# OENOCOCCUS OENI RESPONSE TO WINE STRESS: ROLE OF GLUTATHIONE AND THIOREDOXIN SYSTEMS

**Maria del Mar Margalef Català**

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Mar Margalef Català

# *Oenococcus oeni* response to wine stress: role of glutathione and thioredoxin systems

Doctoral Thesis

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This doctoral research was carried out between 2011-2016 in co-direction between the Lactic Acid Bacteria group of the Oenological Biotechnology research group (Department of Biochemistry and Biotechnology, Faculty of Oenology) at the Universitat Rovira i Virgili (URV) and the Food Microbiology Laboratory (Department of Biotechnology) of the University of Verona (Italy). The doctoral thesis was supervised by Prof. Cristina Reguant, Prof. Albert Bordons and the cotutelle of Prof. Sandra Torriani. The research was financially supported by Spanish Government projects AGL2009-07369ALI and AGL2012-07369 with the pre-doctoral grant from the URV. Apart from the longer stay in the group of the co-supervisor Prof. Sandra Torriani, another international phase was completed at the Institute Jule Guyot (Dijon, France) under the supervision of Prof. Jean Guzzo.

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WE STATE that the present study, entitled "*Oenococcus oeni* response to wine stress: role of glutathione and thioredoxin systems", presented by Maria del Mar Margalef Català for the award of the degree of Doctor, has been carried out under our supervision at the Department of Biochemistry and Biotechnology of Universitat Rovira i Virgili and at the Biotechnology Department of the University degli Study di Verona. This thesis is eligible to apply for the Degree of Doctor with International Mention.

Tarragona, 23rd August 2016

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Prof. Cristina Reguant Prof. Albert Bordons Prof. Sandra Torriani

"When you make the finding yourself – even if you're the last person on Earth to see the light – you'll never forget it".

"Quan fas el descobriment per tu mateix– tot i que siguis l'última persona de la Terra en veure la llum – no l'oblidaràs mai".

*Carl Sagan*

Per la meva mare i l'Alberto.

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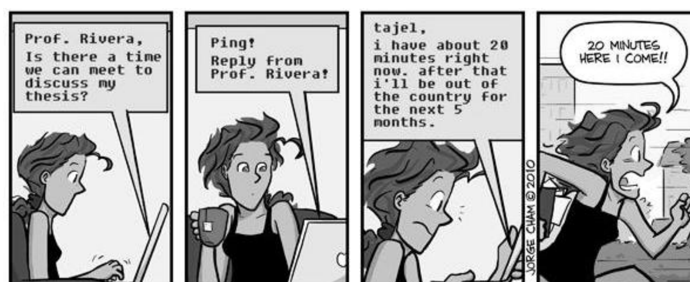


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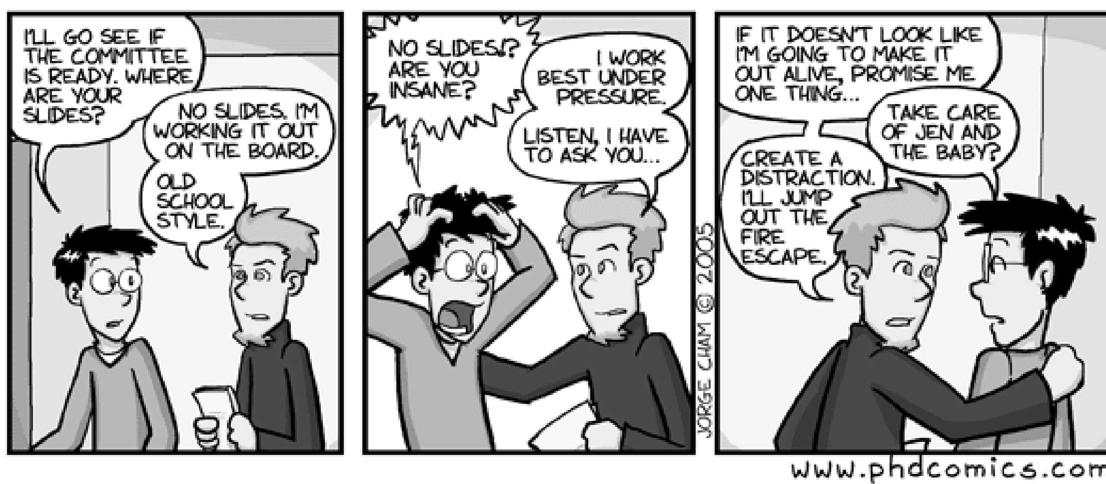


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# SUMMARY

*Oenococcus oeni* has been widely studied for its main role in malolactic fermentation (MLF) in red wine. One of the most relevant topics is its adaptation to wine conditions after the inoculation. The aim of this thesis was to perform a general study of *O. oeni* genes and proteins involved in this acclimation, focusing in two main redox systems, glutathione (GSH) and thioredoxin (Trx). Starting from a genomic approach of *O. oeni* pan-genome, seven plasticity regions were identified and some of them revealed potential candidate genes with interesting oenological relevance. Also, for the first time in *O. oeni*, two complementary proteomic techniques (2D-DIGE and iTRAQ labeling) together with a DNA microarray analysis were used to unveil the crucial first hours of adaptation before MLF starts. Results revealed the up regulation of a great number of genes and some corresponding proteins related to translation. On the other hand, carbohydrate metabolism was repressed leaving the activation of malate and citrate metabolism as the possible source of energy. Cell envelope genes and proteins were activated as a protective response and well known stress targets were identified in the analysis.

The redox systems GSH and Trx were identified in the "omics" analysis and were deepen studied for the first time in *O. oeni*. The uptake of GSH in *O. oeni* was defined as strain-dependent and the beneficial effect during growth was demonstrated. Also, the transcriptional analysis of the complete set of GSH genes was accomplished under wine conditions. Trx system was activated during MLF, being the *trxA3* the one with a higher over-expression in most strains tested. Trx genes are conserved among the different sequenced strains of *O. oeni*, except for one gene enclosed in a genetic horizontal transfer from *Lactobacillus*. However, this gene is still over-expressed in the strains which harboured it under MLF process. These results contribute to a better global view of the response of *O. oeni* during adaptation to wine conditions and the possible future use of those redox systems as targets for strain selection and differentiation.

# RIASSUNTO

*Oenococcus oeni* è una specie ampiamente studiata per il suo ruolo fondamentale nel condurre la fermentazione malolattica (FML) in vino rosso. Lo scopo di questa tesi è stato quello di studiare i geni e le proteine di *O. oeni* coinvolte in questo processo di adattamento, concentrandosi su due principali sistemi redox: glutatione (GSH) e tioredossina (Trx). Partendo da un approccio genomico di pan-genoma di *O. oeni*, sono state identificate sette regione di plasticità e alcune di esse hanno rivelato la presenza di potenziali geni candidati con interessante rilevanza enologica. Inoltre, per la prima volta in *O. oeni*, sono state usate due tecniche proteomiche complementari (2D-DIGE e iTRAQ), insieme con l'analisi microarray del DNA, per svelare le ore iniziali cruciali per l'adattamento prima dell'avvio della FML. I risultati hanno mostrato la sovra-espressione di un elevato numero di geni e di alcune corrispondenti proteine correlate al processo di traduzione e della parete e della membrana cellulare come risposta protettiva. Di contro, il metabolismo dei carboidrati è stato inibito, lasciando come possibile fonte di energia l'attivazione del malato e il metabolismo del citrato.

Nell'analisi "omica" sono stati identificati i sistemi redox GSH e Trx, che sono stati studiati in dettaglio per la prima volta in *O. oeni*. L'incorporazione di GSH in *O. oeni* è stata definita come ceppo-dipendente e ne è stato dimostrato l'effetto benefico durante la crescita. Inoltre, è stata effettuata l'analisi trascrizionale del set completo di geni del sistema GSH in vino. Per quanto concerne il sistema Trx, esso viene attivato durante la FML, e il gene *trxA3* è risultato quello più espresso nella maggior parte dei ceppi testati. I geni Trx sono conservati tra i diversi ceppi sequenzati di *O. oeni*, ad eccezione di un gene presente in un frammento coinvolto in un trasferimento orizzontale genetico da *Lactobacillus*. Tuttavia, questo gene è sovra-espresso nei ceppi durante la FML. I risultati di questa Tesi di Dottorato hanno contribuito ad avere una migliore visione globale della risposta di *O. oeni* durante l'adattamento alle condizioni del vino e hanno suggerito un eventuale uso futuro dei sistemi redox studiati come bersagli per la selezione e la differenziazione dei ceppi.

# RESUM

*Oenococcus oeni* ha estat estudiat pel seu paper en la fermentació malolàctica (FML) en vins negres. L'objectiu d'aquesta tesi era realitzar un estudi general dels mecanismes d'*O. oeni* involucrats en l'aclimatació a les condicions víniques, enfocant gran part del projecte en dos sistemes redox importants, glutatió (GSH) i tioredoxina (Trx). Tenint en compte els genomes disponibles d'*O. oeni* en la base de dades, han estat identificades set regions variables, algunes de les quals contenen gens importants a nivell enològic. Per primera vegada en *O. oeni*, s'han utilitzat dues tècniques proteòmiques (2D-DIGE i marcatge iTRAQ), juntament amb un xip d'ADN, per a l'anàlisi de les primeres hores d'adaptació abans de l'inici de la FML. S'ha observat un augment en la expressió de gens i proteïnes relacionats amb la funció de traducció i una repressió del metabolisme dels carbohidrats, que deixaria l'activació del metabolisme de l'àcid màlic i cítric com a possible font d'energia. A més a més, s'han identificat gens i proteïnes relacionats amb l'embolcall cel·lular i gens diana relacionats amb l'estrés cel·lular ja coneguts, que s'activen com a resposta a les condicions víniques. Els sistemes redox GSH i Trx s'han identificat en l'anàlisi transcriptòmic i proteòmic i per primer cop en *O. oeni* s'ha aprofundit en el seu estudi. La captació de GSH en *O. oeni* s'ha definit com a soca-dependent i ha quedat demostrat el seu efecte beneficiós durant el creixement. Així mateix, s'ha assolit en diferents soques l'anàlisi de l'expressió de tots els gens del sistema GSH en condicions víniques. El sistema Trx s'ha descrit com activat durant la FML, essent el gen *trxA3* el més sobreexpressat en la majoria de soques provades. S'ha pogut comprovar que els gens Trx estan ben conservats entre els diferents genomes seqüenciats d'*O. oeni*, excepte el gen *trxA1* comprès en una regió del genoma provinent d'una transferència horitzontal de *Lactobacillus*. No obstant, aquest gen es segueix expressant al final de la FML en les soques que el contenen. Els resultats d'aquesta tesi contribueixen a una millor visió global de la resposta d'*O. oeni* a les condicions del vi i a un possible ús dels sistemes redox com a objectius per la selecció de soques i la seva diferenciació.

# 1. INTRODUCTION

## 1.1 *Oenococcus oeni* and the malolactic fermentation

Microorganisms are gaining more importance day by day at medicinal and industrial levels. Nowadays, science is aware of the vital impact of microbiological pathways and their role in industrial processes. Natural fermentations have been key to human development and are probably the oldest form of food preservation. In addition to preservation and providing variety to the diet, there are further important consequences of this process, such as the organoleptical changes of the final product. In fact, wine is the product of two fermentations, the alcoholic and the malolactic fermentation carried out by yeasts and lactic acid bacteria, respectively. Expanding our knowledge in all the steps of the process and deeper into the proper action of each microbial actor is important to improve the quality of the final product.

### 1.1.1 Wine and lactic acid bacteria

In the Neolithic village of Jiahu in China, circa 7000 BC, is found the first evidence of production of fermented beverages (McGovern et al., 2004). For winemaking, the earliest evidence is traced to Iran at the Hajji Firuz Tepe site (5400-5000 BC) (This et al., 2006). During the centuries, vineyards and grape wine production gradually spread to adjacent regions and this fermentative process provoked fortuitous domestication of several species (Fay and Benavides, 2005). Winemaking is a microbiological process carried out by a community of yeast and bacteria. After the end of the alcoholic fermentation carried out by yeast, lactic acid bacteria (LAB) perform the malolactic conversion in most wines, especially red wine. LAB are responsible for many fermented foods and they have been isolated from wine at various states of vinification (du Plessis et al., 2004; Gindreau et al., 2001). These bacteria normally undertake the malolactic fermentation (MLF) spontaneously. However, LAB viability depends on the capacity of the cell to adapt to wine conditions. The process is standard for most red wine production and common for some white grape varieties such as Chardonnay, due to the improved organoleptic changes (Gambetta et al., 2014). Even though the existence of

other wine species as *Lactobacillus*, *Pediococcus* and *Leuconostoc* (Wibowo et al., 1985), and despite the fact that recent red wine MLF trials with strains of *Lactobacillus plantarum* (Bravo-Ferrada et al., 2013; Lerm et al., 2011) have been performed, *Oenococcus oeni* (Carr et al., 2002) is the principal bacterium responsible for MLF (Lonvaud-Funel, 1999). As its name implies, *Oenococcus oeni* holds major importance in the field of oenology due to the organoleptic and microbiological changes produced in wine.

### 1.1.2 *O. oeni* characteristics

*Oenococcus oeni*, formely called *Leuconostoc oenos* (Dicks et al., 1995), is a LAB, so it belongs to the phylum *Firmicutes*. This Gram positive bacterium is microaerophilic, meaning that it grows best at low oxygen concentrations, obligately heterofermentative (glucose is fermented to D-(-)-lactic acid, $CO_2$, and ethanol or acetate) and acidophilic (Dicks et al., 1995). The pan-genome of *O. oeni* presents low GC% content, as with other Gram positive bacteria, and it has a small size (1.8 Mb) compared with other LAB species like *Lactobacillus plantarum* (3.3 Mb) or the main model of Gram positive bacteria *Bacillus subtilis* (4.1 Mb). To date, August 2016, 62 different genomes of *O. oeni* are available in the database of NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/). PSU-1 strain was the first sequenced genome from this species (Mills et al., 2005) and nowadays it is the only complete annotated genome published.

Such as other wine LAB, *O. oeni* has fastidious nutritional requirements. However, this species, due to selective pressure, has developed adaptive strategies that enable it to out-compete other potential MLF bacteria during the later stages of vinification and thus dominate in wine. It is, for example, well adapted to high ethanol concentrations (<15% v/v), low pH (as low as 2.9) and limited nutrient availability (Bartowsky, 2005). Marcobal et al. (2008) suggested that the genus *Oenococcus* is hypermutable due to the loss of the mismatch repair pathway (genes *mutS* and *mutL*), which occurred with the divergence away from the *Leuconostoc* branch. This would explain the observed high level of allelic polymorphism (de Las Rivas et al., 2004; Delaherche et al., 2006; Zé-Zé et al., 2008) among known *O. oeni* isolates and likely contributed to the high ecological competitiveness of this genus to acidic and alcoholic environments. Also, the absence of genes for sporulation, catalase, and other key enzymes of oxidative stress response (e.g.,

superoxide dismutase) make the study of this genus interesting because of its capacity to survive harsh environments using other mechanisms.

### 1.1.3 Performance of MLF by *O. oeni*

Yeast and bacteria are both naturally present on grape skins, but are also found in barrels, tanks and the equipment used during vinification (Renouf et al., 2006). Yeasts are better adapted than LAB to grow in grape musts, which are very high in sugar concentrations (>210 g/L) and have a low pH 3.0–3.3. When all reducing sugars are fermented to ethanol, yeast levels decline and LAB growth occurs. Depending on the wine production area, the composition of L-malic acid will be different, from 2 to 10 g/L (Lonvaud-Funel, 1999). The conversion of L-malic acid carried out by *O. oeni*, starts when its population reaches $10^6$ CFU/ml after the alcoholic fermentation. *O. oeni* leads to the transformation of L-malic acid to L-lactic acid through an enzymatic decarboxylation (Peynaud and Domercq, 1968). Also, during its activity in wine, *O. oeni* cells can ferment residual sugars, hexoses and pentoses left by yeasts (Ribéreau-Gayon et al., 2006). Moreover, the production of secondary metabolites increases the sensory qualities of the final product (Malherbe et al., 2012; Sumby et al., 2010).

For wine, as it can be seen in Figure 1A, apart from its deacidification, other benefits have been attached to MLF as the removal of a potential carbon source for spoilage bacteria, which imparts microbial stability to wine (Maicas et al., 1999) and the decrease in titratable acidity, which can influence the sensory properties of wine by decreasing sourness (de Revel et al., 1999). The responsibility for the conversion of malate to lactate is a single enzyme, malate decarboxylase (MleA), which is often referred to the malolactic enzyme (Kunkee, 1991). This conversion indeed produces $CO_2$ which escapes from wine by bubbling. While the active transport of L-malic acid into the cell is performed by malate permease (MleP), the lactate transport out of the cell is still unclear. Moreover, all of the MLF process is regulated by a regulatory protein, MleR (Betteridge et al., 2015). As it is shown in Figure 1B the energy obtained through the use of malate produces a sufficient proton motive force (PMF) for the synthesis of ATP. The increase in the intracellular pH produced by MLF will be discussed in detail in section 1.2.3.

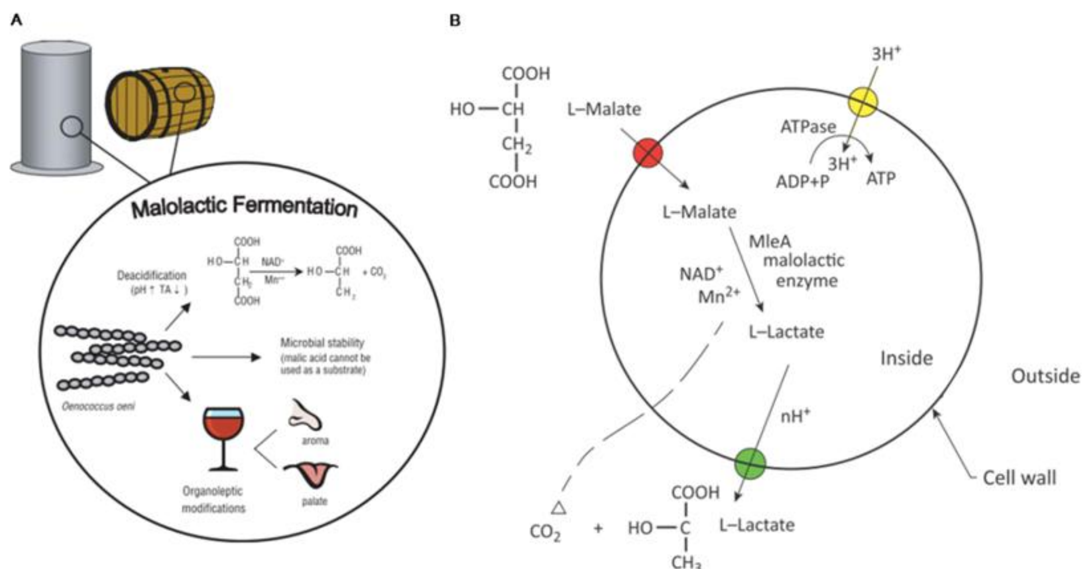


**Figure 1 A)** The three main consequences of MLF: microbial stability of the wine through the removal of a possible carbon source (L-malic acid) for other microorganisms, deacidification of wine (increase in pH [0.1–0.2 units] and decrease in titratable acidity [TA]), and bestows sensory changes (aroma and palate) in the wine (Bartowsky, 2005). **B)** MLF involves the active transport of L-malic acid into the cell by malate permease (MleP, red). Decarboxylation of L-malic acid is facilitated by the malolactic enzyme (MleA) and requires $NAD^+$ and $Mn^{2+}$ as cofactors before lactate is finally transported out of the cell (green). This process is controlled by a regulatory protein, MleR. The increase in the intracellular pH by MLF confers an energy advantage to the cell. The resulting increase in the proton motive force across the cell membrane combined with specific ATPases (yellow) facilitates the production of ATP (Betteridge et al., 2015).

Over centuries of selective pressure, *O. oeni* has adapted to high ethanol concentrations (<15% v/v), low pH (as low as 2.9) and limited nutrient availability; hostile conditions typical of wine. However, despite being *O. oeni* the species more adapted to wine, the induction of this fermentation remains problematic (Reguant et al., 2005). The introduction of commercial freeze-dried bacterial cultures of *O. oeni* for direct inoculation into wine has improved the control of the process ensuring better control of the time of onset and the rate of MLF (Nielsen et al., 1996).

*O. oeni* starters deal with several stresses including low temperature, $SO_2$ concentration, short-chain fatty acid presence, phenolic compounds, low pH and ethanol content (Davis et al., 1988; Lonvaud-Funel, 1999; Spano and Massa, 2006). The action of all these factors on *O. oeni* has been studied in order to enhance the knowledge of the

cellular adaptation this bacterium. Low temperatures affect growth rate and increase lag phase (Fugelsang, 1997). Sulphur dioxide reduces ATPase activity and decreases cell viability (Carreté et al., 2002; Reguant et al., 2005), while phenolic compounds produce breakdown of the LAB cell membrane (García-Ruiz et al., 2011). Yeast fatty acids such as decanoic and dodecanoic acid are powerful inhibitors of LAB growth because, like ethanol, they alter the bacterial membrane (Lonvaud-Funel et al., 1988). Finally, low pH reduces *O. oeni* growth and malolactic activity (Tourdot-Maréchal et al., 1999). Other difficulties in MLF have been ascribed to phage attack (Gindreau and Lonvaud-Funel, 1999; Poblet-Icart et al., 1998). However, as the phages readily disappear through inactivation by wine components, it seems that they are not responsible for influencing MLF (Lonvaud-Funel, 1999). Therefore, a better knowledge of stress physiology may be useful to optimise survival of starter cultures of *O. oeni* and improve the control of MLF in the wine industry (Beltramo et al., 2006).

