, 16.4%	203 out of 4737 background genes, 4.3%	2.13e-06	0.00	0.00	FUR4/YBR021W, CTP1/YBR291C, VBA2/YBR293W, SUL1/YBR294W, PHO89/YBR296C, AGP1/YCL025C, YCF1/YDR135C, GNP1/YDR508C, STL1/YDR536W, HNM1/YGL077C, MEP1/YGR121C, MPC3/YGR243W, DUR3/YHL016C, MUP3/YHL036W, QDR2/YIL121W, GAP1/YKR039W, YCT1/YLL055W, MMP1/YLL061W, ATR1/YML116W, FET3/YMR058W, TAT2/YOL020W, ENB1/YOL158C, DIP5/YPL265W
22857	transmembrane transporter activity	28 out of 140 genes, 20.0%	296 out of 4737 background genes, 6.2%	2.81e-06	0.00	0.00	FUR4/YBR021W, CTP1/YBR291C, VBA2/YBR293W, SUL1/YBR294W, PHO89/YBR296C, AGP1/YCL025C, SNQ2/YDR011W, YCF1/YDR135C, HXT7/YDR342C, GNP1/YDR508C, STL1/YDR536W, FCY2/YER056C, HNM1/YGL077C, MEP1/YGR121C, MPC3/YGR243W, DUR3/YHL016C, MUP3/YHL036W, HXT5/YHR096C, QDR2/YIL121W, GAP1/YKR039W, YCT1/YLL055W, MMP1/YLL061W, ATR1/YML116W, HXT2/YMR011W, FET3/YMR058W, TAT2/YOL020W, ENB1/YOL158C, DIP5/YPL265W
22892	substrate-specific transporter activity	29 out of 140 genes, 20.7%	316 out of 4737 background genes, 6.7%	2.98e-06	0.00	0.00	FUR4/YBR021W, CTP1/YBR291C, VBA2/YBR293W, SUL1/YBR294W, PHO89/YBR296C, AGP1/YCL025C, YCF1/YDR135C, HXT7/YDR342C, GNP1/YDR508C, STL1/YDR536W, FCY2/YER056C, HNM1/YGL077C, YGL114W, MEP1/YGR121C, MPC3/YGR243W, DUR3/YHL016C, MUP3/YHL036W, HXT5/YHR096C, QDR2/YIL121W, GAP1/YKR039W, PTR2/YKR093W, YCT1/YLL055W, MMP1/YLL061W, ATR1/YML116W, HXT2/YMR011W, FET3/YMR058W, TAT2/YOL020W,

							ENB1/YOL158C, DIP5/YPL265W
5215	transporter activity	31 out of 140 genes, 22.1%	370 out of 4737 background genes, 7.8%	7.69e-06	0.00	0.00	FLR1/YBR008C, FUR4/YBR021W, CTP1/YBR291C, VBA2/YBR293W, SUL1/YBR294W, PHO89/YBR296C, AGP1/YCL025C, SNQ2/YDR011W, YCF1/YDR135C, HXT7/YDR342C, GNP1/YDR508C, STL1/YDR536W, FCY2/YER056C, HNM1/YGL077C, YGL114W, MEP1/YGR121C, MPC3/YGR243W, DUR3/YHL016C, MUP3/YHL036W, HXT5/YHR096C, QDR2/YIL121W, GAP1/YKR039W, PTR2/YKR093W, YCT1/YLL055W, MMP1/YLL061W, ATR1/YML116W, HXT2/YMR011W, FET3/YMR058W, TAT2/YOL020W, ENB1/YOL158C, DIP5/YPL265W
15171	amino acid transmembrane transporter activity	9 out of 140 genes, 6.4%	32 out of 4737 background genes, 0.7%	2.78e-05	0.00	0.00	VBA2/YBR293W, AGP1/YCL025C, GNP1/YDR508C, MUP3/YHL036W, GAP1/YKR039W, YCT1/YLL055W, MMP1/YLL061W, TAT2/YOL020W, DIP5/YPL265W
8324	cation transmembrane transporter activity	15 out of 140 genes, 10.7%	145 out of 4737 background genes, 3.1%	0.00255	0.00	0.00	FUR4/YBR021W, PHO89/YBR296C, AGP1/YCL025C, GNP1/YDR508C, STL1/YDR536W, HNM1/YGL077C, MEP1/YGR121C, DUR3/YHL016C, MUP3/YHL036W, QDR2/YIL121W, GAP1/YKR039W, YCT1/YLL055W, FET3/YMR058W, TAT2/YOL020W, ENB1/YOL158C
15179	L-amino acid transmembrane transporter activity	5 out of 140 genes, 3.6%	16 out of 4737 background genes, 0.3%	0.00938	0.00	0.00	AGP1/YCL025C, GNP1/YDR508C, MUP3/YHL036W, GAP1/YKR039W, TAT2/YOL020W

Gene Ontology Enrichment, down-regulated genes, No significant term

Table S4 - Comparison between gene expression levels of the UMCC 2581 and UMCC 855 strains. Gene Ontology (GO) component enriched for over-expressed and under-expressed genes in UMCC 2581 are reported.

Component Ontology

Gene Ontology Enrichment, up-regulated genes

Gene Ontology ID	Gene Ontology term	Cluster frequency	Background frequency	p-value	FDR	Expected False Positives	Genes annotated to the term
71944	cell periphery	42 out of 140 genes, 30.0%	481 out of 4737 background genes, 10.2%	3.21e-09	0.00	0.00	FLR1/ YBR008C, FUR4/ YBR021W, YPC1/ YBR183W, SUL1/ YBR294W, PHO89/ YBR296C, AGP1/ YCL025C, HSP30/ YCR021C, KNH1/ YDL049C, FMP45/ YDL222C, SNQ2/ YDR011W, HXT7/ YDR342C, GNP1/ YDR508C, FIT1/ YDR534C, STL1/ YDR536W, UTR2/ YEL040W, FCY2/ YER056C, STE2/ YFL026W, HNM1/ YGL077C, YGL114W, MTL1/ YGR023W, MEP1/ YGR121C, DUR3/ YHL016C, MYO1/ YHR023W, HXT5/ YHR096C, QDR2/ YIL121W, PRY3/ YJL078C, GAP1/ YKR039W, PTR2/ YKR093W, MMP1/ YLL061W, INA1/ YLR413W, PUN1/ YLR414C, ATR1/ YML116W, HXT2/ YMR011W, FET3/ YMR058W, YNL194C, AGA1/ YNR044W, TAT2/ YOL020W, LDS2/ YOL047C, ENB1/ YOL158C, FIT2/ YOR382W, DIP5/ YPL265W, NCE102/ YPR149W
5886	plasma membrane	33 out of 140 genes, 23.6%	327 out of 4737 background genes, 6.9%	1.70e-08	0.00	0.00	FLR1/ YBR008C, FUR4/ YBR021W, SUL1/ YBR294W, PHO89/ YBR296C, AGP1/ YCL025C, HSP30/ YCR021C, FMP45/ YDL222C, SNQ2/ YDR011W, HXT7/ YDR342C, GNP1/ YDR508C, STL1/ YDR536W, FCY2/ YER056C, STE2/ YFL026W, HNM1/ YGL077C, YGL114W, MTL1/ YGR023W, MEP1/ YGR121C, DUR3/ YHL016C, HXT5/ YHR096C, QDR2/ YIL121W, GAP1/ YKR039W, PTR2/ YKR093W, MMP1/ YLL061W, INA1/ YLR413W, PUN1/ YLR414C, ATR1/ YML116W, HXT2/ YMR011W, FET3/ YMR058W, YNL194C, TAT2/ YOL020W, ENB1/

							YOL158C, DIP5/ YPL265W, NCE102/ YPR149W
16021	integral component of membrane	45 out of 140 genes, 32.1%	817 out of 4737 background genes, 17.2%	0.00105	0.00	0.00	FLR1/ YBR008C, FUR4/ YBR021W, YPC1/ YBR183W, CTP1/ YBR291C, VBA2/ YBR293W, SUL1/ YBR294W, PHO89/ YBR296C, AGP1/ YCL025C, HSP30/ YCR021C, FMP45/ YDL222C, SNQ2/ YDR011W, YCF1/ YDR135C, HXT7/ YDR342C, GNP1/ YDR508C, STL1/ YDR536W, FCY2/ YER056C, STE2/ YFL026W, HNM1/ YGL077C, YGL114W, MTL1/ YGR023W, MEP1/ YGR121C, DUR3/ YHL016C, MUP3/ YHL036W, HXT5/ YHR096C, QDR2/ YIL121W, GUT2/ YIL155C, YJR124C, FAT3/ YKL187C, GAP1/ YKR039W, PTR2/ YKR093W, YCT1/ YLL055W, MMP1/ YLL061W, INA1/ YLR413W, PUN1/ YLR414C, ATR1/ YML116W, HXT2/ YMR011W, FET3/ YMR058W, YNL194C, YOL013W-A, TAT2/ YOL020W, LDS2/ YOL047C, PHM7/ YOL084W, ENB1/ YOL158C, DIP5/ YPL265W, NCE102/ YPR149W
31224	intrinsic component of membrane	45 out of 140 genes, 32.1%	825 out of 4737 background genes, 17.4%	0.00137	0.00	0.00	FLR1/ YBR008C, FUR4/ YBR021W, YPC1/ YBR183W, CTP1/ YBR291C, VBA2/ YBR293W, SUL1/ YBR294W, PHO89/ YBR296C, AGP1/ YCL025C, HSP30/ YCR021C, FMP45/ YDL222C, SNQ2/ YDR011W, YCF1/ YDR135C, HXT7/ YDR342C, GNP1/ YDR508C, STL1/ YDR536W, FCY2/ YER056C, STE2/ YFL026W, HNM1/ YGL077C, YGL114W, MTL1/ YGR023W, MEP1/ YGR121C, DUR3/ YHL016C, MUP3/ YHL036W, HXT5/ YHR096C, QDR2/ YIL121W, GUT2/ YIL155C, YJR124C, FAT3/ YKL187C, GAP1/ YKR039W, PTR2/ YKR093W, YCT1/ YLL055W, MMP1/ YLL061W, INA1/ YLR413W, PUN1/ YLR414C, ATR1/ YML116W, HXT2/ YMR011W, FET3/ YMR058W, YNL194C, YOL013W-A, TAT2/ YOL020W, LDS2/ YOL047C, PHM7/ YOL084W, ENB1/ YOL158C, DIP5/ YPL265W, NCE102/ YPR149W

Gene Ontology Enrichment, down-regulated genes, No significant term

4.3.2 Genomic DNA

Table S5 - Summary of genome sequencing data generated in this study. Statistics from the Ion Torrent sequencing run.

Sample	Bases	>=Q20 bases	Reads	Mean read length
UMCC 855	1.668.850.209	1.411.272.981	11.632.368	143 bp
UMCC 2580	1.233.492.458	1.056.906.818	8.764.338	141 bp
UMCC 2581	1.389.700.415	1.195.000.705	9.860.882	141 bp
UMCC 2584	1.051.785.843	892.738.409	7.309.343	144 bp
UMCC 2585	1.077.515.272	911.723.407	7.461.391	144 bp
ClusterSensitive	820.028.716	695.173.250	5.751.073	143 bp
Cluster21T2	975.820.078	808.919.415	6.507.162	150 bp
ClusterEvolved	1.299.797.429	1.089.163.300	8.834.774	147 bp
ClusterOther	1.483.223.945	1.262.019.784	10.178.880	146 bp

Table S6 - Genome locations and significant genomic variation of genes found in QTL peaks in chromosome 4. Peak 1 and peak 2 are reported, respectively in the upper and lower part of the table. Ref. Allele, reference allele as in S288c reference sequence; Alt. Allele, alternative allele present in UMCC 855/2581 sequences. Het., heterozygous; Hom. Alt., homozygous alternative; Hom. Ref., homozygous reference. SNPs or InDels are considered heterozygous with the calculated allele frequency between 0.25 -0.75 and coverage greater than 20. Predicted effect describes the variant using HGVS notation (http://www.hgvs.org/mutnomen/).

Chromosome 4, Peak 1

Systematic gene name	Standard gene name	End position	Start position	Gene description	Genomic Variation					
					Position	Ref. Allele	Alt. Allele	Predicted effect	UMCC 855	UMCC 2581
YDL018C	ERP3	423510	422833	Protein with similarity to Emp24p and Erv25p	423385	T	G	Missense, p.Glu42Asp	Het.	Hom. Alt.
YDL017W	CDC7	424209	425732	DDK (Dbf4-dependent kinase) catalytic subunit	424905	C	A	Missense, p.Pro233Thr	Hom. Alt.	Hom. Alt.
YDL016C		425872	425570	Dubious open reading frame	\	\	\	\	\	\
YDL015C	TSC13	426934	426002	Enoyl reductase	\	\		\	\	\
					426781	A	T	Missense, p.Ser52Thr	Hom. Alt.	Hom. Alt.
YDL014W	NOP1	427364	428347	Histone glutamine methyltransferase, modifies H2A at Q105 in nucleolus						
YDL013W	SLX5	429067	430926	Subunit of the Slx5-Slx8 SUMO-targeted ubiquitin ligase (STUbL) complex	430473	GA	G	Frameshift, p.Asn471fs	Hom. Ref.	
YDL012C		431517	431108	Tail-anchored plasma membrane protein with a conserved CYSTM module	\	\	\	\	\	
YDL010W	GRX6	432330	433025	Cis-golgi localized monothiol glutaredoxin,	432463	G	A	Missense, p.Ser45Asn	Hom Alt.	Hom Alt.

				binds Fe-S cluster	432487	G	C	Missense, p.Gly53Ala	Hom Alt.	Hom Alt.
					432606	T	A	Missense, p.Ser93Thr	Het.	Hom. Alt.
YDL011C		432631	432308	Dubious open reading frame	432487	G	C	Missense, p.Pro49Ala	Hom Alt.	Hom Alt.
					432606	T	A	Missense, p.Asp9Val	Het.	Hom. Alt.
YDL009C		433248	432925	Protein of unknown function	433160	A	C	Missense, p.Ile30Ser	Hom. Alt.	Hom. Alt.
YDL008W	APC11	433497	433994	Catalytic core subunit, Anaphase-Promoting Complex/Cyclosome (APC/C)	433554	A	G	Missense, p.Ser20Gly	Het.	Hom. Ref.
					433591	T	A	Missense, p.Ile32Asn	Hom. Alt.	Hom. Alt.
YDL007C-A		436824	436567	Putative protein of unknown function	\	\	\	\	\	\
YDL007W	RPT2	438047	439360	ATPase of the 19S regulatory particle of the 26S proteasome	439194	CA	C	Frameshift, p.Lys384fs	Hom. Alt.	Hom. Alt.
YDL006W	PTC1	439909	440754	Type 2C protein phosphatase (PP2C)	\	\	\	\	\	\
YDL005C	MED2	442309	441014	Subunit of the RNA polymerase II mediator complex	441238	T	G	Missense, p.Asn358His	Hom. Alt.	Hom. Alt.
					441267	C	CTGTTGTTGT	Inframe insertion, p.Asn345_Asn347dup	Hom. Ref.	Het.
					441311	GTTATTA	G	Disruptive inframe deletion,	Het.	Hom. Alt.

								p.Asn332_Asn333del		
					441367	C	T	Missense, p.Asp315Asn	Hom. Alt.	Hom. Alt.
					441465	A	G	Missense, p.Ile282Thr	Hom. Alt.	Hom. Alt.
					441636	A	G	Missense, p.Val225Ala	Hom. Alt.	Hom. Alt.
					441646	G	A	Missense, p.Pro222Ser	Het.	Hom. Alt.
					441738	C	T	Missense, p.Ser191Asn	Hom. Alt.	Hom. Alt.
YDL004W	ATP16	443029	443511	Delta subunit of the central stalk of mitochondrial F1F0 ATP synthase	\	\	\	\	\	\
YDL003W	MCD1	444683	446383	Essential alpha-kleisin subunit of the cohesin complex	445472	G	A	Missense, p.Val264Ile	Het	Hom. Alt.
					445581	T	C	Missense, p.Ile300Thr	Hom. Alt.	Hom. Alt.
					445653	A	G	Missense, p.Lys324Arg	Het,	Hom. Ref.
					445817	C	T	Missense, p.Pro379Ser	Hom. Alt.	Hom. Alt.
					446016	G	A	Missense, p.Gly445Asp	Hom. Alt.	Hom. Alt.
					446066	A	G	Missense, p.Arg462Gly	Hom. Alt.	Hom. Alt.
					446126	T	C	Missense, p.Ser482Pro	Het.	Hom. Ref.
					446328	G	A	Missense, p.Gly549Glu	Het.	Hom. Ref.
YDL002C	NHP10	447578	446967	Non-essential INO80 chromatin remodeling complex subunit	\	\	\	\	\	\

YDL001W	RMD1	447984	449276	Cytoplasmic protein required for sporulation	448045	C	G	Missense, p.Thr21Arg	Het.	Hom. Alt.
					448177	C	T	Missense, p.Ser65Leu	Het.	Hom. Alt.
Chromosome 4, Peak 2										
YDR130C	FIN1	716622	715747	Spindle pole body-related intermediate filament protein	\	\	\	\	\	\
YDR131C		718460	716790	F-box protein subunit of SCF ubiquitin ligase complex	717377	G	C	Missense, p.Gln362Glu	Hom. Alt.	Hom. Alt.
					717530	C	G	Missense, p.Glu311Gln	Het.	Hom. Ref.
					717634	C	A	Missense, p.Gly276Val	Hom. Alt.	Hom. Alt.
YDR132C		720303	718816	Protein of unknown function	719391	T	C	Missense, p.Ile305Val	Hom. Alt.	Hom. Alt.
					719745	A	G	Missense, p.Ser187Pro	Hom. Alt.	Hom. Alt.
YDR133C		721297	720962	Dubious open reading frame	720993	C	G	Missense, p.Arg102Pro	Hom. Alt.	Hom. Alt.
					721110	G	A	Missense, p.Pro63Leu	Hom. Alt.	Hom. Alt.
					721119	C	T	Missense, p.Cys60Tyr	Hom. Alt.	Hom. Alt.
					721191	AGTTGGCTTTTCG	A	Disruptive inframe deletion, p.Pro32_Gln35del	Hom. Ref.	Het.
YDR135C	YCF1	727551	723004	Vacuolar glutathione S-conjugate transporter	724610	C	T	Missense, p.Arg981His	Hom. Alt.	Hom. Alt.
					724885	C	A	Missense, p.Gln889His	Het.	Hom. Alt.
					726762	C	G	Missense,	Hom.	Hom.

								p.Glu264Gln	Alt.	Alt.
					727008	T	C	Missense, p.Ser182Gly	Hom. Alt.	Hom. Alt.
YDR137W	RGP1	728259	730250	Subunit of a Golgi membrane exchange factor (Ric1p-Rgp1p)	728391	C	A	Missense, p.His45Asn	Hom. Alt.	Hom. Alt.
					728439	G	A	Missense, p.Val61Ile	Het.	Hom. Alt.
					728470	T	C	Missense, p.Val71Ala	Het.	Hom. Alt.
					728757	G	C	Missense, p.Asp167His	Het.	Hom. Ref.
					729030	G	A	Missense, p.Val258Ile	Hom. Alt.	Hom. Alt.
					730077	A	G	Missense, p.Ile607Val	Hom. Alt.	Hom. Alt.
YDR136C	VPS61	728283	727711	Dubious open reading frame	727773	T	TA	Frameshift, p.Met171fs	Hom. Alt.	Hom. Alt.
					728061	G	A	Missense, p.Leu75Phe	Het.	Hom. Ref.
					728169	C	T	Missense, p.Ala39Thr	Het.	Hom. Ref.
YDR138W	HPR1	730578	732836	Subunit of THO/TREX complexes	730611	A	C	Missense, p.Ile12Leu	Hom. Alt.	Hom. Alt.
					730704	G	A	Missense, p.Val43Ile	Hom. Alt.	Hom. Alt.
					730734	A	G	Missense, p.Lys53Glu	Hom. Alt.	Hom. Alt.
					732005	CA	C	Frameshift, p.Lys477fs	Het.	Hom. Ref.
					732240	G	A	Missense, p.Ala555Thr	Het.	Hom. Ref.
YDR139C	RUB1	733924	733618	Ubiquitin-like protein with similarity to mammalian NEDD8	733663	C	T	Missense, p.Arg63Lys	Hom. Alt.	Hom. Alt.

YDR140W	MTQ2	734138	734803	S-adenosylmethionine-dependent methyltransferase	734692	G	C	Missense, p.Lys185Asn	Het.	Hom. Ref.
YDR141C	DOP1	739997	734901	Golgi-localized, leucine-zipper domain containing protein	736764	CT	C	Frameshift, p.Lys1078fs	Hom. Ref.	Het.
					737762	C	T	Missense, p.Glu746Lys	Het.	Hom. Ref.
YDR142C	PEX7	741600	740473	Peroxisomal signal receptor for peroxisomal matrix proteins	740795	A	G	Missense, p.Leu269Pro	Hom. Alt.	Hom. Alt.
					741082	GT	G	Frameshift, p.Asn173fs	Het.	Hom. Ref.
					741095	C	T	Missense, p.Gly169Asp	Het.	Hom. Ref.
					741116	A	G	Missense, p.Leu162Ser	Het.	Hom. Ref.
YDR143C	SAN1	743874	742042	Ubiquitin-protein ligase	742290	G	GGTTATTGTC	Inframe insertion, p.Asn528_His529insAspAsnAsn	Het.	Hom. Ref.
					742728	C	T	Missense, p.Val383Ile	Hom. Alt.	Hom. Alt.
					742798	TA	T	Frameshift, p.Leu359fs	Hom. Ref.	Het.
					743645	C	T	Missense, p.Ser77Asn	Hom. Alt.	Hom. Alt.
					743850	T	C	Missense, p.Asn9Asp	Hom. Alt.	Hom. Alt.
YDR144C	MKC7	746101	744311	GPI-anchored aspartyl protease	744363	A	C	Missense, p.Leu580Arg	Hom. Alt.	Hom. Alt.
					744456	G	T	Missense, p.Ser549Tyr	Hom. Alt.	Hom. Alt.
					744457	A	G	Missense, p.Ser549Pro	Hom. Alt.	Hom. Alt.
					744475	T	A	Missense,	Hom.	Hom.

								p.Thr543Ser	Alt.	Alt.
					744534	A	G	Missense, p.Phe523Ser	Hom. Alt.	Hom. Alt.
					744552	T	C	Missense, p.Asn517Ser	Hom. Alt.	Hom. Alt.
					745720	TA	T	Frameshift, p.Phe127fs	Het.	Het.
					745998	A	T	Missense, p.Val35Asp	Hom. Alt.	Hom. Alt.
YDR145W	TAF12	746738	748357	Subunit (61/68 kDa) of TFIID and SAGA complexes	747029	T	C	Missense, p.Ser98Pro	Hom. Alt.	Hom. Alt.
					747085	G	GCAA	Disruptive inframe insertion, p.Gln124dup	Het.	Het.
					747674	A	C	Missense, p.Thr313Pro	Hom. Alt.	Hom. Alt.
YDR146C	SWI5	750742	748613	Transcription factor that recruits Mediator and Swi/Snf complexes	749263	C	T	Missense, p.Val494Ile	Hom. Alt.	Hom. Alt.
					750696	A	T	Missense, p.Leu16Gln	Hom. Alt.	Hom. Alt.
YDR147W	EKI1	751631	753235	Ethanolamine kinase	752043	C	T	Missense, p.Thr138Ile	Het.	Hom. Ref.
					752433	T	C	Missense, p.Val268Ala	Het.	Hom. Ref.
					753137	T	A	Missense, p.Ser503Thr	Hom. Alt.	Hom. Alt.
YDR148C	KGD2	755066	753675	Dihydrolipoyl transsuccinylase	\	\	\	\	\	\
YDR150W	NUM1	755628	763874	Protein required for nuclear migration	\	\	\	\	\	\
YDR149C		756262	755555	Dubious open reading frame	755653	AG	A	Frameshift, p.Leu205fs	Hom. Ref	Het.
					755785	A	G	Missense,	Hom.	Hom.

								p.Ser160Pro	Alt.	Alt.
YDR151C	CTH1	765155	764178	Member of the CCCH zinc finger family	764191	CT	C	Frameshift, p.Arg322fs	Het.	Het.
YDR152W	GIR2	765706	766503	Highly-acidic RWD domain-containing protein of unknown function	766262	TC	T	Frameshift, p.His187fs	Het.	Hom. Ref.
YDR153C	ENT5	767971	766736	Protein containing an N-terminal epsin-like domain	767007	C	T	Missense, p.Ser322Asn	Hom. Alt.	Hom. Alt.
					767329	C	G	Missense, p.Asp215His	Het.	Hom. Ref.
					767443	A	G	Missense, p.Ser177Pro	Hom. Alt.	Hom. Alt.
YDR154C		768753	768403	Dubious open reading frame	768483	T	G	Missense, p.Ile91Leu	Het.	Hom. Ref.
YDR155C	CPR1	769000	768512	Cytoplasmic peptidyl-prolyl cis-trans isomerase (cyclophilin)	\	\	\	\	\	\
YDR156W	RPA14	769525	769938	RNA polymerase I subunit A14	\	\	\	\	\	\
YDR157W		769931	770332	Dubious open reading frame	\	\	\	\	\	\
YDR158W	HOM2	770357	771454	Aspartic beta semi-aldehyde dehydrogenase	770441	C	A	Missense, p.His29Asn	Het.	Hom. Ref.
YDR159W	SAC3	771877	775782	mRNA export factor	772436	CA	C	Frameshift, p.Lys188fs	Het.	Het.
					774076	T	G	Missense, p.Leu734Val	Het.	Hom. Alt.
					774133	G	GT	Frameshift, p.Glu753fs	Het.	Hom. Ref
					774251	C	T	Missense, p.Ser792Phe	Het.	Hom. Alt.
YDR160W	SSY1	776163	778721	Component of the SPS plasma membrane amino acid sensor system	\	\	\	\	\	\

YDR161W		779043	780206	Putative protein of unknown function	\	\	\	\	\	\
YDR162C	NBP2	781100	780390	Protein involved in the HOG (high osmolarity glycerol) pathway	\	\	\	\	\	\
YDR163W	CWC15	781423	781950	Non-essential protein involved in pre-mRNA splicing	781568	G	A	Missense, p.Arg49Lys	Het.	Hom. Ref.
					781624	A	G	Missense, p.Met68Val	Het.	Hom. Ref.
					781706	A	C	Missense, p.Gln95Pro	Het.	Hom. Ref.
YDR164C	SEC1	784215	782041	Sm-like protein involved in docking and fusion of exocytic vesicles	783519	G	A	Missense, p.Pro233Ser	Het.	Hom. Ref.
					783867	A	C	Missense, p.Ser117Ala	Het.	Hom. Ref.