## 1.2 Stress response of *Oenococcus oeni* cells

Various bacterial mechanisms recognise different environmental changes and can trigger an appropriate response, which commonly is a simultaneous reaction to a wide variety of stresses. Also, the various cellular systems interact with each other by a complex global regulatory network leading the cell to equilibrium under different conditions.

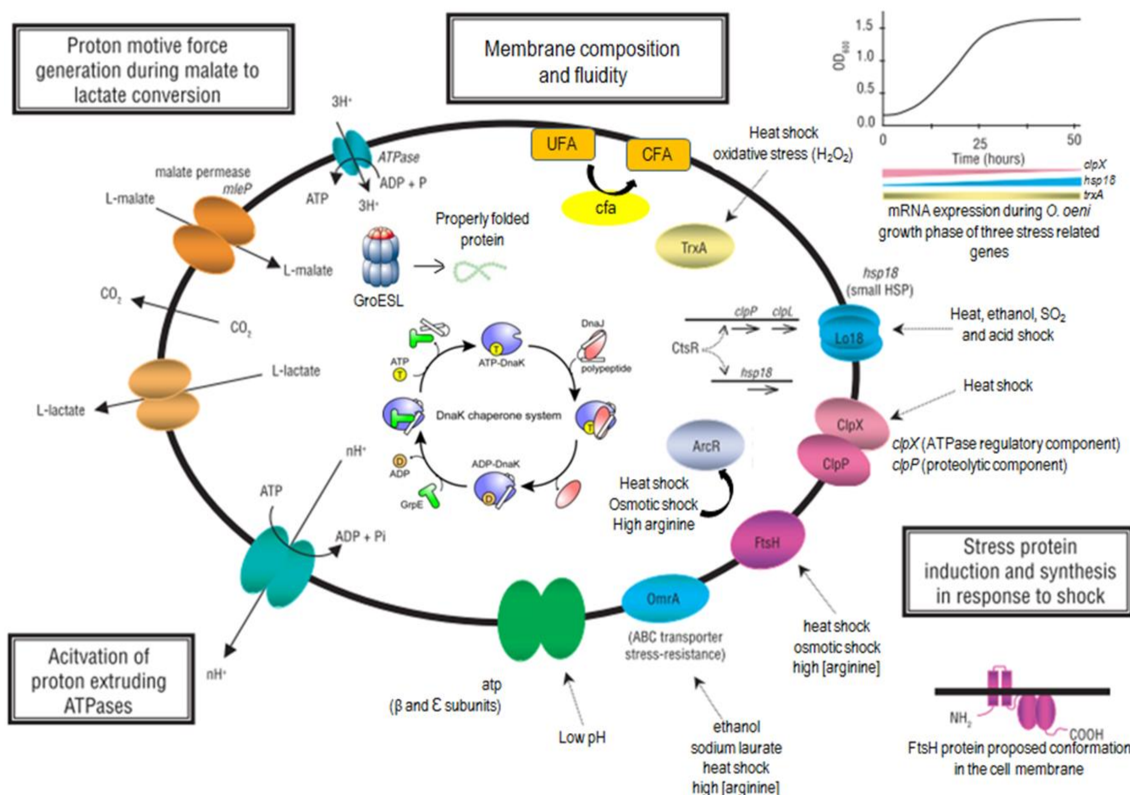


**Figure 2** Mechanisms that are important in conferring, in *O. oeni*, the ability to survive in wine: the membrane composition, the proton motive force generated by malic acid metabolism, the activation of proton-extruding ATPase, and the stress protein induction and synthesis in response to shock. Adapted from Bartowsky (2005).

As mentioned, the multiple stresses contained in wine provoke a complex response of *O. oeni*. Among the stress response mechanisms of *O. oeni* (Figure 2), in this section, it will be considered mainly the effects of two wine parameters , ethanol and low pH, due to their relevant inhibitory effect on *O. oeni*. The ethanol toxicity is generally attributed to the partitioning of ethanol in the hydrophobic lipid bilayer, resulting in a disruption of membrane structure that adversely affects many membrane-associated processes

(Lonvaud-Funel et al., 1988). Low pH appears as a crucial parameter that limits bacterial growth in wine and consequently the desired MLF activity. Moreover, low pH is linked to two requirements for *O. oeni* to survive and function in wine: the activation of a proton-extruding ATPase and the proton motive force (PMF) generated by MLF. The ATPase membrane system is well known for its key role in the acid tolerance of bacteria. Induction of its activity and expression has been associated with increased resistance to low pH (Kobayashi, 1985) due to the maintenance of the intracellular environment and the control of the energetic status of the cell through the PMF. Another important requirement for *O. oeni* cell is the activation of several genes and their corresponding synthesis of stress proteins.

Table 1 summarises most of the studies carried out for *O. oeni* genes involved in adaptation and stress response. Most of them encode for chaperones or are involved in the metabolism of carbohydrates or organic acids. Basically, the expression data in Table 1 has been measured by using RT-qPCR or Northern Blot techniques to unveil the induction of stress related genes under wine conditions, mainly under low pH and presence of ethanol. However, some of the listed references used latest transcriptomic techniques as DNA microarrays. Also, the data available from the few proteomic analysis made on *O. oeni* has been reviewed.

**Table 1** Studies reporting genes of *O. oeni* involved in adaptation and stress response to wine conditions.

| Gene | Gene annotation[a] | Reference | Regulation[b] | Stress involved[c] |
|---|---|---|---|---|
| *ackA* | acetate kinase | Olguín et al. (2009) | ↑ T | low pH and EtOH adapt. |
| *alsS* | acetolactate synthase | Olguín et al. (2009) | ↑ T | low pH and EtOH adapt., during after MLF |
| *alsD* | α-acetolactate decarboxylase | Olguín et al. (2009) | ↑ T | low pH and EtOH adapt., during MLF |
| *arcA* | arginine deiminase | Tonon et al. (2001) | ↑ T | arginine supply |
| *arcB* | ornithine transcarbamylase | Tonon et al. (2001) | ↑ T | arginine supply |
| *arcC* | carbamate kinase | Tonon et al. (2001) | ↑ T | arginine supply |
| *arcR* | regulatory protein, ADI pathway | Tonon et al. (2001) | ↑ T | arginine supply |
| | | Bourdineaud (2006) | ↓T / ↑ T | low pH / HS, OS, arginine supply |
| *atpα* | ATP synthase subunit α | Fortier et al. (2003) | ↑ T | low pH |
| *atpβ* | ATP synthase subunit β | Beltramo et al. (2006) | ↑ T | adaptative response at low pH |
| *cfa* | cyclopropane-fatty-acyl-phospholipid synthase | Beltramo et al. (2006) | ≈ T / ↑ T | low pH, inoculation to WLM |
| | | Grandvalet et al. (2008) | ↑ T | low pH, EtOH, Sp |
| *citE* | citrate lyase | Olguín et al. (2009) | ↑ T | low pH and EtOH adapt. |
| | | Costantini et al. (2015) | ↓ P | WLM adapt. EtOH 8% |
| *citI* | citrate lyase | Olguín et al. (2009) | ↑ T | low pH and EtOH adapt. |
| *clpC* | ATP-dependent Clp protease ATP-binding protein | Grandvalet et al. (2005) | | cotranscribed with the upstream *ctsR* |
| | | Olguín et al. (2015) | ↓ P | growing with EtOH |
| *clpP* | ATP-dependent Clp protease proteolytic subunit | Beltramo et al. (2004) | ↑ T | HS, EtOH shock |
| | | Desroche et al. (2005) | ↑ T | HS |
| | | Costantini et al. (2015) | ↑ T | WLM adapt. EtOH 12% |

| | | | | |
|---|---|---|---|---|
| *clpL1* | ATP-dependent Clp protease ATP-binding protein | Beltramo et al. (2004) | ↑ T | at Sp |
| | | Desroche et al. (2005) | ↑ T | HS, at Sp |
| | | Beltramo et al. (2006) | ↑ T | adaptative response at low pH |
| *clpL2* | Clp protease ClpX | Grandvalet et al. (2005) | ↑ T | HS, EtOH, low pH |
| | | Desroche et al. (2005) | ↑ T | HS, at Sp |
| | | Beltramo et al. (2006) | ↑ T | adaptative response at low pH |
| | | Costantini et al. (2015) | ↑ P | WLM adapt. EtOH 12% |
| | | Olguín et al. (2015) | ↓ P | growing with EtOH |
| *clpX* | ATP-dependent protease | Jobin et al. (1999b) | ↑ T | in the early phase of growth, HS |
| | | Guzzo et al. (2000) | ↑ T | at exponential phase |
| | | Desroche et al. (2005) | ↑ T/ ↓ T | HS / at Sp |
| *ctsR* | transcriptional repressor | Desroche et al. (2005) | ↑ T | HS |
| | | Grandvalet et al. (2005) | ↑ T | HS, EtOH, low pH |
| | | Beltramo et al. (2006) | ≈ T | during MLF |
| *ddl* | D-alanine--D-alanine ligase | Silveira et al. (2004) | ↑ P | EtOH adapt. |
| *dnaK* | molecular chaperone DnaK | Olguín et al. (2015) | ↓ P | growing with EtOH |
| *dnaJ* | molecular chaperone DnaJ | Grandvalet et al. (2005) | | |
| *ftsH* | cell division protein FtsH | Bourdineaud et al. (2003) | ↑ T | HS, OS |
| | | Bourdineaud (2006) | ↑ T | arginine supply |
| *ggpps* | geranylgeranyl pyrophosphate synthase | Cafaro et al. (2014) | ↑ T | EtOH shock |
| *groES* | molecular chaperone GroES | Grandvalet et al. (2005) | ↑ T | HS and EtOH shock |
| | | Desroche et al. (2005) | ↑ T | HS |
| *groEL* | molecular chaperone GroEL | Costantini et al. (2015) | ↓ P | WLM adapt. EtOH 8% |

| | | | | |
|---|---|---|---|---|
| *grpE* | protein GrpE | Grandvalet et al. (2005) | ↑ T | HS, EtOH and low pH |
| | | Desroche et al. (2005) | ↑ T | HS |
| *hdcA* | histidine decarboxylase | Beltramo et al. (2006) | not expressed | adaptative response at low pH |
| *hsp18* | heat-shock protein Hsp20 (Lo18) | Jobin et al. (1997) | ↑ T | transition to Sp, HS, low pH, sulphite, EtOH |
| | | Guzzo et al. (1997) | ↑ T | EtOH, low pH |
| | | Guzzo et al. (2000) | ↑ T | entrance to Sp |
| | | Desroche et al. (2005) | ↑ T | HS, at Sp |
| | | Beltramo et al. (2006) | ↑ T | adaptative response at low pH |
| | | Darsonval et al. (2015) | ↑ T | HS, low pH |
| | | Olguín et al. (2015) | ↑ T | EtOH shock |
| | | Costantini et al. (2015) | ↑ P | WLM adapt. EtOH 12% |
| *maeP* | malate permease | Olguín et al. (2009) | ↑ T | combined low pH and EtOH adapt. |
| | | Costantini et al. (2015) | ↓ T | WLM adapt. EtOH 8% / 12% |
| *mleA* | malolactic enzyme | Beltramo et al. (2006) | ↑ T | low pH, inoculation to WLM |
| | | Costantini et al. (2015) | ↑ T | WLM adapt. EtOH 8% / 12% |
| *mleP* | malate transporter | Augagneur et al. (2007) | ↑ T | at low pH with the presence of L-malate |
| | | Costantini et al. (2011) | ↑ T | after rehydration |
| | | Costantini et al. (2015) | ↑ T | WLM adapt. EtOH 8% / 12% |
| *omrA* | multidrug ABC transporter | Bourdineaud et al. (2004) | ↑ T | HS and OS |
| | | Bourdineaud (2006) | ↑ T | arginine supply |
| *rmlB* | dTDP-glucose 4,6-dehydratase | Silveira et al. (2004) | ↑ P | EtOH adapt. |
| *tig* | trigger factor | Jobin et al. (1999b) | ≈ T | HS and the addition of chloramphenicol |
| *trxA* | thioredoxin | Guzzo et al. (2000) | ≈ T / ↑ T | all stages of growth / HS |

| | | | |
|---|---|---|---|
| | Jobin et al. (1999a) | ≈ T / ↑ T | all growth stages / hydrogen peroxide, HS |
| | Beltramo et al. (2006) | ↑ T | adaptative response at low pH |
| | Costantini et al. (2015) | ↓ T / ↑ T | WLM adapt. EtOH 8% / 12% |

ADI: Arginine Deiminase

[a] Based on PSU-1 genome

[b] Regulation: Transciptomic and Proteomic Profile; ≈ no significant variation in the gene expression; ↑T/P: gene/protein induced expression, ↓ T/P gene/protein repressed expression

[c] EtOH: ethanol; adapt.: adaptation; Sp: stationary phase; WLM: wine like media; HS: heat shock; OS: osmotic shock; MLF: malolactic fermentation

### 1.2.1 Membrane composition

Cell membranes protect and organise cells. All cells have a cytoplasmic membrane which regulates not only what enters the cell, but also how much of any given substance comes in. This selective barrier has a specialised structure that facilitates its gatekeeping function, maintains homeostasis and consequently has an important role in adaptation to environmental changes (Šajbidor, 1997). Its composition of proteins and lipids makes the membrane a good target for ethanol toxicity. The motion of phospholipid molecules within the lipid bilayer can be rotational or lateral and it is named fluidity. Also, this motion depends on the classes of phospholipids present, their fatty acid (FA) composition and the degree of unsaturation of the acyl chains. Lipid packing can affect the rotation and diffusion of proteins and other biomolecules within the membrane, thereby affecting their functions. Ethanol toxicity is now generally attributed to its interaction with membranes at the aqueous interface, resulting in a perturbed membrane structure and function (Weber and de Bont, 1996) which leads to a leakage of intracellular compounds, including enzymatic cofactors and ions essential for cell growth and fermentation as well as dissipation of the electrochemical gradient (da Silveira et al., 2002). As a result, membrane composition is adjusted to counteract the increase in membrane fluidity, like changing phospholipid content (Grandvalet et al., 2008; Teixeira et al., 2002).

The membrane phospholipids incorporate FA of varying length and saturations. The lipid structure and the portion of saturated (SFA), unsaturated (UFA) and cyclopropane fatty acids (CFA) influence the fluidity of biological membranes. Low pH produced an increase of the ratio of UFA to SFA in membranes of *O. oeni* (Drici-Cachon et al., 1996a). On the other hand, *O. oeni* cells respond to ethanol by increasing their CFA content, specifically lactobacillic acid (Teixeira et al., 2002), by activating the gene ***cfa*** (encoding a CFA synthase) responsible for the conversion of UFA to CFA (Grandvalet et al., 2008). The *cfa* gene been related to the presence of ethanol and low pH (Grandvalet et al., 2008). CFAs in the membrane were suggested to reduce proton permeability (da Silveira et al., 2002) and increase membrane rigidity (da Silveira et al., 2003).

The fluidity of a membrane has been measured for *O. oeni* using electro spin resonance (ESR) (da Silveira et al., 2003) and fluorescence anisotropy (Chu-Ky et al., 2005;

Couchenev et al., 2005; Maitre et al., 2012; Tourdot-Maréchal et al., 2000; Weidmann et al., 2010). In ESR, rotational correlation time of spin probes is used to characterise how much restriction is imposed on the probe by the membrane. Fluorescence anisotropy measures the light emitted by a fluorophore inserted (probe) in the membrane (anisotropy value). The steady-state anisotropy of the probe gives information about the microstructure of the membrane. The first work focusing on the role of the membrane fluidity as a determinative factor in stress tolerance on *O. oeni*, presented the instantaneous variations of anisotropy values caused by heat (42ºC), acid (pH 3.2) and ethanol (10% v/v) shocks (Tourdot-Maréchal et al., 2000). Heat or acid shocks decreased the anisotropy values (fluidising effects), whereas an ethanol shock increased the membrane rigidity. Chu-Ky et al. (2005) (Figure 3) showed that cold shocks strongly rigidified plasma membrane. Ethanol shocks (10–14% v/v) induced instantaneous membrane fluidisation followed by rigidification and acid shocks (pH 4.0 and pH 3.0) exerted a rigidifying effect on membrane without affecting cell viability.

Other membrane fluidisation studies (Coucheney et al., 2005; Maitre et al., 2012; Weidmann et al., 2010) have been focused in the role of the small heat shock protein (sHsp) **Lo18** in the membrane. sHsps bind membranes to regulate bilayer fluidity (Horváth et al., 2008; Nakamoto and Vígh, 2007) and Lo18, under ethanol exposure, was shown to interact with the cytoplasmic membrane and to stabilise liposome fluidity (Coucheney et al., 2005).

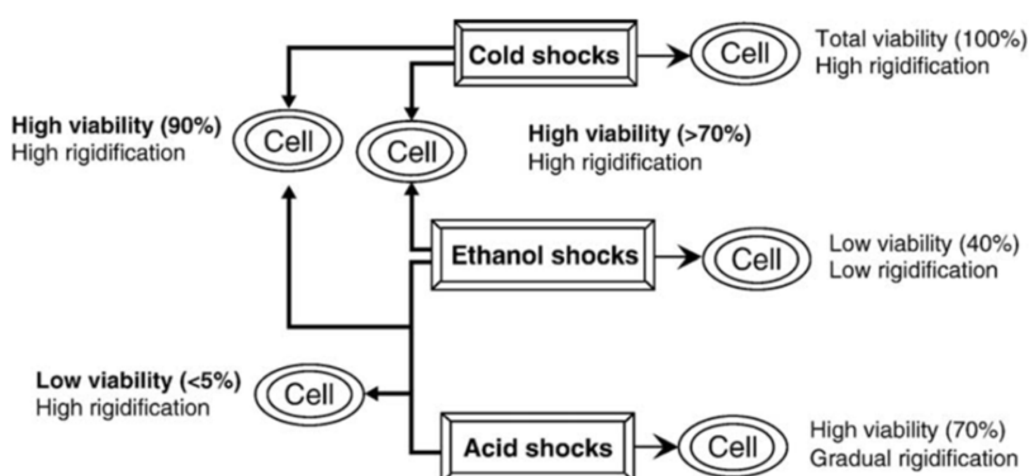


**Figure 3** Schematic representation of effects of single and combined cold, ethanol and acid shocks on the physical state of the membrane and on the cell viability of *O. oeni* (Chu-Ky et al., 2005).

Apart from the fluidity experiments, the ethanol effect has been widely studied and alterations in protein membrane patterns of *O. oeni* cells stressed with 12% ethanol for 1 h and cells grown in the presence of 8% ethanol have already been reported (Silveira et al., 2004). The enzymes dTDT-glucose-4,6-dehydratase (encoded by ***rmlB***) and D-alanine--D-alanine ligase (encoded by ***ddl***), were increased in ethanol-adapted cells. These enzymes are known to be involved in lipopolysaccharide and peptidoglycan biosynthesis, respectively, suggesting that the cell wall is modulated during ethanol adaptation (Silveira et al., 2004). Moreover, Elahwany (2012) reported an increase in the intracellular levels of amino acids under ethanol stress and aggregation of bacterial cells.

### 1.2.2 ATPase system

ATP synthase or ATPase is a membrane-bound enzyme complex and ion transporter which combines ATP synthesis and/or hydrolysis with the transport of protons across the membrane. Using the proton motive force (PMF) produced in the cell, the ATPase generates ATP (Poolman et al., 1991). Depending on the particular organism and the conditions of growth, the enzyme functions in the direction of either ATP synthesis or ATP hydrolysis (Futai and Kanazawa, 1983). ATPase is well known for its key role in the acid tolerance of bacteria. Induction of the activity and expression has been associated with increased resistance to low pH (Kobayashi, 1985; Kullen and Klaenhammer, 1999). Cox and Henick-Kling (1989) proposed that the malolactic conversion of L-malic acid to L-lactic acid, which is not energetic by itself, provides a proton motive force that is sufficient to drive ATP synthesis by membrane ATP synthase.

Currently, in the genome of *O. oeni* strain PSU-1 there are annotated several genes for the ATPase subunits. Due to partial cloning of the genes encoding the β-subunit and the Ɛ-subunit, a typical complex $F_1F_0$-ATPase genetic organisation in *O. oeni* has been suggested (Fortier et al., 2003): a $F_1$ cytoplasmic complex (α, β, δ, Ɛ and γ) which contains the catalytic site for ATP hydrolisis and a $F_0$ integral membrane complex (A, B and C) which forms a proton channel. The same authors reported that in the absence of malic acid, ATPase of *O. oeni* is induced at low pH and its regulation seems to occur at transcription level. In *O. oeni*, no significant increase of ATPase activity was measured at low pH although, mutants of the $F_1F_0$-ATPase isolated during a long term survival

screen at pH 2.6, were able to grow in acidic media and were characterised by a high H+-ATPase activity at low pH (Drici-Cachon et al., 1996b). In addition, preliminary results suggested the existence of several ATPases with different optimum pHs (Guzzo et al., 2000) and a K+-ATPase involved in pH homeostasis (Guchte et al., 2002).

Tourdot-Maréchal et al. (1999) suggested that ATPase and malolactic activities of *O. oeni* are linked to each other and play a crucial role in the mechanism of resistance to an acid stress. This link was demonstrated later by Galland et al. (2003). On the other hand, ATPase activity can be inhibited by $SO_2$, pesticides as copper and fatty acids; moreover, this effect increases with ethanol presence (Carreté et al., 2002).

Although in all LAB studied the bidimensional proteomic analysis of logarithmic growth an adaptive response always revealed all or some of the subunits of the cytoplasmic F1 complex among the up-regulated proteins (Champomier-Vergès et al., 2002), there is still no evidence in *O. oeni*. The γ chain of the $F_0F_1$-ATPase from *Lactobacillus plantarum* 423 was reported less abundant in acid-stressed cells (Heunis et al., 2014). *Lactococcus lactis* $F_0F_1$-ATPase has been shown to be essential for the growth (Koebmann et al., 2000), except when an electron transfer chain is active (Blank et al., 2001; Duwat et al., 2001). Recently, few “omics” studies have been undertaken with *O. oeni.* Regarding the subunits of the $F_0F_1$-ATPase of *O. oeni*, transcriptomic studies have reported a non-differential expression during ethanol presence in growth media (Olguín et al., 2015). However, α-subunit codifying gene was activated in ethanol acclimation in wine like media (WLM) at pH 3.5 (Costantini et al., 2015) and the ***atpB*** gene encoding the β-subunit of the $F_1F_0$ ATPase was induced three-fold after acidic adaptation (Beltramo et al., 2006).

### 1.2.3 Proton motive force (PMF)

Except for some species of the genera *Lactobacillus*, *Leuconostoc* and *Oenococcus*, LAB are neutrophiles (i.e., optimal pH for growth between 5 and 9). Therefore, *Oenococcus* is suitable for the wine-making process. The acids present in the outside of the cell can diffuse through the membrane in its non-dissociated form due to the low external pH (pHe). After the entry into the cytoplasm, they rapidly dissociate into protons and charged derivatives to which the cell membrane is impermeable (Presser et al., 1997) provoking a decrease in the intracellular pH (pHi). This fact has a detrimental effect on cellular physiology (Presser et al., 1997) reducing the activity of acid-sensitive

enzymes and damaging proteins and DNA, perhaps through a chelating interaction with these essential elements. Also, the change in the transmembrane pH gradient (ΔpH) contributes to the PMF used as an energy source in numerous transmembrane transport processes. The mechanism by which the electrochemical proton gradient is generated during MLF in *O. oeni* has been inferred from transport studies with membrane vesicles (Salema et al., 1996).

In wine two substrates induce PMF, malic and citric acid. It is believed that the only way *O. oeni* can generate ATP at the low pH of wine, is through **malic metabolism** because glycolysis is effectively switched off (Bartowsky, 2005). MLF pathway includes the active uptake of L-malate into the cell by malate permease (MleP) (Cox and Henick-Kling, 1989). The gene ***mleP*** is over-expressed due to the presence of L-malate in the media (Augagneur et al., 2007a) and due to acclimation (1 day) to ethanol 8% and 12% (Costantini et al., 2015). After this step, the decarboxylation of L-malic acid takes place, which is facilitated by the malolactic enzyme (MleA). Finally, the cell proceeds to the excretion of the end products, lactate and $CO_2$. All this process is regulated by a regulatory protein, MleR, but it is still not clear how lactate extrusion is carried out (Loubiere et al., 1992; Salema et al., 1994).

Beltramo et al. (2006) reported that the relative expression levels of *mleA* increased more than threefold after acidic adaptation (pH 3.5) and this genes was induced after wine inoculation. Also, Costantini et al. (2015) showed the induction of *mleA* using microarrays in cells adapted to ethanol.

The **metabolism of citric acid** is usually sequential to malic acid in wine during MLF, and is not initiated until more than half of the malic acid has been metabolised (Nielsen and Richelieu, 1999). Citrate uptake is catalyzed by the CitP or MaeP carrier in an electrogenic precursor/product (citrate/lactate) antiport, which generates an electrochemical gradient over the membrane (Konings, 2002; Marty-Teysset et al., 1996; Ramos et al., 1994). The electrochemical gradient is similar to that from MLF. Citrate fermentation in *O. oeni* is, in addition to MLF, important for wine production since citrate is a precursor of aroma compounds due to the production of volatile compounds such as diacetyl and acetic acid (Bartowsky and Henschke, 2004). This process has been associated to a possible acidic stress response in other LAB species (Martín et al., 2004; Tourdot-Maréchal et al., 1999; van Bokhorst-van de Veen et al.,

2011). Ramos et al. (1994) studied the effect of the pH gradient and the transmembrane electrical potential on citrate uptake using membrane vesicles, revealing that the membrane potential is generated at the level of citrate uptake into the cell. At a molecular level and as described by Bekal-Si Ali et al. (1999) the absence of intergenic regions between *citC*, *citD*, *citE*, and *citF* suggested that all of these genes could be expressed in an large polycistronic mRNA. Olguín et al. (2009) suggested the involvement of citrate metabolism in cell adaptation to wine stress conditions by the activation of some of these genes affected by ethanol exposure at low pH. In the presence of ethanol, metabolic and transcriptional behaviour were different than observed when ethanol was absent. The expression of citrate pathway genes was mainly affected by ethanol, while pH showed a lower effect (Olguín et al., 2009). Several citrate genes were inhibited in the recent microarrays analysis with ethanol shock (Olguín et al., 2015) and WLM adapted cells to ethanol (Costantini et al., 2015). In this latter investigation, at protein level, citrate lyase alpha and β subunits showed reduced abundance in cell adapted to ethanol 8%.

Other studies have combined the effect of both acids due to their involvement in the generation of ATP via $F_0F_1$-ATPase. Generally, L-malic and citric acids favour *O. oeni* growth in nutritional stress conditions. Specifically citric acid is involved in the biosynthesis of the aspartate-derived essential amino acids (Saguir and Manca De Nadra, 2002). On the other hand, the presence of phenolic acids affects malate and citric metabolism in a strain-dependent manner (Campos et al., 2009; Reguant et al., 2000). It has been shown that L-malate enhanced the growth yield at pH equal or below 4.5 while the presence of citrate in media led to a complete and unexpected inhibition of the growth at pH 3.2 (Augagneur et al., 2007a). Nevertheless, the same authors exhibited that whatever the growth conditions, both L-malate and citrate participated in the enhancement of the transmembrane ΔpH, whereas the membrane potential decreased with the pH. Their results showed that it was not citrate that was directly responsible for the inhibition observed in cultures done at low pH, but its end products.

**The arginine metabolism** has been studied for its role against the damaging effects of acidic environments, specifically, the arginine deiminase pathway (ADI) which has been considered as a protector for other LAB species (Champomier Vergès et al., 1999; Rallu et al., 1996) and *O. oeni* (Tonon and Lonvaud-Funel, 2000). Also, the presence of

different *arc* genes of the ADI pathway in *O. oeni* was reported in different strains and despite their presence in the genomes, considerable variability in the ability to degrade arginine among strains was observed. Therefore, despite the presence of the *arc* genes in all strains, the expression patterns of individual genes must be strain dependent and influenced by the different wine conditions (Araque et al., 2016). Expression of three arc genes (***arcB***, ***arcC*** and ***arcR***), and particularly that of ***arcA***, was positively affected by arginine supplementation confirming the enzymatic results obtained by Tonon et. (2001). The work of Bourdineaud (2006) with *O. oeni* showed that arginine, in combination with fructose, triggers the expression of a subset of stress-responsive genes (*arcR*, ***omrA***, and ***ftsH***). Also, cultivation of *O. oeni* in a fructose and arginine supplemented medium prior to wine exposure protects bacteria against subsequent wine shock, and this acquired stress resistance is independent of pH. The *O. oeni ftsH* gene, encoding a protease belonging to the ATP-binding cassette protein superfamily, and the *omrA* gene, which encodes a protein belonging to the ATP-binding cassette superfamily of transporters, are induced at high temperature and under osmotic shock (Bourdineaud et al., 2003, 2004).

### 1.2.4 Stress proteins

Microorganisms undergo complex programs of differential gene expression, involving a rapid increase in the concentrations of specific sets of proteins to counteract the harsh environmental conditions. These stress proteins are named according to their molecular weight in kilodaltons. Some Hsps from operons GroESL (*groES – groEL*) and DnaK (*grpE – dnaK – dnaJ*) and small Hsps, like Lo18 protein (Hsp18), perform chaperone functions, by stabilising new proteins to ensure correct folding or by helping to refold proteins that were damaged by the cell stress. Other members have protease activity such as the Clp ATP-dependent protease, HtrA and FtsH, which degrade incorrectly folded proteins (Grandvalet et al., 2005). Moreover, apart from the known chaperones, several enzymes involved in stress response have been studied like the ***hdcA*** gene (encoding a histidine decarboxylase). It was important for its role in histamine production, a biogenic amine. However, no expression could be detected under studied conditions (Beltramo et al., 2006).

The mechanisms of stress response regulation among the different Gram positive bacteria are different. In *B. subtilis* three different classes of stress-inducible genes can

be defined by their common regulatory characteristics: the expression of class I genes involves an inverted repeat, called CIRCE element, which is the binding site for the HrcA repressor; the regulation by sigma factor $\sigma^B$ of the majority of general stress genes (class II), and class III genes which are controlled by CtsR (class three stress genes repressor), which binds to a specific direct repeat referred to as the CtsR box (Grandvalet et al., 2005; Hecker and Völker, 1998; Hecker et al., 1996; Spano and Massa, 2006). For *O. oeni* the master regulator for many molecular chaperone genes is CtsR (Figure 4) (Grandvalet et al., 2005). Indeed, in the genomes of strain PSU-1 and strain ATCC BAA-1163 there are no genes encoding for other main transcriptional regulators, such as hrcA or alternative sigma factors, and no CIRCE elements were found (Beltramo et al., 2004; Grandvalet et al., 2005; Mills et al., 2005).

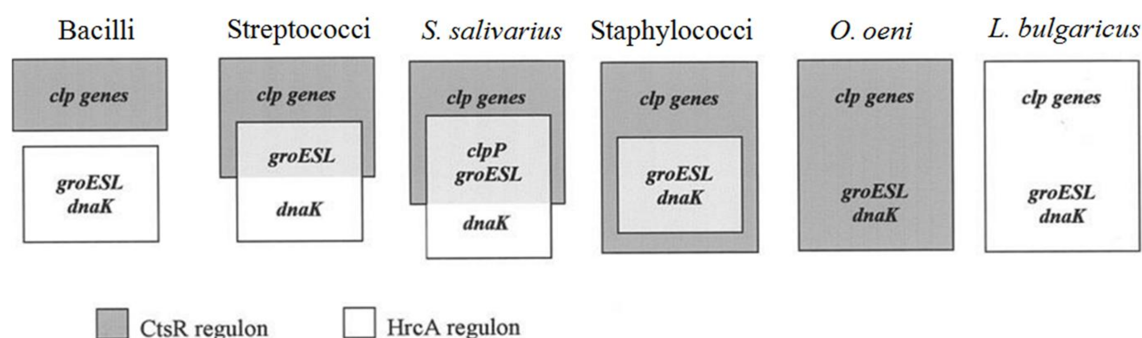


**Figure 4** Dual regulation by CtsR and HrcA in different Gram-positive bacteria. In many Gram-positive bacteria, the CtsR and the HrcA regulons coexist. In *B. subtilis* and closely related bacilli (*B. anthracis*, *B. stearothermophilus*, *B. halodurans*, *C. acetobutylicum*, *C. difficile*, *C. perfringens*, *L. monocytogenes*, and *L. innocua*), the two regulons are distinct, whereas in the streptococcal group (*S. pneumoniae*, *L. lactis*, and *S. salivarius*), they partially overlap, and the HrcA regulon is entirely embedded within the CtsR regulon in *S. aureus*. *O. oeni* and *L. bulgaricus* have original heat shock gene regulation with a predominant control of molecular chaperone genes by either CtsR or HrcA, respectively (Grandvalet et al., 2005).

Gaining a better knowledge of the biological significance of these regulations in response to wine conditions is important in order to determine the complex biological role of CtsR-mediated stress response in *O. oeni*. Therefore, the study of the genetic expression under wine conditions has been key to establish basic stress characters in order to select better starters which can overcome cell mortality after inoculation into wine. Adaptation processes like heat-shock at 42 ◦C (Guzzo et al., 1994) or growth in

the presence of ethanol (8%, vol/vol) (da Silveira et al., 2002) have been shown to enhance the survival of *O. oeni* cells to stress conditions in wine.

As it has been commented above, the ***cts*R gene** encodes the transcriptional regulator of several chaperones annotated in strain PSU-1. Specifically, the transcription of *groESL* and *dnaK* operons, *clpP*, *clpC*, *clpL2* and *hsp18* genes (codifying for Lo18 protein), is regulated by CtsR (Grandvalet et al., 2005). All these genes are heat inducible, but also differential expression was observed during growth for some of them (Beltramo et al., 2004; Jobin et al., 1997). While *clpX* is preferentially expressed at the beginning of the exponential phase, *clpP* has been activated during all stages of growth reaching its maximum expression at the exponential phase. The expression of *hsp18* has been only at the end of the exponential phase.

The increase in cell survival is linked to stress response mechanisms: heat treatment has been shown to increase the synthesis of Hsps, notably the Lo18 (Guzzo et al., 1997), and growth of *O. oeni* in the presence of ethanol leads to a modification in membrane composition (da Silveira et al., 2003). These stress-induced proteins could be used as positive indicators for a culture that is fully adapted to resisting an upcoming stress condition. The subclass of Hsp, **small Hsp** (12 - 43 kDa), can form large oligomers with molecular chaperone activity that protects polypeptides from aggregation (Narberhaus, 2002). The synthesis of sHsp is induced by various stresses, such as heat, ethanol and low pH, in prokaryotes and plays a role in the acquisition of thermotolerance in many organisms (Horwitz, 1992). The most known sHsp in *O. oeni* is Lo18 protein. This protein is coded by the *hsp18* gene and its expression seems to be controlled at the transcriptional level (Coucheney et al., 2005). This protein synthesis was determined after heat (42°C), acid (pH 3) and ethanolic shock (12% v/v) (Guzzo et al., 1997; Jobin et al., 1997) and in the stationary growth phase (Guzzo et al., 1997). The over expression of the gene *hsp18* was also confirmed under heat shock and during the stationary phase in strain ATCC BAA-1163 (Desroche et al., 2005). sHps can bind membranes to regulate bilayer fluidity (Horváth et al., 2008; Nakamoto and Vígh, 2007). Since Lo18 protein was found to be peripherally associated with the membrane of *O. oeni* by Jobin et al. (1997), several studies have been focused on its role in this cellular barrier. Delmas et al. (2001) showed that the prokaryotic Lo18 can function as a molecular chaperone *in vitro* and its membrane association depends on the temperature

upshift. Also, expression of this sHSP was induced by administration of a membrane fluidiser, the benzyl alcohol, suggesting that Lo18 expression could be regulated by the level of membrane fluidity. Coucheney et al. (2005) suggested that Lo18 could be involved in an adaptive response allowing the maintenance of membrane integrity during temperature ramping in presence Lo18 protein in *O. oeni* cells. More recently Weidmann et al. (2010) suggested, using transformed *E. coli* cells under heat shock, that a major part of Lo18 is associated with the cytoplasmic membrane. The same group has reported Lo18 preventing the thermal aggregation of proteins and playing a crucial role in membrane quality control (Maitre et al., 2014).

Other authors have focused the attention on the expression of *hsp18* under harsh conditions as an indicator of wine stress response (Beltramo et al., 2006; Olguín et al., 2010). In the thesis of Stefanelli (2014) it is shown that this gene was over expressed in pH 3.5 + 10% EtOH and at 42°C. Olguín et al. (2015) and Costantini et al. (2015) have detected *hsp18* as over-expressed using microarray analysis due to ethanol addition (12% v/v) after 1h and in WLM acclimation containing 8% ethanol, respectively. Also, the latter proteomic study found the Lo18 protein more abundant in cells adapted in WLM containing 8% and 12% ethanol.

Other Hsp involved in a great diversity of functions are the Hsp100 or **Clp proteins**. They are ATPase involved in the increased tolerance to high temperatures, promotion of proteolysis of specific cellular substrates and regulation of transcription. Many Clp proteins play a part in determining the half-life of regulatory proteins and are divided into two classes. Members of the first class (ClpA, ClpB, ClpC, ClpD, ClpE, and ClpL) contain two ATP nucleotide-binding domains (NBDs), while the second class includes smaller proteins containing only one NBD, such as ClpX (Schirmer et al., 1996).

The *clpX* gene of *O. oeni*, which is preferentially expressed at the beginning of the exponential phase, has been reported as heat inducible. Also, it has been suggested that the long 5'-untranslated region could be involved in stability of mRNA (Jobin et al., 1999a). It is worth noting that ClpP is a stress responsive protease that can act independently or in association with ClpX ATPase to degrade larger specific substrates (Makovets et al., 1998). The different behaviour observed in the transcriptional evolution of these genes, *clpP* and *clpX*, in Olguín et al. (2010) during MLF can be explained by the fact that *clpP* is regulated by *ctsR*, whereas *clpX* may depend on other

mechanisms, not yet characterised (Beltramo et al., 2004). In some bacteria, the *clpX* gene belongs to a cluster that also contains the gene encoding the trigger factor (***tig***). In *E. coli* the trigger factor is a peptidyl-prolyl isomerase able to catalyse protein folding *in vitro*, to associate with nascent polypeptides on ribosomes, and to cooperate with the GroEL chaperone in promoting degradation of some unstable proteins *in vivo* (Hesterkamp and Bukau, 1996). Moreover, recent "omic" studies have confirmed the activation of *clp* genes and the abundance of ClpP and ClpL2 proteins in adapted ethanol cells (Costantini et al., 2015).

The class I heat shock genes of *B. subtilis* (***groESL*** and ***dnaK***operons) were identified in PSU-1 strain (Mills et al., 2005) and potential CtsR operator sites were found upstream from these operons (Grandvalet et al., 2005). The induced transcription of their first genes of these operons, *groES* and *grpE* respectively, was reported under heat shock (42°C), ethanol 11% v/v and for *grpE* also under pH 3.6 (Desroche et al., 2005; Grandvalet et al., 2005). The recruitment of DnaK chaperone to the membrane, as described for *B. subtilis* after short-term ethanol stress (Seydlová et al., 2012) was suggested as an explanation for the decrease in DnaK concentration in the cytosolic fraction after ethanol shock (12%) (Olguín et al., 2015). A similar phenomenon of membrane association has been described for Lo18, as mentioned above, for *O. oeni* (Weidmann et al., 2010). On the contrary, in WLM adapted cells at ethanol 12% during 1 day, DnaK chaperone was found to be more abundant (Costantini et al., 2015). However, these authors reported the chaperone GroEL less abundant in ethanol (8%) adapted cells.

### 1.2.5 Redox systems

All bacteria have developed sensory systems that facilitate adaptation to changes in the environmental conditions. Redox systems are often related to oxygen ($O_2$) and are widely distributed along many microorganisms (Cash et al., 2007). There is strong evidence that reactive oxygen species (ROS) and free radicals play an important role in cell survival, and have been reported to appear under ethanol exposure and other toxics (Zeller and Klug, 2006). In *O. oeni*, the geranylgeranyl pyrophosphate synthase (**GGPPS**) is an enzyme implicated in the biosynthesis of secondary metabolites, mainly in terpenoids, steroids and membrane lipids biosynthesis. Carotenoids, as terpenoids, are lipophilic agents that are incorporated into the bacterial membrane and its

production in LAB is considered to play a role in the elimination of oxygen radicals (Cafaro et al., 2014). This gene has been over-expressed under ethanol exposure (Table 1).

It is known from *in vitro* studies that LAB strains possess antioxidant mechanisms and inactivate ROS via enzymatic mechanisms, e.g. by a coupled NADH oxidase/peroxidase system, superoxide dismutase and catalase (Bruno-Bárcena et al., 2004; Cabiscol et al., 2000; Hertel et al., 1998; Kullisaar et al., 2010). Also, other small proteins, such as thioredoxins (Trx) or low molecular-weight thiols including glutathione (GSH), play a key role in maintaining a reducing environment in the cell. Trx is ubiquitous in bacteria, whereas GSH is ubiquitous in eukaryotes and in Gram-negative bacteria. GSH antioxidant system and catalase is lacking in some specific bacteria. While catalase is present in most of Gram-negative bacteria, the GSH system is absent in many Gram-positive and some Gram-negative bacteria (Lu and Holmgren, 2014; Serata et al., 2012; Vido et al., 2005). However, it has been proposed that some Gram-positive organisms with no GSH synthesis capacity have the machinery to use it (Fernándes and Steele, 1993). Therefore, for some bacteria the thioredoxin system is presumed to be essential (Vido et al., 2005). Prinz et al. (1997) showed the contribution of both systems to the recovery after oxidative stress and the reduction of disulfide of cytoplasmic proteins in *E. coli*.

Nowadays, little is known about these redox systems in *O. oeni* and their role during wine adaptation. Since one of the greatest inhibitors in wine is the ethanol content which provokes more oxidative processes (Maitre et al., 2014; van Bokhorst-van de Veen et al., 2011), redox systems may be key for cell survival. Up to now, only some transcriptional studies (Bordas et al., 2015; Costantini et al., 2015; Guzzo et al., 2000; Olguín et al., 2010) have revealed the activation of specific genes of these systems and some proteomic analysis (Cecconi et al., 2009; Silveira et al., 2004) have identified those proteins under wine stress conditions in *O. oeni*.

### 1.2.5.1 Glutathione system

The tripeptide GSH (L-γ-glutamyl-L-cysteinyl-glycine, molecular mass 307 daltons) as a major natural component of many plants and foods (Noctor and Foyer, 1998; Son et al., 2001) was discovered in grapes on 1989 (Cheynier et al., 1989). These authors reported GSH concentrations (56 to 372 µmol/kg) in the berries of 28 grape varieties

from different locations and in the corresponding white musts (46 to 333 mM GSH). Nevertheless, there has been little written about assaying this compound in must and wine (Okuda and Yokotsuka, 1999; Park et al., 2000). Lavigne et al. (2007) presented a methodology to detect GSH in wine by capillary electrophoresis coupled with fluorimetric detection. These results showed the presence of important quantities of GSH in reduced form (65µmol/L) in white grape must and also GSH content variability at the end of alcoholic fermentation depending on the yeast strain used.