Table S7 - Genome locations and significant genomic variation of genes found in QTL peak in chromosome 6. Ref. Allele, reference allele as in S288c reference sequence; Alt. Allele, alternative allele present in UMCC 855/2581 sequences. Het., heterozygous; Hom. Alt., homozygous alternative; Hom. Ref., homozygous reference. SNPs or InDels are considered heterozygous with the calculated allele frequency between 0.25 -0.75 and coverage greater than 20. Predicted effect describes the variant using HGVS notation (http://www.hgvs.org/mutnomen/).

Chromosome 6, Peak

Systematic gene name	Standard gene name	End position	Start position	Gene description	Genomic Variation					
					Position	Ref. Allele	Alt. Allele	Predicted effect	UMCC 855	UMCC 2581
YFL037W	TUB2	56336	57709	Beta-tubulin	56468	G	C	Missense, p.Glu45Gln	Het.	Hom. Ref.
					57672	A	G	Missense, p.Gln446Arg	Het.	Hom. Alt.
YFL036W	RPO41	58782	62837	Mitochondrial RNA polymerase	59556	T	A	Missense, p.Ser259Thr	Het.	Hom. Alt.
					60234	G	A	Missense, p.Gly485Ser	Hom. Alt.	Hom. Alt.
					60870	CT	C	Frameshift, p.Leu698fs	Het.	Het.
					61110	T	A	Missense, p.Ser777Thr	Hom. Alt.	Hom. Alt.
YFL034C-B	MOB2	63993	63016	Activator of Cbk1p kinase	63136	T	A	Missense, p.His248Leu	Het.	Hom. Ref.
					63199	T	G	Missense, p.His227Pro	Het.	Hom. Ref.
					63394	C	G	Missense, p.Arg162Pro	Hom. Alt.	Hom. Alt.
					63422	T	C	Missense, p.Ile153Val	Het.	Hom. Ref.
YFL034C-A	RPL22B	64932	64243	Ribosomal 60S subunit protein L22B	64475	G	A	Missense, p.Ala46Val	Het.	Hom. Ref.

YFL034W		65477	68698	Putative integral membrane protein that interacts with Rpp0p	65504	G	A	Missense, p.Val10Ile	Hom. Alt.	Hom. Alt.
					65754	G	T	Missense, p.Cys93Phe	Het.	Hom. Ref.
					65802	T	G	Missense, p.Ile109Ser	Hom. Alt.	Hom. Alt.
					66428	A	G	Missense, p.Ile318Val	Het.	Hom. Alt.
					66782	C	T	Missense, p.Pro436Ser	Hom. Alt.	Hom. Alt.
					67184	A	G	Missense, p.Ile570Val	Het.	Hom. Alt.
YFL033C	RIM15	74427	69115	Protein kinase involved in cell proliferation in response to nutrients	69214	T	G	Missense, p.Lys1738Asn	Het.	Hom. Ref.
					70239	G	C	Missense, p.Pro1397Ala	Het.	Hom. Alt.
					70295	G	A	Missense, p.Thr1378Ile	Hom. Alt.	Hom. Alt.
					70534	GT	G	Frameshift, p.Asn1298fs	Het.	Het.
					70707	C	T	Missense, p.Gly1241Ser	Het.	Hom. Ref.
					71231	GA	G	Frameshift, p.Ser1066fs	Hom. Ref.	Het.
					72216	G	A	Missense, p.Pro738Ser	Het.	Hom. Alt.
					72261	T	A	Missense, p.Thr723Ser	Hom. Alt.	Hom. Alt.
					72603	T	A	Missense, p.Thr609Ser	Hom. Alt.	Hom. Alt.
					72607	T	G	Missense, p.Glu607Asp	Hom. Alt.	Hom. Alt.
					72636	T	C	Missense, p.Ser598Gly	Het.	Hom. Ref.

	72652	G	T	Missense, p.Asn592Lys	Het.	Hom. Alt.
	72876	C	T	Missense, p.Glu518Lys	Het.	Hom. Ref.
	72919	G	C	Missense, p.His503Gln	Het.	Hom. Alt.
	73433	A	T	Missense, p.Leu332His	Het.	Hom. Ref.
	74036	C	T	Missense, p.Ser131Asn	Het.	Hom. Ref.

Table S8 - Genome locations and significant genomic variation of genes found in QTL peak in chromosome 12. Ref. Allele, reference allele as in S288c reference sequence; Alt. Allele, alternative allele present in UMCC 855/2581 sequences. Het., heterozygous; Hom. Alt., homozygous alternative; Hom. Ref., homozygous reference. SNPs or InDels are considered heterozygous with the calculated allele frequency between 0.25 -0.75 and coverage greater than 20. Predicted effect describes the variant using HGVS notation (http://www.hgvs.org/mutnomen/).

Chromosome 12, Peak

Systematic gene name	Standard gene name	End position	Start position	Gene description	Genomic Variation					
					Position	Ref. Allele	Alt. Allele	Predicted effect	UMCC 855	UMCC 2581
YLR007W	NSE1	164392	165402	Component of the SMC5-SMC6 complex	164546	A	G	Missense, p.Asn52Ser	Het.	Hom. Ref.
					164564	A	T	Missense, p.Gln58Leu	Het.	Hom. Ref.
					164617	A	T	Missense, p.Asn76Tyr	Het.	Hom. Ref.
					164865	G	T	Missense, p.Met158Ile	Het.	Hom. Ref.
YLR008C	PAM18	166083	165577	Subunit of the import motor (PAM complex)	\	\	\	\	\	\
YLR009W	RLP24	166537	167136	Essential protein required for ribosomal large subunit biogenesis	166651	G	A	Missense, p.Ala39Thr	Het.	Hom. Ref.
YLR010C	TEN1	167802	167320	Protein that regulates telomeric length	167601	AT	A	Frameshift, p.Met68fs	Het.	Het.
YLR011W	LOT6	169103	169678	FMN-dependent NAD(P)H:quinone reductase	169223	C	T	Stop gained, p.Gln41*	Het.	Hom. Ref.
YLR012C		170281	169982	Putative protein of unknown function	\	\	\	\	\	\
YLR013W	GAT3	171339	171764	Protein containing GATA family zinc finger motifs	\	\	\	\	\	\

YLR014C	PPR1	174982	172268	Zinc finger transcription factor	172947	AC	A	Frameshift, p.Val679fs	Hom. Ref.	Het.
					173892	A	T	Missense, p.Val364Glu	Hom. Alt.	Hom. Alt.
					174927	CT	C	Frameshift, p.Arg19fs	Het.	Het.
YLR015W	BRE2	175227	176744	Subunit of COMPASS (Set1C) complex	175450	T	C	Missense, p.Phe75Ser	Het.	Hom. Ref.

Table S9 - Genome locations and significant genomic variation of YAP1 gene. Ref. Allele, reference allele as in S288c reference sequence; Alt. Allele, alternative allele present in UMCC 855/2581 sequences. Het., heterozygous; Hom. Alt., homozygous alternative; Hom. Ref., homozygous reference. SNPs or InDels are considered heterozygous with the calculated allele frequency between 0.25 -0.75 and coverage greater than 20. Predicted effect describes the variant using HGVS notation (http://www.hgvs.org/mutnomen/).

Chromosome 13, YAP1

Systematic gene name	Standard gene name	End position	Start position	Gene description	Genomic Variation					
					Position	Ref. Allele	Alt. Allele	Predicted effect	UMCC 855	UMCC 2581
YML007W	YAP1	253848	255800	Basic leucine zipper (bZIP) transcription factor, required for oxidative stress tolerance	254293	A	T	Missense, p.His149Leu	Het.	Hom. Ref.
					254427	A	G	Missense, p.Asn194Asp	Hom. Alt	Hom. Alt
					255034	G	GTAGCACTGA	Disruptive inframe insertion, p.Asp399_Thr401dup	Het.	Het.

Perspectives

For the important contribution in stability and protection that GSH exerts on musts and wine, it has been the subject of an ever-growing interest in winemaking. However, the GSH content in these matrices is highly variable and depends on multiple factors, among which the *Saccharomyces cerevisiae* fermenting strains play a key role.

In this work we successfully applied an evolution-based strategy for the purpose to generate wine yeast strains with an enhanced GSH production. We obtained in particular two evolved strains, UMCC 2581 and UMCC 2585, able to enhance the GSH content in wine with an increase of 100% and 36%, respectively, compared to the parental strain UMCC 855. Furthermore, we found that UMCC 2581 was particularly able to produce GSH indifferently to the fermentative media used, making it the most suitable candidate for technological applications.

In order to identify the genetic changes underlying the improved phenotype of the evolved strains we exploited the potential of genomic DNA and transcriptome sequencing. We applied a three-step process for quantitative trait loci (QTL) mapping to identify genes and pathways responsible for the Mo(VI) resistance trait, and consequently for high GSH production. We identified four peaks within 11 candidate genes in QTL analysis and 296 genes differentially expressed between parental and UMCC 2581 evolved strain that result in a presumed additive phenotype effects. The impaired Yap1p transcription factor together with over-expression of amino acids permeases and biosynthetic enzymes are probably the reasons of the high GSH production phenotype observed.

Further investigations are needed to verify the correlation between each genomic variation on the candidate genes identified and the evolved phenotypes. A deeper insight on these aspects will allow a more rapid identification of new evolved strains that carry the alleles of interest through a marked-assisted selection strategy.

Overall, the evolution-based strategy has proven to be effective for selecting improved wine strains and it can be applied for a wide variety of desired traits with a significant probability of success as the evolved strains are quickly recognized through the expression of selectable phenotypes.

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Appendix 2: Papers, oral communications and posters

International Paper

FEMS YEAST RESEARCH

RESEARCH ARTICLE

Evolved *Saccharomyces cerevisiae* wine strains with enhanced glutathione production obtained by an evolution-based strategy

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Saccharomyces cerevisiae; evolutionary strategies; glutathione; molybdate resistance; wine strain.

Abstract

In winemaking, the application of glutathione (GSH) has been the subject of ever-growing interest because of its important role in limiting must and wine oxidation and in protecting various aromatic compounds. Glutathione concentration in wine is highly variable, involving as it does several factors from must, through alcoholic fermentation, to yeast strain activity. Consequently, the development of new wine yeast strains able to improve flavor stability is in great demand. To generate evolved *Saccharomyces cerevisiae* strains with enhanced GSH production, we have applied an evolution-based strategy that combines the sexual recombination of spores with the application of molybdate, which is toxic for the cells at high concentration, as specific selective pressure. Eight molybdate-resistant strains were selected and further screened for GSH production in synthetic grape must and in microvinification assay. By this nongenetically modified strategy, we obtained two evolved strains, Mo21T2-5 and Mo21T2-12, both able to enhance GSH content in wine with an increase of 100% and 36%, respectively, compared with the parental strain 21T2, and 120% and 50% compared with initial GSH content in the must.

Introduction

Glutathione (GSH) is an important antioxidant widely distributed in living cells (Sies, 1999). From a chemical point of view, GSH is a nonprotein thiol synthesized from L-glutamate, L-cysteine, and glycine, which can occur in the cell as the reduced form of GSH mostly (> 90%) and in the oxidized form as glutathione disulfide (GSSG) (Li *et al.*, 2004).

Glutathione has an essential role in several processes, including the control of redox potential, protection against oxidative stress, detoxification of endogenously and exogenously derived toxins, protein folding, and transport of organic sulfur (Meister & Anderson, 1983; Penninckx, 2002; Suzuki *et al.*, 2011).

Recently, the function of GSH in must and wine has received growing attention, primarily because of its ability to control oxidative spoilage damage and to limit the amount of browning pigments by competitive o-quinones reduction (Li *et al.*, 2008; Sonni *et al.*, 2011; Kritzinger *et al.*, 2013a). Furthermore, GSH avoids the formation of sotolon and other atypical aging characters (Dubourdieu & Lavigne, 2004; El Hosry *et al.*, 2009) as well as exerting a protective effect on various aromatic compounds in wine (Roussis *et al.*, 2007, 2009; Papadopoulou & Roussis, 2008).

Glutathione concentration in wine is highly variable and several factors are involved, from must to alcoholic fermentation. Different musts are characterized by different initial GSH concentrations, according to oxygen exposure, tyrosinase activity, and grape skin maceration during the prefermentation stage and pressing. The evolution of GSH during alcoholic fermentation can either increase or decrease depending on the initial GSH content and the yeast strain activity (Park *et al.*, 2000; Lavigne *et al.*, 2007; Fracassetti, 2010; Kritzinger *et al.*, 2013b). It has been postulated that *Saccharomyces cerevisiae* could possibly alter the GSH content in wines by utilizing and secreting GSH during fermentation and that differences between strains in the quantities assimilated or secreted may result in various GSH levels in wine (Kritzinger *et al.*, 2013a).

Although GSH has been widely used in medicine, as a food additive, and in the cosmetic industry (Yoshida *et al.*, 2002; Wei *et al.*, 2003), the addition of a commercial food-grade preparation of GSH to wine prior to bottling is not allowed by current wine regulation (Ugliano *et al.*, 2011). Nevertheless, inactive dry yeast (IDY) preparations are commonly used within the enological industry even though both the precise amount of GSH released from IDY and available in the wine, and the required optimum dose of these preparations to achieve a positive effect in preserving wines from oxidation are unclear (Ángeles Pozo-Bayón *et al.*, 2009; Kritzinger *et al.*, 2013a). The use of active yeast strains able to increase the GSH content in wine would be more worthwhile; indeed, different strategies applied to obtain an enhanced GSH production have been recently reported (Nisamedtinov *et al.*, 2011). Some of these strategies are based on the modulation of precursor amino acids, in particular cysteine, to overcome substrate limitation (Alfafara *et al.*, 1992; Wen *et al.*, 2006; Wang *et al.*, 2007; Liang *et al.*, 2008). Other strategies consist in the development of genetically engineered strains overexpressing GSH biosynthetic enzymes, or the key enzymes in sulfur assimilation pathways, to increase cysteine biosynthesis (Grant *et al.*, 1997; Hara *et al.*, 2012). In addition, several mutation strategies, based on physical or chemical treatments, have been described in the literature relating to the isolation of GSH over-accumulating variants (Lai *et al.*, 2008; Chen *et al.*, 2012).

Even though recombinant DNA techniques are widely used in biotechnological production of GSH (Li *et al.*, 2004; Hao *et al.*, 2012; Hara *et al.*, 2012), the use of genetically modified (GM) yeast strains for winemaking does not seem to be particularly appreciated by consumers and legal restrictions limit their use in foods and beverages in many parts of the world (Grossmann *et al.*, 2011). On the other hand, wine strains obtained by adaptive evolutionary approaches have high public acceptance (Çakar *et al.*, 2005; Kutyna *et al.*, 2010). Approaches that simply mimic nature by random mutation or genetic recombination of the microorganisms, followed by selection under conditions suitable to the desired phenotype, have been extensively applied to the generation of wine yeast with improved enological properties (Kuyper *et al.*, 2005; Stanley *et al.*, 2010; Cadière *et al.*, 2011; Çakar *et al.*, 2012; Kutyna *et al.*, 2012). In our evolution-based strategy, recently proposed, we have exploited the resistance to chromate Cr(VI) and molybdate Mo(VI) (toxic sulfate analogs), as selectable phenotypes to rapidly select *S. cerevisiae* strains impaired in the sulfate assimilation pathway (De Vero *et al.*, 2011). These toxic metals enter a yeast cell through Sul1p and Sul2p high-affinity sulfate permeases (Fig. 1); mutation in these permeases is one of the most important events, conferring resistance to sulfate toxic analogs (Cherest *et al.*, 1997; Tamás *et al.*, 2006; Wysocki & Tamás, 2010). However, other mechanisms could be involved, in fact, another important function of the sulfate assimilation pathway is the GSH biosynthesis, which has an essential role in the defense against oxidative stress and metal toxicity (Grant, 2001; Wysocki & Tamás, 2010). In particular, GSH biosynthesis in *S. cerevisiae* takes place in two ATP-dependent steps. In the first, cysteine is linked with glutamate by γ-glutamylcysteine synthetase (encoded by *GSH1*) to form γ-glutamylcysteine. In the second step, glycine is added to this intermediate product by glutathione synthetase (encoded by *GSH2*) to form the final product (Li *et al.*, 2004; Zechmann *et al.*, 2011). In scientific literature, it has been reported that GSH is able to chelate heavy metals by forming complexes (metal-GSH complex) that are actively transported into the vacuole or removed from the cell by specific transporters such as Ycf1p and Gex1p (Li *et al.*, 1996; Mendoza-Cózatl *et al.*, 2005; Paumi *et al.*, 2009; Dhaoui *et al.*, 2011).

The aim of this work was to generate evolved *S. cerevisiae* strains with enhanced GSH production. To achieve this, we applied our evolution-based strategy that combines sexual recombination and the application of molybdate as specific selective pressure. The molybdate-resistant strains selected were further screened for GSH production in synthetic grape must and in microvinification assay.

Materials and methods

Yeast strains and growth conditions

The *S. cerevisiae* 21T2 parental strain, previously selected in our laboratory (De Vero & Giudici, 2011), was used to obtain the evolved strains in this work. The yeast strains were routinely cultured on YPD agar plates or slants (2% glucose, 2% peptone, 1% yeast extract, and 2% agar), incubated at 27 °C for 48 h, and stored at 4 °C. For long-term preservation, all the strains were maintained in the University of Modena and Reggio Emilia (Unimore) Microbial Culture Collection (UMCC), Reggio Emilia, Italy, stored at −80 °C in cryovials supplemented with glycerol (final concentration 25%).

Evolution-based strategy and screening of molybdate-resistant strains

To obtain strains improved in GSH production, the evolution-based strategy previously described by De Vero *et al.* (2011) was applied to the parental strain 21T2 with few modifications. This strain was induced to sporulate on acetate medium (0.5% sodium acetate and 2% agar).

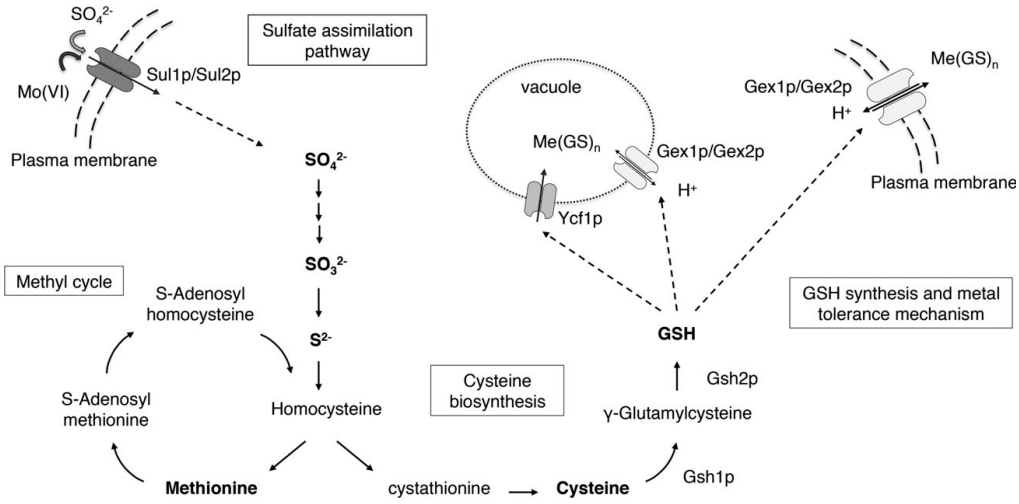

Fig. 1. A schematic model for sulfur metabolism, glutathione synthesis, and GSH-mediated metal tolerance mechanism in *Saccharomyces cerevisiae*. The sulfur metabolism can be divided into three parts: the sulfate assimilation pathway, the methyl cycle, and the cysteine biosynthesis (Tamás *et al.*, 2006). Glutathione is synthesized in two consecutive reactions and GSH free or conjugate with metal are transported into the vacuole or out of the cell. Sul1p/Sul2p: sulfate transporters; Gsh1p: γ-glutamylcysteine synthetase; Gsh2p: glutathione synthetase; Gex1p/Gex2p: yeast glutathione exchangers; Ycf1p: vacuolar glutathione S-conjugates pump; $Me(GS)_n$: Metal–GSH complex.

After tetrad formation, asci and cells were digested in 0.5 mL distilled water containing 0.5 mg mL^{-1} of Zymoliase 20T (Seikagaku Corp., Tokyo, Japan). The spores, recovered by centrifuging, were resuspended in 0.5 mL YPD and periodically monitored under a microscope to check the restoration of diploid state by the conjugation of gametes. After that, 10 μL of this suspension was inoculated in 50 tubes containing YPD medium supplemented with 10 mM ammonium molybdate (Carlo Erba, Rodano, Italy). The high Mo(VI) concentration was chosen to lead to significant growth inhibition within 10 days of incubation at 27 °C in YPD. From each tube where cell growth was observed, 100 μL of culture was withdrawn and plated on the YPD agar with Mo(VI) 10 mM. Then, from each plate, a single colony was re-isolated and purified. The resistant strains selected were subsequently screened on YNB minimal medium plates (0.17% yeast nitrogen base w/o amino acids and ammonium sulfate, DIFCO, Detroit, MI), supplemented with 2% agar, 2% glucose, 100 μM ammonium sulfate (Sigma-Aldrich, St. Louis, MO), and different concentrations of molybdate, resulting in a medium (pH 5) containing either 0, 1.0, 2.5 or 5.0 mM Mo(VI). Strain precultures were obtained in tubes with 5 mL YNB after 24 h at 27 °C. From each tube, 5 μL of 1×10^6 cells mL^{-1} and fivefold dilution series were spotted on the plates. Colony growth was observed after 4 days of incubation at 27 °C.

Evaluation of glutathione production in synthetic grape juice

The total GSH (GSH + GSSG) produced by the parental and selected strains was determined with the enzymatic Glutathione Assay Kit (Sigma-Aldrich) following the manufacturer's instructions. The strains were precultured, for 24 h at 27 °C, in 5 mL synthetic grape juice prepared according to Giudici & Kunkee (1994), with complete YNB containing amino acids and ammonium sulfate. The cells were then inoculated at an optical density of 0.05 at 600 nm ($OD_{600\ nm}$) into Erlenmayer flasks, each containing 50 mL synthetic medium, and incubated at 27 °C under shaking conditions (150 r.p.m.). The cells grown until the exponential phase ($OD_{600\ nm}$ of 4) were harvested and centrifuged at 1000 ***g*** for 5 min at 4 °C. For each sample, the supernatant and the pellet were processed, separately, to analyze the extracellular and intracellular GSH, respectively. The supernatant was concentrated by freeze-drying equipment (FREEZONE 1L Labconco, Kansas City, MO) before using the Glutathione Assay Kit. The extracellular GSH concentration was expressed as mg GSH L^{-1}.

The pellet was washed three times with ice-cold distilled water, and the intracellular GSH was extracted with 100 μL ethanol 25% according to Xiong *et al.* (2009). After 1 h of incubation at room temperature in a rotary

shaker, each sample was centrifuged at 18 000 ***g*** for 10 min at 4 °C. Then 25 μL 5-sulfosalicylic acid 5% was added to the recovered supernatant, mixed, and incubated for 10 min at 4 °C. Finally, after centrifugation at 8000 ***g*** for 10 min at 4 °C, the supernatant was recovered and transferred to a fresh tube; GSH was then detected with the enzymatic kit. Intracellular GSH content was expressed as a percentage (%) of GSH concentration (mg L^{-1}) divided by dry cell weight (DCW) (mg L^{-1} at $OD_{600\ nm}$ of 4). DCW was measured after washing the cell pellet twice with distilled water and drying the wet cells at 105 °C for 4 h.

Microvinification assay and analytical methods

Microvinification assay was carried out under static conditions at 25 °C in 250-mL sterile flasks filled with 200 mL pasteurized Trebbiano grape juice (pH 3.44, titratable acidity 8.68 g L^{-1}, reducing sugars 166.59 g L^{-1}) without added sulfites. All yeast strains were precultured in 5 mL synthetic grape juice for 24 h at 27 °C and inoculated in Trebbiano grape juice at a final concentration of 1×10^6 cells mL^{-1}. Flasks were sealed with cotton plugs, and the fermentation process was evaluated daily by the weight loss due to the evolved CO_2. At the end of the fermentation (constant weight for three consecutive days), the samples were refrigerated at 4 °C or racked and stored at −20 °C until required for chemical analysis. Titratable acidity and pH were measured using TitroLine easy titrator (Schott Instruments, Mainz, Germany). Reducing sugars were determined by the Fehling method. Enzymatic kits (Megazyme, Bray, Ireland) were used to quantify the concentrations of ethanol (K-ETOH) and total and free SO_2 (K-SULPH) according to the manufacturer's instructions.

The fermentation efficiency was expressed as a percentage (%), calculated from the formula $[(Y_{Eth}/Y_{Eth\ Theor}) \times 100]$, where Y_{Eth} indicates the detected ethanol yield, and $Y_{Eth\ Theor}$ indicates the theoretical ethanol yield as determined by the Gay–Lussac equation.

Glutathione concentration was determined in each wine sample, as previously described on the basis of extracellular GSH analysis.

Evaluation of hydrogen sulfide production

Hydrogen sulfide (H_2S) production by the yeast strains was qualitatively evaluated on bismuth–glucose–glycine–yeast (BiGGY) agar medium (Oxoid Co., Hampshire, UK). Five microlitre of each strain cultured in YPD medium until $OD_{600\ nm}$ of 1 was spotted on BiGGY agar and incubated for 4 days at 27 °C. On this differential medium, the colony color turns dark, brown, or remains white, depending on the amount of sulfide produced by the yeast. The qualitative amount of H_2S formed during microvinification assay was detected by lead acetate paper strips soaked with 50 μL of 5% lead acetate solution. Throughout the fermentation process, the strips were attached to the top of each flask, away from the liquid. The degree of blackening of the strips is correlated with the amount of H_2S produced during fermentation (Giudici & Kunkee, 1994).

Statistical analysis

Statistical analysis was carried out by analysis of variance (ANOVA) using GRAPHPAD PRISM v. 6.0 (GraphPad Software Inc., San Diego, CA). Tukey's multiple comparison test was used to identify significant differences between strains ($P < 0.05$). All experiments were performed in triplicate.

Result

Selection of molybdate-resistant strains

We used the *S. cerevisiae* 21T2 strain to obtain Mo(VI)-resistant strains by the evolution-based strategy described above. This parental strain, selected in our laboratory, was chosen on the basis of its high percentage of asci conversion and its specific enological aptitude (De Vero & Giudici, 2011; Gobbi *et al.*, 2014).

The 21T2 strain was induced to sporulate then, after sexual conjugation of gametes, a cell suspension was inoculated in tubes containing YPD medium with Mo(VI) as specific selective pressure. Fifteen evolved strains, codified as Mo21T2 followed by a sequential number (from 1 to 15), were obtained in the preliminary screening. The Mo (VI) resistance of these strains was further assessed on YNB minimal medium plates with a low sulfate concentration (100 μM) and different Mo(VI) concentrations (Fig. 2).

The screening on minimal YNB medium allowed for a more stringent selection compared with that made on YPD; in fact, although the sulfate concentration used (100 μM) was not limiting for yeast growth, as observed in the control plates, on YNB, there was a marked growth inhibition when Mo(VI) was supplied. This was in alignment with the experiments of Pereira *et al.* (2008) who investigated chromate toxicity by spotting yeast cells on YNB plates containing different concentration of Cr(VI) and sulfur source. He found that chromate toxicity increased at low sulfur concentration (100 μM) and that it was strongly dependent on the sulfur source and its concentration. In our screening, we observed that the low Mo(VI) concentration (1 mM) was not limiting for either the parental strain or the fifteen evolved strains previously

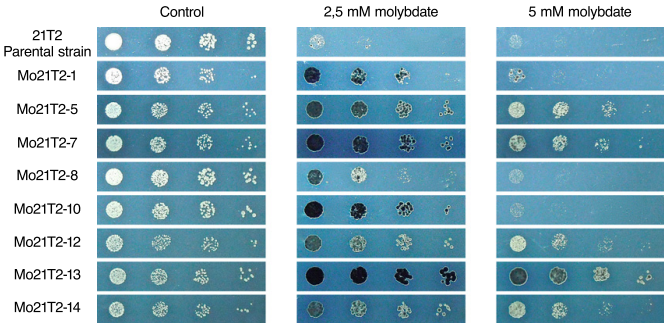

Fig. 2. Molybdate resistance screening of the parental and evolved strains. Fivefold dilution series of the strains, starting from 1×10^6 cell mL^{-1} on the left to the right, were spotted on YNB plates supplemented with 2% glucose, 100 μM of ammonium sulfate, and Mo(VI) at the concentrations of 0 (control plates), 2.5, and 5.0 mM as indicated. Plates were photographed after 4 days of incubation at 27 °C.

Table 1. *Saccharomyces cerevisiae* parental strain and evolved strains obtained by evolution-based strategy

Strain	Description	UMCC code	Reference
21T2	Laboratory yeast strain	UMCC 855	De Vero & Giudici (2011)
Mo21T2-1*	Molybdate-resistant strain	UMCC 2580	This study
Mo21T2-5*	Molybdate-resistant strain	UMCC 2581	This study
Mo21T2-7*	Molybdate-resistant strain	UMCC 2582	This study
Mo21T2-8*	Molybdate-resistant strain	UMCC 2583	This study
Mo21T2-10*	Molybdate-resistant strain	UMCC 2584	This study
Mo21T2-12*	Molybdate-resistant strain	UMCC 2585	This study
Mo21T2-13*	Molybdate-resistant strain	UMCC 2586	This study
Mo21T2-14*	Molybdate-resistant strain	UMCC 2587	This study

In bold characters is the *S. cerevisiae* parental strain.
UMCC – Unimore Microbial Culture Collection, University of Modena and Reggio Emilia, Reggio Emilia, Italy.
*Evolved strains generated from 21T2.

selected on YPD (data not shown). However, in the plates with Mo(VI) 2.5 mM, there was strong inhibition and only eight evolved strains were able to grow (Fig. 2).

The Mo(VI) concentration of 5 mM was limiting for almost all the strains, with the exclusion of Mo21T2-13, especially when the lowest cell concentration (8×10^3 cells) was spotted on YNB. The Mo(VI)-resistant strains, selected on the basis of this screening, are listed in Table 1. These evolved strains were further investigated for their ability to produce GSH and for their enological aptitude.

Intra- and extracellular GSH production in synthetic grape juice

The native capability of the parental 21T2 and the eight evolved strains to produce GSH was preliminarily evaluated in a defined synthetic grape juice. The enzymatic determination of intra- and extracellular total GSH was assessed in culture samples harvested at the exponential phase.

The highest values of intracellular GSH content in synthetic grape juice were observed for the strains Mo21T2-1 (0.35%) and Mo21T2-12 (0.44%), which were, respectively, 2.1-fold and 2.6-fold higher than those of the strain 21T2 (0.17%) (Fig. 3a).

The other evolved strains gave a lower content of GSH ranging from 0.13% to 0.26% with differences not statistically significant compared with the parental strain.

Extracellular GSH analysis in synthetic medium made it possible to point out a great difference among almost all the evolved strains and 21T2 (Fig. 3b). For the latter, in particular, it was not possible to detect the GSH value as it did not reach the detection limit (0.96 μg mL^{-1}) of the enzymatic kit used. The same condition was observed for Mo21T2-13. The highest extracellular GSH amounts were obtained with the strains Mo21T2-5 (0.65 mg L^{-1}), Mo21T2-10 (0.65 mg L^{-1}), and Mo21T2-14 (0.63 mg L^{-1}). The remaining strains tested showed extracellular GSH concentrations ranging from 0.32 to 0.46 mg L^{-1} with not statistically significant differences.

Microvinification assay: GSH production and analysis of enological traits

The microvinification assay was performed in Trebbiano white must, inoculated with the parental and the evolved strains, to evaluate their ability to produce extracellular GSH in wine and to assess their enological aptitude. The must used for the laboratory-scale fermentation showed an initial GSH amount of 2.5 mg L^{-1}. At the end of the fermentation process, the GSH concentration was detected in all the wine samples that had been fermented with the strains tested (Fig. 4).

Compared with the GSH amount of the parental strain (2.75 mg L^{-1}), Mo21T2-5 produced a double amount of GSH (5.5 mg L^{-1}), while Mo21T2-12 showed GSH increasing by 0.36-fold (3.75 mg L^{-1}). On the other hand, Mo21T2-7, Mo21T2-8, Mo21T2-10, and Mo21T2-14 strains displayed a lower GSH production in wine

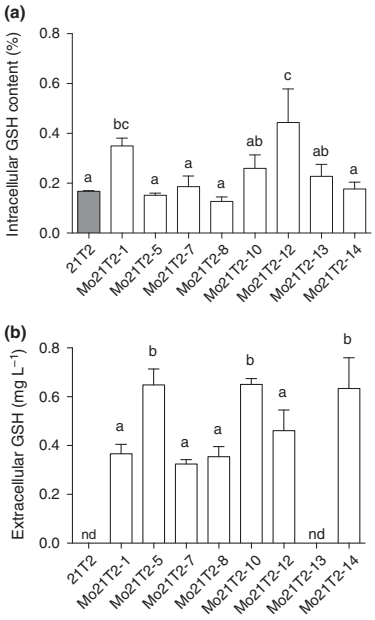

Fig. 3. Intracellular and extracellular total glutathione concentration in synthetic grape juice. (a) Intracellular GSH content (%) is expressed as GSH concentration divided by DCW × 100. (b) Extracellular GSH concentration is expressed as mg L^{-1}. nd = not detected. Graph shows means with SD (n = 3) of 21T2 parental strain (gray column) and evolved strains (white columns). Means with different letters are significantly different from each other as determined by Tukey's test ($P < 0.05$).

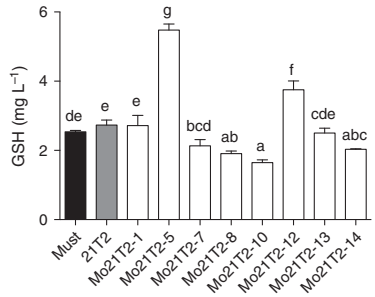

Fig. 4. Extracellular total glutathione concentration after microvinification. Graph shows means with SD (n = 3) of must (black column), 21T2 parental strain (gray column), and evolved strains (white columns). Means with different letters are significantly different from each other as determined by Tukey's test ($P < 0.05$).

compared with 21T2 and, apart from Mo21T2-7, showed a significantly lower amount of GSH also compared with the initial content in the must. For Mo21T2-10, in particular, the total resultant GSH was 0.35-fold less than the amount in the must.

Table 2. Main enological traits of *Saccharomyces cerevisiae* parental and evolved strains

Strains	pH	Titratable acidity (g L^{-1} tartaric acid)	Ethanol yield (% v/v)	Fermentation efficiency (%)
21T2	3.37^{abc}	9.64^{bcd}	11.18^{bc}	88.34^{bc}
Mo21T2-1	3.31^{a}	9.89^{cde}	11.08^{bc}	87.59^{bc}
Mo21T2-5	3.35^{ab}	9.91^{de}	11.40^{c}	90.09^{c}
Mo21T2-7	3.39^{abc}	9.24^{ab}	11.06^{bc}	87.38^{bc}
Mo21T2-8	3.43^{bc}	9.65^{cd}	10.99^{bc}	86.85^{bc}
Mo21T2-10	3.44^{c}	8.87^{a}	11.27^{c}	89.04^{c}
Mo21T2-12	3.40^{bc}	9.51^{bc}	10.67^{ab}	84.33^{ab}
Mo21T2-13	3.39^{bc}	10.21^{e}	10.35^{a}	81.81^{a}
Mo21T2-14	3.41^{bc}	10.14^{e}	10.61^{ab}	83.86^{ab}

In bold characters is the 21T2 parental strain. Data are mean of three independent experiments. Standard deviation (SD) did not exceed 3% for any of the values. Means with different letters are significantly different from each other as determined by Tukey's test ($P < 0.05$). The titratable acidity was measured at the pH end-point of 7.2. Fermentation efficiency is expressed as $[(Y_{Eth}\ Y_{Eth\ Theor}{}^{-1}) \times 100]$, where Y_{Eth} indicates the detected ethanol yield and $Y_{Eth\ Theor}$ the theoretical ethanol yield.

All the evolved strains detected in the laboratory-scale fermentation showed notable fermentative aptitudes, comparable to those of the parental strain. After 25 days, all fermentations reached dryness with < 2 g L^{-1} of reducing sugars (data not shown), indicating the completed fermentation of the must with all the strains used. The enological traits detected in the microvinification assay, including pH, titratable acidity, ethanol production and fermentation efficiency, are reported in Table 2.

The pH analysis displayed a range of values from 3.31 to 3.44 with no significant differences between parental and evolved strains. Titratable acidity analysis (expressed as g L^{-1} of tartaric acid) showed a wider variability with values ranging from 8.87 to 10.21 g L^{-1} even if only the strains Mo21T2-10, Mo21T2-13, and Mo21T2-14 were significantly different from 21T2 (9.64 g L^{-1}). The fermentation efficiency observed was close to 90% with no significant differences compared with 21T2, except for Mo21T2-13 (81.81%).

The total SO_2 analysis showed that all strains, parental and evolved, produced almost nil amounts of sulfites, with the exceptions of Mo21T2-5 and Mo21T2-12 that produced 33.3 and 43.1 mg L^{-1}, respectively (Fig. 5). Interestingly, Mo21T2-5 and Mo21T2-12 were the highest GSH-producing strains in wine. Nevertheless, all strains showed an almost zero amount of free SO_2 (< 2 mg L^{-1}) with no statistically significant difference.

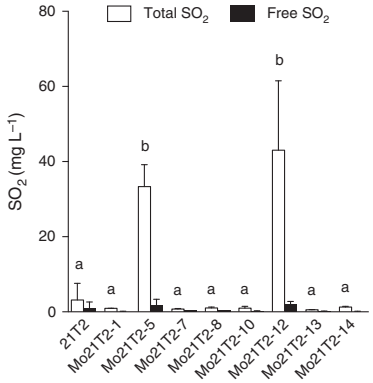

Fig. 5. Total and free SO_2 detected after microvinification. Graph shows means with SD ($n = 3$). Means with different letters are significantly different from each other as determined by Tukey's test ($P < 0.05$). No significant differences were found among all free SO_2 values.

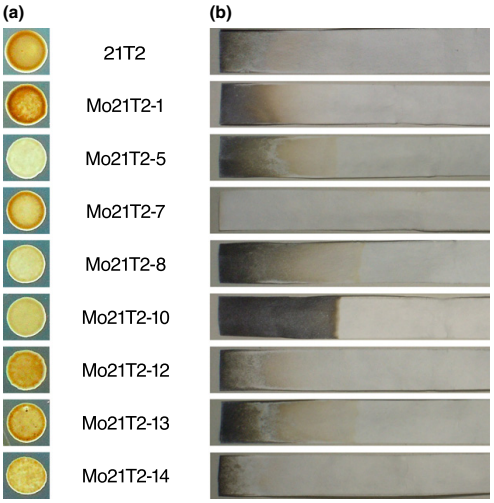

Fig. 6. Qualitative evaluation of hydrogen sulfide on BiGGY agar and on lead acetate strips. (a) The colony color of parental and evolved strains was observed after 4 days incubation. BiGGY agar assay was performed in triplicate. (b) Blackening degree of lead acetate strips after microvinification assay. It is showed one representative replicate of three independent experiments.

Evaluation of H_2S production in BiGGY agar and wine

Hydrogen sulfide formation by parental and evolved strains was qualitatively evaluated on BiGGY agar and on lead acetate strips (Fig. 6a and b). The spotted strains exhibited four different colony color types from white to brown. In particular, we observed white colonies for Mo21T2-5, Mo21T2-8, and Mo21T2-10, light tan colonies for Mo21T2-14, tan colonies for the parental and Mo21T2-7, Mo21T2-12, Mo21T2-13 strains, and brown colonies for Mo21T2-1.

A qualitative measurement of the H_2S produced during fermentation was achieved by the extent of blackening of the lead acetate strips (Fig. 6b). In some cases, we encountered a moderate variability within the three replicates performed; therefore, only one representative replicate was considered. The 21T2, Mo21T2-7, Mo21T2-12 and Mo21T2-14 strains appeared to be nil or low producers of H_2S, with a strip blackening ranging from 0 to 1 cm. Four strains, Mo21T2-1, Mo21T2-5, Mo21T2-8, Mo21T2-13, were moderate producers (ranging from 1 to 2 cm). Mo21T2-10 was the highest H_2S-producer, as it blackened the strip completely (3 cm).

Interestingly, it was observed that in some cases, there was no correlation between colony color and H_2S formation during fermentation; however, this discrepancy has been already reported by other authors (Spiropoulos *et al.*, 2000; Kumar *et al.*, 2010). In particular, the Mo21T2-5, Mo21T2-8, Mo21T2-10 strains gave white colonies on BiGGY agar, although they resulted from moderate or high H_2S producers during microvinification. On the other hand, the Mo21T2-7 strain showed tan colonies on the BiGGY medium but was the lowest H_2S-producing strain in wine.

Discussion

In this study, we used the *S. cerevisiae* 21T2 strain, previously screened by our laboratory and appreciated for its excellent fermentative and enological ability, to obtain evolved strains improved for GSH production. To achieve this aim, we applied an evolution-based strategy that combines the random hybridization of spores and the application of Mo(VI) as specific selective pressure. Particularly, the resistance to this analog of sulfate, toxic for the cells at high concentrations (Millson *et al.*, 2009), was performed as a rapid and high-throughput screening method for strains with an increased GSH production. In addition, we exploited the genome plasticity of wine yeasts, which are often diploid, heterozygous and homothallic, with a large capacity for genome reorganization through chromosome rearrangements (Sipiczki *et al.*, 2004; Borneman *et al.*, 2011). The eight evolved strains selected showed a wide variability of the phenotype, particularly regarding the production of sulfites, H_2S, and GSH. This confirms the effectiveness of meiosis in

providing clones with different and frequently better properties than their parental strain, in accordance with the findings of several authors (Pretorius, 2000; Marullo *et al.*, 2004; Giudici *et al.*, 2005).

The assessment of intra- and extracellular GSH in synthetic grape juice underlined the variation of the native capability of GSH production in evolved strains. Above all, we obtained two high intracellular GSH-producing strains, Mo21T2-1 and Mo21T2-12, compared with 21T2. Furthermore, all the evolved strains were able to produce extracellular GSH, the only exception being the Mo21T2-13 strain. The highest extra GSH-producing strains were Mo21T2-5, Mo21T2-10 and Mo21T2-14. These data are particularly notable if we consider that, as reported by Perrone *et al.* (2005), GSH excretion starts at a distinct growth stage, during the end of the exponential phase when the intracellular GSH level has reached a maximum. However, it may be that certain mutations give rise to an altered GSH metabolism with an increased excretion. The genetic characterization of the evolved strains, in particular regarding the expression of genes involved in the GSH metabolism, could be helpful in understanding their different behavior on a molecular level. Even though much work has been done to increase GSH production in *S. cerevisiae* for biotechnological purposes, only one attempt, to our knowledge, has been made with the *S. cerevisiae* wine strain (Hao *et al.*, 2012). Hao *et al.* constructed a recombinant *S. cerevisiae* strain by self-cloning *GSH1* genes and increasing GSH production by 19% in model wine. Approaching from a different direction, we did not use the recombinant DNA technique; instead, we employed an evolution-based strategy more suitable for complex enological traits. It is remarkable that by this strategy, we obtained two evolved strains, Mo21T2-5 and Mo21T2-12, able to enhance GSH content at the end of the fermentation process with an increase of 100% and 36%, respectively, compared with the parental strain, and 120% and 50% compared with initial GSH content in the must.

The reasons for the variation in final GSH levels in different fermentation media remain unexplained, but, as also reported by Kritzinger *et al.* (2013a), they are probably linked to grape juice complexity. However, comparing the GSH assay results obtained in synthetic grape juice and in microvinification, it was remarkable that Mo21T2-5 was the highest GSH-producing strain in wine and one of the best extracellular GSH-producing strains in a synthetic medium. Regarding the Mo21T2-12 strain, which improved the content of GSH in wine by up to 0.36-fold compared with the parental strain, it also showed a high intracellular GSH production in addition to good GSH excretion in a synthetic medium. Overall, our data seem to point to the importance of a homeostasis shifted toward extracellular excretion to obtain wine with an increased GSH content. In the case of Mo21T2-5, the Gex1p glutathione exchanger might play an important role in this shifted homeostasis, according to the behavior of Gex1p-overproducing strains described by Dhaoui *et al.* (2011). Moreover, overproduction of GSH may be favored by the release of γ-glutamylcysteine synthetase (Gsh1p) from feedback inhibition by GSH at low intracellular concentrations (Richman & Meister, 1975; Nisamedtinov *et al.*, 2011).

Regarding sulfite and hydrogen sulfide formation during must fermentation, we also observed that the two highest GSH-producing strains (Mo21T2-5 and Mo21T2-12) were at the same time moderate SO_2-producing strains ($SO_2 < 45$ mg L^{-1}). These strains were also moderate H_2S producers in must fermentation; therefore, we could assume that a fully active sulfate assimilation pathway was involved in the increased GSH metabolism. On the other hand, GSH may also be a source of hydrogen sulfide (Swiegers *et al.*, 2005), following the degradation of cysteine by the cysteine desulfhydrase enzyme to form H_2S. This could be consistent with the Mo21T2-5 screening on the BiGGY medium, where the colony color of the strain was completely white.