Regarding the presence of **GSH in microorganisms**, it has been found in eukaryotic and Gram-negative bacteria, but it is not so commonly found in Gram positive bacteria (Fahey et al., 1978). LAB species presented a widely varied GSH concentration through strains tested (Fernándes and Steele, 1993). These authors reported highest concentrations in strains of *Lactococcus lactis* ssp. *cremoris* and *Streptococcus thermophilus*, followed by *Leuconostoc mesenteroides* ssp. *cremoris*. On the other hand, GSH was not detected in *Lactococcus lactis* ssp. *lactis*, micrococci, pediococci, and lactobacilli (except *Lactobacillus helveticus* CNRZ 32) cultures.

The **biosynthesis of GSH** as a peptide takes place in two ATP-dependent steps (Anderson and Meister, 1983) using two enzymes: γ-glutamylcysteine synthetase (GshA) and glutathione synthetase (GshB). The dipeptide γ-glutamyl- cysteine (γ-GC) is first synthesised from L-glutamic acid and L-cysteine by GshA. In the second step, catalysed by GshB, glycine is added to the C-terminal end of γ-GC to form GSH. The gene *gshA* is annotated in several sequenced genomes of *Lactobacillus* and *Leuconostoc* but it is not found in most of LAB, including *Oenococcus*. The gene *gshB* is not found in any sequenced LAB species. However, in several *Streptococcus*, *Enterococcus* and *Lactobacillus* it is detected a gene named *gshF*, which would be the fusion of *gshA* and *gshB* (Kim et al., 2008).

Most of LAB species which **use GSH**, must capture it from the media. Many organisms can transport the tripeptide GSH from the medium and utilise it for various cellular reactions (Smirnova and Oktyabrsky, 2005). Its transport in prokaryotes is known to be carried by CydDC (consisting of two subunits CydC and CydD), a heterodimeric ATP-binding cassette type transporter (Pittman et al., 2005). The transport mechanism of GSH has been studied in *Streptococcus mutans* (Sherrill and Fahey, 1998) also reporting a protective role of this antioxidant against the thiol-oxidizing agent diamide.

Wiederholt and Steele (1994) reported the accumulation of GSH in a strain of *L. lactis* ssp. *cremoris* under oxidative stress.

The **antioxidant action of GSH** comes from the thiol group in the amino acid cysteine. GSH is involved in numerous metabolic functions using the disulphide bond between both cysteines to reduce certain proteins (Masip et al., 2006). This reduction is catalysed by the glutathione peroxidase enzyme (Gpo) and leads to the oxidised glutathione form (GSSG). This form is highly toxic because it easily reacts with free sulfhydryl groups. Therefore, glutathione reductase (GshR) catalyzes the reduction of GSSG to GSH using NADPH as an electron donor (Figure 5). GshR seems to play a crucial role in evolutionary adaptation of organisms to atmospheric oxygen (Smirnova and Oktyabrsky, 2005).

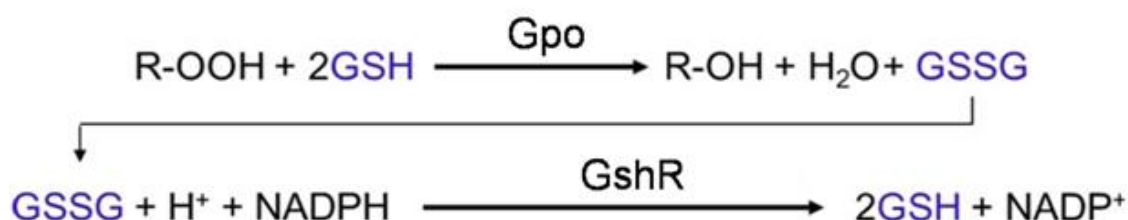


**Figure 5** General reaction mechanism for antioxidant glutathione peroxidase (Gpo) and glutathione reductase (GshR). Gpo catalyses the chemical reduction of lipid peroxides or $H_2O_2$ to respective alcohols and water by glutathione (GSH) which forms glutathione disulfide (GSSG). GshR catalyses the reduction of GSSG back to GSH in the presence of NADPH (Mattmiller et al., 2013).

Related to GSH system, it is well known in *E. coli* the **glutaredoxin (Grx) system** which also contributes to reduce disulfide bonds of cytoplasmic proteins and it is composed by GshR, GSH and different Grxs (Prinz et al., 1997). In this case, GSH is used as a cofactor for the oxidoreductases Grx (Figure 6). These enzymes are oxidised by substrates including at least one compound devoid of thiol groups and reduced due to GSH binding. Structurally, Grxs belong to the Trx fold family of proteins. This motif consists of a four stranded β-sheet surrounded by three α-helices (Eklund et al., 1984). Dithiol Grxs contain the characteristic C-X-X-C active site motif. However, a second group of Grxs, commonly named monothiol Grxs, lacks the C-terminal active site thiol in its C-X-X-S active site, but contains all structural and functional elements to bind and utilise GSH as substrate (Lillig et al., 2008). Therefore, two distinct but functionally

connected reaction mechanisms evolved, the dithiol and the monothiol mechanism, both relying on the proteins' inherent affinity for the GSH moiety (Bushweller et al., 1994; Holmgren, 1989).

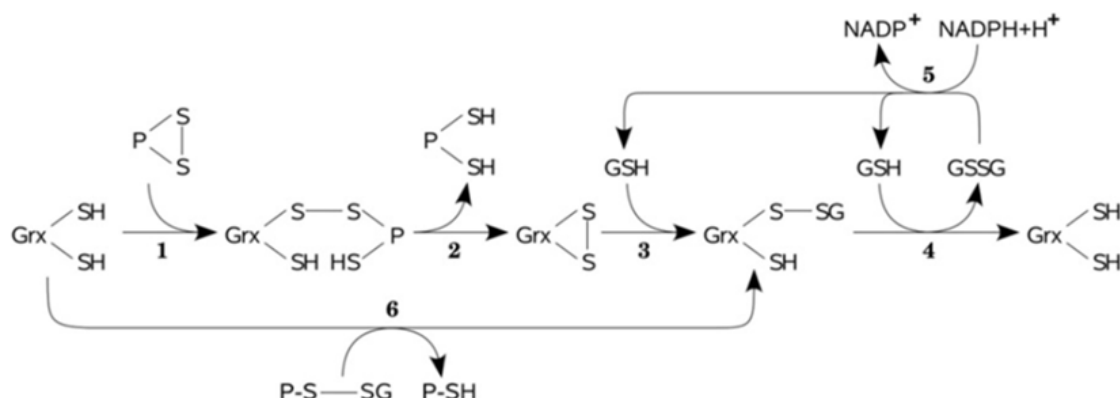


**Figure 6** Reaction mechanisms of glutaredoxins (Grx). Grx catalyse the reversible reduction of protein disulfides utilising both of their active site cysteinyl residues (reactions 1–4). Disulfides between GSH and proteins (P) or low molecular weight compounds are reduced in the monothiol mechanism that requires only the more N-terminal active site cysteinyl residue (reactions 6 and 4). In either case, GSSG in the reaction is reduced by GshR at the expense of NADPH (reaction 5) (Lillig et al., 2008).

Pophaly et al. (2012) exemplifies a putative **GSH system and its role in LAB** (Figure 7). However, as it has been mentioned above, not all LAB and closely related Gram-positive organisms possess GSH pathway synthesis, but utilisation machinery. The only study of the complete GSH system in a probiotic LAB strain and with GSH synthesis demonstrated has been for *Lactobacillus fermentum* ME-3 (Kullisaar et al., 2010). For other LAB species the studies have been focused only on GSH concentration and the protective role of this system under stress conditions like oxidative, acidic, cold and osmotic stress (Fernándes and Steele, 1993; Jänsch et al., 2007; Kim et al., 2012; Lee et al., 2010; J. Zhang et al., 2010; Y. Zhang et al., 2010).

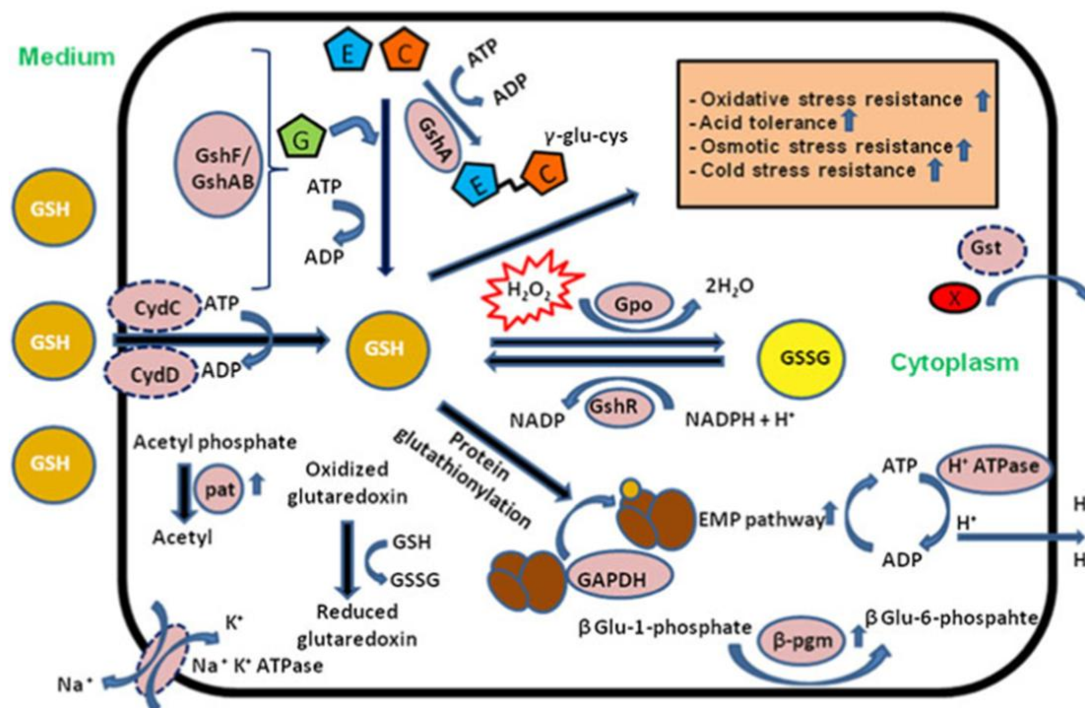


**Figure 7** Schematic diagram showing a putative GSH system and its role in LAB. Genes or proteins whose activity is not yet established in LAB are shown with dotted circle. Glutathione (GSH) is made-up of three amino acids viz. glutamic acid (E), cysteine (C) and glycine (G). The enzymes of glutathione system are γ-glutamylcystiene synthetase (GshA), glutathione synthetase (GshB), glutathione bifunctional fusion protein (GshAB/GshF), glutathione reductase (GshR/Gor), and glutathione peroxidase (Gpo). Glutathione-S-transferases (GSTs) are a class of enzymes which are involved in cellular detoxification of xenobiotics (X) using reduced glutathione. The exact cellular role of GST in LAB is not yet established. GSH also carries glutathionylation of key proteins of EMP pathway (e.g. GAPDH) and helps to maintain ATP production at required levels during stress conditions (Pophaly et al., 2012).

**For *O. oeni*** no value of intracellular GSH has not been reported yet and no annotated genes related to the GSH biosynthesis are present in the available genomes. However, several genes related to the use of GSH, *gshR*, *gpo*, three possible *grxs* and the two subunits genes *cydC* and *cydD* of the putative GSH transporter, are annotated in the genome of PSU-1 strain (Pophaly et al., 2012). As far as we are concerned, Rauhut et al. (2004) presented the only work related to the growth of *O. oeni* and MLF performance using GSH as a sulfur and cysteine source. These preliminary results showed that the addition of cysteine and GSH to a fermented wine after alcoholic fermentation can promote the development of LAB and MLF. The addition of cysteine seemed to slow down the degradation of L-malate, whereas the addition of GSH promoted MLF.

The first proteomic studies done with *O. oeni*, revealed GshR was more abundant in ethanol-adapted cells (Cecconi et al., 2009; Silveira et al., 2004). According to these results, the activation of *gshR* in different *O. oeni* strains in wine was reported as strain-dependent (Bordas et al., 2015). Moreover, as the detected over expressions were at the end of MLF, the authors suggested that the *gshR* gene would be not involved in the early stress response, like *hsp18*, *clpP* and *citE*, but in the long-term adaptation of *O. oeni* to wine conditions. Recently, microarrays analysis showed *gshR* over expression under wine acclimation (Costantini et al., 2015), but the activation of other GSH related genes has not been reported.

### 1.2.5.2 Thioredoxin system

The thioredoxin is a small oxidoreductase (12 kDa), which is present in mostly all organisms and acts as the antioxidant GSH. Due to its active site C-G-P-C, Trx can neutralise ROS and can maintain the reduced state of intracellular proteins (Zeller and Klug, 2006). This reduction takes place with a thioldisulfide interchange from the amino acid cysteine. The reduction of oxidised Trx depends on NAD(P)H and the enzyme Trx reductase (TrxR) (Holmgren, 1985) (Figure 8).

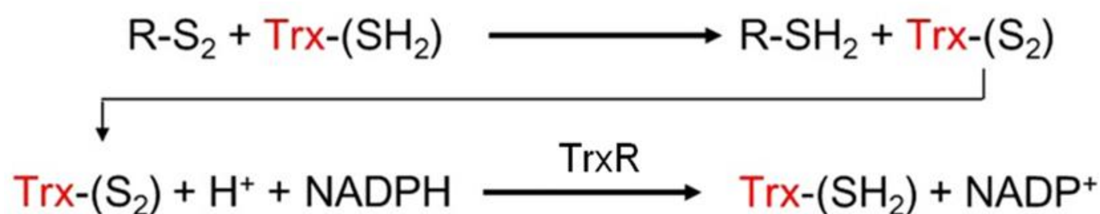


**Figure 8** Oxidised protein disulfides and other free radicals are reduced to their corresponding thiols by thioredoxin (Trx). TrxR then catalyses the reduction of oxidised Trx in the presence of NADPH. Adapted from Mattmiller et al. (2013).

**The Trx/TrxR system in Gram-positive bacteria** is generally essential as a redox system, more than GSH system. This fact is explained because some species must uptake this last compound from the media. The *trx* gene content can vary depending on the species. Among LAB, for *Lb. plantarum* WCFS1, Trx system was described as a key response against oxidative stress due to the over-expression of the three reported genes of *trx* and the *trxB1* (Serrano et al., 2007). Moreover, a mutant over expressing *trxB* presented a better recovery after an oxidative treatment together with an induction

of other 16 genes related to better survival. The *Lb. casei* Shirota strain has four thioredoxin genes (*trxA1*, *trxA2*, *trxA3* and *trxA4*) and one putative *trxB* (Serata et al., 2012). The generation of *Lb. casei* gene disruption mutants for different *trx* genes revealed that the Trx system is essential for aerobic growth and is the major system used to maintain the intracellular thiol/disulphide balance (Serata et al., 2012). However, Vido et al. (2005) reported the viability of *L. lactis* mutant for the gene thioredoxin reductase (*trxB*) under oxidative stress. These authors suggested the existence of another redox system and GSH was not considered because this bacterium cannot synthesise it. Also, the two Trx have different functions in stress resistance in *L. lactis*. While *trxA* seems to be involved in responses to oxidative stress, *trxD* appears to be important for resistance towards arsenate and tellurite (Efler et al., 2015).

**Regarding *O. oeni*,** in the strain ATCC BAA-1163, a gene codifying for a functional Trx, named *trxA*, was reported over expressed under hydrogen peroxide and heat shock (Guzzo et al., 2000; Jobin et al., 1999b). Unlike stress genes like *hsp18* and *clpX*, *trxA* presented no difference in its expression during all stages of growth. The same group continued the study of this *trxA* an reported its activation under low pH conditions (pH 5.3 to 3.5) and at the end of MLF in wine (Beltramo et al., 2006). The relation of thioredoxins to oxidative stress has been reported for many other microorganisms (Kuge and Jones, 1994; Scharf et al., 1998; Wieles et al., 1997) and for *B. subtilis trxA* was also found induced by heat shock (Scharf et al., 1998). For PSU-1 (= ATCC-BAA331) genome (Mills et al., 2005) there are annotated three *trx* and one of them was used by Renouf et al. (2008) as a genetic oenological marker in *O. oeni* strains. This gene has been located in a described plasticity region of the genome of *O. oeni* (Bon et al., 2009) which also harboured a gene codifying for a Dps-like protein. This latter protein is as well particularly important during oxidative stress or depletion of environmental nutrients (Nair and Finkel, 2004). Also, recent transcriptomic analysis showed for the first time that *trx* is associated with alcohol stress response (Costantini et al., 2015). For *O. oeni* there is no work elucidating the complete Trx system role during other conditions or even MLF.

## 1.3 Functional "omics" in *O. oeni*

Recent technological advances, combined with the development of bioinformatic tools and high-throughput molecular techniques, colloquially called the "omics" methods, allow scientists to better address biological questions. This novel, comprehensive perspective addresses the identification, characterisation and quantification of the whole repertoire of genes, proteins and metabolites occurring in living organisms. Recent advances in "omics" and integrated functional genomic analysis involving transcriptomics, proteomics, secretomics, metabolomics, and interactomics have accelerated the research into deciphering specific mechanisms for commensal and probiotic functionality: within the gastrointestinal tract (Baugher and Klaenhammer, 2011), probiotics to mitigate infectious diseases (Rebollar et al., 2016) and for microorganisms of dairy industry (Sohier et al., 2014). Unlike other LAB, *O. oeni* -omic studies are scarce but preliminary results have started to emerge related to stress response in wine conditions. This more holistic approach could base further selection of strains in the knowledge rather than trial and error strategies.

The following sections will describe functional "omics" focused mainly in comparative genome analysis, transcriptomic studies using microarrays and proteomic reports and techniques available. It is worth noting that metabolomics has been used in the analysis of volatile compounds specifically in MLF (Lee et al., 2009; Pozo-Bayón et al., 2005; Ugliano and Moio, 2005).

### 1.3.1 Genomics

Related bacterial strains can differ significantly in their genotype and phenotype, and features from one bacterial strain or species cannot necessarily be applied to a related one. Since the first bacterial genome (*Haemophilus influenzae*) was completely sequenced in 1995 (Fleischmann et al., 1995), sequencing technology has experienced an exponential increase in processing speed, at significantly lowered costs, yielding more than 8319 completed bacterial genome sequences (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/). Placing genomes into an evolutionary framework has proved useful for understanding the functioning of organisms. Comparative studies of genomes have revealed that bacterial chromosomes are under selective pressures that have deeply shaped their organisation. The processes

of replication, transcription and the regulation of gene expression all impact how genes are arranged along the genome (Abby and Daubin, 2007).

Genome-wide comparative genomics analysis have been undertaken in different species of lactobacilli (Douillard et al., 2013; Klaenhammer et al., 2008; Liu et al., 2010; O'Sullivan et al., 2009; Sun et al., 2015) in order to identify gene targets for functional analysis and to determine a niche-specific gene set. Also, genome comparison has been used in other LAB species (Endo et al., 2015), LAB adapted to beer (Snauwaert et al., 2015), *Lactococcus* (Kok et al., 2005) and *Enterococcus* species (Palmer et al., 2012).

Nowadays, 63 genome assemblies of *O. oeni* can be used from the database. The first determined *O. oeni* genome, PSU-1 strain (Mills et al., 2005), marked a significant new phase for wine-related research on LAB in which the physiology, genetic diversity and performance of starter cultures could be more rigorously examined. Before the availability of the *O. oeni* genomes, Zé-Zé et al. (2000, 1998) performed a detailed genome mapping on two strains (PSU-1 and GM), providing insight into the genetic organisation of the bacterium. Important phenotypes, such as malate degradation, citrate metabolism and diacetyl production, were mapped. This comparative analysis also revealed extensive conservation of loci order. However, further studies have indicated the genomic heterogeneity of *O. oeni* strains, also due to its loss of mismatch DNA repair (Marcobal et al., 2008).

The comparative genome study of the MLF capacity of over 70 *O. oeni* strains (Bon et al., 2009) in three wines indicated the presence of eight stress-responsive genes which could be associated with high MLF performance. Borneman et al. (2010) started using array-based comparative genome hybridisation and genome sequencing of three *O. oeni* strains (PSU-1, BAA-1163, and AWRIB429) revealing a 10% of genomic diversity. This variation in the *O. oeni* species protein coding capacity would presumably be key to phenotypic differences between strains (Bartowsky and Borneman, 2011). Also, Borneman et al., (2010) in order to ascertain the basis of these phenotypic differences mapped the genomic content of ten wine strains of *O. oeni*. These strains comprised a genomically diverse group in which large sections of the reference genome were often absent from individual strains. The same authors (Borneman et al., 2012) provided 11 sequenced strains by whole-genome sequencing, which enhanced a broad insight into the genetic variation present within *O. oeni*.

Other works have been focused on the presence of specific traits related to wine conditions. *O. oeni* genome comprises different genes related to the synthesis of exopolysaccharides (EPS). Different comparative genome studies have been carried out using multilocus sequence typing (MLST) revealing the importance of EPS for the adaptation of the bacteria to wine and providing an inventory of the genes potentially involved in this biosynthesis (Dimopoulou et al., 2016, 2014, 2012). Similar studies were conducted for the study of substrate transport and phosphorylation (Jamal et al., 2013). These authors reported that as a part of the core genome, the phosphotransferase genes may contribute to the perfect adaptation of *O. oeni* to its singular ecological niche.

A big study (Campbell-Sills et al., 2015) involved 50 genomes from *Oenococcus* genus isolated from different wine products (red wine, white wine, champagne, and cider), different regions (France, Australia, Lebanon, United States, Italy and England) and from different years. Phylogenomic and population structure analyses revealed two major groups of strains, one related to wine and champagne and another related to wine and cider. The authors suggested that ancestral *O. oeni* strains were adapted to a low-ethanol containing environment such as overripe fruits, being domesticated to cider and wine, while another group suffered a process of further domestication to specific wines such as champagne.

Some of these mentioned studies have revealed that the major factor that affects genomic diversity is the presence of prophages, which generate large-size structural polymorphisms among *O. oeni* genomes (Bon et al., 2009; Borneman et al., 2010; Zé-Zé et al., 2008). Also, insertion sequences (IS) represent another class of variable genetic elements found in the pangenome of *O. oeni* (El Gharniti et al., 2012; Zé-Zé et al., 2008). IS are autonomous transposable elements (Mahillon and Chandler, 1998) and most of the IS-related sequences available in the species *O. oeni* have been characterised because they were linked to strain-specific genes involved in stress response and/or persistence in the niche, such as the ferritin-like protein $dps_B$ (Bon et al., 2009), the ornithine decarboxylase *odc* (Marcobal et al., 2006) and the glucosyltransferase *gtf* genes (Dols-Lafargue et al., 2008). IS elements may disrupt genes, but may also activate downstream genes (Treangen et al., 2009), as well as increasing the propensity of acquiring further adaptive mechanisms. Moreover, as

observed in *Escherichia coli*, IS play a role in the inactivation and immobilisation of incoming phages and plasmids (Ooka et al., 2009).

More extensive comparative genomics are needed in order to study the link between industrial characteristics and genomic features. Also, further phylogenomics analysis involving a whole genome data could reconstruct the evolutionary history of microorganisms, such as *O. oeni*, and their acquired capacities related to its specific niche.

### 1.3.2 Transcriptomics

The static view of a microorganism comes from its genome sequence, whereas the transcriptome introduces a dynamic vision of RNA variation throughout cell life or period. Quantification of mRNA has been employed to characterise gene expression changes in response to a great diversity of altered environmental conditions, and recently in LAB, using microarrays technique and RNA sequencing (RNA-seq). Transcriptional studies help to identify candidate genes underpinning stress responses, as well as to predict putative functions through the association of co-expressed or differentially-expressed genes.

#### 1.3.2.1 Microarrays and RNA sequencing techniques

**DNA microarrays** employ nucleic acid probes, typically 60 oligomers, covalently bound to glass slides. Fluorescently labeled target sequences of DNA, which may be reverse-transcribed from mRNA, are then hybridized to the probes and scanned. The images are then converted to signal intensities and the data is processed using software specific to the application of the array (Maughan et al., 2001; Nakanishi et al., 2001). **RNA sequencing (RNA-seq)** sequences labeled cDNA in parallel and multiple times, sometimes several million times over. The technique requires fragmenting RNA prior to reverse transcription and labeling with adapter sequences. The sequenced fragmented transcripts are typically 50–500 bp. The read sequences are then counted and assembled into full length transcripts (Marguerat and Bähler, 2010; Wang et al., 2009). Figure 9 exemplifies briefly both proteomic techniques already mentioned.

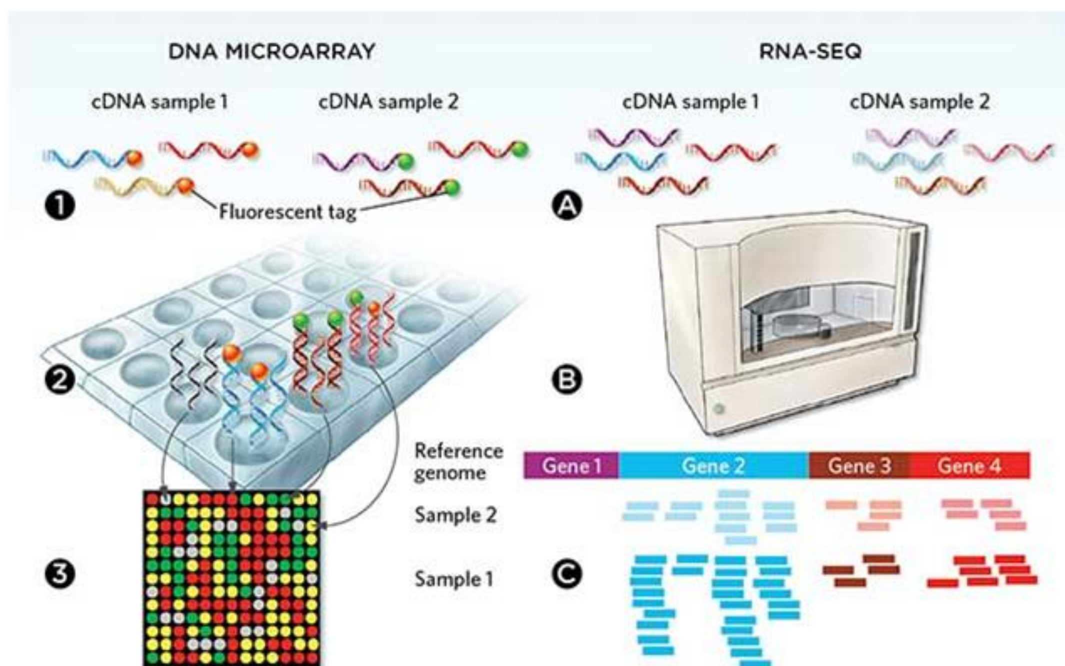


**Figure 9** DNA microarrays (above, left) consist of nucleic acid probes affixed to a surface. First, RNA is extracted from samples and converted into complimentary DNA (cDNA), which is labeled with fluorescent tags (1). Next, labeled cDNA fragments hybridize with the nucleic acids on the array (2). Scanning the microarray measures the fluorescence level at each spot, revealing levels of gene expression (3). In RNA-seq, RNA is also extracted from samples, fragmented, and converted into cDNA in preparation for sequencing (A). Next, the cDNA library is sequenced (B). The resulting reads are mapped to the genome and gene expression is quantified (C). Available at: http://www.the-scientist.com/?articles.view/articleNo/43063/title/An-Array-of-Options/.

Microarrays are a robust, reliable method proven over decades of use, and nowadays they are more economical than RNA-seq. Thus, they provide significant advantages in working on a big project with a large number of samples. As microarrays are designed on hybridization probes, these must be based on prior sequence knowledge. Therefore, they cannot detect structural variations or discover novel genes or transcripts. The hybridization method limits the sensitivity, which means that differences in expression between very similar sequences such as isoforms cannot be detected. Additionally, microarrays can only produce relative expression levels not absolute quantification values (Mantione et al., 2014).

The sequencing strategy of RNA-seq provides a comprehensive view of the transcriptome because it doesn't depend on any prior sequence. Instead, every single

transcript in the sample is sequenced. This enables the identification of structural variations such as gene fusions and alternative splicing events as well as novel genes and transcripts (Marioni et al., 2008). While microarrays measure probe intensities, sequencing quantifies discrete digital reads aligned to a particular sequence, therefore it is more sensitive and can detect low abundance transcripts. However, the analysis is much complex and specialised computing infrastructure and personnel are required.

#### 1.3.2.2 Transcriptomics in LAB and *O. oeni*

*Lactobacillus plantarum* WCFS1 was selected as a model organism to validate the reliability of RNA-seq technology compared with the use of DNA microarrays in prokaryotes (Leimena et al., 2012). Moreover, genes associated with the utilization of tetrasaccharides by *Lactobacillus ruminis* L5 were identified by RNA-seq (Lawley et al., 2013). Some authors have used RNA-seq to unveil the general transcriptome of *Lactococcus lactis* (Golomb and Marco, 2015; van der Meulen et al., 2016), the sugar effect on the gene expression of *Streptococcus mutans* (Zeng and Burne, 2015) and the genetic response to calcium and sodium lactate on *Bacillus coagulans* (Qin et al., 2015). Moreover, regarding dairy LAB species, the industrial features of these bacteria have been studied (Monnet et al., 2016; Zheng et al., 2016). There is no report about the use of RNA-seq with *O. oeni* yet.

DNA microarray technique has been widely used in LAB. *Lb. plantarum* has been studied under low growth rate using transcriptomics and metabolomics techniques (Goffin et al., 2010), while Bron et al. (2012) reported transcriptome signatures underlying physiological variations imposed by different fermentation conditions. *Lb. casei*, commonly found in gastrointestinal tract of animals, changes its transcriptome and proteome under the exposure to bile revealing the involvement of stress response pathways, fatty acid and cell wall biosynthesis, metabolism of carbohydrates, transport of peptides, coenzyme levels and membrane H+-ATPase (Alcántara and Zúñiga, 2012). For *Lactococcus lactis* microarrays were used to determine the expression profile in mixed cultures (Nouaille et al., 2009), under low growth rates (Ercan et al., 2015) and under isoleucine starvation (Dressaire et al., 2011). As it has been mentioned, several studies used different -omic approaches to unveil their objectives. This combination is even more common and powerful (Di Cagno et al., 2011; Kim et al., 2008; Li et al., 2012).

Apart from the specific transcriptomic analysis mentioned for *O. oeni* (Table 1) in which RT-qPCR has been mostly used, few transcriptomic studies determining the patterns of gene expression under stress conditions have been assessed. For *O. oeni* only two published microarray works (using a commercial wine starter and the strain PSU-1) are available in which proteomic techniques were used too (Costantini et al., 2015; Olguín et al., 2015). Also, the PhD thesis of Stefanelli (2014) reports the transcriptome data from PSU-1 strain under wine conditions (low pH and ethanol) and heat shock. This latter DNA microarray obtained an expression atlas for the four condition tested (pH 3.5, 10% EtOH, pH+EtOH and 42°C). The analysis provided a pan transcriptome of 766 genes differentially expressed and among them 28 were modulated in all the four stresses. This approach generated a general framework for further studies in which transcriptional regulators and thioredoxins were involved due to their presence in the microarray data. Regarding the other two transcriptomic works published, the presence of ethanol was mostly evaluated. Olguín et al. (2015) evaluated the effect of ethanol addition (12% vol/vol) on PSU-1 strain revealing that the main functional categories of the genes affected by ethanol were metabolite transport and cell wall and membrane biogenesis. It was also observed that known stress genes were over-expressed in response to ethanol stress, like *hsp18*.

The last transcriptomic work published for *O. oeni* studied the addition as a starter into wine. The influence of two ethanol concentrations (8% and 12%) in wine-simulated conditions was evaluated. Following this acclimation a significant over expression of genes related to MLF and stress response occurred. The stress response was mainly focused on the control of envelope composition (membrane, cell wall and EPS), and general stress proteins such as chaperones and proteases. Moreover, the authors reported that different ethanol concentration differentially modulate bacterial responses. For instance adaptation to the lower ethanol content induced surface modifications such as EPS layer production, whereas adaptation to the higher ethanol concentrations induced structural changes such as membrane composition modification.

It is clear that for *O. oeni* the transcriptomic era using high-throughput techniques is just beginning. The global view obtained trough transcriptomics could confirm the induction of different cellular response pathways and uncover new ones.

### 1.3.3 Proteomics

While genetic information is indicative of the microbial potential and transcriptomics elucidates the set of mRNA expressed, functional proteomics reveals the final protein synthesis. It has been shown that the expression level of mRNA cannot predict the amount of protein synthesized (Gygi et al., 1999). For this reason, different –omic techniques, normally proteomics, are used with transcriptomics. This enables scientists to make a clearer map of the response given in a certain time or under certain conditions.

The literature available for proteomic studies of LAB species demonstrates that these approaches provide a unique supplement to genomic data and add new levels of information to interpret the genomic sequences. Laboratory conditions and wine-like conditions elucidate the specific mechanisms of adaptation, which attain to several phenotype traits (carbohydrates utilisation, energy metabolism, stress resistance, etc.). Although proteomic studies are frequently used to identify stress resistance biomarkers, the increased availability of genome sequences and, more recently, the suitability of mass spectrometric (MS) platforms and gel-free proteomics could markedly speed the process of protein identification (De Angelis and Gobbetti, 2004). These, integrated with bioinformatics tools to re-construct metabolic pathways, open the way to uncover the mechanisms of adaptation of *O. oeni* to wine.

#### 1.3.3.1 Proteomic techniques

##### 1.3.3.1.1 Gel techniques

The traditional method for relative quantitative proteome analysis combines protein separation for their isoelectric point and protein mass. This **two-dimensional gel electrophoresis** (2DE) is coupled to mass spectrometric (MS) or tandem mass spectrometric (MS/MS) identification of selected stained protein spots detected from the gel. 2DE is widely used in bacterial proteomics, when the complexity of the sample is low enough to make the limits of gel-based proteomics less acute (Hecker et al., 2008). Although the name of 2DE electrophoresis suggests that it is a two-step process, it is indeed a five-step process starting from sample preparation to the protein detection (Figure 10).

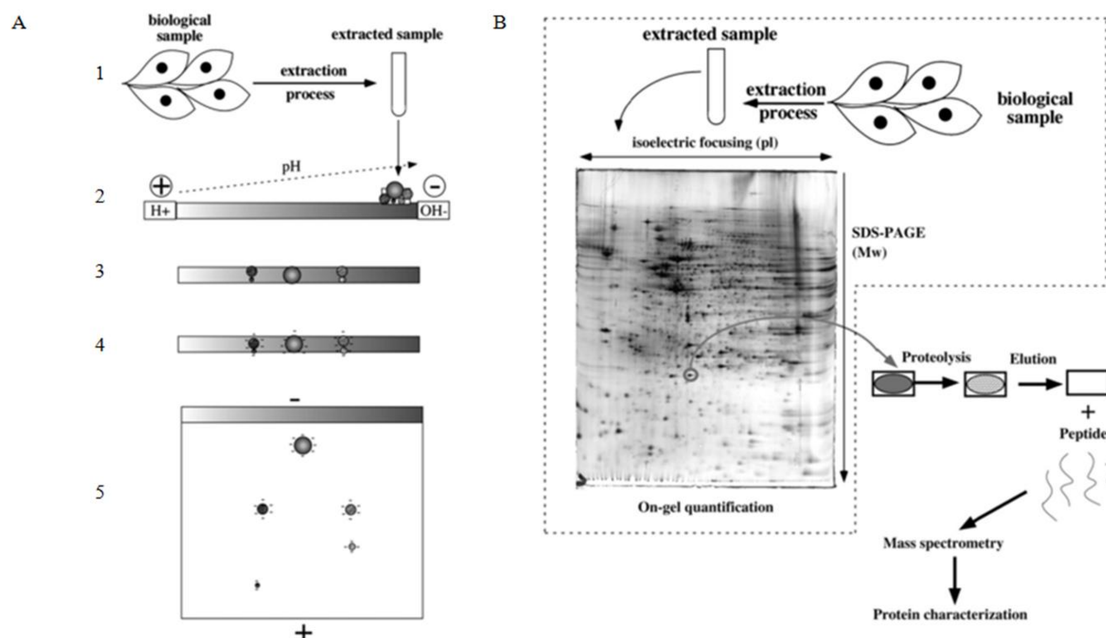


**Figure 10** A) Scheme of principle of 2D gel electrophoresis. The total process starts with the extraction of proteins from the biological sample to get an IEF-compatible sample (1). The sample is then loaded onto a pH gradient (2) oriented with the acidic side at the anode and the basic side at the cathode. After the IEF step, the proteins have reached their pI and thus have no remaining electrical charge (3). The strip is then equilibrated in a SDS-containing buffer, so that all proteins become strongly negatively charged (4). The IEF gel is then loaded on top of a SDS PAGE gel, and the proteins are separated according to their molecular masses (5). After this step, the proteins are detected directly on the gel. B) After the detection of the proteins in the gels, the resulting images are quantitatively analysed to determine the spots of interest. Those spots are then excised and submitted to in-gel digestion (generally with trypsin). The resulting peptides are then eluted and analysed by mass spectrometry, leading to protein identification and characterisation. The dotted box shows the part of 2D electrophoresis in the whole process, and it can be easily seen that key steps, including sample preparation and quantitative analysis, take place during this process (Rabilloud and Lelong, 2011).

Generally, the 2DE is called as 2DE-PAGE (two dimensional polyacrylamide gel electrophoresis) in which the order of separation is IEF (isoelectric focusing) first, which separates species based on their net charge, or isoelectric point (pI); and SDS (sodium dodecyl sulfate) electrophoresis second, which separates proteins based on molecular weight in the presence of denaturing conditions (Lilley and Friedman, 2004). This technique has been used to compare relative abundances of proteins in related samples, such as control and treatment or stressed condition, allowing the response of classes of proteins to be determined. The main problem has been the irreproducibility of

2DE gels, as there can be considerable gel-to-gel variation, and corresponding proteins between two gels must be carefully and often laboriously matched prior to quantification. Finally, normalisation has proved challenging, especially in the case of silver staining where staining is protein dependent. A combination of these factors adds variability to the system, which makes it unsuitable for accurate quantification (Lilley and Friedman, 2004).

**Difference gel electrophoresis (DIGE)** circumvents many of the issues associated with traditional 2DE-PAGE, such as reproducibility and limited dynamic range, and allows a more accurate and sensitive proteomic studies. This technique (Figure 11) relies on pre-electrophoretic labeling of samples with one of three spectrally-resolvable fluorescent CyDyes (Cy2, 3 and 5) allowing multiplexing of samples into the same gel.

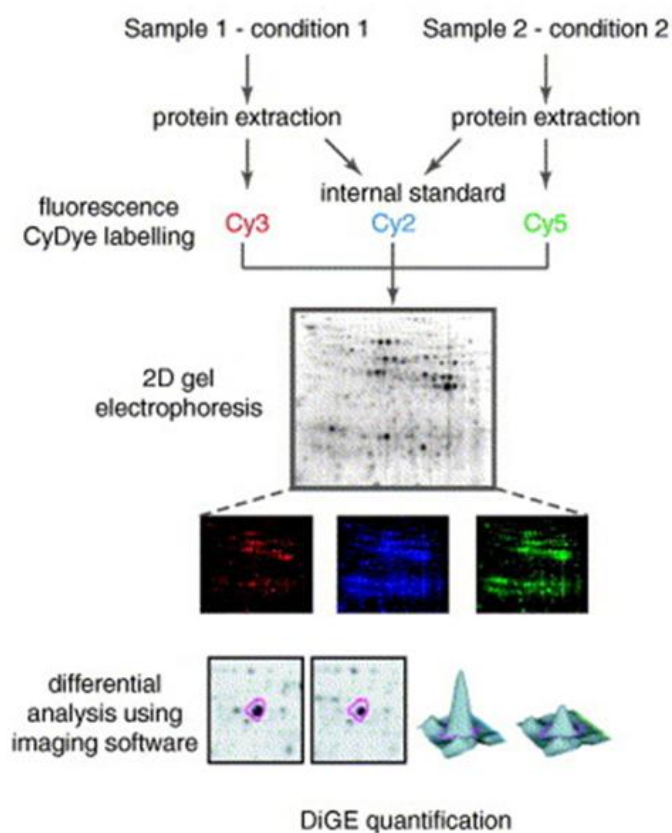


**Figure 11** Two-dimensional differential in gel electrophoresis (2D-DIGE). 2D-DIGE is used to analyse more than one sample on a single 2D gel. This method employs pre-electrophoretic labeling of proteins with different spectrally resolvable fluorescent cyanide dyes (e.g. Cy2, Cy3, Cy5). Proteins common to the three samples are subsequently labeled with three structurally similar tags, which are size and charge matched. Identical proteins have the same mobility regardless of the dye and will migrate to a single spot on a 2D gel. (Kolkman et al., 2005).

Samples to be compared are labeled with either Cy3 or Cy5 CyDye DIGE fluors, whereas the Cy2 CyDye DIGE fluor is employed to label a pooled sample comprising equal amounts of each of the samples within the study, and acts as an internal standard. The internal standard ensures that all proteins present in the samples are represented, assisting both inter- and intra-gel matching (Lilley and Friedman, 2004).

#### 1.3.3.1.2 Gel-free techniques

**Gel-free MS-based** technologies allows the identification of proteins which are not detected by 2DE (e.g., low abundant proteins, several membrane proteins, proteins with "extreme" molecular weight and/or pI) resulting in a significant increase of the proteome coverage (Kolkman et al., 2005). Depending on the labeling of the sample used it can be defined the ***in vivo*** **labeling** harboring SILAC (Stable Isotope Labeling with Amino acids in Cell culture), $^{15}N$ label and SIP (Stable Isotope Probing) techniques; and ***in vitro*** **labeling** in which ICAT (Isotope-Coded Affinity Tags) and iTRAQ reagents (Isobaric Tags for Relative and Absolute Quantitation) are used. Also, **label-free** quantification workflows have become exceedingly popular in MS-based proteomics. Though not as accurate as isotope labeling based strategies, label-free proteomics has proven to be reliable in quantitation, especially for the comparison of larger cohorts of samples (Otto et al., 2014).

Although these novel approaches were initially pitched as replacements for gel-based methods, they should probably be regarded as complements to rather than replacements of 2-DE. There are many points of comparison and contrast between the standard 2-DE and shotgun analyses, such as sample consumption, depth of proteome coverage, analyses of isoforms and quantitative statistical power. The choice between the different platforms is often determined by the biological question addressed (Abdallah et al., 2012). Representative workflows for the SILAC, ICAT, and iTRAQ technologies are shown in Figure 12. The above strategies are widely used in protein quantitation because of their accuracy; unfortunately, they entail the use of expensive molecules and of specific software to analyze data. Moreover, the number of samples that can be simultaneously analyzed is limited by the number of available labels, and not all strategies can be applied to all types of samples (Neilson et al., 2011).

As for LAB species the main gel-free techniques used has been iTRAQ (De Angelis et al., 2016) it will be described in more detail down below.