In summary, the evolution-based strategy applied in this work was successful for the purpose of generating yeast strains with enhanced GSH production in wine. This strategy, unlike the standard evolutionary approaches, has the advantage of not requiring multiple rounds of screening and extensive cultivation periods because the evolved strains are recognized through a selectable phenotype. In particular, Mo(VI) resistance has proved to be effective for the selection of the desired evolved strains, probably by activating the yeast common metal response that involves sulfur assimilation and GSH biosynthesis.

Acknowledgement

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>> **SPECIALE/** BIOTECNOLOGIE

LIEVITI ENOLOGICI, QUESTIONE DI DESIGN

di Paolo Giudici, Tommaso Bonciani, Alexandra Verspohl, Francesco Mezzetti, Lisa Solieri, Luciana De Vero*.

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In cantina, per scongiurare il pericolo di "stallo" si cercano venti di cambiamento, senza la consapevolezza della rotta da prendere. Così la progettazione tecnologica di ceppi altamente performanti deve essere orientata ai gusti dei consumatori, alle esigenze dei produttori e alle strategie di mercato

L'uso di lieviti selezionati nell'enologia italiana moderna è una pratica consolidata da diverso tempo, tanto da ritenerne qualsiasi perorazione del tutto inutile e marginale. Tuttavia, le caratteristiche o tratti dei ceppi di lievito da impiegare nell'enologia odierna sono oggetto di ricerca e innovazione continua: in particolare, le mutate condizioni commerciali ed enologiche delineano i tratti e su di essi si improntano le strategie idonee all'ottenimento dei nuovi ceppi altamente performanti.

Creare valore aggiunto

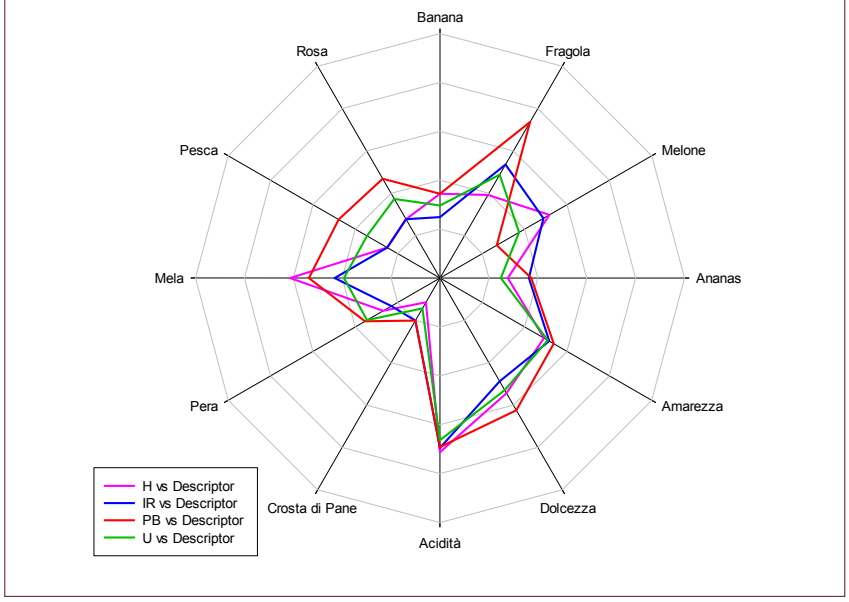
Ai lieviti odierni, oltre alle caratteristiche già delineate e riportate in precedenti pubblicazioni (Giudici et al 2005, 2006; Fleet 2008), sono richiesti tratti nuovi ma non ancora completamente palesati: in breve, si avverte la necessità di qualcosa di diverso e nuovo senza avere la piena consapevolezza di cosa sia necessario e possibile ottenere. In un recente incontro pubblico (con operatori del settore ed enologi) la domanda su che caratteristiche dovesse possedere un lievito ha dato seguito a molteplici risposte, tutte riguardanti tratti ben noti e consolidati o ambiziosamente olistiche, fatto esempio il super-ceppo che fermenta in tutte le condizioni e con risultati sensoriali eccezionali. A rigore scientifico ed intellettuale, un ceppo vigoroso, rustico e che non produca profumi anomali ed alte acidità volatili è già di per se un ottimo ceppo; le restanti caratteristiche sono elementi aggiuntivi che, spesso, possono essere esaltate o ridotte in relazione a parametri tecnologici come composizione del mosto, nutrizione e condizioni di processo. Il primo aspetto che emerge è, dunque, che le caratteristiche più sofisticate o di sintonizzazione raffinata dei ceppi di lievito non possono essere disgiunte dalle condizioni nelle quali i ceppi andranno ad operare, per cui ha sempre più importanza la progettazione o, per usare un termine inglese di moda, *design* del lievito in funzione di ciò che si vuole ottenere nelle specifiche condizioni di esercizio.

Limiti normativi

Il secondo aspetto da sviluppare sono le tecniche praticabili per ottenere i ceppi con i fenotipi desiderati (FD). Il settore enologico nazionale non

>> Prova di degustazione di vini organizzata dal gruppo UMCC (Unimore Microbial Culture Collection).

>> Fig.1 - Plot di analisi sensoriale su 12 descrittori in un test di rifermentazione in bottiglia con 4 ceppi di lievito: PB2033 (rosso), IperR (blu), IperR x PB2033_8 (viola), UMCC2575 (verde)



consente, sia per ragioni legali che commerciali, l'impiego di tecniche di modificazione genetica (GM) e il conseguente impiego di starters OGM. Per la legislazione Europea (Dir. 2001/18/EC) sono da considerarsi tecniche di modificazione genetica tutte quelle che alterano il materiale genetico in maniera diversa da quella naturale, che avviene tramite tecniche di mutagenesi o sfruttando la ricombinazione genetica e il ciclo sessuale di *Saccharomyces cerevisiae*: ciò consente, per assurdo, tecniche che inducono un alto tasso di mutazione come le radiazioni e i composti chimici mutageni. Tenendo conto delle limitazioni legislative ed anche della percezione negativa che il consumatore ha per alcune pratiche anche non GM, le tecniche di miglioramento genetico per l'ottenimento dei FD si riducono alla selezione clonale, alla costituzione di ibridi e alla mimica delle selezione naturale.

La fase commerciale

Il terzo aspetto da considerare sono le strategie di mercato necessarie, compatibili ed utili per l'affermazione del prodotto. Un buon ceppo di lievito deve, in primo luogo, essere prodotto come preparato commerciale e venduto ai produttori di vino e, solo in un secondo momento, il vino prodotto sarà oggetto di vendita e di scelta da parte dei consumatori. In breve, il primo e fondamentale attributo di un buon ceppo di lievito è quello di essere traducibile in un preparato commerciale in grado di soddisfare i desiderata degli enologi e dei cantinieri.

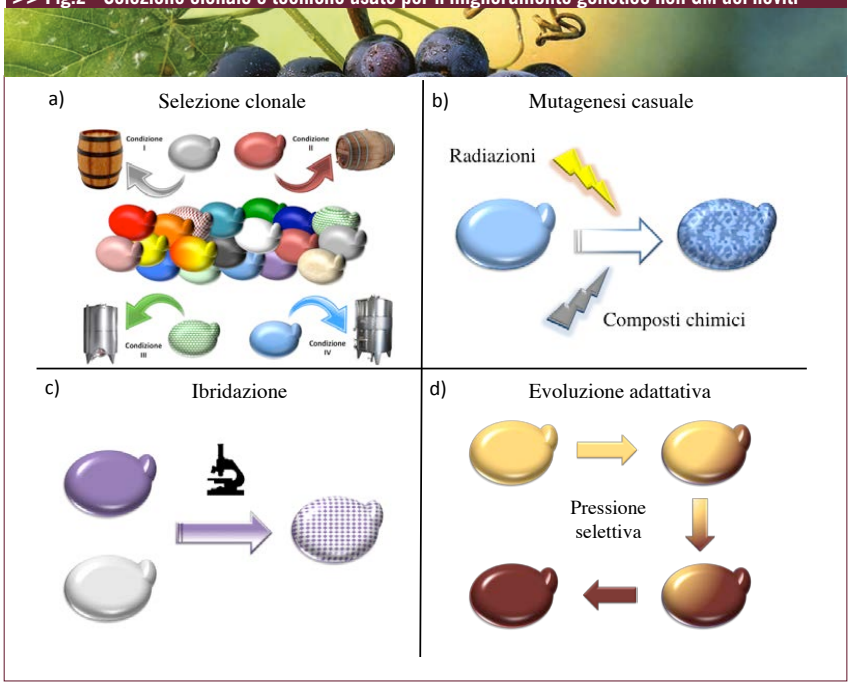
I tratti dei nuovi potenziali lieviti

Il *design* di nuovi ceppi di lievito deve necessariamente considerare i *desiderata*, spontanei o indotti, dei consumatori di vino e i vantaggi tecnologici che ne derivano dal loro uso in cantina. Per quanto riguarda il primo aspetto, non sempre le richieste dei consumatori, che non sempre si traducono in preferenze di acquisto, sono ascrivibili a due linee principali: la salutistica e la "riconoscibilità". Parlare di aspetti salutistici delle bevande alcoliche è un ossimoro evidente e sul quale non vale la pena di spendere parole. Tuttavia, la divulgazione capillare degli effetti positivi sulla salute, più o meno dimostrati, di molecole presenti anche nel vino (e.g. i polifenoli) fa da stimolo alla progettazione di ceppi di lievito che tutelano la quantità e la qualità di questi composti. Numerose evidenze dimostrano che antociani e polifenoli antociani e polifenoli hanno, *in primis*, un importantissimo ruolo nella stabilità del colore e nella qualità sensoriale del vino, mentre più limitata è la disponibilità di ricerche e dati sull'effetto dei lieviti su queste componenti dell'uva, e in particolare sul miglioramento genetico e la costituzione di ceppi enologicamente interessanti.

Il fenotipo "colore" e gli aspetti genetici su questo carattere sono stati studiati, mettendo in evidenza che il carattere è geneticamente trasmissibile (Caridi 2007). La capacità di stabilizzare il colore e, più in generale, la capacità di incidere sul potenziale redox del vino in tutto il suo percorso produttivo è un attributo auspicabile per i lieviti enologici, specie in condizioni operative a basso contenuto di solfiti. Il potenziale redox incide significativamente sulle proprietà sensoriali, basti pensare a due termini sensoriali molto diffusi, *ossidato* e *ridotto*. Le reazioni di ossidoriduzione interessano molti composti del vino e spesso sono reazioni esponenziali ed irreversibili, a meno di trattamenti fortemente invasivi. Lieviti in grado di limitare l'ossidazione e la polimerizzazione di polifenoli ed antociani

senza indurre la formazione di prodotti sensorialmente negativi (e.g. solfuri, etanale, ect.) possono rappresentare un potenziale strumento alla riduzione dell'impiego della solforosa. La "riconoscibilità", ovvero, un'impronta facilmente individuabile, è una proprietà che influenza in modo molto positivo il giudizio dei consumatori, per la semplice ragione che i consumatori tendono ad auto gratificarsi per la loro abilità nel riconoscimento sensoriale. Più un vino è riconoscibile, più tendono a preferirlo rispetto ad altri più anonimi o semplicemente più difficili da descrivere. Per analogia, i ceppi di lievito con forte impatto positivo, olfattivo o sensoriale in generale, risultano facilmente riconoscibili e tendono a dare vini più graditi dai consumatori. In una recente prova di degustazione condotta su vini ottenuti con diversi ceppi di lievito, realizzata con più repliche e opportunamente bilanciata per mettere in evidenza l'influenza del ceppo, è risultato un gradimento maggiore per i ceppi con l'impatto olfattivo più forte (**Fig. 1**).

>> Fig.2 - Selezione clonale e tecniche usate per il miglioramento genetico non GM dei lieviti

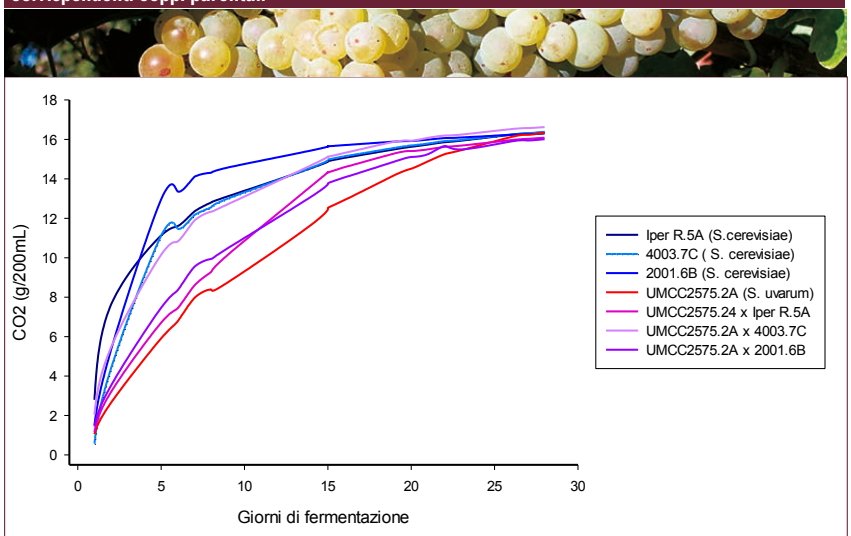


Tecniche di selezione e miglioramento genetico (non GM)

La selezione clonale ha costituito per lungo tempo il modo prevalente per l'individuazione di colture starter ad uso enologico ed ancora attualmente rappresenta una pratica consolidata per l'ottenimento di lieviti con specifici FD, senza il ricorso a nessuna tecnica di manipolazione genetica. Lo screening di ceppi isolati a fine fermentazione costituisce una procedura efficace di selezione di colture starter in quanto sfrutta la pressione selettiva esercitata dal vino, che permette la crescita di ceppi molto competitivi per via dell'alta energia fermentativa e tolleranza all'etanolo. Ciò avviene grazie a riarrangiamenti genetici e mutazioni che sono prontamente selezionati dall'ambiente di fermentazione, orientando il processo evolutivo verso le necessità di interesse enologico. Sebbene molti cambiamenti genici possano risultare neutrali o nocivi, altri, invece, possono essere benefici per la fermentazione modificando l'attività di uno o più geni o alterando indirettamente l'espressione di un più grande gruppo di geni di importanza enologica (Cavalieri et al., 2000). Pertanto la selezione clonale è fondamentale per l'implementazione del materiale biologico da impiegare nei processi di miglioramento genetico e, in questo contesto, le collezioni microbiche o biobanche assolvono all'importante ruolo di preservare nel tempo la purezza, la vitalità e la stabilità genetica delle bioresorse. Grazie alle competenze multidisciplinari nelle diverse aree della biologia, le collezioni microbiche sono in grado di fornire servizi e ricerca ad alti livelli e di soddisfare le aspettative del mercato, selezionando i ceppi adeguati per ogni specifica richiesta. Infatti, le collezioni non sono solo la sede fisica di conservazione del materiale biologico, bensì anche le annesse banche dati contenenti informazioni di tipo genomico, proteomico e metabolomico necessarie per la caratterizzazione dei ceppi, per la definizione dei caratteri di potenziale interesse e la messa a punto delle strategie di selezione e miglioramento genetico idonee all'ottenimento dei caratteri desiderati.

A parte la selezione clonale, che a rigore non è una tecnica di miglioramento genetico, altre tecniche non GM tra cui quelle basate sull'incrocio o ibridazione di ceppi di lievito e sulla mimica delle selezione naturale o evoluzione adattativa sono risultate idonee per l'ottenimento di lieviti ottimizzati (Giudici et al. 2005; De Vero et al., 2011; Mezzetti et al., 2014). Tentativi sono stati fatti anche inducendo mutazio-

>> Fig. 3 - Andamento della CO_2 svolta da 4 ibridi interspecifici S. cerevisiae x S. uvarum e i corrispondenti ceppi parentali



Nota: Analisi effettuata in 200 ml di mosto durante 28 giorni di microfermentazione a 18°C. In parentesi è indicato la specie di appartenenza dei ceppi parentali.

ni con composti chimici (Giudici e Zinnato 1983) e radiazioni anche se queste ultime non hanno dato risultati spendibili in enologia (**Fig.2**). Altre tecniche sono potenzialmente sfruttabili, ma non sono applicate in modo sistematico e efficace.

Ibridazione

L'ibridazione si basa sull'incrocio di ceppi di lievito ai fini di combinarne i tratti tecnologici. *S. cerevisiae* e altre specie a esso affini, se poste in condizioni di stress nutrizionale o mancanza di fonti azotate, avviano il processo di sporificazione. I lieviti, caratterizzati da un genoma diploide (2n), producono attraverso la divisione meiotica associata alla sporificazione 4 spore aploidi (n) a 2 a 2 appartenenti a due tipologie cellulari distinte, ovvero le cellule **a** e le cellule α. Ciascuna di queste due tipologie presenta pattern di espressione proteica caratteristici: le cellule di tipo a e α producono delle molecole segnale di natura proteica, dette fattore **a** e α rispettivamente. Inoltre, ciascuna tipologia di cellula produce un recettore di membrana ferormone-specifico, il quale riconosce il fattore α nelle cellule di tipo **a** e il fattore a nelle cellule di tipo α. Il segnale reciproco tra una cellula **a** e una α innesca il processo di coniugazione o "mating", ovvero la costituzione di uno zigote ibrido e quindi della diploidia. Questa arma di tipo evolutivo può prontamente essere sfruttata dal miglioratore di lieviti, che la può applicare al fine di migliorare tratti industriali associati ai lieviti. Tale procedimento viene attuato tramite l'accostamento fisico delle spore, derivate da ceppi parentali appositamente selezionati, attraverso uno strumento detto micromanipolatore. L'accostamento è quindi volto a massimizzare, ammesso che si tratti di gameti di tipologia opposta, la possibilità dell'evento di coniugazione. Questa tecnica può essere applicata per costituire ceppi che integrino i tratti dei ceppi parentali appartenenti alla stessa specie *S. cerevisiae* o anche a specie affini caratterizzate anch'esse da un ciclo sessuale, come *S. uvarum* e *S. bayanus*. Ciò è utile per esempio ai fini dell'integrazione di tratti specie-specifici, combinando come per esempio la performance fermentativa di ceppi commerciali di *S. cerevisiae* con tratti tipici di *S. uvarum* come la fermentazione a bassa temperatura (**Fig.3**). Quest'ultimo è di fatto un ceppo interessante per l'industria enologica, in quanto conferisce vantaggi in termini di criotolleranza e produzione di glicerolo (Rainieri et al., 1999; Salvado et al. 2011; Solieri et al., 2015). Anche a livello intraspecifico si riscontra tra i lieviti il fenomeno del "vigore ibrido" o eterosi, in cui il ceppo F1 risulta caratterizzato da tratti superiori, grazie alla complementazione reciproca dei geni deleteri dei parentali (**Fig.4**). Questo fenomeno può, ovviamente, essere sfruttato ai fini del miglioramento di particolari tratti industriali, quali la capacità di fermentare in condizioni di carenza nutrizionale o la produzione di determinati profili sensoriali, in virtù della eccezionale divergenza evolutiva che si riscontra anche tra ceppi diversi appartenenti alla stessa specie (Wang et al. 2012).

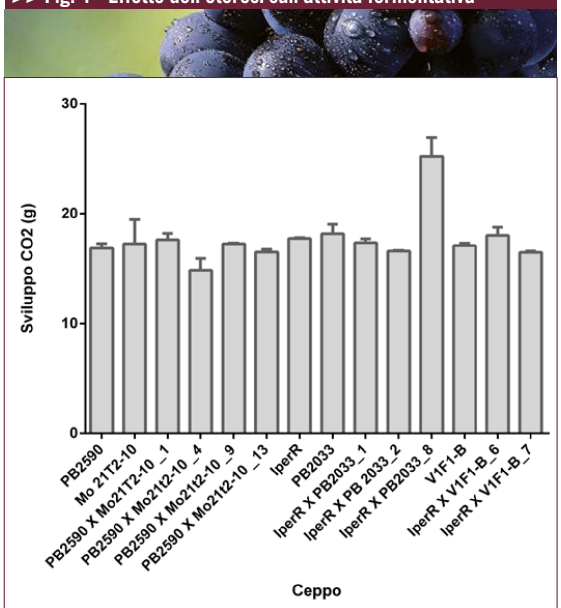
Mimica della selezione naturale o evoluzione adattativa

Sebbene le tecniche di ibridazione risultino efficaci per l'ottenimento di lieviti migliorati, possono risultare talvolta dispendiosi in termini di tempo in quanto necessitano screening di conferma dei FD. Un approccio alternativo valido per il miglioramento dei lieviti ad uso industriale è quello dell'evoluzione adattativa. Attraverso questa tecnica, un organismo è soggetto a una coltura seriale o continua per diverse generazioni ed è sottoposto a condizioni non ottimali di crescita alle quali riesce a sopravvivere solo grazie a mutazioni casuali o ad eventi ricombinatoriali vantaggiosi per la cellula (Brown et

al., 1998; Ferea et al. 1999; Foster, 1999). Come tecnica di ottimizzazione dei ceppi, l'evoluzione adattativa ha il vantaggio di non richiedere necessariamente precedenti modificazioni genetiche e neppure la scelta e l'applicazione di complicati metodi per identificare il tratto d'interesse o la conoscenza dei geni coinvolti in tale attributo.

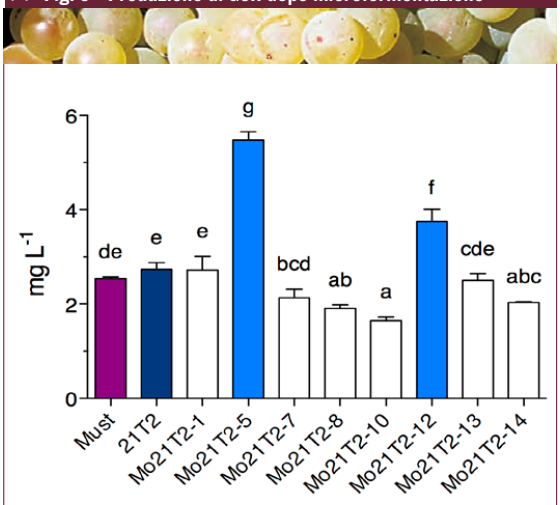
Le strategie di evoluzione adattativa sono ampiamente utilizzate per il miglioramento dei lieviti enologici, riscuotendo anche il consenso dei consumatori, dato che non portano alla produzione di organismi geneticamente modificati. Alcuni esempi delle caratteristiche di interesse enologico ottenute con queste strategie, riguardano la produzione di ceppi di *S. cerevisiae* basso produttori di solfiti e solfuri, alto produttori di glicerolo o di ceppi con un accresciuta *pathway* dei pentosi fosfati a vantaggio della riduzione dei livelli di acetato, delle prestazioni fermentative e della sintesi di prodotti aromatici (De Vero et al., 2011; Cadière *et al.,* 2011; Kutyna *et al.,* 2012). Più recentemente sono stati ottenuti ceppi di *S. cerevisiae* alto produttori di glutatione (GSH) sottoponendo un ceppo parentale dotato di ottime performance fermentative, a elevate concentrazioni di molibdato che è tossico per la cellula nella sua forma ionica esavalente (Mezzetti et al., 2014). In particolare, con la strategia di evoluzione adattativa usata, si ottengono ricombinanti casuali attraverso l'induzione della meiosi e la coniugazione delle spore, infine si individuano i ricombinanti resistenti al molibdato da testare per la produzione di GSH. In tal modo la selezione di un carattere non direttamente selezionabile, quale la produzione di GSH, viene bypassata sfruttando la resistenza al molibdato quale metodo di screening rapido dei ceppi con un metabolismo alterato relativamente alla via di assimilazione dei solfati e della biosintesi del GSH. Il molibdato, infatti, essendo dal punto di vista strutturale analogo del solfato, attraversa la cellula, utilizzandone le stesse permeasi di membrana, e attiva la via metabolica di assimilazione dei solfati fino a indurre una maggior produzione di GSH che, tra i suoi molteplici ruoli, ha anche quello di intervenire nei processi di detossificazione della cellula. I ceppi selezionati per la produzione di GSH (**Fig.5**) sono attualmente depositati nella Unimore Microbial Culture Collection (UMCC) del Dipartimento di Scienze della Vita dell'Università di Modena e Reggio Emilia (*www.umcc.unimore.it*).

>> Fig. 4 - Effetto dell'eterosi sull'attività fermentativa



Nota: (CO_2 svolta per 200 ml di mosto Chardonnay a 20° C) di sei ibridi intraspecifici di S. cerevisiae (PB2590xMo21T2-10_1, IperR x PB2033_1, 2 e 3, IperR x V1F1-B_6 e 7), dei corrispondenti ceppi parentali e di tre colture da singola spora derivate dal ceppo parentale Mo21T2-10 (der_1, Der_2, e Der-3).

>> Fig. 5 - Produzione di GSH dopo microfermentazione



Nota: mosto Trebbiano inoculato con ceppi evoluti di S. cerevisiae (Mo21T2-) e con il ceppo parentale 21T2. L'incremento di GSH osservato con il ceppo Mo21T2-5 è del 100% rispetto al parentale e del 120% rispetto al mosto di partenza, mentre con il ceppo Mo21T2-12 è rispettivamente del 36% e del 50%.

Dalla coltura cellulare al preparato commerciale

Il lievito è generalmente fruibile come sotto forma di lievito secco attivo (LSA), ovvero un preparato di cellule e materiale di supporto disidratati. La vitalità delle cellule, il costo del processo di preparazione e la facilità d'uso sono i tre fattori che maggiormente influenzano il successo commerciale di un

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preparato LSA. La moltiplicazione cellulare ed il successivo processo di disidratazione sono due step chiave nella preparazione di un LSA. Un ceppo di lievito che non riesca a garantire alte rese in massa cellulare ed elevata sopravvivenza dopo disidratazione, limita il suo impiego a preparazioni di *pied de cuve* in cantina.

I costi inerenti la fase di moltiplicazione del lievito, sono inversamente proporzionali alla resa cellulare, cioè la massa di lievito per unità di substrato fermentato, e alla capacità di utilizzare risorse di basso costo. Per quanto riguarda la resa cellulare, basse concentrazioni di glucosio e la presenza di ossigeno dovrebbero favorire il metabolismo respirativo, incrementando la produzione di biomassa. Tuttavia, sulla base del noto effetto *Crabtree*, in presenza di ossigeno e a dosi relativamente basse di glucosio, *S. cerevisiae* tende ad inibire la respirazione, orientando il proprio catabolismo verso la via fermentativa. Fra i ceppi della specie esiste una limitata variabilità all'effetto *Crabtree*, che può comunque essere investigata per selezionare ceppi in grado di dare maggiori rese cellulari a parità di concentrazione di glucosio nel mezzo. Se nell'ambito produttivo dei LSA alti coefficienti respiratori garantiscono maggiori rese in biomassa, in ambito enologico, è importante che il lievito abbia un coefficiente respiratorio molto basso, per assicurare elevate rese alcoliche e bilanci ossido-riduttivi che minimizzano i valori di acidità volatile. Comparando il processo produttivo dei LSA che avviene in condizioni che favoriscono la respirazione e di conseguenza la resa cellulare, e le esigenze di cantina che richiedono cellule in pieno catabolismo fermentativo, ne deriva che la riattivazione del preparato commerciale deve essere condotta in modo da riorientare il metabolismo cellulare dalla respirazione verso la fermentazione. Un obiettivo futuro nella preparazione di starter enologici potrebbe essere quello di avere LSA utilizzabili direttamente in autoclave, perché la semplicità d'uso e l'affidabilità nel condurre una buona fermentazione, a prescindere dalla composizione del mezzo, sono le due caratteristiche che maggiormente determinano il successo di un preparato commerciale. Per concludere, il vino deve piacere al consumatore mentre il lievito deve essere gradito all'enologo.

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DOCUMENTO TECNICO

VERSO LA "PROGETTAZIONE PERSONALIZZATA"
DEI LIEVITI STARTER PER I PROCESSI FERMENTATIVI IN ENOLOGIA

Le innovazioni biotecnologiche permettono oggi una modellazione degli starters sulla base delle esigenze dettate dall'impiego di specifiche tipologie di mosti. Le strategie di evoluzione adattativa applicate a *Saccharomyces cerevisiae*, sono risultate valide per l'ottenimento di lieviti con performance ottimizzate anche in condizioni fortemente stressanti.

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(Da sinistra nella foto)

INTRODUZIONE

La selezione di starter enologici progettati per la realizzazione di specifiche tipologie di vino è una conseguenza delle disponibilità di nuove tecnologie ed è, allo stesso tempo, prerequisito per incrementare la complessità e la qualità sensoriale dei vini. Tre fattori concorrono reciprocamente in questo processo di innovazione: i trends del mercato, l'innovazione di processo e la diffusione di strumenti biotecnologici atti alla selezione di nuovi starter enologici. I consumatori orientano il mercato spostando le loro preferenze verso vini di alta qualità e sensorialmente differenziati; l'innovazione tecnologica consente il controllo e la "modulazione" dei parametri di processo, abbassando i costi di produzione e rendendo disponibili vini differenziati in termini di qualità e complessità sensoriale per ogni fascia di prezzo; nuovi strumenti biotecnologici ad alto rendimento consentono di accelerare il processo di costituzione e selezione genetica di starter con definite caratteristiche tecnologiche e sensoriali **(Fig. 1).**

DOCUMENTO TECNICO

Fig. 1 - Rappresentazione grafica dei fattori che concorrono alla realizzazione di nuovi starter enologici

IL MIGLIORAMENTO GENETICO E I FENOTIPI DESIDERATI

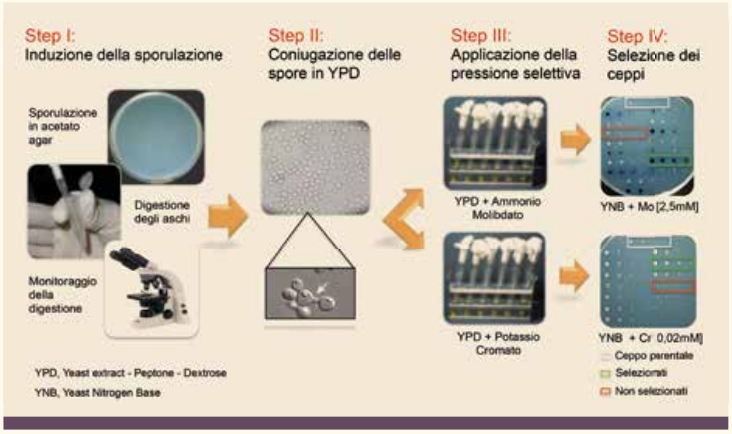
Saccharomyces cerevisiae è il principale biocatalizzatore del processo fermentativo in enologia e rappresenta il fattore che maggiormente ne influenza il successo e la riproducibilità. Oltre alla funzione di catalizzare un'efficiente, completa e rapida conversione degli zuccheri in alcool senza lo sviluppo di aromi sgradevoli, gli starter enologici devono possedere un set complesso di caratteri di qualità che contribuiscono ad aumentare il valore aggiunto del prodotto finale e a definirne un suo profilo sensoriale caratteristico e facilmente riconoscibile sul mercato. Questo complesso set di criteri di selezione è stato inizialmente definito da Giudici e Zambonelli nel 1992, poi ripreso e attualizzato da numerosi autori (Head, 1999; Rainieri & Pretorius, 2000; Giudici *et al.*, 2006). La maggior parte dei caratteri, fra i quali anche quelli prettamente tecnologici come il vigore fermentativo, la resa e la tolleranza all'etanolo o la temperatura di crescita, variano in maniera quantitativa nella popolazione e dipendono da più loci (Quantitative Trait Loci o QTL) distribuiti nel genoma e ciascuno dei quali responsabile di un piccolo contributo al fenotipo finale (Pretorius, 2000). Per caratteri regolati da QTL, le variabili genetiche e le loro interazioni sono così complesse e numerose che la costituzione di ceppi geneticamente migliorati è praticabile solo attraverso approcci stocastico-combinatoriali, capaci di agire a livello dell'intero genoma (Giudici *et al.*, 2005). Tali steps di randomizzazione del genotipo sono seguiti da screening fenotipici atti alla selezione dei microrganismi più performanti a livello tecnologico.

Fra gli approcci stocastico-combinatoriali, le strategie di evoluzione adattativa si prestano al miglioramento di fenotipi quantitativi sensibili alla pressione selettiva esercitata dal mezzo e dalle condizioni di crescita. Mentre la resistenza alla SO_2, l'alcol tolleranza o l'osmotolleranza sono tratti quantitativi direttamente selezionabili, altre caratteristiche, come la produzione di composti sensorialmente attivi, non rappresentano fenotipi direttamente selezionabili su cui sia possibile operare approcci diretti di evoluzione adattativa. Tuttavia, il problema può essere aggirato individuando nella via biosintetica del metabolita di interesse, uno o più step in cui applicare la pressione selettiva in modo da selezionare i ceppi evoluti potenzialmente in grado di esprimere il fenotipo desiderato (De Vero *et al.*, 2013). Recentemente ceppi di lievito basso produttori di solfiti e solfuri e ceppi alto produttori di glutatione (GSH) sono stati selezionati mediante evoluzione adattativa applicando come pressione selettiva alte concentrazioni di cromato e molibdato, tossici per le cellule nella forma ionica esavalente (De Vero *et al.* 2011; Mezzetti *et al.*, 2014). Tale strategia si basa sulla formazione casuale di ricombinanti attraverso l'induzione della meiosi e dell'accoppiamento e sulla successiva individuazione dei ricombinanti resistenti a cromato e molibdato. Questi metalli, strutturalmente analoghi del solfato, possono attraversare la cellula utilizzandone le stesse permeasi di membrana e stimolando la produzione di GSH che, tra i suoi molteplici ruoli, ha anche quello di intervenire nei processi di detossificazione della cellula. Ne deriva che la resistenza al cromato e al molibdato consente di selezionare ricombinanti con un metabolismo alterato relativamente alla via di assimilazione dei solfati e della biosintesi del GSH **(Fig. 2)**.

L'INTERAZIONE LIEVITO-AMBIENTE GUIDA LA SELEZIONE

La "personalizzazione" del ceppo di lievito va intesa come la progettazione di ceppi in grado di valorizzare le specifiche potenzialità dei singoli mosti di partenza, superando eventuali deficit nutrizionali che gli stessi possono presentare. L'interazione tra ceppo di lievito e mosto è un processo complesso e di difficile predizione a causa dell'estrema variabilità compositiva dei mosti che dipende sia dalle condizioni climatico-ambientali che dalle operazioni tecnologiche effettuate in cantina (Manea, 2013). Conseguentemente, un ceppo di lievito idoneo a fermentare un mosto fresco può essere completamente inadeguato

Fig. 2 - Rappresentazione schematica della strategia di evoluzione adattativa



per la fermentazione dello stesso mosto, prima mutizzato e poi desolforato. Bassi livelli di azoto prontamente assimilabile sono il principale fattore responsabile degli arresti fermentativi (Bisson, 1999). A partire dai primi lavori di Agenbach del 1977, sono stati progressivamente messe in rilievo le ripercussioni delle carenze delle fonti azotate sugli aspetti fisiologici dello sviluppo di *S. cerevisiae* (Sablayrolles *et al.*, 1996; Bezenger & Navarro, 1987; Bell & Henschke, 2005), sulle proprietà sensoriali dei prodotti finali (Ough & Lee, 1981; Rapp & Versini, 1991) e sulla produzione di idrogeno solforato (Giudici & Kunkee, 1994; Jiranek *et al.*, 1995; Spiropoulos *et al.*, 2000).

■ A fronte della consistente letteratura sull'influenza della quantità e del tipo di fonte azotata nei processi fermentativi, non corrisponde un pari ed approfondito studio su altre esigenze nutrizionali dei lieviti. In condizioni di aerobiosi e in mezzi non selettivi questi microorganismi presentano limitate esigenze nutrizionali, circoscritte ad una fonte di carbonio e di azoto assimilabile, alcune vitamine e fattori di crescita (biotina, acido pantotenico, acido folico, niacina, inositolo, p-Amino benzoico, piridossina, riboflavina, tiamina) e microelementi (boro, iodio, ferro, rame, manganese, molibdeno, zinco, fosforo, magnesio, calcio). Lo stato di ossidazione di quest'ultimi ne determina la biodisponibilità e conseguentemente la funzionalità nell'ottica del metabolismo microbico.

■ Nelle fermentazioni vinarie, caratterizzate da condizioni anaerobiche e fortemente limitanti, *S. cerevisiae* presenta maggiori esigenze nutrizionali. I trattamenti tecnologici dei mosti possono alterare significativamente lo stato di ossidoriduzione dei microelementi e dei fattori di crescita, riducendone la biodisponibilità e determinando possibili blocchi della fermentazione. Un altro parametro importante è il rapporto tra steroli e acidi grassi (alternativamente di loro precursori), in quanto l'assenza di ossigeno molecolare non consente la ciclizzazione dello squalene. Nei mezzi di laboratorio l'aggiunta di Tween 80 o altre fonti di acidi grassi, di preparati a composizione nota di vitamine e fattori di crescita, o più genericamente di estratto di lievito, consente di sopperire a queste esigenze nutrizionali. In campo enologico, il problema nutrizionale è stato affrontato empiricamente attraverso l'adozione di prodotti a composizione indefinita commercializzati come "attivanti della fermentazione" che forniscono una base di azoto prontamente assimilabile (APA), una componente di derivazione organica (lisati cellulari, scorze di lievito od altro), più o meno addizionati indirettamente di vitamine, steroli, sali minerali, e materiale inerte con funzione dispersiva ed antiflocculante.

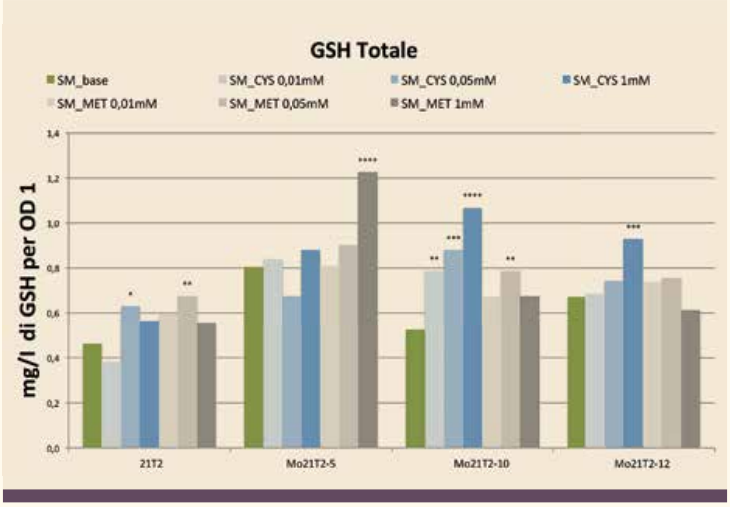
LE PRATICHE TECNOLOGICHE ACUISCONO LE ESIGENZE NUTRIZIONALI

■ La meccanizzazione della raccolta delle uve ha ampliato significativamente l'impiego della solfitazione (1800-2000 ppm) come tecnica per la conservazione dei mosti, consentendo la dilazione nel tempo dei processi di vinificazione (Giudici *et al.*, 2014). La tecnica della mutizzazione trova impiego in numerosi processi produttivi, ed al momento è esclusa solo per i vini a denominazione protetta che ne fanno esplicito divieto nei rispettivi disciplinari di produzione. Il vantaggio più consistente è legato ad una complessiva riduzione dei costi di produzione derivata da un minor investimento in autoclavi termo-condizionate e dalla distribuzione del lavoro in un arco temporale maggiore. A tali vantaggi prettamente tecnologici fanno da contraltare gli effetti dell'anidride solforosa sui composti biologicamente attivi del mosto e, di conseguenza, sull'interazione lievito/mosto. La conservazione dei mosti per lunghi periodi mediante aggiunta di solfiti non è solo responsabile della degradazione di composti biologicamente attivi e di alcune vitamine come la tiamina (Leichter & Joslyn, 1969), ma anche dell'aumento consistente dei solfati nei mosti desolforati corrispondenti, a causa della ossidazione a solfati dei solfiti in presenza di ossigeno molecolare o di altri accettori di elettroni come i chinoni e le catechine (Makhotkina & Kilmartin, 2009).

COME FERMENTARE IL MOSTO DESOLFORATO

■ Gli alti livelli di solfati e la generale povertà in molecole biologicamente attive e fattori di crescita rendono i mosti desolforati un ambiente estremamente sfavorevole per la crescita dei lieviti. La costituzione di ceppi idonei

Fig. 3 - Glutatione totale prodotto dopo 7 giorni di fermentazione in simil-mosto (SM) base e SM arricchito con diverse concentrazioni di cisteina e metionina.
* Livello di significatività determinato con il Dunnett test < del 5%, ** < 1%, *** < 0,1%, **** < 0,01%.
Per ogni ceppo il terreno di riferimento è il rispettivo SM_base



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alla fermentazione di tali mosti rappresenta pertanto una nuova sfida per l'industria biotecnologica. Il primo carattere necessario, per dei potenziali ceppi personalizzati per questo tipo di mosti, è quello di non avere esigenze nutrizionali marcate anche in condizioni stressanti, mantenendo allo stesso tempo elevate performance fermentative. Il secondo carattere è legato al metabolismo degli aminoacidi solforati, perché l'alto valore dei solfati e le deficienze nutrizionali aumentano la produzione di composti solforati indesiderati (Eschenbruch, 1974).

■ Sulla base di questi due tratti desiderati sono stati condotti test con l'obiettivo di individuare i ceppi più efficienti in possesso di potenziali tratti tecnologici esprimibili in condizioni limitanti. In primo luogo, ceppi evoluti ottenuti con la strategia di evoluzione adattativa sono stati testati per la produzione di solfuri e di glutatione, rispettivamente utilizzati come indicatore negativo e positivo dei prodotti legati al metabolismo degli aminoacidi solforati. Successivamente, i ceppi evoluti sono stati ulteriormente ottimizzati tramite la creazione di derivati meiotici o ibridandoli con ceppi commerciali ed è stato valutato se la buona performance fermentativa e la produzione di GSH venivano mantenuti in condizioni di forte stress.

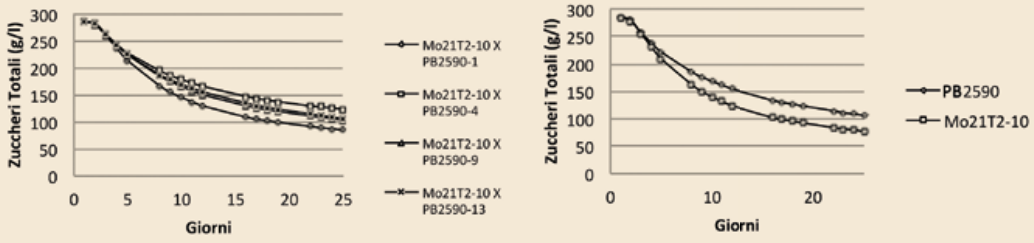
■ A partire dal ceppo parentale 21T2, che possedeva un buon vigore fermentativo nella fermentazione dei mosti, sono stati ottenuti per evoluzione adattativa tre ceppi (rispettivamente Mo21T2-5, -10, -12) che sono stati testati per la produzione di solfuri e di glutatione. Infatti, alte produzioni di solfuri sono indice di un'assimilazione e riduzione dei solfati eccedente il fabbisogno in aminoacidi solforati, mentre un'elevata quantità di glutatione è una sorta di stoccaggio di aminoacidi solforati da parte delle cellule, ed è un carattere desiderato per la sua funzione regolatoria del potenziale di ossidoriduzione dei vini. Allo scopo sono state considerate 7 condizioni colturali diverse: simil-mosto come condizione mimanti scarsità di nutrienti e simil-mosto arricchito con tre diverse concentrazioni (0.01, 0.05 e 1 mM) di cisteina e metionina. I ceppi testati nelle diverse tipologie di terreno hanno mostrato una produzione di GSH diversificata in relazione alla specifica tipologia di terreno. In particolare, i ceppi Mo21T2-5 e Mo21T2-12 mostrano una produzione di GSH sostanzialmente elevata e costante mentre il ceppo Mo21T2-10, che ha prodotto GSH in concentrazione doppia rispetto al controllo in simil-mosto arricchito con cisteina, ha mostrato una maggiore variazione in risposta al mezzo utilizzato **(Fig. 3)**. Relativamente alla produzione di solfuro, sono state ottenute per tutti i ceppi produzioni nulle di solfuri con la sola eccezione del ceppo evoluto Mo21T2-10 che ha mostrato una minima produzione di H_2S nei terreni arricchiti di metionina.

■ Per testare la performance fermentativa e la produzione di GSH in condizioni molto stressanti, il ceppo Mo21T2-10, tre derivati meiotici (Mo21T2-10_4, Mo21T2-10_9 e Mo21T2-10_13) e un ibrido intraspecifico (PB2590 x Mo21T2-10_1) sono stati testati in

Tab. 1 - Produzione di Glutatione, Solfiti e H_2S al termine delle prove di microvinificazione

Ceppo	Descrizione	Glutatione (mg/l)	Solfiti Totali (mg/l)	H_2S
PB2590	Parentale commerciale	0.6	62.3	-
Mo21T2-10	Ceppo evoluto di 21T2	1.9	39.6	+
PB2590 x Mo21T2-10_1	Ibrido	1.2	61.7	+
Mo21T2-10_4 Mo21T2-10_9 Mo21T2-10_13	Derivati meiotici di Mo21T2-10	0.8 1.2 1.0	32.6 39.5 48.9	- - -

Fig. 4 - Cinetica del consumo di zuccheri totali (glucosio e fruttosio) in esperimenti di microvinificazione effettuati con i ceppi PB2590, Mo21T2-10 e i suoi derivati meiotici. La concentrazione residua degli zuccheri è stata calcolata indirettamente dal calo in peso in CO_2. I migliori risultati sono stati ottenuti con l'ibrido intraspecifico Mo21T2-10xPB2590_1



mosto con un contenuto zuccherino complessivo del 40% (w/v). Una delle principali cause di arresti fermentativi è infatti uno sbilanciamento del rapporto tra fonti carboniose e fonti azotate nei mosti a favore delle prime. I risultati ottenuti sono riportati nella tabella 1. Il ceppo ibrido PB2590 x Mo21T2-10_1 ha prodotto GSH in quantità intermedie rispetto ai ceppi parentali, mentre il contenuto finale dei solfiti totali è stato simile a quello del ceppo parentale PB2590. La cinetica di consumo degli zuccheri da parte del ceppo ibrido ha mostrato un andamento simile a quella del ceppo commerciale PB2590 tecnologicamente più performante rispetto a Mo21T2-10_1. A differenza del ceppo Mo21T2-10, i derivati meiotici Mo21T2-10_4, Mo21T2-10_9 e Mo21T2-10_13 erano incapaci di produrre H_2S, ma hanno prodotto quantità inferiori di GSH, mentre le quantità di solfiti totali sono rimaste invariate. Tuttavia nessun ceppo testato è riuscito a portare a termine la fermentazione a causa della carenza di fonti azotate rispetto all'eccesso di fonti carboniose. Pertanto questo studio ha riconfermato l'importanza della disponibilità di una fonte di azoto assimilabile ai fini di una corretta fermentazione. Di fatti le microvinificazioni effettuate con un mosto addizionato di zuccheri (non compensata da alcuna aggiunta di fonte azotata), caratterizzato da circa 28° Brix finali, non hanno portato il vino a secco dopo un periodo di 25 giorni.

CONSIDERAZIONI CONCLUSIVE

L'innovazione tecnologica nei processi fermentativi e nel campo delle biotecnologie offre oggi la possibilità di una sempre più concreta "personalizzazione" degli starter enologici andando incontro alle esigenze dei produttori e alle richieste dei consumatori. Ceppi di lievito ottimizzati possono rappresentare una valida risposta a pratiche ampiamente diffuse in enologia quali la mutizzazione dei mosti che alterano e accentuano le esigenze nutrizionali dei lieviti durante la fermentazione.