Nowadays, the most successful chemical tags are the commercially available isobaric mass tags (TMTs) and **iTRAQ** (Otto et al ., 2014) which are only different in chemical structure. **iTRAQ** tags are isobarics and primarily designed for the labelling of peptides rather than proteins. These reagents are amine-specific and covalently attach isobaric mass labels to the N-terminus and to lysine side chains of peptides. They can be used to label all the peptides in up to eight different biological samples simultaneously. The derivatized precursor peptides are identical in mass and thus indistinguishable in MS spectra but produce strong diagnostic MS–MS signature ions (Ross et al., 2004). This approach is not limited to cysteine-containing peptides and therefore provides a more comprehensive analysis well as increasing the likelihood of analysing post-translational modifications (Lilley and Friedman, 2004).

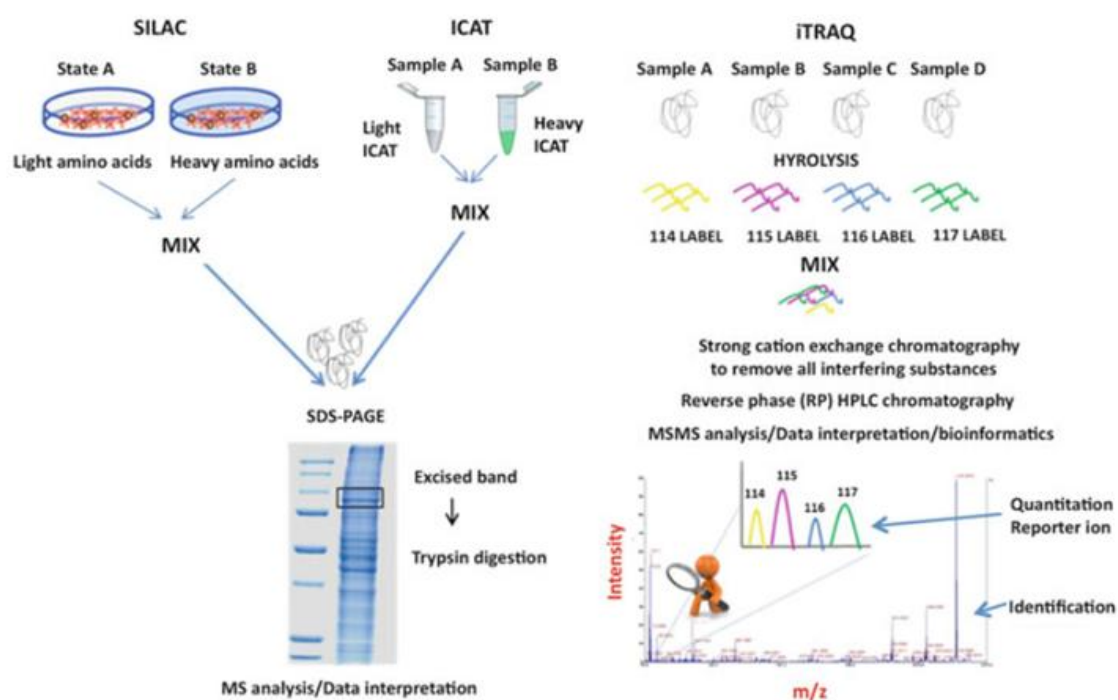


**Figure 12** Representative workflows for SILAC, ICAT, and iTRAQ. The main differences among labeling techniques are (i) SILAC and ICAT labeling are applied on intact proteins, while iTRAQ labeling is performed on peptides, and (ii) in the case of SILAC and ICAT, peptides are quantified during MS analysis, while in the case of iTRAQ, quantitation occurs during fragmentation, i.e., MS/MS analysis (Salvatore et al., 2015).

### 3.3.2 Proteomics in LAB and *O. oeni*

Despite 2DE gel electrophoresis has some drawbacks, largely resulting from experimental variation in gel-to-gel reproducibility, the first proteomic studies of LAB species such as *O. oeni*, used this technique. However, both stable isotope labeling and DIGE currently appear to be the methods of choice for reliable protein expression quantification. Moreover, in the stable isotope approach, the reliability of the quantification increases with the number of peptides of the same protein that are used for quantification (Kolkman et al., 2005). However, classical proteomic techniques have been powerful tools to analyse food bacteria properties in order to choose the best strain (Mangiapane et al., 2015). Also, proteomics has been indispensable to elucidate the proteome diversity, and the mechanisms of regulation of a complex panel of proteins and survival of *Lactobacillus* strains in different ecological niches (De Angelis and Gobbetti, 2004; De Angelis et al., 2016).

2DE proteome mapping of lactobacilli was mainly used to compare proteomes of the same strain grown under different environmental conditions, during various phases of growth or to map proteomes of different strains (see review De Angelis et al., 2016). Gel-free techniques have been used already for this LAB species in order to study cells under bile exposure, low pH and starvation stress (Hussain and Natt, 2014; Hussain, 2015; Lee et al., 2013). Also, these techniques are applied to detect bacterial exoproteome and surface-associate proteome (Espino et al., 2015; Solis and Cordwell, 2011).

Unlike other LAB species in which the amount of proteomic data is significant huge, the use of these techniques with *O. oeni* is still in its infancy. Guzzo et al., (1997, 1994) started using radio labeling with [35S] methionine and 2D-PAGE in the study of protein profiles under stress conditions (during heat shock at 42°C, ethanol and acid shock at pH 3). Also, immunoblotting was used to study the expression of specific proteins, like the malolactic enzyme (Galland et al., 2003; Labarre et al., 1998), $H^+$ATPase (Tourdot-Maréchal et al., 1999) and Lo18 (Guzzo et al., 1997; Jobin et al., 1997).

Currently, only few comparative proteomic analyses of *O. oeni* have been reported (Table 2). Silveira et al., (2004) presented the first global proteomic overview providing evidence for and active ethanol adaptation response of *O. oeni* at the cytoplasmic and

membrane levels. Also, these authors identified some interesting proteins involved in this adaptation to ethanol like glutathione reductase (GshR). The same year, the work of *O. oeni* during starvation revealed differential protein expression in cultures differentially aged (Zapparoli, 2004). However, none of the differently expressed spots were identified. The physiological adaptation of *O. oeni* to wine conditions was studied reporting different protein behaviour from non-acclimated and ethanol-acclimated cells after their inoculation into wine (Cecconi et al., 2009). These variations were mainly attributed to differential modulation of specific proteins involved in stress response and in sugar and amino acid metabolism.

More recently, a cytoplasmic and membrane proteome reference map for *O. oeni* was reported (Mohedano et al., 2014) with the identification of 203 spots an additional 21 spots for the membrane fraction (finally corresponding to 152 unique proteins). The most abundant group contained proteins involved in metabolism (55% of the total), especially in the metabolism of amino acids and the pathways related to energy production and conversion. Also, the importance of malate and citrate metabolism in this species was underlined.

A combined proteomic (2DE-PAGE) and transcriptomic analysis showed that several proteins are affected by the presence of ethanol (Olguín et al., 2015). Functions related to protein synthesis and stability were the main target of ethanol damage. A diminution of the chaperone DnaK and the stress proteins with ATPase activity (Clp and ClpE) was observed after a 12% ethanol shock. Also, a down-regulation of proteins related to murein biosynthesis and translation was reported. Moreover, in this work the decrease in protein concentration due to growth with ethanol was suggested as a relocation of cytosolic proteins in the membrane, as a protective mechanism. Another work which has combined these two “omics” (Costantini et al., 2015) applied the differential gel electrophoresis (DIGE) technique for the first time in *O. oeni*, which allowed a more accurate and sensitive quantitative proteomic analysis. Their aim was focused on describing the physiological behaviour of *O. oeni* when adapted to different media prior to inoculation in wine. To this purpose a rich medium was compared to wine-like media with different ethanol content (8% and 12% respectively). The most significant response of *O. oeni* in media containing ethanol was the increased transcription of stress

related genes and an enhanced production of stress related proteins, as ClpL2 protein, Lo18 and ATP-binding subunit of Clp protease and DnaK/ DnaJ chaperones.

As mentioned above, 2-DE has provided valuable information on the *O. oeni* response and proteomic profile changes under external conditions. This conventional method involving the 2-DE as the final purification step followed by gel digestion and mass spectrometric analysis (MS) has some drawbacks (Fountoulakis et al., 1997; Rabilloud and Lelong, 2011; Tonella et al., 2001). However, MS methodologies still provide a rapid and sensitive tool for the identification and quantification of metabolites, amino acids, and proteins and their post-translational modification (Mazzotti et al., 2012a, 2012b; Napoli et al., 2010). In this sense, to improve the separation of peptides, recently liquid chromatography (LC) has been applied by Napoli et al. (2014) to an isolated *O. oeni* wine strain. Their data set represented the *in vivo* phosphoproteome, since bacterial cells were grown in normal laboratory conditions and were not treated prior to harvesting. Despite some interesting phosphorylated proteins like Cfa were identified, there it is still a lack of information on how phosphorylation affects the function of glycolytic enzymes in bacteria. However, MS/MS analysis identified proteins involved in amino acid metabolism/catabolism, glycolysis, pyruvate and carbohydrate metabolism, and stress response.

Like transcriptomic studies, proteomic works on *O. oeni* are now starting to emerge. However, the perspectives offered by gel-free techniques and all the data obtained from other LAB using proteomics, lead us to hope that in a near future great developments on the knowledge of these species and their adaptation to wine can be made.

**Table 2** Proteomic studies applied to *O. oeni* strains.

| Strain | Origin[a] | Studied conditions[b] | Separation and detection method[c] | Identification method[c] | References |
|---|---|---|---|---|---|
| ATCC BAA-1163 | UBo & ISVV, Red wine, Bordeaux, F | Stress shock: 42ºC, pH 3, EtOH 12% | 2DE, [35 S] methionine-labelled proteins, Coomassie staining | MS | Guzzo et al. (1997, 1994) |
| GM | Microlife Technics, Sarasota, Fla. | Growth in rich medium, EtOH shock and acclimation | 2DE, Coomassie staining | MS | Silveira et al. (2004) |
| VP01 | Valpolicella wine, Verona, I | Growth in rich medium | 2DE, SYPRO Ruby staining | Evaluation of spot intensity (PDQuest software from Bio-Rad) | Zapparoli (2004) |
| VP41 | Lalvin, LA | Acclimated cells to WLM | 2DE, Coomassie staining | Nanoflow-HPLC-ESI-MS/MS | Cecconi et al. (2009) |
| ATCC BAA-1163 | UBo & ISVV, Red wine, Bordeaux, F | Growth in rich medium, total and membrane proteins | 2DE, SYPRO Ruby staining | MALDI-TOF/TOF analysis PMF and MS/MS | Mohedano et al. (2014) |
| ITEM 8261 | Primitivo wine, Apulia, I | Growth in rich medium | HPLC fractionation | MALDI-TOF/TOF MS/MS analysis | Napoli et al. (2014) |
| PSU-1 (=ATCC BAA-331) | Red wine, Pennsylvania State University, US | EtOH shock 12% | 2DE, Coomassie staining | MALDI-TOF MS | Olguín et al. (2015) |
| ICV Elios 1 | LA | EtOH (8% and 12%) acclimation in WLM | 2D-DIGE, Coomassie staining | MALDI-TOF PMF and MS/MS | Costantini et al. (2015) |

[a] ATCC: American Type Culture Collection; UBo: Université de Bourgogne, Dijon, France; ISVV: Institut des Sciences de la Vigne et du Vin, Université de Bordeaux, France; LA: Lallemand Inc., Montreal, Canada; US: United States of America; F: France; I: Italy.

[b] EtOH: ethanol; WLM: wine like media

[c] 2DE, two-dimensional gel-electrophoresis; MALDI, matrix-assisted laser desorption/ionisation; TOF-MS, time-of-flight mass spectrometer; 2D-DIGE, fluorescence difference gel electrophoresis; MS, mass spectrometry; LC, liquid chromatography; ESI-MS, electrospray ionisation mass spectrometry; PMF, peptide mass fingerprinting; HPLC, high performance liquid chromatographic system; iTRAQ, isobaric tags for relative and absolute quantitation

# 2. HYPOTHESIS AND OBJECTIVES

Since I joined the URV LAB research team, the main target of study has been the isolation and the study of *Oenococcus oeni* strains under wine conditions. After the alcoholic fermentation, in order to promote *O. oeni* development, some cases selected starters are inoculated. Unfortunately, due to the wine harsh conditions (low pH, high ethanol content, presence of $SO_2$ and temperature) the loss of viability is a fact (Reguant et al., 2005). The recent research on *O. oeni* has been focused on the isolation and characterization of promising starters due to their potential characteristics (Bordas et al., 2013; Capozzi et al., 2010; Marques et al., 2012; Mesas et al., 2011; Solieri et al., 2010). Moreover, the response to the stress suffered by *O. oeni* has been studied in order to understand the differences between strains. Indeed, some pathways (Olguín et al., 2010, 2009), genes (Beltramo et al., 2006; Guzzo et al., 2000) and proteins (Silveira et al., 2004) were hypothesized some years ago as stress-related. Recently, there has been an increase of global studies with the intention to reveal key proteins and genes involved in stress response. Different parameters of acclimation and adaptation to wine conditions have been studied from an "omics" approach (Cecconi et al., 2009; Costantini et al., 2015; Mohedano et al., 2014; Napoli et al., 2014; Olguín et al., 2015).

Despite being still quite few global studies, some bacterial redox systems appear unequivocally relevant under stress conditions. Among them, glutathione system appears together with thioredoxin (Trx) system as two key antioxidant systems in defence against oxidative stress (Lu and Holmgren, 2014; Smirnova and Oktyabrsky, 2005). As other studies had revealed GSH system as related to wine stress conditions (Cecconi et al., 2009; Costantini et al., 2015; Silveira et al., 2004), it could have a protective role in *O. oeni* response. Thus, glutathione (GSH) as a common antioxidant could be a possible candidate to enhance *O. oeni* adaptation to wine. Moreover, little is known about the action an effect of GSH in this bacterium but the effect of GSH on *O. oeni* and the duration of MLF (Rauhut et al., 2004). Regarding Trx system, several

thioredoxins have been described for different lactic acid bacteria (Serrano et al., 2007; Vido et al., 2005). However, there is only scarce transcriptional information for some genes in *O. oeni* (Guzzo et al., 2000; Jobin et al., 1999) and their use as a genetic marker (Renouf et al., 2008). Thus, none complete study for GSH or Trx systems has yet been established for *O. oeni*.

Within this framework, the **hypothesis** of this thesis was that **multiple molecular mechanisms of *O. oeni* are involved in the stress response to wine conditions, playing glutathione and thioredoxin systems a significant role.**

Thus, the main **objective** of this thesis was the study of *O. oeni* general response and the role of glutathione and thioredoxin systems during wine adaptation and the characterization of all the genes involved.

In order to assess the established assumption the following **specific objectives** were attained to:

1. Assess a global and current view of all stress genes annotated and their prevalence through sequenced *O. oeni* genomes. (CHAPTER I)
2. Establish the adaptation mechanisms of *O. oeni* at transcriptomic and proteomic level during the acclimation to wine before the beginning of MLF. (CHAPTER II)
3. Characterize the effect of GSH addition during growth in different *O. oeni* strains and during an acclimation growth under wine stress conditions. (CHAPTER III)
4. Identify the genomic GSH system in *O. oeni* and its transcriptional expression under stress wine conditions. (CHAPTER IV)
5. Detect the variability of the different thioredoxin genes present in several *O. oeni* strains and their expression in wine conditions. (CHAPTER V)

The results of this thesis could enhance the general knowledge of the main pathways and cellular functions involved in the adaptation of *O. oeni* to wine, which could increase their survival after inoculation into harsh wine conditions and perform a faster MLF. Also, they could provide information about the role of the two main redox systems described above. The possible applications of this thesis go from the basic genetic knowledge of a model bacterium to the improvement of successful MLF.

# CHAPTER I

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## Insights in the comparative pan-genome analysis of *Oenococcus oeni* and its intra-strain variability

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## Abstract

The lactic acid bacterium *Oenococcus oeni* is the most important species involved in malolactic fermentation due to its capability to survive in presence of ethanol and in the acidic environment of wine. In order to identify novel genes involved in adaptation to wine, a genome-wide analysis was performed in strain *O. oeni* PSU-1, and 106 annotated stress genes were identified. The *in silico* analysis revealed the high similarity of all those genes through 57 *O. oeni* genomes available in the NCBI, but seven regions of genomic plasticity could be determined for their different presence observed among these strains. Regions 3 and 5 had the typical hallmarks of horizontal transfer, suggesting that the strategy of acquiring genes from other bacteria enhanced the fitness of *O. oeni* strains. Certain genes related to stress resistance were described in these regions, and similarities of putative acquired regions with other lactic acid bacteria species were found. Some genomic fragments present in all the strains were described and another new genomic island harbouring a threonine dehydrogenase was found. The association of selected sequences with adaptation to wine was assessed by screening 31 *O. oeni* strains using PCR of single genes, but no sequences were found to be exclusive to highly performing malolactic fermentation strains.

## Keywords

*Oenococcus oeni* – Stress genes – Genomic island – PCR – Malolactic fermentation - Wine

## Introduction

*Oenococcus oeni* is a Gram-positive lactic acid bacterium (LAB) isolated mainly from winemaking process (Bordas et al. 2013; González-Arenzana et al. 2013; Wibowo et al. 1985) and it is responsible for the malolactic fermentation (MLF) in red wines. Since *O. oeni* is the LAB species most resistant to wine conditions, its physiological and molecular responses to wine stress have been widely studied (Bartowsky 2005; Beltramo et al. 2006; Bourdineaud et al. 2003; Guzzo et al. 2000; Renouf et al. 2008) in order to unveil strain-specific phenotypes linked to genomic features (Sumby et al. 2014). Recently, combined “omics” approaches have elucidated specific gene activation and protein regulation during stressful wine conditions (Costantini et al. 2015; Margalef-Català et al. 2016a; Olguín et al. 2015).

Genome analyses have indicated *O. oeni* has a compact genome of 1.8 Mb which presumably reflects its adaptation to the ecologically restricted niche of fermenting grape juice and wine (Borneman et al., 2010). The hypermutable status of the genus *Oenococcus* due to the lack of mismatch repair (MMR) – i.e., the genes *mutS* and *mutL* – explains the observed high level of allelic polymorphism among known *O. oeni* isolates and likely contributes to the unique adaptation of this genus to acidic and alcoholic environments (Marcobal et al. 2008). Moreover, recent genomic analyses have explored the molecular mechanisms involved in the fitness and genomic diversity of this bacterium (Borneman et al. 2012; Campbell-Sills et al. 2015; El Gharniti et al. 2012; Sternes and Borneman 2016). Mobile genetic elements, like plasmids, transposable bacteriophage DNAs, transposable elements, genomic islands (GI), and many other specialized genetic elements face bacteria with continuous challenges to genomic stability, promoting evolution through horizontal gene transfer (HGT) (Abby and Daubin, 2007; Darmon and Leach, 2014). Favier et al. (2012) revealed a family of related plasmids associated with industrial starters and indigenous strains of *O. oeni* performing spontaneous MLF that possibly contribute to the technological performance of strains in wine. GIs are typically recognized as DNA segments found in some closely related strains. It is thought that their formation contributes to the diversification and adaptation of microorganisms, thus having a significant impact on the genome plasticity and evolution (Juhas et al., 2009).

Renouf et al. (2008) and Bon et al. (2009) used genomic subtractive hybridization between two isolates of *O. oeni* with differing oenological potential to elucidate the genetic bases of this intra-strain diversity and it was suggested that the selection of *O. oeni* industrial starters could be based on the presence of specific genetic markers (Renouf et al. 2008). In particular, six regions of genomic plasticity were revealed together with novel genes involved in adaptation to wine (Bon et al. 2009). A large proportion of these ORFs resembled genes involved in carbohydrate transport and metabolism, cell wall/membrane/envelope biogenesis, and replication, recombination and repair. More approaches to identify the genomic diversity of *O. oeni* strains and mobile elements have been used like looking up for specific homolog sequences related to stress (Athané et al., 2008), insertion sequences (IS) (El Gharniti et al., 2012) and transposases annotations (Stefanelli, 2014).

The major aim of this study was to identify putative flexible genomic regions through the *O. oeni* available genomes starting from the stress gene annotation of PSU-1 strain. Comparative genome analyses were used to identify genetic differences between the 57 *O. oeni* strain genomes isolated from different world areas, and the results showed seven plasticity regions, highlighting the role of some genes harboured in these fragments. We have confirmed some flexible genomic regions and we have added the current genetic annotation based on the strain PSU-1. As a result, we report an *in silico* approach to detect possible genes and fragments linked to positive oenological characteristics.

## Materials and Methods

### O. oeni *strains and gene sequences*

The *in silico* analysis of the presence of several genes related to stress was carried out with the available 57 different genomes of *O. oeni* sequenced strains (Table S1 in the supplementary data). The nucleotide sequences of stress-related genes were obtained from the complete sequenced genome of strain PSU-1 (accession number NC_008528) in the National Center for Biotechnology Information (NCBI) web page (http://www.ncbi.nlm.nih.gov/), by using the following key words: stress, oxidative, redox, peroxidase, damage, shock, oxidase, redoxin and reductase.

Moreover, 31 *O. oeni* strains were used to deepen the analysis of some genomic regions (Table 1) *in vivo*. Among them, 19 strains were isolated from red wines from south Catalonia, in NE Spain. Other nine strains were commercial starters and three strains with available genome were chosen as controls. Stock cultures (kept frozen at -80ºC) were grown in MRS broth medium (De Man et al., 1960) supplemented with 4 g/l L-malic acid and 5 g/l fructose (MRSmf) at pH 5.0 at a constant 28ºC in a $CO_2$ incubator. Cells were collected at the end of the exponential phase ($OD_{600nm}$ = 1.6) and harvested pellet was kept at -20ºC in order to extract the DNA.