Nel presente lavoro sono stati selezionati ceppi migliorati caratterizzati da alte produzioni di GSH e dalla contemporanea bassa o nulla produzione di H_2S che hanno dimostrato di esprimere questi caratteri anche in condizioni di forte stress. I risultati dello studio mettono poi in luce come i ceppi evoluti Mo21T2-5 e Mo21T2-12 siano i più costanti nell'esprimere i fenotipi desiderati (non produzione di H_2S ed alta produzione di GSH) indipendentemente dalla condizioni di fermentazione testate, rendendoli dei buoni candidati per la fermentazione di mosti desolforati.

RIASSUNTO

Le innovazioni apportate dalle biotecnologie nel campo dei processi fermentativi permettono oggi una modellazione degli starters sulla base delle esigenze dettate dall'impiego di specifiche tipologie di mosti, quali quelli mutizzati e desolforati. Se queste pratiche permettono la soppressione dei microorganismi competitori nel mosto e la conservazione a lungo termine con conseguente uso dilazionato nel tempo, d'altra parte inaspriscono le già limitanti condizioni di fermentazione nelle quali i lieviti operano: l'utilizzo di mosti desolforati in seguito a mutizzazione con SO_2 porta spesso a una alta concentrazione di solfati, causata dall'ossidazione dei solfiti, e a una diminuita presenza di composti biologicamente attivi, che vengono degradati durante il processo. Più in particolare queste condizioni tendono sia a favorire la produzione di composti solforati indesiderati, sia a ostacolare più in generale la performance fermentativa: per ovviare a tale problematica si è qui tentato una strategia di evoluzione adattativa su *Saccharomyces cerevisiae*, atta a incrementare la produzione di glutatione a scapito di quella di solfuri. Si è inoltre valutata la performance fermentativa e la produzione di composti solforati dei ceppi ottenuti da tale strategia e dei loro derivati, operando fermentazioni in condizioni fortemente stressanti e caratterizzate da marcati squilibri in termini di rapporto tra fonti carboniose e azotate. ■

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La selezione di lieviti migliorati per la produzione di glutatione

Negli ultimi anni le tendenze del mercato enologico sono state fortemente influenzate dalle esigenze dei consumatori sempre più attenti alla qualità dei vini e, sulla scia di queste tendenze, oltre che delle innovazioni tecnologiche di produzione, sono stati improntati i nuovi criteri di selezione dei lieviti enologici da utilizzare come coltura starter per i processi fermentativi.

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La selezione di una coltura starter, come di qualsiasi altro *biocatalizzatore*, è un processo multi-disciplinare che non include solo l'isolamento, l'identificazione e la caratterizzazione della performance metabolica e fermentativa dei ceppi, ma anche lo studio dei determinanti genici di ogni carattere e l'implementazione di tecniche di miglioramento genetico.

Le caratteristiche principali richieste a un ceppo di lievito da impiegare in vinificazione sono sensibilmente cambiate nel tempo. I primi ceppi di lievito selezionati negli anni '50-'60 dovevano possedere caratteristiche fondamentali quali una buona tolleranza alla SO_2, una buona energia fermentativa, tempi brevi di fermentazione ed assenza di aromi sgradevoli. Queste caratteristiche erano certamente in sintonia con le tecnologie e le esigenze dell'enologia del passato. Più attuali sono invece le richieste di ceppi criotolleranti, da impiegare su larga scala nelle fermentazioni a bassa temperatura, di ceppi basso produttori di etanolo, per la diffusione sul mercato internazionale dei *low-alcohol wine* o di ceppi che non producono solfiti, i cui effetti negativi sulla salute sono noti ai ben informati consumatori.

Perché il glutatione è interessante

Recentemente è anche aumentato l'interesse per i lieviti in grado di produrre elevate quantità di glutatione (GSH), poiché i ceppi con queste caratteristiche possono potenzialmente rappresentare un'alternativa all'impiego dell'anidride solforosa, limitatamente al suo ruolo antiossidante. Dal punto di vista chimico, il glutatione è un tripeptide sintetizzato a partire dagli amminoacidi cisteina, glicina e acido glutammico ed è presente in tutte le cellule viventi, principalmente nella sua forma ridotta.
Il ruolo cruciale che il GSH svolge nei lieviti, nei mammiferi e in molti organismi procarioti è dato principalmente dal suo gruppo sulfidrilico libero (-SH) che gli conferisce proprietà nucleofiliche e ossidoriduttive uniche. Grazie a queste particolari proprietà, il GSH ha trovato largo impiego in molte applicazioni biotecnologiche che vanno dalla produzione di alimenti salutistici ai preparati farmaceutici, fino ad arrivare alle bevande fermentate. In particolare, nei mosti e nei vini bianchi, la notorietà del GSH deriva dalla sua capacità di controllare i danni ossidativi e di limitare l'imbrunimento tramite la riduzione competitiva degli *o*-chinoni prodotti dall'azio-

ne della polifenolo ossidasi sugli acidi idrossi-cinnamil tartarici. Queste azioni si traducono, fondamentalmente, in un rallentamento della formazione del sotolone e degli altri caratteri di invecchiamento atipico, e in una effettiva protezione nei confronti dei diversi composti aromatici del vino.

I fattori che influenzano la concentrazione di GSH nel vino

Il raggiungimento di efficaci concentrazioni di GSH nel vino non è però di facile ottenimento. L'apporto iniziale dato dalle uve, i processi tecnici usati in cantina e il ceppo di lievito impiegato nella fermentazione sono tutti fattori capaci di introdurre una forte variabilità nella concentrazione finale.

In primo luogo, il contenuto di GSH evidenziato nelle diverse varietà di *Vitis vinifera* è estremamente variabile in funzione della componente genetica, del livello di maturazione dell'uva, della nutrizione o degli stress ambientali, tanto per citare alcuni dei fattori determinanti.

Nel mosto, i fattori che possono alterare la concentrazione del GSH sono l'esposizione all'ossigeno, l'attività tirosinasica dell'uva, le operazioni di pigiatura e la macerazione delle bucce durante il periodo pre-fermentativo.

Anche durante la fase fermentativa è possibile osservare una variazione del contenuto di GSH legata all'attività del lievito: evidenze sperimentali riportano un diverso comportamento dei ceppi di *Saccharomyces cerevisiae* relativamente alla capacità di assimilare o rilasciare GSH nel mezzo, sostenendo l'importanza della scelta del lievito giusto da utilizzare. Ciò nonostante, allo stato attuale sono ancora pochi i lavori scientifici relativi all'ottenimento di lieviti per uso enologico alto produttori di glutatione, in grado di combinare questa prerogativa, oltre che alle immancabili performance fermentative, alla non produzione di composti olfattivi indesiderati. Tra questi, quelli chiamati maggiormente in causa sono i solfuri, direttamente coinvolti, insieme ai solfiti e al GSH, nella *pathway* metabolica dei solfati .

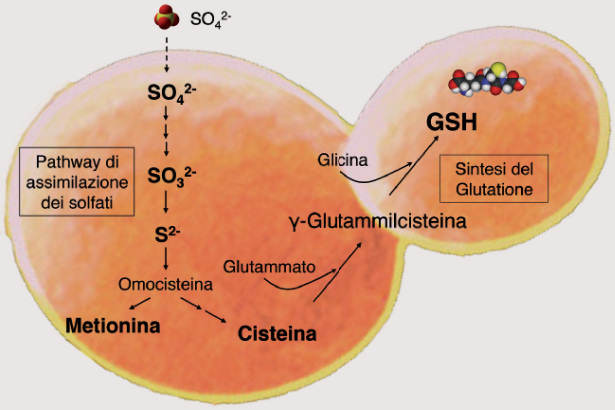
Saccharomyces cerevisiae e glutatione

In *S. cerevisiae* il GSH è naturalmente presente in concentrazioni elevate, che vanno dallo 0,1% all'1% del peso secco cellulare, rappresentando più del 90% dei tioli a basso peso molecolare.

Il GSH ha un ruolo fondamentale per i lieviti stessi, in quanto coinvolto in molte funzioni cellulari essenziali come il controllo del potenziale ossidoriduttivo, l'azione antiossidante e la capacità detossificante di xenobiotici e metalli pesanti. Proprio in virtù delle sue molteplici implicazioni, modifiche nel metabolismo del GSH non sono semplici da ottenere, e gli effetti indiretti non sempre prevedibili in tutti i loro aspetti. Ad esempio, i lieviti capaci di apportare significativi aumenti nelle quantità finali di GSH durante la fermentazione di un determinato mosto spesso non confermano questa attitudine al variare del mezzo fermentativo. Le ragioni di questa variabilità, ancora oggi non del tutto chiare, sono legate con buona probabilità alla complessità metabolica del mosto e alle articolate interazioni che intercorrono tra

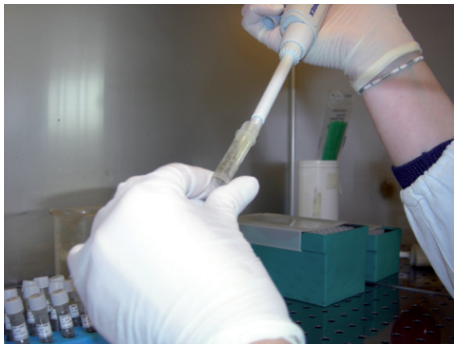
IL METABOLISMO DEGLI AMMINOACIDI SOLFORATI E DEL GLUTATIONE

La biosintesi del GSH nei lieviti coinvolge il metabolismo dello zolfo e degli amminoacidi solforati metionina e cisteina. La *pathway* di assimilazione dei solfati prevede il loro ingresso nella cellula attraverso due specifiche permeasi di membrana (*SUL1* e *SUL2*) e l'attivazione attraverso due reazioni enzimatiche sequenziali: la prima consiste nella fosforilazione dei solfati con formazione di adenosina-5'-fosfosolfato (APS), la seconda in una ulteriore fosforilazione con formazione di 3'-fosfoadenosina-5'-fosfosolfato (PAPS). I solfati attivati vengono ridotti a solfiti dall'enzima solfito reduttasi e successivamente a solfuri. Infine, lo zolfo ridotto è inserito nella molecola omocisteina, coinvolta nella biosintesi della metionina e della cisteina. Quest'ultima forma insieme al glutammato, la γ-Glutammilcisteina, ad opera dall'enzima Glutammato-Cisteina Ligasi (GSH1). La formazione finale del GSH avviene con l'aggiunta della glicina per mezzo della Glutatione Sintetasi (GSH2).



L'USO DEI LIEVITI SECCHI INATTIVI

Un discorso a parte merita l'uso di specifiche formulazioni di lieviti secchi inattivi (LSI), pratica largamente diffusa nell'industria enologica. Partendo dal presupposto che la regolamentazione vigente non permette di addizionare direttamente glutatione prima dell'imbottigliamento, si è cercato di sfruttare al meglio le matrici estratte dai lieviti secchi inattivi per arricchire i mosti in glutatione. Tuttavia, se da un lato l'uso di queste formulazioni si è dimostrato efficace in determinate condizioni, in altre non garantisce i risultati desiderati; ad esempio, posticipando l'applicazione dopo l'inizio della fermentazione i risultati non sembrano apprezzabili. Inoltre, la quantità di GSH rilasciato e biodisponibile nel vino, così come la dose ottimale di LSI da usare e l'effetto di altri parametri - come la temperatura, SO_2 e pH - non sono ancora ben definiti, per cui il rischio di ottenere risultati scarsi ed inefficaci risulta ancora fondato.



composizione iniziale del mosto, componente genetica del ceppo di lievito e parametri ambientali quali pH, temperatura e pressione osmotica, che influenzano la crescita dei microrganismi.

Selezione di lieviti alto produttori di glutatione

I primi approcci per una produzione di GSH su grande scala prevedevano la via chimica o enzimatica, tecniche successivamente abbandonate in favore della produzione per via fermentativa, decisamente più economica, che vede il coinvolgimento dei lieviti. Le metodiche oggi impiegate per la produzione massiva di cellule di lievito con aumentata produzione di GSH si basano sostanzialmente sull'ottimizzazione e sul controllo del processo fermentativo. Punto centrale è la scelta dei nutrienti e la loro concentrazione. Ad esempio, implementando la cisteina nel mezzo di crescita, la concentrazione di GSH aumenta ma un suo eccessivo incremento porta all'inibizione della crescita cellulare.

Diverse strategie di miglioramento genetico dei lieviti, che implicano le tecniche di mutagenesi e di ingegneria genetica, sono state ampiamente descritte per l'ottenimento di lieviti alto produttori di glutatione.

In particolare, le tecniche di mutagenesi puntano ad inserire mutazioni casuali nel genoma per poi selezionare i ceppi migliori mediante screening successivi.

Un approccio opposto è quello dell'ingegneria genetica, con cui grazie alle tecniche del DNA ricombinante si *over*-esprimono i geni chiave delle vie metaboliche di interesse. Nel caso del GSH, i risultati migliori sono stati ottenuti *over*-esprimendo, oltre ai due enzimi direttamente implicati nella biosintesi, anche gli enzimi chiave della *pathway* di assimilazione dei solfati, in virtù della stretta relazione tra queste due vie metaboliche.

La maggior parte delle strategie impiegate per le produzioni industriali non sono però applicabili all'industria enologica a causa di limitazioni giuridiche, culturali, scientifiche o di processo.

Le strategie di evoluzione adattativa, invece, sono ampiamente utilizzate per il miglioramento dei lieviti enologici, riscuotendo anche il consenso dei consumatori, dato che non portano alla produzione di organismi geneticamente modificati. Alcuni esempi delle caratteristiche di interesse enologico ottenute con queste strategie riguardano la produzione di ceppi di *S. cerevisiae* alto produttori di glicerolo o di ceppi con un accresciuta *pathway* dei pentosi fosfati, a vantaggio della riduzione dei livelli di acetato, delle prestazioni fermentative e della sintesi di prodotti aromatici.

I vantaggi delle strategie di evoluzione adattativa sono quelli di non introdurre geni ricombinanti estranei nel genoma dei microrganismi e di non richiedere una preventiva conoscenza dei geni coinvolti nell'espressione dei fenotipi desiderati. Infatti, sottoponendo i microrganismi a colture seriali o continue per diverse generazioni, in presenza di una specifica pressione selettiva, si selezionano i ceppi evoluti esprimenti il carattere d'interesse. Tuttavia, poiché lo screening dei microrganismi evoluti richiede necessariamente l'espressione di fenotipi selezionabili, ossia facilmente riconoscibili, tali strategie non sono applicabili per l'ottenimento di caratteristiche enologiche legate a variazioni e ricombinazioni genetiche non direttamente selezionabili. Esempi specifici di tali limiti riguardano l'ottenimento di ceppi alto produttori di GSH, ceppi con attività β-glicosidasica o con alta capacità di produrre composti sensorialmente attivi, per i quali ogni singola variante ottenuta mediante randomizzazione deve essere testata individualmente per il fenotipo di interesse, con un dispendio di tempo consistente.

Ciò nonostante questo limite è superabile individuando, nella *pathway* biosintetica del metabolita di interesse, uno o più step in cui applicare la pressione selettiva in modo da selezionare i ceppi evoluti potenzialmente in grado di esprimere il fenotipo desiderato. Questa strategia *evolution-based* è stata recentemente messa a punto e applicata per la selezione di ceppi di lievito basso produttori di solfiti e solfuri. La strategia descritta si basa sulla formazione di ricombinanti attraverso la riproduzione sessuale e sulla successiva individuazione dei ricombinanti di interesse attraverso uno screening rapido e altamente selettivo. In particolare, i metalli come il cromato e il molibdato, tossici per le cellule nella forma ionica esavalente, applicati come pressione selettiva, consentono di selezionare i ceppi evoluti con un metabolismo alterato relativamente alla *pathway* di assimilazione dei solfati e della biosintesi del GSH. Questi metalli, infatti, essendo strutturalmente analoghi del solfato, possono attraversare la cellula utilizzando le stesse specifiche permeasi di membrana e stimolare la produzione di GSH che, ricordiamolo, tra i suoi vari ruoli ha anche quello di intervenire attivamente nei processi di detossificazione della cellula. Il GSH prodotto viene principalmente concentrato nei mitocondri, dove svolge importanti azioni antiossidanti, ma è presente in quasi tutti i compartimenti cellulari, in particolar modo nel vacuolo, dove viene immagazzinato, se in eccesso, o inglobato in forma complessata a metalli pesanti o xenobiotici.

Nuovi ceppi con la strategia *evolution-based*

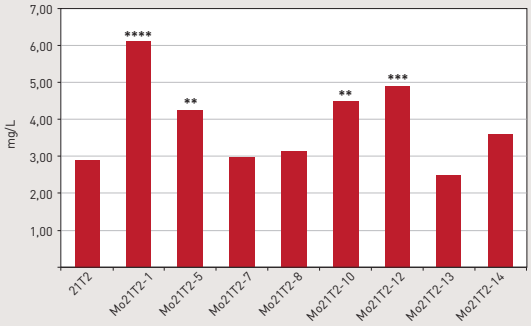
La strategia *evolution-based*, applicata recentemente nel nostro laboratorio con la finalità di ottenere ceppi alto produttori di GSH, ha dato risultati molto promettenti. Infatti, la sperimentazione effettuata, in via preliminare su campioni di mosto sintetico, ha permesso di individuare, tra i ceppi evoluti resistenti a elevate concentrazioni di molibdato (Mo^{6+}), quelli migliorati per la formazione di GSH.

In particolare, 4 ceppi di *Saccharomyces cerevisiae* (Mo21T2-1, Mo21T2-5, Mo21T2-10, Mo21T2-12) hanno mostrato valori di GSH totale (sia ossidato che ridotto) significativamente più elevati rispetto al ceppo parentale 21T2. Questi ceppi selezionati sono stati depositati nella collezione microbica UNIMORE del Dipartimento di Scienze della Vita (Unimore Microbial Culture Collection –UMCC - website: www.umcc.unimore.it) e saranno oggetto di sperimentazione in cantina per verificare su larga scala gli effetti benefici dell'aumentato rilascio di GSH, sia relativamente alla prevenzione dell'imbrunimento dei vini bianchi, sia relativamente alla protezione dei composti aromatici. La valutazione degli andamenti fermentativi su mosti diversi, i risultati analitici del vino ottenuto e un'accurata analisi sensoriale consentiranno di completare e validare la procedura di selezione dei ceppi alto produttori di GSH. ■

www.vitevinoqualita.it/VGhPN

LE PERFORMANCE DEI NUOVI CEPPI

I 4 ceppi di *Saccharomyces cerevisiae* Mo21T2-1, Mo21T2-5, Mo21T2-10, Mo21T2-12 danno valori di GSH totale (sia ossidato che ridotto) significativamente più elevati rispetto al ceppo parentale 21T2. Nel grafico sono riportati i valori di glutatione totale intra- ed extracellulare ottenuto su mosto sintetico con i ceppi di lievito evoluti. Il ceppo Mo21T2-1, in particolare, ha mostrato una concentrazione di GSH circa doppia rispetto al ceppo 21T2.



**** Livello di significatività dello 0,01%, *** 0,1%, ** 1%.

Ringraziamenti:
La ricerca qui presentata è stata svolta, in parte, con il contributo di AEB (Brescia).

La mimica della selezione naturale nel miglioramento dei lieviti

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Per le sue caratteristiche di alcol-tolleranza e per la generale purezza fermentativa, la specie d'elezione per la conduzione della fermentazione alcolica in enologia è *S. cerevisiae*. Tuttavia, in passato sono state proposte altre specie di lievito, recentemente riconsiderate per alcuni caratteri tecnologici e sensoriali.

Tra le specie non-*Saccharomyces* più interessanti per la loro influenza positiva sul profilo aromatico dei vini possiamo citare *Candida zemplinina, Hanseniaspora uvarum, Metschnikowia pulcherrima, Toluraspora delbrueckii* e *Zygosaccharomyces bailii*. Tali specie sono perlopiù proposte per fermentazioni in cocoltura o in successione scalare con *S. cerevisiae,* per compensare le eventuali difficoltà a completare la fermentazione.

Perché migliorare geneticamente i lieviti?

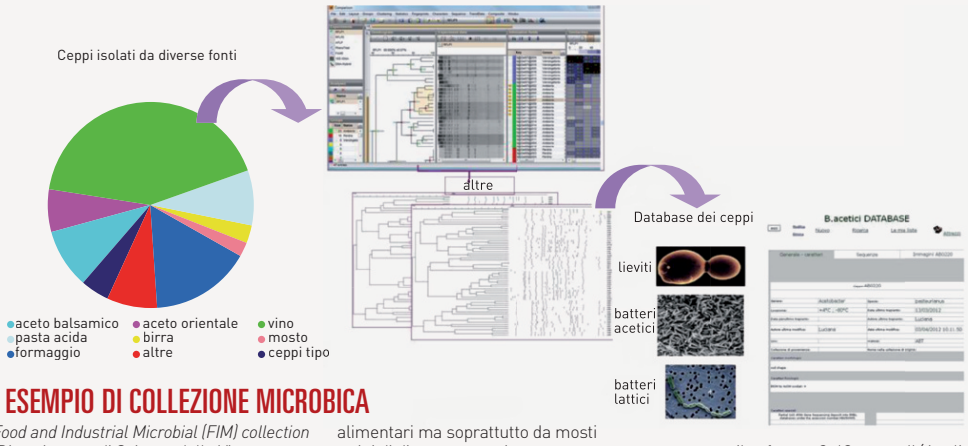
La scelta del ceppo di lievito ad uso enologico è il fattore che maggiormente influenza il successo della fermentazione alcolica dei vini, per cui è necessario che il ceppo impiegato possegga le caratteristiche o i tratti desiderati. In natura è difficile trovare i ceppi dotati della combinazione di tutti i tratti desiderati, perciò è necessario attuare programmi di selezione e miglioramento genetico. Tali strategie si basano su analisi genetiche e screening funzionali puntuali, che esulano dalla mera provenienza geografica dei lieviti. Nel caso della vinificazione, i criteri di selezione sono stati inizialmente definiti da Giudici e Zambonelli nel 1992, poi ripresi e attualizzati da numerosi Autori (Head, Rainieri e Pretorious, Giudici). La continua rivisitazione dei criteri di selezione, ovvero dei tratti enologici richiesti ad un buon ceppo di lievito enologico, è dovuta al fatto che la lista dei tratti desiderati è aperta ed in continuo divenire, perché alcuni tratti perdono di importanza e nuove caratteristiche vengono richieste, sulla scia delle innovazioni tecnologiche e dei desiderata dei consumatori.

Le collezioni microbiche

Un qualsiasi progetto di miglioramento genetico non può prescindere dalla disponibilità dell'adeguato materiale biologico, che nel caso dei lieviti enologici, è reperibile isolando direttamente da vini a fine fermentazione e ottenuti senza l'aggiunta volontaria di lieviti selezionati. Infatti, le condizioni estreme del mosto a fine fermentazione esercitano una forte pressione selettiva, operando di fatto una preselezione a favore dei ceppi con caratteristiche tecnologiche interessanti, quali la buona capa-

POTENZIALITÀ DEGLI OGM

L'uso di OGM in enologia non è consentito, per cui le *engineering strategy* che impiegano tecniche del DNA ricombinante sono qui riportate più per dovere di cronaca che per un attuale impiego enologico. Va tuttavia ricordato che esperienze di laboratorio hanno ampiamente dimostrato l'utilità delle tecnologie del DNA ricombinante per il miglioramento dei lieviti enologici. Ad esempio sono stati costituiti ceppi più efficienti nell'utilizzo degli zuccheri, con capacità di chiarificazione dei vini, con aumentata attività peptolitica, glucanolitica o xilanolitica e influenti sull'aroma del prodotto finale per l'espressione dell'attività β-(1,4)-endoglucanasica.