In order to find if there was some correlation between the presence of some genes and oenological characteristics, these 31 strains were assayed in wine like media (WLM) (Bordas et al. 2013) with 4 g/l L-malic acid and ethanol (12-14%, v/v), and time for complete consumption of L-malic acid was evaluated.

*DNA extraction*

The genomic DNA extraction was performed with Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's protocols. Then the DNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany) and the samples were diluted to the concentration of about 10 ng/µl.

*Detection of specific genes by PCR amplification*

Primers used in this study are reported in Table S2 (in the supplementary data). PCR reaction for the amplification of single genes were performed with the GoTaq® DNA polymerase reagents (Promega, USA), whereas the intergenic PCRs were conducted using GoTaq® Long PCR Master Mix (Promega, USA) (Stefanelli, 2014). The amplifications were performed in a thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, USA). In the case of single genes, reactions mix were prepared in a final volume of 20 µl containing 4 µl buffer 5X, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 0.025 U/µl of polymerase, and about 10 ng of DNA. The amplifications were carried out with the following protocol: 5 min at 94°C, 30 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and 72°C for 7 min. For the intergenic PCRs, 0.2 µM of each primer and about 10 ng of DNA were added to 10 µl of GoTaq® Long PCR Master Mix. The protocols of amplifications were: 2 min at 94°C, 35 cycles at 94°C for 30 s, 54-60°C (depending on the genes) for 30 s, 65°C for 1 min for each

kbp, and 72°C for 10 min. The PCR products were verified by electrophoresis on agarose gel at 0.8-1%, coloured with EuroSafe (Euroclone, Italy), and using the marker O'GeneRuler™ DNA (Thermo Scientific, USA).

*Bioinformatics tools*

BLAST (Basic Local Alignment Search Tool) programs (Altschul et al., 1990) -in particular, BLASTN and BLASTX- at the National Center for Biotechnology Information were used to evaluate sequence conservation and the presence or absence of genes in defined NCBI databases (using Whole Genome Shotgun) or local databases (created for CLC Genomics Workbench 7.0.4). After all BLAST analyses hits with significant similarities (90-100% coverage and identity) were sorted or manually curated. Coverage and identity are referred in the text as *cov* and *ide*, respectively. Microbial genomic viewer (v.2.0) (Overmars et al., 2013) was used to extract the GC% of genes and to visualize the genomic regions. The CiVi (Circular genome Visualization) program (Overmars et al., 2015) was used to construct the circular map of PSU-1.

*Phylogenetic tree analysis*

All protein sequences used for phylogenetic analysis were obtained from the NCBI All protein sequences used for phylogenetic analysis were obtained from the NCBI database. Each dataset was aligned using the Muscle computer program (Edgar, 2004) and was manually adjusted with Jalview 2.6.1 (Waterhouse et al., 2009). Phylogenetic analyses were performed using the MEGA v6.0 software package (Tamura et al., 2011), and the neighbour-joining method was used for tree reconstruction (Saitou and Nei, 1987). The statistical reliability of phylogenetic tree topology was evaluated by bootstrapping with 1000 replicates (Felsenstein, 1985).

**Table 1** PCR detection of genomic region R5 in strains of *O. oeni*.

| Strains | R5 | | | MLF finished[a] | | Source[b] | Isolation: wine and place[c] |
|---|---|---|---|---|---|---|---|
| | OEOE_RS03535 | OEOE_RS03560 | OEOE_RS03585 | 12% | 14% | | |
| PSU-1 (=ATCC BAA-331) | + | + | + | + | + | ATCC | Red wine, Pennsylvania State University, USA |
| CECT 217[T] (=ATCC 23279) | + | + | + | - | - | CECT | Red wine, Bordeaux, France |
| ATCC BAA-1163 | + | + | + | | | UBo & ISVV | Red wine, Bordeaux, France |
| 1Pw13 | + | + | + | + | + | URV | Carignan wine, Priorat, S (Franquès, J., unpublished data) |
| 2Pw1 | - | - | - | + | + | URV | Carignan grape, Priorat, S (Franquès, J., unpublished data) |
| 2Pw2 | + | - | + | + | + | URV | Carignan grape, Priorat, S (Franquès, J., unpublished data) |
| 2Pw3 | + | - | + | + | + | URV | Black Grenache grape, Priorat, S (Franquès, J., unpublished data) |
| 2Pw4 | + | - | + | + | + | URV | Carignan grape, Priorat, S (Franquès, J., unpublished data) |
| 2Pw15 | + | - | + | + | + | URV | Black Grenache wine, Priorat, S (Franquès, J., unpublished data) |
| Vi2FB1 | + | + | + | - | - | URV | Black Grenache wine, Priorat, S (Franquès, J., unpublished data) |
| 2T1 | + | - | + | + | - | URV | Grenache wine, Tarragona, S (Bordas et al., 2013) |
| 2T2 | + | - | + | - | - | URV | Grenache wine, Tarragona, S (Bordas et al., 2013) |
| 3T1 | + | - | + | + | - | URV | Grenache wine, Tarragona, S (Bordas et al., 2013) |
| 1P1 | + | - | + | + | - | URV | Grenache wine, Priorat, S (Bordas et al., 2013) |
| 1P2 | - | - | - | + | + | URV | Grenache wine, Priorat, S (Bordas et al., 2013) |
| 1P3 | + | - | + | - | - | URV | Grenache wine, Priorat, S (Bordas et al., 2013) |
| 2P2 | + | - | + | + | - | URV | Cabernet Sauvignon wine, Priorat, S (Bordas et al., 2013) |
| 3P2 | + | - | + | + | + | URV | Cabernet Sauvignon wine, Priorat, S (Bordas et al., 2013) |
| Mf3 (=CECT 7441) | - | + | - | + | + | URV | Grenache wine, Tarragona, S (Reguant and Bordons, 2003) |
| Mf6 (=CECT 7440) | + | + | + | + | + | URV | Tempranillo wine, Tarragona, S (Reguant and Bordons, 2003) |
| UB51 | + | - | + | + | + | URV | Tempranillo wine, Conca de Barberà, S (Masqué and Bordons, 1996) |
| Fn42 | + | + | + | + | + | URV | Monastrell wine, Priorat, S (Masqué and Bordons, 1996) |
| Lactoenos 450 PreAc® (Lo450) | + | + | + | + | + | LAF | Not specified |
| Microoenos MBR B1 (MO) | + | + | + | + | + | LAF | Not specified |
| Enoferm Alfa® (Alfa) | + | - | + | + | + | LA | Not specified |
| Lalvin VP41® (VP41) | + | - | + | + | + | LA | Not specified |
| Viniferm OE 104 (Agv) | + | - | + | + | + | AG | Not specified |
| Viniflora® Oenos (VO) | + | - | + | + | + | CH | Not specified |
| Viniflora® CH11 (CH11) | + | - | + | + | + | CH | Not specified |
| Viniflora® CH16 (CH16) | + | + | + | + | + | CH | Not specified |
| Vitilactic® F (VL) | - | - | - | + | + | MV | Not specified |

[a] Malolactic fermentation (consumption of 2 g/L L-malic acid) finished in WLM (+) or not (-).

[b] ATCC: American Type Culture Collection; CECT: Colección Española de Cultivos Tipo, València, Spain; UBo: Université de Bourgogne, Dijon, France; ISVV: Institut des Sciences de la Vigne et du Vin, Université de Bordeaux, France; URV: Universitat Rovira i Virgili, Tarragona, Spain; Laffort, Bordeaux, France; LA: Lallemand Inc., Montreal, Canada; AG: Agrovin, Alcázar de San Juan, Spain; CH: Chr. Hansen A/S, Hørsholm, Denmark. LAF; MV: Martin Vialatte, Épernay, France.

[c] US: United States of America; F: France; S: Spain.

## Results

In silico *analysis of annotated stress genes in* O. oeni *PSU-1*

This analysis was carried out before the PSU-1 genome annotation update in NCBI database (February 2015). The list of all the ORFs, with their correspondence to the current annotation and its location in the genome, are available in Table S3 (in the supplementary data). In this text, the current annotations OEOE_RS have been abbreviated using RS followed by the four last numbers.

A subset of 106 genes (Table 2) was picked up using the computational annotation prediction in the NCBI, according to their stress-related annotation in *O. oeni* PSU-1 strain. These sequences were used to unveil the genetic variability among all the genomes available for *O. oeni*. The complete assembled genome of strain PSU-1 allowed the localization of the 106 genes in its genome (grey circles of ring 1 in Figure 1). Most of them are distributed around all the genome, but there are some areas denser than others.

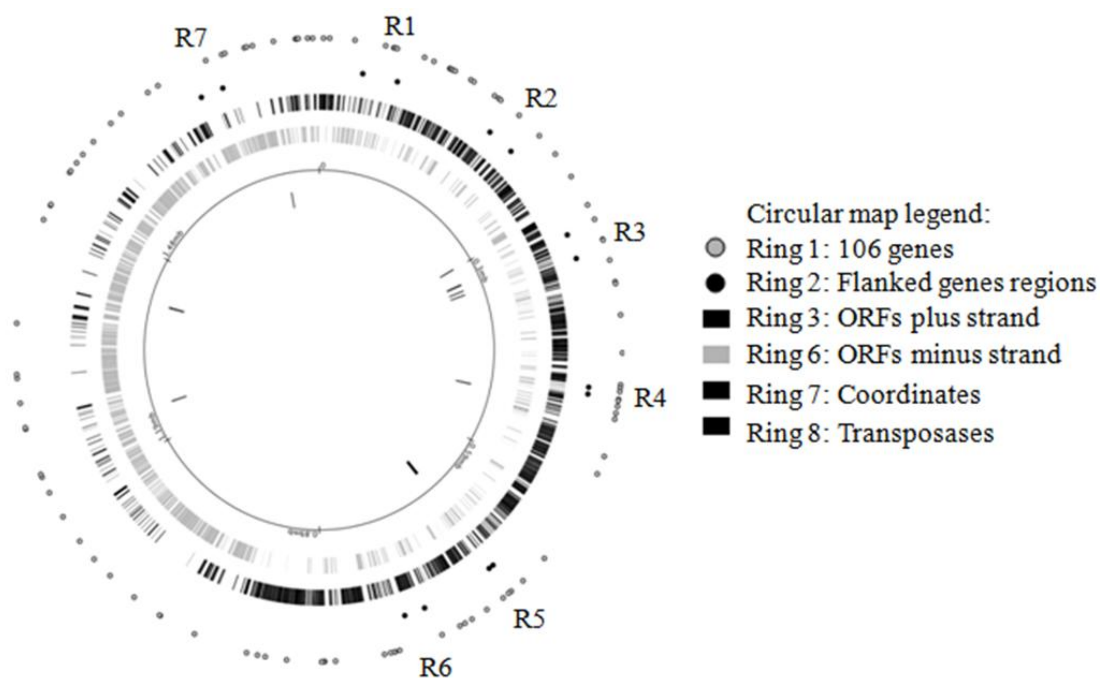


**Figure 1** Localization of the 106 genes related to stress, the flanked genes of the seven genomic regions and transposase genes on *O. oeni* PSU-1 genome. The rings indicated in the legend correspond from outside to inside.

The presence or the absence of all these 106 genes was looked up in 57 *O. oeni* strains (Table S1 in the supplementary data) available in NCBI. The number of strains that harboured each gene, with a high coverage (*cov*) and identity (*ide*), was represented in Figure 2. From this *in silico* analysis, seven regions (R1-R7) appeared as putative genomic flexible regions, because of their absence in at least 15% of the *O. oeni* genomes.

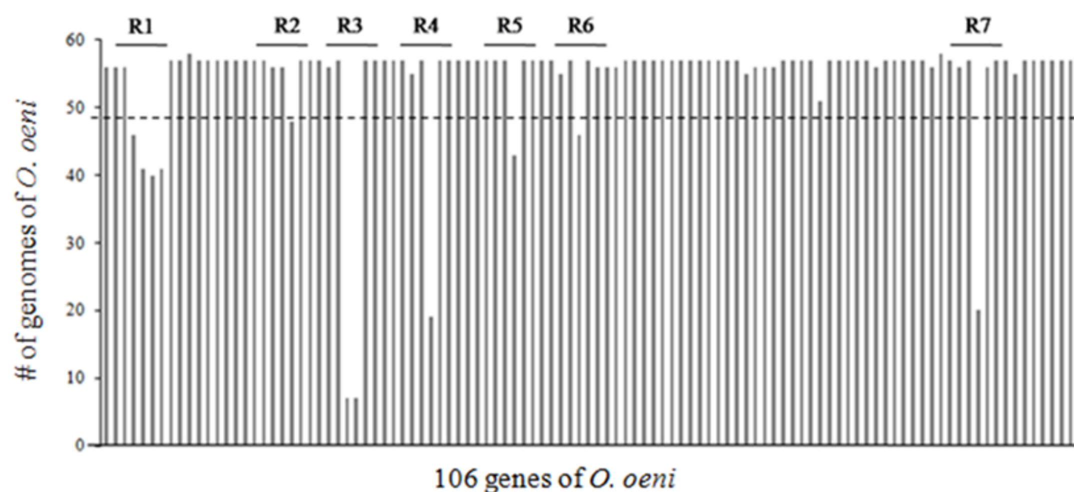


**Figure 2** Number of *O. oeni* genomes in database presenting gene sequences with a high identity and coverage with the 106 genes selected from strain PSU-1. The identified genomic regions (R1-R7) enclosed fragments absent in at least 15% of the genomes tested.

**Table 2** The 106 selected genes of *O. oeni* PSU-1 according to their annotation related to stress.

| Old gene code | Old annotation | G+C% | No. of strains harbouring | New gene code | Current annotation |
|---|---|---|---|---|---|
| OEOE_0005 | DNA gyrase subunit B | 43 | 56 | OEOE_RS00025 | DNA gyrase subunit B |
| OEOE_0009 | putative multicopper oxidase | 43 | 56 | OEOE_RS00040 | multicopper oxidase |
| OEOE_0036 | aldo/keto reductase | 41 | 56 | OEOE_RS00165 | oxidoreductase ion channel protein IolS |
| OEOE_0070 | aldo/keto reductase related protein | 42 | 46 | OEOE_RS00320 | 2,5-diketo-D-gluconic acid reductase |
| OEOE_0077 | 3-oxoacyl-ACP reductase | 38 | 41 | OEOE_RS00350 | diacetyl reductase [(S)-acetoin forming] |
| OEOE_0078 | short chain dehydrogenase | 37 | 40 | OEOE_RS00355 | short-chain dehydrogenase |
| OEOE_0081 | NADPH:quinone reductase or related Zn-dependent oxidoreductase | 43 | 41 | OEOE_RS00370 | NADPH:quinone reductase |
| OEOE_0106 | PAS/PAC sensor signal transduction histidine kinase | 40 | 57 | OEOE_RS00490 | histidine kinase |
| OEOE_0107 | trypsin-like serine protease | 43 | 57 | OEOE_RS00495 | serine protease |
| OEOE_0120 | Signal transduction histidine kinase | 38 | 58 | OEOE_RS00555 | histidine kinase |
| OEOE_0138 | ribonucleotide reduction protein | 32 | 57 | OEOE_RS00640 | ribonucleotide reductase |
| OEOE_0139 | ribonucleoside-diphosphate reductase class Ib glutaredoxin subunit | 37 | 57 | OEOE_RS00645 | glutaredoxin NC_008528.1 |
| OEOE_0140 | ribonucleotide-diphosphate reductase subunit alpha | 41 | 57 | OEOE_RS00650 | ribonucleotide-diphosphate reductase subunit alpha |
| OEOE_0141 | ribonucleotide-diphosphate reductase subunit beta | 38 | 57 | OEOE_RS00655 | ribonucleoside-diphosphate reductase |
| OEOE_0143 | Signal transduction histidine kinase | 39 | 57 | OEOE_RS00665 | histidine kinase |
| OEOE_0159 | ribose-5-phosphate isomerase A | 43 | 57 | OEOE_RS00750 | ribose 5-phosphate isomerase A |
| OEOE_0163 | thioredoxin reductase | 42 | 57 | OEOE_RS00770 | ferredoxin--NADP reductase |
| OEOE_0184 | ribosome-associated heat shock protein implicated in the recycling of the 50S subunit S4-like protein | 37 | 57 | OEOE_RS00875 | hypothetical protein |
| OEOE_0189 | disulfide bond chaperones of the HSP33 family | 38 | 56 | OEOE_RS00900 | disulfide bond formation protein |
| OEOE_0191 | hypothetical protein | 36 | 56 | OEOE_RS00910 | hypothetical protein |
| OEOE_0217 | threonine dehydrogenase or related Zn-dependent dehydrogenase | 42 | 48 | OEOE_RS01025 | threonine dehydrogenase |
| OEOE_0242 | Short-chain alcohol dehydrogenase | 43 | 57 | OEOE_RS01155 | 3-beta-hydroxysteroid dehydrogenase |
| OEOE_0264 | dihydroorotate dehydrogenase 1A | 38 | 57 | OEOE_RS01265 | dihydroorotate dehydrogenase |
| OEOE_0289 | heat shock protein Hsp20 | 38 | 57 | OEOE_RS01385 | heat-shock protein Hsp20 |
| OEOE_0318 | DNA repair protein RadA | 44 | 56 | OEOE_RS02615 | DNA repair protein RadA |
| OEOE_0331 | dihydrolipoamide dehydrogenase | 44 | 57 | OEOE_RS01585 | dihydrolipoamide dehydrogenase |
| OEOE_0350 | Thiol-disulfide isomerase and thioredoxin | 45 | 7 | OEOE_RS01675 | thioredoxin NC_008528.1 |
| OEOE_0353 | DNA-binding ferritin-like protein (oxidative damage protectant) | 44 | 7 | OEOE_RS01690 | DNA-binding protein |
| OEOE_0373 | Short-chain alcohol dehydrogenase | 45 | 57 | OEOE_RS01790 | 3-beta-hydroxysteroid dehydrogenase |
| OEOE_0394 | threonine dehydrogenase or related Zn-dependent dehydrogenase | 43 | 57 | OEOE_RS01895 | threonine dehydrogenase |
| OEOE_0396 | carbonyl reductase | 39 | 57 | OEOE_RS01905 | carbonyl reductase |
| OEOE_0427 | NAD-dependent DNA ligase | 41 | 57 | OEOE_RS02055 | DNA ligase |
| OEOE_0459 | flavodoxin | 41 | 57 | OEOE_RS02210 | flavodoxin |
| OEOE_0489 | Signal transduction histidine kinase | 31 | 55 | OEOE_RS02335 | histidine kinase |
| OEOE_0492 | ferric reductase | 31 | 57 | OEOE_RS02350 | iron reductase |
| OEOE_0495 | NADPH:quinone reductase or related Zn-dependent oxidoreductase | 35 | 19 | OEOE_RS02365 | NADPH:quinone reductase |
| OEOE_0508 | peptide methionine sulfoxide reductase | 38 | 57 | OEOE_RS02415 | peptide methionine sulfoxide reductase |
| OEOE_0513 | transcriptional repressor of class III stress genes | 38 | 57 | OEOE_RS02440 | hypothetical protein |
| OEOE_0518 | aldo/keto reductase | 43 | 57 | OEOE_RS02465 | 2,5-diketo-D-gluconic acid reductase |
| OEOE_0527 | alcohol dehydrogenase | 44 | 57 | OEOE_RS02510 | hypothetical protein |
| OEOE_0566 | thioredoxin reductase | 43 | 57 | OEOE_RS02695 | thioredoxin reductase |
| OEOE_0584 | NADPH:quinone reductase or related Zn-dependent oxidoreductase | 40 | 57 | OEOE_RS02785 | NADPH:quinone reductase |
| OEOE_0693 | acetoin reductase | 42 | 57 | OEOE_RS03325 | acetoin reductase |
| OEOE_0727 | putative NADPH-quinone reductase (modulator of drug activity B) | 34 | 57 | OEOE_RS03470 | NADPH-quinone reductase |
| OEOE_0745 | threonine dehydrogenase or related Zn-dependent dehydrogenase | 39 | 43 | OEOE_RS03560 | threonine dehydrogenase |
| OEOE_0749 | putative NADH-flavin reductase | 39 | 57 | OEOE_RS03585 | NADH-flavin reductase |
| OEOE_0755 | nitroreductase | 41 | 57 | OEOE_RS03615 | nitroreductase |
| OEOE_0775 | dihydrodipicolinate reductase | 38 | 57 | OEOE_RS03710 | dihydrodipicolinate reductase |