UN ESEMPIO DI COLLEZIONE MICROBICA

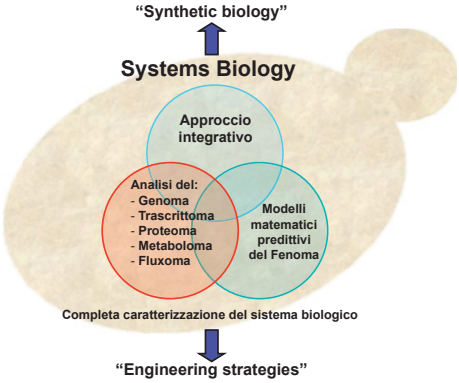
La *Food and Industrial Microbial (FIM) collection* del Dipartimento di Scienze della Vita dell'Università di Modena e Reggio Emilia dispone di circa 2.700 ceppi, di cui circa 1.600 ceppi di lievito isolati da diverse matrici alimentari ma soprattutto da mosti e vini di diversa provenienza.
Tutti i ceppi in collezione sono sottoposti ad accurata caratterizzazione fenotipica e molecolare e le informazioni sono elaborate con il software GelCompar II (Applied Maths), che consente di normalizzare e comparare fra loro i dati di fingerprinting raccolti su ceppi isolati da matrici differenti.

cità fermentativa, la tolleranza all'alcol e, se usata, la resistenza alla solforosa. Le operazioni di campionamento di ceppi da mosti in fermentazione, di caratterizzazione genetico-funzionale e di screening vanno sotto il nome di *selezione clonale*. La selezione clonale non solo rappresenta il punto di partenza per la realizzazione di nuovi starter enologici, ma soprattutto consente di costituire il pool di materiale biologico (e di caratteri ad esso associati) su cui operare la *progettazione* di nuovi ceppi ricombinanti attraverso strategie di miglioramento genetico. Le collezioni microbiche, o *biobanche*, mettono a disposizione competenze e servizi non solo per la semplice conservazione dei ceppi isolati e selezionati, ma anche per l'archiviazione, con specifiche banche dati informatizzate, delle informazioni ad essi correlati: più informazioni associate ai rispettivi ceppi sono disponibili, maggiore è la qualità e l'utilità della collezione. In particolare, i programmi di miglioramento genetico necessitano di informazioni di tipo genomico, trascrittomico, proteomico e metabolomico, perché consentono di prevedere e/o programmare il risultato finale, attraverso il ricorso a tecniche computazionali di *systems biology*.

Gli approcci di *systems biology*

L'elevato numero e la complessità genetica di molti caratteri riducono la possibilità di riscontrare in un unico ceppo la combinazione dei determinanti genetici e dei rispettivi caratteri fenotipici desiderati, per cui è necessario ricorrere a tecniche di miglioramento genetico per ottenere i ceppi evoluti in grado di esprimere i caratteri d'interesse. Diverse tecniche di miglioramento dei lieviti sono state ideate e applicate con successo, tra cui: induzione di mutazioni, ibridizzazione, *rare-mating*, fusione di sferoplasti, *over-expression* e/o delezione di geni esistenti e introduzione *ex-novo* di geni eterologhi mediante clonazione e ricombinazione omologa. L'ingegneria metabolica si fonda su solide conoscenze delle vie biochimiche, oggetto della manipolazione genica, e la fase analitica – ossia la conoscenza delle attività cellulari – precede la fase sintetica, ovvero la modificazione genetica vera e propria, che avviene attraverso approcci diretti e mirati quali modificazioni del tipo o della lunghezza dei promoter genici, delezioni o inserzioni di nuovi geni coinvolti nel metabolismo d'interesse.
Questi approcci trovano largo impiego in biotrasformazioni industriali, ad esempio per la produzione di metaboliti o farmaci, ma non sono impiegati in ambito enologico per diverse ragioni, fra le quali il rifiuto da parte dei consumatori ad accettare prodotti ottenuti con organismi geneticamente modificati (OGM).
S. cerevisiae è da tempo usato come modello per lo studio e la descrizione quali/quantitativa dei processi

STRATEGIE DI MIGLIORAMENTO GENETICO DEI LIEVITI



cellulari: è facile da coltivare e da manipolare geneticamente, ha un genoma relativamente piccolo (6000 geni privi di introni) ed è stato la prima cellula eucariotica il cui genoma è stato completamente sequenziato. Tutto ciò fa si che le informazioni su *S. cerevisiae* siano moltissime e disponibili in diverse banche dati fra le quali, *Saccharomyces Genome Database* (SGD-http://www.yeastgenome.org) e Yeastnet (yeast metabolic network - http://yeast.sourceforge.net). Questa disponibilità di informazioni, nonché la sua efficienza come biocatalizzatore di numerosi processi fermentativi, ha determinato la scelta di *S. cerevisiae* come organismo modello per gli approcci di *systems biology*. Le strategie di ingegneria genetica vanno intese nella loro accezione ampia ed includono la *metabolic engineering*, la *synthetic biology* e la *evolutionary engineering*; una sintesi sul significato dei termini è riportato in tabella.

CONFRONTO TRA DIVERSE STRATEGIE DI INGEGNERIA GENETICA

Tipologia	Definizione	Vantaggi	Limiti applicativi
Metabolic engeneering	Strategia basata sulle modificazioni genetiche di specifiche e note reazioni biochimiche mediante approcci diretti e mirati, come modificazioni del tipo o della lunghezza dei promotori genici, delezioni o inserzioni di nuovi geni	Consente di agire in modo mirato sui geni responsabili del fenotipo di interesse	Richiede solide conoscenze delle vie biochimiche oggetto della manipolazione genica. Utilizza le tecniche del DNA ricombinante
Synthetic biology	Strategia che combina la scienza e l'ingegneria genetica per sintetizzare funzioni biologiche originali, progettando e fabbricando componenti e sistemi biologici non ancora esistenti in natura e/o riprogettando sistemi biologici già presenti in natura	Consente di ottenere nuovi microrganismi, tecnologicamente evoluti, dotati delle caratteristiche desiderate	Non è attualmente possibile predire gli effetti dei microrganismi sintetici sulla salute umana e sull'ambiente
Evolutionary engeneering	Strategia basata sui processi di evoluzione adattativa, finalizzata all'ottenimento dei fenotipi desiderati mediante l'applicazione di idonee pressioni selettive	Consente di selezionare, in modo casuale, i microrganismi evoluti, senza richiedere una preventiva conoscenza dei geni coinvolti nell'espressione dei fenotipi complessi desiderati	Lo screening dei microrganismi evoluti richiede l'espressione di fenotipi selezionabili, ossia facilmente riconoscibili

Evoluzione adattativa e QTL

La maggior parte dei caratteri, fra i quali anche quelli prettamente tecnologici come il vigore fermentativo, la resa e la tolleranza all'etanolo o la temperatura di crescita, variano in maniera quantitativa nella popolazione e dipendono da più loci (*Quantitative Trait Loci* o QTL), distribuiti nel genoma e ciascuno dei quali responsabile solo di una piccola parte della proprietà fenotipica quantitativa. Ad esempio, è stato osservato che in *S. cerevisiae* il carattere tolleranza all'etanolo coinvolge più di 250 geni. Nonostante i recenti successi nel mappaggio dei QTL, grazie all'impiego di nuove tecniche, come l'analisi dei microsatelliti o del polimorfismo di singoli nucleotidi (SNPs), l'identificazione delle basi genetiche di caratteri complessi è considerata a tutt'oggi come uno dei traguardi più ardui della ricerca genetica. Nel caso dei QTL il numero delle variabili genetiche in gioco è così elevato e le interazioni fra queste così complesse e difficili da predire che la costituzione di ceppi geneticamente migliorati è più facilmente praticabile attraverso approcci combinatoriali ciechi, capaci di agire a livello dell'intero genoma. Le strategie di *evolutionary engineering* o evoluzione adattativa si prestano al miglioramento di QTL sensibili a una eventuale pressione selettiva. In breve, un organismo è soggetto a una coltura seriale o continua per diverse generazioni, in condizioni non ottimali di crescita (sotto pressione selettiva), al fine di selezionare i ceppi evoluti esprimenti il fenotipo d'interesse. L'evoluzione adattativa privilegia le mutazioni che risultano vantaggiose per la cellula sottoposta a condizioni di stress. Le mutazioni, per definizione, determinano la formazione di nuovi alleli nella popolazione e forniscono la variabilità genetica su cui agisce la selezione. Pertanto, l'evoluzione adattativa determina l'aumento nella popolazione delle cellule varianti che più si adattano all'ambiente caratterizzato dalla pressione selettiva. La procedura è efficace, ma poiché è basata sull'occorrenza occasionale di mutazioni durante la riproduzione mitotica può richiedere tempi lunghi. L'evoluzione adattativa, di fatto, è il risultato della selezione di fenotipi selezionabili, cioè sensibili alla pressione selettiva esercitata dal mezzo e dalle condizioni di crescita. Caratteristiche quali la resistenza alla SO_2, l'alcol tolleranza o la capacità di sviluppare in presenza di elevate pressioni osmotiche sono legate a ricombinanti direttamente *selezionabili*. Tuttavia molte altre caratteristiche di interesse enologico tra cui, ad esempio, la produzione di composti sensorialmente favorevoli, sono legate a variazioni e ricombinazioni genetiche che non esprimono fenotipi direttamente selezionabili, su cui non è possibile operare approcci di evoluzione adattativa.

La mimica della selezione naturale

Un altro limite intrinseco degli approcci di evoluzione adattativa è la loro applicabilità a fenotipi selezionabili, cioè sensibili alla pressione selettiva esercitata dal mezzo e dalle condizioni di crescita. Approcci di evoluzione adattativa non sono applicabili a caratteristiche di interesse enologico tra cui, ad esempio, la produzione di composti sensorialmente attivi, che sono legate a variazioni e ricombinazioni genetiche che non esprimono fenotipi direttamente selezionabili. In laboratorio è possibile attuare pressioni selettive indirette anche su fenotipi non selezionabili, nel caso si riescano ad individuare uno o più step, nella pathway biosintetica del metabolita di interesse, sottoponibili a pressione selettiva. Un esempio concreto è riportato di seguito e riguarda la non produzione di solfiti e solfuri e l'elevata produzione di glutatione, sfruttando la capacità dei ceppi evoluti di svilupparsi in presenza di metalli pesanti, quali cromo e molibdato, molto tossici per le cellule nella forma ionica esavalente.

Metalli pesanti come fattore di pressione selettiva

La resistenza al cromato e al molibdato costituisce lo step selezionabile per l'individuazione dei ceppi caratterizzati da un alterato metabolismo dei solfati. In particolare, la resistenza delle cellule ad alte concentrazione di ioni Cr^{6+} e Mo^{6+} può essere dovuta ad almeno due meccanismi: 1) una mutazione a carico dei geni responsabili della codifica delle proteine trasportatrici degli ioni Cr^{6+} e Mo^{6+}, le stesse proteine di membrana che mediano l'entrata del solfato; 2) un aumento nella sintesi del glutatione, un tiolo costituito da cisteina, glicina e acido glutammico, che svolge un'importante azione antiossidante ed è in grado di chelare i metalli pesanti formando complessi che vengono trasferiti attivamente nel vacuolo tramite trasportatori presenti nella membrana del vacuolo stesso. Nel vacuolo i complessi metallo-glutatione vengono degradati dagli enzimi litici e gli amminoacidi e i metalli liberati vengono immagazzinati in questo organello per poi essere riutilizzati o eliminati dalla cellula. La riduzione dei solfati a solfiti e solfuri sono step necessari della sintesi degli aminoacidi solforati cisteina e metionina. La degradazione di quest'ultimi determina la produzione del glutatione. In sintesi, abbiamo osservato che i nuovi ricombinanti ottenuti, resistenti al molibdato e al cromato, producevano quantità bassissime di solfiti e solfuri, oppure producevano quantità elevate di glutatione, importante antiossidante naturale (vedere approfondimento sul web). La strategia descritta ci ha consentito di ottenere un ceppo con buonissima attitudine enologica, basso produttore di solfiti e solfuri e non producente tioli sensorialmente sgradevoli. ■

La ricerca qui presentata è stata svolta con il contributo di AEB (Brescia).

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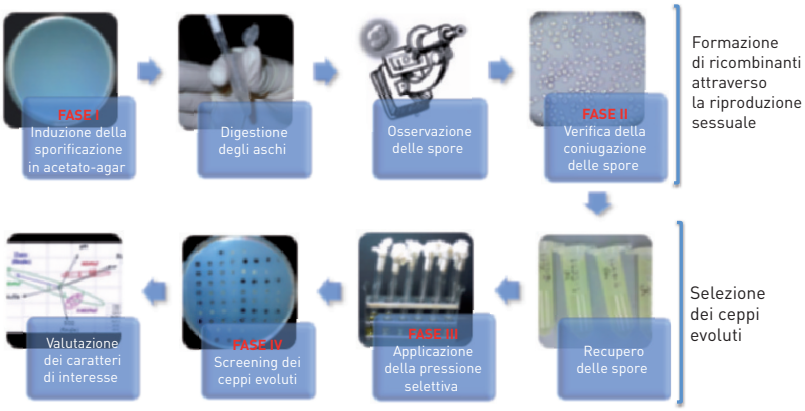
LA PRESSIONE SELETTIVA INDIRETTA



EVOLUZIONE ADATTATIVA E LIEVITI AUTOCTONI

In apparenza i principi che regolano l'evoluzione adattativa, *in primis* la pressione selettiva esercitata dall'ambiente – nel nostro caso dal sistema vigneto-cantina-mosto sulla popolazione dei lieviti – supporta la tesi, tanto cara a molti Autori, della presenza dei lieviti autoctoni.
Quei ceppi di lievito che nel tempo si sono evoluti e selezionati in un determinato territorio, ceppi che sono in grado di valorizzare al meglio le note di tipicità del vino del territorio di riferimento. In realtà quanto esposto in questo articolo sulla evoluzione adattativa confuta in modo documentato e concreto tale convinzione. Infatti, l'evoluzione adattativa funziona per i caratteri selezionabili e quindi sensibili alla pressione selettiva esercitata dall'ambiente circostante. La domanda che viene spontanea è: qual è la pressione selettiva che privilegia i ceppi che migliorano la tipicità territoriale dei rispettivi vini di riferimento?
Non c'è alcuna pressione selettiva, ma se talora capita di isolare un ceppo in grado di avere tante caratteristiche desiderate ed anche quella di essere eccellente per la fermentazione dei vini del proprio territorio, è solo perché il caso è un burlone e si prende gioco di noi presentandoci anche gli eventi meno probabili.

Scopri gli altri contenuti sul web.

PT 6. **Yeast and industrial biotechnology: From fermented foods to Cell Factories**

Exploitation of a Evolution Strategy to Select Yeast Strains Improved in Glutathione Production

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Yeasts have been largely explored as cell factories to produce substances for food and industrial biotechnological applications. Among these chemicals, glutathione (GSH) is an important antioxidant molecule involved in several processes, including the control of redox potential, protection against oxidative stress, detoxification and transport of organic sulfur. Due to its functional roles, GSH is widely used in the pharmaceutical, food and cosmetic industries. Recently, GSH has received growing attention also in the winemaking field, to control oxidative spoilage damage; to limit the amount of browning pigments; to avoid the formation atypical aging characters; and to exert a protective effect on various aromatic compounds. At present GSH is successfully produced on an industrial scale through fermentation by high GSH-producing *Saccharomyces cerevisiae* strains, and several methodological tools have been reported for increasing efficiency and yield of the bioprocess.
In this study, we have applied an evolution-based strategy that combines the sexual recombination of spores with the application of molybdate Mo(VI), a sulfate analogue toxic for the cells at high concentration, as specific selective pressure, to generate evolved *S. cerevisiae* strains with enhanced GSH production. To achieve this aim we used the 21T2 wine strain from the Unimore Microbial Culture Collection (UMCC) and we exploited its resistance to Mo(VI) as a rapid and high-throughput screening method for the selection of the evolved strains improved in GSH production. By this strategy, we obtained two evolved strains, Mo21T2-5 and Mo21T2-12, both able to enhance GSH content in wine with an increase of 100% and 36%, respectively, compared with the parental strain 21T2, and 120% and 50% compared with initial GSH content in the must.
Our strategy, unlike the standard evolutionary approaches, has the advantage of not requiring multiple rounds of screening and extensive cultivation periods because the evolved strains are recognized through a selectable phenotype. The Mo(VI) resistance has proved to be effective for the selection of the desired evolved strains, probably by activating the yeast common metal response that involves sulfur assimilation and GSH biosynthesis.

Keywords:
glutathione
adaptive evolution
molybdate resistance

Abstract

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Implementation of the Unimore Microbial Culture Collection with novel wine yeast strains obtained by an evolution-based strategy

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Type : Poster
Theme : Poster

Microbial collections are an essential support for selection of functional starter cultures useful in food and industrial fermentation processes. The University of Modena and Reggio Emilia (Unimore) Microbial Culture Collection (UMCC) is designed to offer a useful service for several purposes such as research, teaching, screening assay, knowledge-based bioeconomy and biotechnological applications. The UMCC holds about 2700 strains including yeasts, lactic acid bacteria and acetic acid bacteria isolated from must and wines, beer, vinegar, sourdough and other fermented products. Among the yeast cultures collected, there are several strains which are of oenological interest and some of them are commercialized by the AEB Group (Brescia, Italy), as active dry yeasts. Currently the UMCC has been implemented with wine yeast strains obtained by an evolution-based strategy that combines spores sexual recombination and application of a specific selective pressure in order to rapidly select evolved strains with desired oenological characteristics. In particular, with this non-genetically modified strategy, novel *Saccharomyces cerevisiae* strains with low or nil sulfites and sulfides production, which are attractive phenotypes in winemaking to reduce allergenic risks and off-flavours compounds, respectively, have been selected (De Vero et al., 2011). Moreover, evolved strains with enhanced glutathione (GSH) production have been obtained (Mezzetti et al., 2014). These strains can be also useful in winemaking, for the important role of the GSH in limiting must and wine oxidation and in protecting various aromatic compounds. The strains have been characterized by a polyphasic approach and all the information has been managed in the UMCC Database (http://biolomics.umcc.unimore.it) by the BioloMICS NET software (BioAware).

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Mezzetti F., De Vero L. & Giudici P., 2014. Evolved *Saccharomyces cerevisiae* wine strains with enhanced glutathione production obtained by an evolution-based strategy. FEMS Yeast Research, 14(6), pp.977-987.

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20th Workshop on the *Developments in the Italian PhD Research on Food Science Technology and Biotechnology,* University of Perugia, Perugia, September 23rd-25th, 2015

Selection and molecular characterization of *Saccharomyces cerevisiae* wine strains improved in glutathione production by an evolution-based strategy

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This work has focused on the selection and characterization of wine yeast strains improved in glutathione (GSH) production. An evolution-based strategy was applied on *Saccharomyces cerevisiae* UMCC 855 wine strain and the eight evolved strains selected were tested for the production of GSH in synthetic must with or without the addition of methionine and cysteine and in microvinification assay. We obtained one evolved strain, UMCC 2581, with a high production of GSH in wine and in almost all the tested synthetic media. A deep understanding of the modification involved in the different phenotypes was obtained through a comprehensive molecular characterization of selected strains.

Selezione e caratterizzazione molecolare di ceppi enologici di *Saccharomyces cerevisiae* ottenuti tramite una strategia di evoluzione adattativa e migliorati per la produzione di glutatione

Scopo di questo lavoro è stato la selezione e caratterizzazione di ceppi di lievito enologici migliorati per la produzione di glutatione (GSH). Sul ceppo enologico di *Saccharomyces cerevisiae* UMCC 855 è stata applicata una strategia di evoluzione adattativa e gli otto ceppi evoluti selezionati sono stati testati per la produzione di GSH in mosto sintetico con o senza l'aggiunta di metionina e cisteina e in esperimenti di microvinificazione. Abbiamo ottenuto un ceppo evoluto, UMCC 2581, con un'alta produzione di GSH in vino e in quasi tutti i mezzi sintetici testati. Una profonda comprensione delle modificazioni responsabili dei differenti fenotipi è stata ottenuta attraverso una completa caratterizzazione molecolare dei ceppi selezionati.

Key words: Glutathione; *Saccharomyces cerevisiae*; wine strain; evolutionary strategies; RNA-seq; genomic DNA.

1. Introduction

In the never-end process of wine improvement, *Saccharomyces cerevisiae* oenological strains play a main role. The yeast wine strains are indeed the principal responsible of the fermentation process and act also as a biocatalyst able to modify flavour and chemical complexity of the final product through the production of functional metabolites. Among them, glutathione (GSH) is the main nonprotein thiol (L-γ-glutamyl-L-cysteinyl-glycine) in yeasts involved in the response to different nutritional and oxidative stresses (Penninckx, 2000). Recently GSH has received a growing interest in the winemaking field for its role in limiting the amount of browning pigments by competitive o-quinones reduction, avoiding the formation of sotolon and other atypical aging characters and exerting a protective effect on various aromatic compounds (Kritzinger *et al.*, 2013). Although it has been postulated that the final GSH amount in wine is dependent of wine strains (Lavigne *et al.*, 2007), few works have been reported regarding the development of new non-genetically modified wine yeast strains, able to produce high amount of GSH. The first main aim of the PhD thesis project has been the selection of evolved wine yeast strains, high GSH producers, obtained through the application of an evolution-based strategy. Subsequently metabolic and molecular characterizations have been performed in order to deep understand the different phenotypic expression and the genetic modification among parental and evolved strains. Increase knowledge about the complex metabolic processes behind these different phenotypes could provide new opportunities for a further future development of optimized strains.
In this oral communication are shown the main results of the following activities, previously described in the PhD thesis project (Mezzetti, 2013):

A2) Application of evolution-based strategy and selection of improved wine yeast strains with high production of GSH;
A3.2) Metabolic analysis performed by using different media to characterise phenotypic and quantitative aspects of selected evolved strains;
A3.3) Molecular characterization including the strain typing, the karyotyping, the analysis of RNA (RNAseq) and the genomic DNA sequencing to understand the phenotypic differences between parental and evolved strains.

2. Materials and methods

2.1 Evolution-based strategy

The *Saccharomyces cerevisiae* wine strain UMCC 855, selected in our lab and deposited in the Unimore Microbial Culture Collection, has been chosen as parental strain for its good oenological aptitude (Gobbi *et al.*, 2014). Starting from this strain, the evolution-based strategy developed by De Vero *et al*. (2011) was applied using 10 mM of Mo(VI) as specific selective pressure. The molybdate, analogue of sulphate and toxic for the cells at high concentrations, has been chosen as indirect screening method for strains with an increased GSH production, exploiting the yeast common metal response that involve the GSH biosynthesis. The following selection has been carried out on YNB minimal medium plates supplemented with 0, 1, 2.5, 5 mM Mo(VI). The most resistance strains on molybdate have been tested for the production of GSH in two tests. In the first test the selected strains were inoculated into Erlenmayer flasks with 50 mL of chemically defined synthetic must (SM) prepared according to Giudici and Kunkee (1994). Cells, reached the early exponential phase, have been collected and the supernatant and the pellet have been separated and differently processed in order to analyse extracellular and intracellular GSH. The amounts of both, extra- and intracellular GSH, were quantified with the enzymatic Glutathione Assay Kit (Sigma-Aldrich) following the manufacturer's instructions. The extracellular content was expressed in mg L^{-1} at OD_{600} of 4, while the intracellular content was expressed as percentage of GSH (mg L^{-1}) divided by dry cells weight (mg L^{-1} at OD_{600} of 4). The second test consisted in a microvinification assay carried out in 250 mL sterile flask with pasteurised Trebbiano must. At the end of fermentation, extracellular GSH concentration was determined as previously described.

2.2 Evaluation of sulphur amino acids on GSH production

The total GSH production (extra- and intracellular) was also evaluated in SM with the addition of the sulphur amino acids methionine and cysteine. Parental strain, along with UMCC 2581 and 2585, selected as high producers of GSH in wine, and UMCC 2584, selected as low GSH producer in wine, were inoculated in tubes filled with 10 mL of SM with or without the addition of methionine and cysteine at the different concentrations of 0.01, 0.05 and 1 mM. These three different values correspond respectively to low, medium and high concentrations compared to the average concentration of methionine and cysteine found in musts. After 7 days of fermentation, cells and supernatant were collected and processed as previously described to assess the total GSH content.

2.3 Strains molecular characterization

The strain typing was performed through a multi-approach that included PCR amplification of the interdelta region and the $(GTG)_5$-fingerprint analysis. Genomic DNA was extracted from 5 mL of YPD (2% glucose, 2% peptone, 1% yeast extract) cultures using the standard phenol-chloroform extraction method as described by Hoffman and Winston (1987). PCR amplifications were carried out in 25 µL of reaction volumes containing 25 µg of yeast DNA and oligonucleotide primer of the delta family, for the amplification of the interdelta regions, and with primer $(GTG)_5$ for the amplification of the microsatellite regions (Legras and Karst, 2003; Baleiras Couto *et al.*, 1996). Amplification products were separated by electrophoresis run on 1.5% agarose gel for 2h at 80V. Karyotype analyses of parental and evolved strains were performed using pulse-field gel electrophoresis (PFGE). Chromosomes were first prepared from overnight cultures in agarose plugs and then separated with a CHEF DRII apparatus (Bio-Rad) as described by Solieri *et al.* (2015). Agarose gel was then stained with ethidium bromide, washed in TBE buffer and visualized under UV light.

2.4 RNA-Seq and genomic DNA sequencing

To capture gene expression changes between the parental and evolved strains, we performed RNA-Seq experiments. Along with UMCC 855 parental strain and UMCC 2581, 2584, 2585 also UMCC 2580 evolved strain has been included in this analysis as high intracellular GSH producer. Selected strains were inoculated in Erlenmayer flasks filled with 100 mL of SM as described above and cell growth was monitored by measuring the optical density at 600 nm hourly until reaching the end of exponential phase. At each time point the cells were harvested and centrifuged, then supernatant was removed and the pellet was immediately frozen in liquid nitrogen and stored at -80°C until sample analysis. For each sample, the time point corresponding to the ¾ of exponential phase was chosen and the total RNA was extracted with hot acidic phenol (Ausubel *et al.*, 2002). mRNA was purified from total RNA using Ambion Dynabeads mRNA Direct Micro Kit (Life Technologies) and RNA-Seq library was prepared from mRNA using Ion Total RNA-seq kit v2 (Life Technologies). The libraries were sequenced by Ion Torrent Proton instrument (Life Technologies). Bowtie 2 (Langmead and Salzberg, 2012) was used to align each sample's read to the reference genome, while DESeq (Anders and Huber, 2010) was used to measure differential expression.

Since the parental strain UMCC 855 is heterozygote, the identification of the mutations effectively involved in the different phenotypes through a general genomic comparison between parental and evolved strains, would be hard to carried out. To overcome this problem an approach similar to that proposed by Parts *et al.* (2011) had

been applied in order to map trait loci involved in the phenotype of interest (QTL mapping) and simplify the following analysis. Briefly, 69 spores were generated by dissection of the parental strain UMCC 855 tetrads. All the monosporic cultures (MSCs) were then subjected to a phenotypic test on the same condition used for the selection of the evolved strains and described above. According to the phenotype showed, the MCSs were clustered as similar to parental phenotype, similar to evolved phenotype or other phenotypes. Finally, genomic DNA was extracted from each MSC using the ZR fungal/bacterial DNA miniprep kit (Zymo Research), pooled in equimolar amount in the respective cluster and sequenced.

3. Results and Discussion

3.1 Evolution-based strategy

The application of the evolution-based strategy followed by a more stringent selection on minimal medium supplemented with molybdate, allowed a selection of eight evolved strains able to grown on 2.5 mM Mo(VI) (Fig.1).

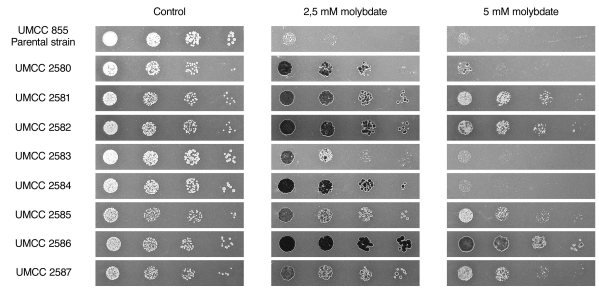

Figure 1 *Molybdate resistance screening. Parental and evolved strains were spotted on YNB plates containing 2% of glucose, 100 µL of ammonium sulphate and different concentrations of Mo(VI). Plates were scanned after 4 days of incubation at 27 °C. Image modified from Mezzetti et al. (2014).*

The 2.5 mM Mo(VI) concentration was chosen as reference concentration since the minimum concentration of 1 mM was not limiting for none of the strains tested (data not shown), while the maximum concentration of 5 mM was severely limiting for the majority of the evolved strains. The resistant strains selected were initially tested for their native ability to produce GSH in a chemically defined must. The highest intracellular GSH contents were reached by the evolved strains UMCC 2580 and 2585 (respectively 2.1 and 2.6-fold higher than UMCC 855), while the GSH contents for the other evolved strains were not statistically different compared to parental strain (Fig. 2a). On the other hand, the extracellular GSH values showed a great difference among all the evolved and the parental strain, that did not reach the detection limit of the kit used, with the only exception of UMCC 2586 (not detectable value) (Fig. 2a). The evaluation of GSH production on SM underlined the effectiveness of the applied strategy since all evolved strains were able to produce an increased GSH amount compared to parental strain, with the only exception of UMCC 2586 strain. The microvinification assay was carried out to further investigate the ability of the evolved strains to produce extracellular GSH in wine. At the end of fermentation process, UMCC 2581 and 2585 strains showed an increased GSH content of 100% and 36% respectively, compared to parental strain (Fig. 2b). These data are particularly notable if we consider that, contrary to what obtained for the intracellular content, the UMCC 2581 strain was one of the best extracellular GSH producer in SM underlined the importance of an extracellular excretion to obtain wine with an increased GSH content.

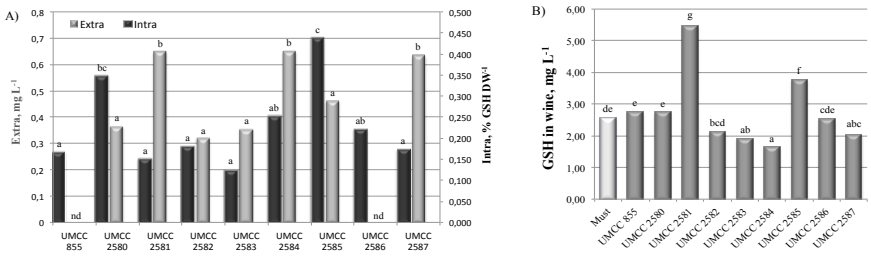

Figure 2 *GSH content in synthetic must and wine. (A) Intracellular (%) and extracellular GSH concentration in synthetic must. nd = not detected. (B) Glutathione concentration in wine. Graph shows means (n = 3) of UMCC 855 parental strain and evolved strains. Means with different letters are significantly different from each other as determined by Tukey's test (P< 0.05).*

3.2 Evaluation of sulphur amino acids on GSH production

How the oenological yeast strains react to stress situations during wine production is an important aspect for their future technological application. It is known that the high value of sulphates and nutritional deficiencies increase the production of undesirable sulphur compounds (Eschenbruch, 1974), but also that in the presence of sulphate as a nutrient, most of the excess sulphur is incorporated into GSH (Elskens *et al.*, 1991). To verify whether the evolved strains selected were able to store the excess of sulphur amino acids in GSH, tests in SM with the addition of cysteine and methionine were carried out. The strains tested showed a differentiated production of GSH in relation to the specific type of media (Fig. 3).

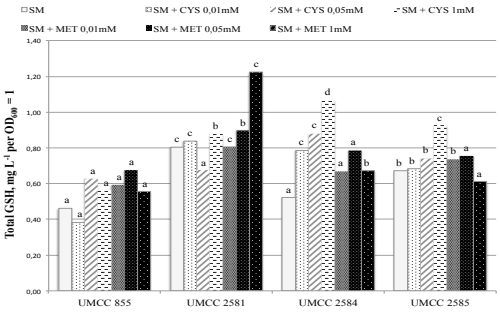

Figure 3 *GSH content in synthetic must with and without the addition of methionine and cysteine at different concentrations. Graph shows means (n = 3) of UMCC 855 parental strain and selected evolved strains. For each media, means with different letters are significantly different from each other as determined by Tukey's test (P< 0.05).*

Compared to parental strain, a constantly high amount of GSH was observed for the UMCC 2581 in almost all the tested media. Differently, for the strain UMCC 2584 a progressively increased production of GSH was achieved only with the addition of cysteine with an increase of 89% in the media with 1 mM of cysteine. An intermediate behaviour was obtained for the evolved strain UMCC 2585. The results suggest that the evolved strain UMCC 2581 is the most consistent in the expression of the desired phenotype almost regardless of the fermentation media.

3.3 Strains molecular characterization

The strain typing of parental and all evolved strains, was performed by interdelta region and $(GTG)_5$-fingerprint analyses. As expected, each evolved strain has shown a different electrophoretic profile compare to the parental strain and to the other evolved strains (data not shown).

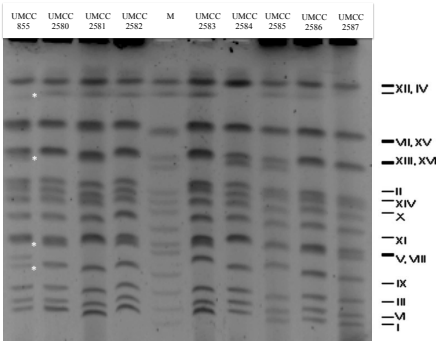

Figure 4 *Karyotype profiles of UMCC 855 and evolved strains. S. cerevisiae YNN 295 (Bio-Rad Laboratories) was used as reference strain (lane M). Polymorphic bands are marked with white stars.*

The karyotyping, performed by PFGE, underlined the effect of sexual recombination and the large capacity of genome reorganization through chromosome rearrangements of wine yeast (Borneman *et al.*, 2011). Indeed, the gel showed multiple polymorphic bands between parental and evolved strains corresponding to chromosomes IV, IV, XVI, V, VIII, IX (indicated by white star in Fig. 4).

3.4 Total RNA and genomic DNA sequencing

The main part of RNA-Seq and genomic DNA computational analyses are still under investigation. Nevertheless, the first results of RNA-Seq revealed that a great number of genes were differentially expressed between parental and evolved strains (Fig. 5). In particular, the differentially expressed genes in the evolved strains were 392 in UMCC 2581, 198 in UMCC 2580, 118 in UMCC 2584 and 86 in UMCC 2585. Among these, only 6 genes were significant in all four evolved strains.

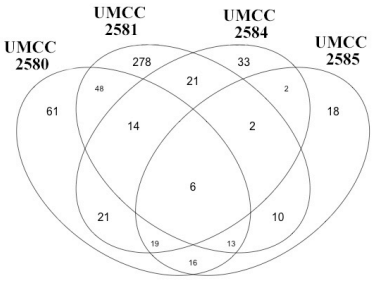

Figure 5 *A Venn diagram summarizing the overlap between the evolved strains gene called as differentially expressed from the parental strain (Negative Binomial Test, p values < 0.05). The number of differentially expressed genes shared by two or more evolved strains is indicated by the overlap between two or more circles.*

4. Conclusions and future perspectives

This study proved the effectiveness of the evolution-based strategy applied for the purpose to generate wine yeast strains with an enhanced GSH production. Furthermore, for the evolved strain UMCC 2581 the experimental results indicated a good stability of the desired phenotype in different media, making it the most suitable candidate for future technological applications.

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Selection of evolved wine yeast strains improved in glutathione production

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This report shows the main results of the activities developed during the PhD thesis project. An evolution-based strategy was applied on *Saccharomyces cerevisiae* 21T2 wine strain to obtain glutathione (GSH) high-producer strains. Eight evolved strains were selected after screening on minimal medium supplemented with molybdate, used as selective pressure. The novel yeasts were tested for the production of GSH in synthetic juice and in microvinification assay. We obtained two evolved strains, Mo21T2-5 and -12, able to enhance GSH content in wine with an increase of 100% and 36%, respectively, compared to the parental strain.

Selezione di ceppi di lievito enologici evoluti con alte produzioni di glutatione

Questo report presenta i principali risultati ottenuti nel corso del progetto di tesi di dottorato. Per ottenere lieviti con alte produzioni di glutatione (GSH), sul ceppo enologico di *Saccharomyces cerevisiae* 21T2 è stata applicata una strategia di evoluzione adattativa. Otto ceppi evoluti sono stati selezionati, dopo uno screening su terreno minimo addizionato con molibdato usato come pressione selettiva, e poi testati per la produzione di GSH in mosto sintetico e in esperimenti di microvinificazione. Abbiamo ottenuto due ceppi evoluti, Mo21T2-5 e -12, capaci di incrementare il GSH nel vino rispettivamente del 100% e del 36% in confronto al ceppo parentale.

Key words: *Saccharomyces cerevisiae*, evolution-based strategy, glutathione, sulfite, wine strains.

1. Introduction

The activities of the PhD thesis project described in this report are concerning the selection of novel wine yeast strains with high production of glutathione (GSH), according to the activities A2 (Evolution-based strategy) and A3 (System Biology approach) indicated in the previous report (Mezzetti, 2013). To achieve this aim, it has been applied an evolution-based strategy that combines sexual recombination of spores and application of molybdate Mo(VI), toxic for the cells at high concentration, as specific selective pressure (A2). The evolved strains were tested in synthetic must to evaluate the production of GSH and in Trebbiano grape must to evaluate their fermentation performance as well as the production of GSH and sulfites at the end of the microvinification assay (A3.1, A3.2 Microbiology and Metabolic analysis).

2. Materials And Methods

The evolution-based strategy, described by De Vero *et al.* (2011), was applied on the *S. cerevisiae* parental strain 21T2 by using 10 mM of Mo(VI) as specific selective pressure. The resistant strains obtained were, subsequently, screened on YNB minimal medium plates supplemented with 0, 1, 2.5, 5 mM Mo(VI). Selected strains were inoculated into Erlenmayer flask with 50 mL of defined synthetic juice. Cells grown until exponential phase were harvested and the supernatant and the pellet were separately processed to analyse the extracellular and intracellular GSH respectively. Total GSH (reduced and oxidized form) was quantified with the enzymatic Glutathione Assay Kit (Sigma-Aldrich) following the manufacturer's instructions. Intracellular GSH content was expressed as percentage (%) of GSH concentration (mg/L) divided by dry cell weight (mg/L at OD_{600} of 4). Microvinification assay was carried out under static conditions at 25 °C in 250 mL sterile flask filled with 200 mL of pasteurised Trebbiano grape juice without added sulfites. At the end of fermentation, an enzymatic kit (Megazyme) was used to quantify the concentration of total sulfites according to the manufacturer's instructions.

3. Results And Discussion

3.1 Selection of evolved strains resistant to molibdate

The screening on plates with YNB minimal medium and Mo(VI) allowed a stringent selection of the evolved strains. In fact, we observed a strong inhibition in the plates with Mo(VI) 2.5 mM where only eight evolved strains were able to grow.

3.2 GSH production in synthetic juice

The native capability to produce GSH of the parental 21T2 and the eight evolved strains was preliminarily evaluated in a chemically defined synthetic juice. The highest values of intracellular GSH content were observed for the strains Mo21T2-1 and -12 which were, respectively, 2.1 and 2.6-fold higher than 21T2 while the other strains were not statistically different compared to the parental strain (Fig. 1A). Extracellular GSH analysis allowed to underline a great difference among all the evolved strains (GSH concentrations ranging from 0.32 to 0.65 mg/L), with the exception of Mo21T2-13, and the 21T2 that did not reach the detection limit of the enzymatic kit (Fig. 1B). The assessment of intra- and extra-cellular GSH underlined the variation of the native capability of GSH production in evolved strains and the effectiveness of the applied strategy.

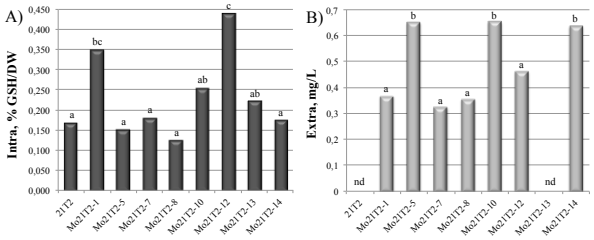

Fig. 1 *Intracellular and extracellular concentration in synthetic juice. (A) Intracellular GSH content (%). (B) Extracellular GSH concentration. nd = not detected. Graph shows means (n = 3) of 21T2 parental strain and evolved strain. Means with different letters are significantly different from each other as determined by Tukey's test (P< 0.05).*

3.3 GSH and sulfites production in microvinification assay

The microvinification assay was performed in order to evaluate the ability of parental and evolved strains to produce extracellular GSH in wine and to assess the relation between GSH production and sulfur metabolism. Mo21T2-5 and -12 strains showed a GSH amount increased of 100% and 36%, respectively, compared to parental strain (Fig. 2A). It was remarkable that the highest GSH-producing strain in wine, Mo21T2-5 was one of the best extracellular GSH-producing strain in synthetic medium, pointed out the importance of a homeostasis shifted toward extracellular excretion to obtain wine with an increased GSH content. Total sulfite analysis showed that all strains produced almost nil amount of sulfites with the exceptions of Mo21T2-5 and -12, which were the highest GSH producing strains in wine (Fig. 2B). We could assume that a fully activated sulfate assimilation pathway was involved in the increased GSH metabolism.

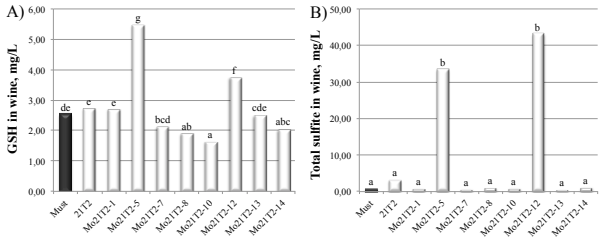

Fig. 2 *Glutathione and sulfite in wine. (A) Extracellular total GSH concentration and (B) Total sulfite detected after microvinification. Graph shows means (n = 3) of must, 21T2 parental strain and evolved strain. Means with different letters are significantly different from each other as determined by Tukey's test (P< 0.05).*

The evolution-based strategy applied has been successful for the purpose to generate yeast strains with an enhanced GSH production. Furthermore, Mo(VI) resistance has proved to be effective for selection of the desired evolved strains by activating, probably, the yeast common metal response that involve the GSH biosynthesis. In conclusion, the experimental results obtained agree with the expected ones. Moreover, since the GSH exerts protective effects on various aromatic compounds in wine (Kritzinger *et al.*, 2013), the research activities of this second year can be included in the original PhD project plan (A2.1, .2, .3). The future research activities will focus on the optimization of the fermentation media composition in order to identify the yeasts best conditions for high amount of GSH production. Further researches will be carry out to better understand the expression of the genes involved in GSH metabolism of the selected strains (A3.3, A3.4).

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Evolutionary strategies to improve *Saccharomyces cerevisiae* wine strains and their characterization through "systems biology" approaches

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The aim of this PhD research project is to apply evolution-based strategies for the development of adaptively evolved wine yeast strains. Evolutionary strategies might be particularly advantageous for selection of complex desirable phenotypes which involve many genes or more than one operon.
The combination of evolutionary strategies and systems biology approaches may contribute to advance knowledge of complex metabolic pathways and provide opportunities for the development of improved strains with the desirable phenotype.

Strategie evolutive per il miglioramento di ceppi enologici di *Saccharomyces cerevisiae* e loro caratterizzazione mediante approcci di "systems biology"

L'obiettivo di questo progetto di dottorato è lo sviluppo di ceppi enologici di lievito evoluti tramite l'applicazione di strategie basate sull'evoluzione adattativa. Le strategie evolutive sono particolarmente vantaggiose per la selezione di fenotipi complessi che coinvolgono molti geni o più di un operone.
Le strategie evolutive in combinazione con gli approcci di systems biology possono contribuire ad incrementare le conoscenze relative ai processi metabolici complessi e fornire nuove opportunità per lo sviluppo di ceppi ottimizzati con il fenotipo desiderato.

1. State-of-the-Art

Traditionally, 'clonal selection' process from spontaneous fermenting musts and wines has been the main method to select *Saccharomyces cerevisiae* wine strains with desirable traits. However it is hard to find wild strains with all required oenological and technological properties for winemaking. With the recent knowledge in genomic, proteomic and metabolic fields it has been possible to develop new genetic improvement strategies that are summarized in Table 1.

Table 1 *Summary of the genetic improvement strategies.*

Name	*Strategy*	*Aims*
Evolutionary engineering	Strategy based on adaptative evolution process: suitable selective pressure is applied to obtain desired phenotype.	Develop of evolved microorganisms with the desired phenotype.
Metabolic engineering	Manipulation (insertion\deletion of genes, promoter region modification) of a specific metabolic pathway by recombinant DNA technologies.	Inserting\modulating new or already present function.
Synthetic biology	Generate new biological function designing or redesigning, by recombinant DNA technologies, biological systems that not already exist in nature.	Improving or obtaining new function or desired phenotype.

A "systems biology" approach is the finest way to choose the best strategy to apply. Indeed "systems biology" is an inter-disciplinary method to study all relations and interactions in a complex biologic structure in order to understand and characterise the whole system. In particular the best strategy depends on genetic nature of desirable function (monogenic or polygenic), the knowledge of the genes involved and the aims of manipulation (Giudici *et al.* 2005). The most important traits in wine strains are multiple polymorphic loci (Quantitative Trait Loci, Marullo *et al.* 2004) and in this, or in similar cases, a whole genome approach is necessary. Furthermore genetically modified yeasts obtained through recombinant DNA technologies are subjected at many legal restrictions and consumers do not appreciate them. For these reasons evolutionary approaches has been preferred. An evolution-based strategy has been developed to combines random hybridization of spores and application of specific selective pressure to obtain wine yeasts with improved genetic mutation or recombination. With the aim to generate novel wine yeast strains characterized by low production of sulfites and H_2S or by high production of glutathione, resistance to heavy metals, toxic analogues of sulfate, was used as a high-throughput

and rapid screening method (De Vero *et al.* 2011). In the first PhD year, the best-evolved strains obtained with this strategy are selected and studied through inter-disciplinary "systems biology" approach. Microbiology and metabolic analysis are carried out to understand mechanisms involved in heavy metals resistance and to characterise phenotypic and quantitative traits of the evolved strains.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

Table 2 *Gantt diagram for the PhD research activities* (Planned period: September 2013-December 2015)

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
A1)	***Clonal selection and strains characterization***																												
A2)	***Evolution-based strategies***																												
	1) High terpenes production																												
	2) High aromatic thiols production																												
	3) High esters production																												
	4) Evolved strain selection																												
A3)	***Systems Biology approach***																												
	1) Microbiology analysis																												
	2) Metabolic analysis																												
	3) Genetic analysis																												
	4) Proteomic analysis																												
A4)	***Papers & Posters Preparation***																												
A5)	***PhD Thesis***																												

A1) Clonal selection and strains characterization; continuous activity consisting in yeast strains isolation from grape musts and wines. The isolated yeast strains are identified by phenotypical and molecular techniques and further characterised to evaluate their thecnological properties. The clonal selection allows the constitution of a bioresource and it is always a fundamental step in any yeast improvement genetic program.
A2) Evolution-based strategy for selecting wine strains improved in production of important compounds for wine aroma: terpenes (A2.1), aromatic thiols (A2.2) and esters (A2.3).
Evolved strains will be screened to choice the best strains which show the desired characteristics.
A3) Systems Biology approach to understand the cellular system involved in improvement of important traits responsible for wine aroma. Microbiology (A3.1) and Metabolic (A3.2) analysis are needed to characterise phenotypic and quantitative aspects of selected evolved strains; Genetic (A3.3) and Proteomic (A3.4) assays will be also carry out to investigate differences between parental and evolved strains in order to identify the main aspects amended and selected by evolution strategy.
A4) Paper & Poster Preparation: writing and editing of scientific papers, posters and oral communications at scientific meetings.
A5) PhD Thesis: writing of PhD thesis.

3. Selected References

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