| | | | | | |
|---|---|---|---|---|---|
| OEOE_0790 | ribulose-5-phosphate 3-epimerase | 40 | 57 | OEOE_RS03595 | ribulose-phosphate 3-epimerase |
| OEOE_0799 | methylated DNA-protein cysteine methyltransferase | 34 | 55 | OEOE_RS03830 | methylated DNA-protein cysteine methyltransferase |
| OEOE_0807 | stress response membrane GTPase | 42 | 57 | OEOE_RS03875 | GTP-binding protein |
| OEOE_0826 | aldo/keto reductase | 41 | 46 | OEOE_RS03970 | 2,5-diketo-D-gluconic acid reductase |
| OEOE_0875 | flavoprotein | 38 | 57 | OEOE_RS04190 | FMN reductase |
| OEOE_0880 | ribonucleoside-diphosphate reductase class Ib glutaredoxin subunit | 30 | 56 | OEOE_RS04215 | ribonucleoside-diphosphate reductase |
| OEOE_0881 | Acyl carrier protein phosphodiesterase | 34 | 56 | OEOE_RS04220 | ACP phosphodiesterase |
| OEOE_0884 | membrane signal transduction histidine kinase | 40 | 56 | OEOE_RS04235 | sensor histidine kinase |
| OEOE_0888 | glutathione peroxidase | 37 | 57 | OEOE_RS04255 | glutathione peroxidase |
| OEOE_0936 | pyruvate oxidase | 41 | 57 | OEOE_RS04485 | pyruvate oxidase |
| OEOE_0945 | hypothetical protein | 45 | 57 | OEOE_RS04530 | hypothetical protein |
| OEOE_0946 | pyrroline-5-carboxylate reductase | 40 | 57 | OEOE_RS04535 | pyrroline-5-carboxylate reductase |
| OEOE_0948 | gamma-glutamyl phosphate reductase | 38 | 57 | OEOE_RS04545 | gamma-glutamyl phosphate reductase |
| OEOE_0987 | metal-dependent hydrolase | 37 | 57 | OEOE_RS04740 | endoribonuclease YbeY |
| OEOE_1006 | ribosomal large subunit pseudouridine synthase B | 36 | 57 | OEOE_RS04830 | ribosomal large subunit pseudouridine synthase |
| OEOE_1013 | dihydrofolate reductase | 35 | 57 | OEOE_RS04865 | dihydrofolate reductase |
| OEOE_1026 | DNA topoisomerase IV subunit B | 42 | 57 | OEOE_RS04930 | DNA gyrase subunit B |
| OEOE_1079 | ribosomal large subunit pseudouridine synthase D | 40 | 57 | OEOE_RS05185 | RNA pseudouridine synthase |
| OEOE_1115 | ribonucleoside-triphosphate reductase class III activase subunit | 41 | 57 | OEOE_RS05360 | anaerobic ribonucleoside-triphosphate reductase activating protein |
| OEOE_1116 | anaerobic ribonucleoside triphosphate reductase | 41 | 57 | OEOE_RS05365 | ribonucleoside triphosphate reductase |
| OEOE_1140 | cell division protein | 38 | 57 | OEOE_RS05485 | cell division protein |
| OEOE_1169 | UspA family nucleotide-binding protein | 40 | 55 | OEOE_RS05630 | universal stress protein UspA |
| OEOE_1191 | glutathione reductase | 42 | 56 | OEOE_RS05740 | glutathione reductase |
| OEOE_1228 | RNA-binding protein | 38 | 56 | OEOE_RS05915 | RNA-binding protein |
| OEOE_1258 | Lon-like protease | 39 | 56 | OEOE_RS06065 | peptidase NC_008528.1 |
| OEOE_1290 | NADPH:quinone reductase or related Zn-dependent oxidoreductase | 38 | 57 | OEOE_RS06215 | NADPH:quinone reductase |
| OEOE_1308 | DnaJ-like molecular chaperone | 42 | 57 | OEOE_RS06305 | molecular chaperone DnaJ |
| OEOE_1310 | molecular chaperone GrpE (heat shock protein) | 43 | 57 | OEOE_RS06315 | protein GrpE |
| OEOE_1358 | NADPH:quinone reductase or related Zn-dependent oxidoreductase | 40 | 57 | OEOE_RS06535 | NADPH:quinone reductase |
| OEOE_1360 | sigma 54 modulation protein / SSU ribosomal protein S30P | 36 | 51 | OEOE_RS06545 | raiA ribosome-associated inhibitor A |
| OEOE_1376 | cold-shock DNA-binding protein family protein | 40 | 57 | OEOE_RS06620 | cold-shock protein |
| OEOE_1394 | 30S ribosomal protein S4 | 43 | 57 | discontinued | |
| OEOE_1398 | redox-sensing transcriptional repressor Rex | 40 | 57 | OEOE_RS06735 | transcriptional regulator |
| OEOE_1445 | dTDP-4-dehydrorhamnose reductase | 38 | 57 | OEOE_RS06980 | dTDP-4-dehydrorhamnose reductase |
| OEOE_1538 | peptide methionine sulfoxide reductase | 44 | 57 | OEOE_RS07415 | peptide methionine sulfoxide reductase |
| OEOE_1553 | aldo/keto reductase | 42 | 56 | OEOE_RS07490 | 2,5-diketo-D-gluconic acid reductase |
| OEOE_1554 | peptide methionine sulfoxide reductase domain-containing protein | 41 | 57 | OEOE_RS07495 | methionine sulfoxide reductase B |
| OEOE_1589 | 3-oxoacyl-ACP reductase | 40 | 57 | OEOE_RS07665 | 3-ketoacyl-ACP reductase |
| OEOE_1591 | dioxygenase | 45 | 57 | OEOE_RS07675 | 2-nitropropane dioxygenase |
| OEOE_1602 | acetoin reductase | 44 | 57 | OEOE_RS07730 | diacetyl reductase |
| OEOE_1611 | ribose-5-phosphate isomerase A | 40 | 57 | OEOE_RS07770 | ribose 5-phosphate isomerase |
| OEOE_1625 | Thiol-disulfide isomerase and thioredoxin | 40 | 56 | OEOE_RS07835 | thiol-disulfide isomerase |
| OEOE_1646 | universal stress protein UspA-like nucleotide-binding protein | 39 | 58 | OEOE_RS07940 | universal stress protein UspA |
| OEOE_1658 | UDP-N-acetylmuramate dehydrogenase | 41 | 57 | OEOE_RS08000 | UDP-N-acetylenolpyruvoylglucosamine reductase |
| OEOE_1690 | threonine dehydrogenase or related Zn-dependent dehydrogenase | 42 | 56 | OEOE_RS08155 | threonine dehydrogenase |
| OEOE_1702 | Thiol-disulfide isomerase and thioredoxin | 39 | 57 | OEOE_RS08215 | thioredoxin NC_008528.1 |
| OEOE_1750 | DNA-binding ferritin-like protein (oxidative damage protectant) | 41 | 20 | OEOE_RS08440 | ferritin NC_008528.1 |
| OEOE_1767 | putative NADPH-quinone reductase (modulator of drug activity B) | 37 | 56 | OEOE_RS08520 | oxidoreductase NC_008528.1 |
| OEOE_1772 | Signal transduction histidine kinase | 38 | 57 | OEOE_RS08540 | histidine kinase |
| OEOE_1791 | universal stress protein UspA-like nucleotide-binding protein | 40 | 57 | OEOE_RS08630 | universal stress protein UspA |
| OEOE_1794 | universal stress protein UspA-like nucleotide-binding protein | 38 | 55 | OEOE_RS08645 | universal stress protein UspA |

| | | | | | |
|---|---|---|---|---|---|
| OEOE_1799 | 3-hydroxy-3-methylglutaryl-coenzyme A reductase | 42 | 57 | OEOE_RS08670 | 3-hydroxy-3-methylglutaryl-CoA reductase |
| OEOE_1818 | pyruvate oxidase | 40 | 57 | OEOE_RS08770 | pyruvate oxidase |
| OEOE_1836 | cytochrome bd-type quinol oxidase, subunit 2 | 37 | 57 | OEOE_RS08865 | cytochrome C oxidase assembly protein |
| OEOE_1837 | cytochrome bd-type quinol oxidase, subunit 1 | 40 | 57 | OEOE_RS08870 | cytochrome D ubiquinol oxidase subunit |
| OEOE_1838 | NADH dehydrogenase, FAD-containing subunit | 39 | 57 | OEOE_RS08875 | pyridine nucleotide-disulfide oxidoreductase |
| OEOE_1852 | redox protein, regulator of disulfide bond formation | 39 | 57 | OEOE_RS08940 | osmotically inducible protein C |
| OEOE_1858 | aldo/keto reductase | 37 | 57 | OEOE_RS08970 | 2,5-diketo-D-gluconic acid reductase |

*Analysis of high-plasticity genomic regions in* O. oeni

Genomic regions were further analysed. All the gene sequences harboured in each marked region identified (Figure 2) were used as queries in a new BLASTN analysis against all the 57 genomes of *O. oeni*. From this deeper analysis the preliminary regions were shortened and their prevalence among all the genomes was represented in Figure 3. Also, black circles in Figure 1 indicate the flanked location of genomic regions in PSU-1 genome. As can be seen, the seven regions are quite dispersed throughout the genome, except for the third quadrant, which does not contain any of them.

In the following sections each plasticity region is analysed in detail, reporting previous studies related to it, the most relevant oenological genes harboured, and the further analysis undertaken. Also, a point is made in the presence of transcriptional regulators (TR) due to their possible conjugative elements, the hypothetical proteins (HP), genes annotated as transposases (T) and described IS elements, because they have been described as genetic mobility elements (Juhas et al., 2009). A summary of each genomic region with their more relevant genes is displayed in Table 3.

**Table 3** Summary of the relevant genes harboured in the seven flexible genomic regions found in *O. oeni* and related studies.

| Regions | Relevant genes | Current annotation | No. of strains harbouring | Main changes found under wine-related conditions (References)[a] |
|---|---|---|---|---|
| R1 | RS0230 | NAD(P)-dependent oxidoreductase | 42 | |
| | RS0240 | hypothetical protein | 19 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS0260 | transcriptional regulator | 42 | |
| | RS0285 | transcriptional regulator | 27 | |
| | RS0290 | hypothetical protein | 27 | ↑ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS0295 | phytoene synthase | 27 | |
| | RS0300 | pseudo | 27 | |
| | RS0305 | oxidoreductase | 27 | |
| | RS0310 | hypothetical protein | 26 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS0315 | membrane protein | 26 | |
| | RS0320 | 2,5-diketo-D-gluconic acid reductase | 46 | ↓ P ethanol presence 12% (Olguín et al., 2015)<br>↓ T and ↑ P WLM acclimation (Margalef-Català et al. 2016a)<br>↑ T in 8% and 12% ethanol in WLM (Costantini et al., 2015) |
| | RS0325 | general stress protein | 26 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS0330 | pseudo | 25 | |
| | RS0335 | hypothetical protein | 29 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS0340 | MFS transporter | 57 | GM M11 (Renouf et al., 2008)<br>GM M14 (Renouf et al., 2008) |
| | RS0345 | MFS transporter | 39 | ↑ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS0350 | diacetyl reductase [(S)-acetoin forming] | 41 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS0355 | short-chain dehydrogenase | 40 | ↑ T combined stress condition of 10% ethanol and pH 3.5 (Stefanelli, 2014), GM M9 (Renouf et al., 2008) |
| | RS0360 | transcriptional regulator | 42 | |
| | RS0370 | NADPH:quinone reductase | 41 | |
| | RS0375 | MarR family transcriptional regulator | 42 | |
| | RS0380 | hypothetical protein | 42 | ↑ T WLM acclimation (Margalef-Català et al. 2016a)<br>GM M8 (Renouf et al., 2008) |
| | RS0385 | hypothetical protein | 41 | ↑ T WLM acclimation (Margalef-Català et al. 2016a) |
| R2 | RS0980 | 1,4-beta-N-acetylmuramidase | 48 | ↓ T ethanol presence 12% (Olguín et al., 2015) |
| | RS0995 | AraC family transcriptional regulator | 44 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS1000 | hypothetical protein | 44 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS1015 | D-alanyl-D-alanine dipeptidase | 48 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS1020 | TetR family transcriptional regulator | 48 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |

| | | | | |
|---|---|---|---|---|
| | RS1025 | threonine dehydrogenase | 50 | |
| | RS1030 | glycosyltransferase | 42 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS1045 | PTS sugar transporter subunit IIA | 54 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS1050 | PTS cellobiose transporter subunit IIA | 50 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS1060 | 6-phospho-beta-glucosidase | 53 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS1065 | transcriptional regulator | 54 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS1070 | GntR family transcriptional regulator | 56 | |
| | RS1080 | araD pseudo | 58 | |
| | RS1105 | transcriptional antiterminator | 54 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| R3 | RS1600 | transposase | 37 | |
| | RS1605 | transposase | 31 | |
| | RS1620 | PTS sugar transporter | 27 | |
| | RS1625 | PTS cellobiose transporter subunit IIA | 27 | |
| | RS1645 | PTS fructose transporter subunit IIC | 27 | |
| | RS1655 | acetyltransferase | 26 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS1660 | Transposase | 6 | |
| | RS1665 | haloacid dehalogenase | 7 | GM M1 (Renouf et al., 2008) |
| | RS1670 | glycerol uptake permease | 7 | GM M13 (Renouf et al., 2008) |
| | RS1675 | thioredoxin | 8 | ↑ T WLM 12% ethanol pH 3.4 (Margalef-Català et al., 2016b)<br>GM M12(Renouf et al., 2008) |
| | RS1680 | hypothetical protein | 8 | |
| | RS1685 | hypothetical protein | 8 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS1690 | DNA-binding protein | 8 | ↓ T acid shock (Bon et al., 2009) GM M2(Renouf et al., 2008) |
| | RS1695 | copper chaperone | 7 | ↑ T acid shock (Bon et al., 2009) GM M10 (Renouf et al., 2008) |
| | RS1700 | Crp/Fnr family transcriptional regulator | 7 | ↓ T acid shock ↑ G heat shock (Bon et al., 2009)<br>GM M5 (Renouf et al., 2008) |
| | RS1705 | transposase | 22 | |
| | RS1710 | metal transporter CorA | 52 | |
| | RS1715 | pseudo | 13 | |
| | RS1720 | hypothetical protein | 11 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS1725 | pseudo | 6 | |
| | RS1730 | pseudo | 17 | |
| | RS1735 | pseudo | 24 | |
| R4 | RS2365 | NADPH:quinone reductase | 19 | ↑ T at 42°C (Stefanelli, 2014) |
| | RS2370 | hypothetical protein | 19 | ↑ T at 42°C (Stefanelli, 2014) |
| | RS2375 | ArsR family transcriptional regulator | 19 | |
| | RS2380 | pseudo | 19 | |
| | RS2385 | pseudo | 19 | ↑ T 10% ethanol (Stefanelli, 2014) |
| | RS2390 | transcriptional regulator | 19 | ↑ T at 42°C (Stefanelli, 2014) |
| | RS2395 | Hypothetical protein | 19 | ↑ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS2400 | MFS transporter | 19 | ↑ T 10% ethanol (Stefanelli, 2014)<br>↑ T WLM acclimation (Margalef-Català et al. 2016a) |
| R5 | RS3550 | transposase | 37 | |
| | RS3555 | transposase | 31 | |
| | RS3560 | threonine dehydrogenase | 43 | ↓ T 10% ethanol and at 42ºC (Stefanelli, 2014) |
| | RS3565 | hypothetical protein | 43 | |
| | RS3570 | transposase | 31 | |
| | RS3575 | transposase | 37 | |
| | RS3585 | NADH-flavin reductase | 57 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| R6 | RS4000 | hypothetical protein | 57 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS4005 | hypothetical protein | 35 | |
| | RS4010 | acetyltransferase | 57 | ↑ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS4015 | membrane protein | 57 | ↑ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS4020 | haloacid dehalogenase | 57 | ↑ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS4025 | hypothetical protein | 57 | ↑ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS4035 | leucine--tRNA ligase | 57 | |
| | RS4040 | peptide ABC transporter substrate-binding protein | 49 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS4045 | peptide ABC transporter substrate-binding protein | 44 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| R7 | RS8360 | RNA polymerase I and III, subunit | 57 | ↑ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS8365 | glucosaminidase | 26 | ↓ T ethanol 12% (Olguín et al., 2015) |
| | RS8370 | polysaccharide biosynthesis export protein | 12 | GM M3 (Renouf et al., 2008) |
| | RS8375 | glycosyl transferase | 12 | |
| | RS8385 | glycosyl transferase | 14 | |
| | RS8390 | hypothetical protein | 14 | |
| | RS8395 | capsular polysaccharide biosynthesis protein | 14 | |
| | RS8405 | glycosyl transferase | 14 | |
| | RS8425 | methionyl-tRNA synthetase | 57 | ↓ P WLM acclimation (Margalef-Català et al. 2016a) |
| | RS8435 | HD family phosphohydrolase | 57 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS8440 | ferritin | 20 | ↓ T low pH (3.5) (Stefanelli, 2014)<br>↑ P WLM acclimation (Margalef-Català et al. 2016a)) |
| | RS8445 | hypothetical protein | 19 | ↓ T WLM acclimation (Margalef-Català et al. 2016a) |
| | RS8450 | transposase | 44 | |

[a] ↑ T : over-expressed gene; ↓ T: repressed gene; ↑ P: up-regulated protein; ↓ P: down-regulated protein; GM: Genetic Marker; WLM: wine-like-media

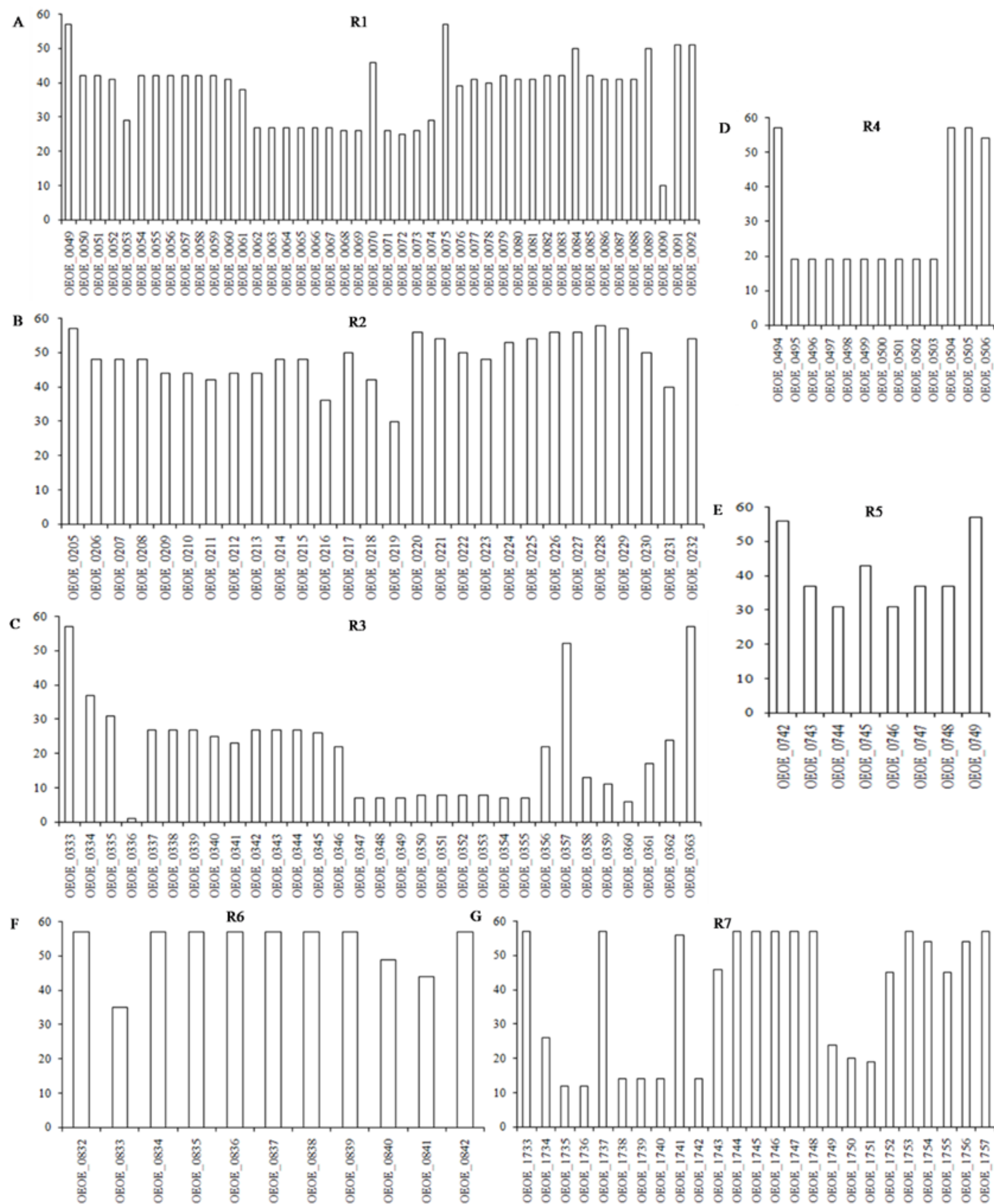


**Figure 3** Number of *O. oeni* genomes (vertical axis) in database presenting gene sequences with a high identity and coverage with the genes from the strain PSU-1 harboured in the seven identified genomic regions in Figure 2.

*Genetic study of genomic region R1 (RS0225–RS0420)*

The genomic region R1 of *O. oeni* is the biggest region found in this research, and it comprises 49 ORFs whose presence among the studied genomes is represented in Figure 3A. The main flexible region is located in the central part of the R1. This fragment is present only in 27 strains, with the exception of the gene RS0320, which codifies for a 2,5-diketo-D-gluconic acid reductase, which is widespread among 46 strains. Specifically, the region RS0275-RS0405 was well reported by Bon et al. (2009), being referred by them as region 2. Due to the low presence of this part in *O. oeni* strains we analysed the similarity of RS0285-RS0335 with other LAB species. The BLASTN analysis indicated the highest match for *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC8293 (48% *cov* / 69% *ide*).

R1 harbours a variety of genes, including those coding for proteins involved in carotenoid biosynthesis and various genes dedicated to unspecific transport, used before as genetic markers for oenological *O. oeni* strains by Renouf et al. (2008) (Table 3). R1 also contains genes encoding for enzymes involved in oxidation and reduction reactions, 13 HP whose function have yet to be elucidated, four TR and seven genes related to stress. The genes RS0230, RS0305, RS0320 and RS0370 code for an oxidoreductase activity, thus it is difficult to establish a more specific action. RS0325 is currently described as coding for a general stress protein, but it is worth noting that this gene was previously studied due to its former annotation associated to exopolysaccharide (EPS) production (Costantini et al., 2015). One of the key enzymes of citrate metabolism, a diacetyl reductase [(S)-acetoin forming] (RS0350), is located in this R1 and harboured in 71% of the genomes studied here.

We analysed the possible identity of one TR (RS0260) with other LAB species. The BLASTX analysis matched a general stress protein from *Enterococcus raffinosus* (93% *cov* / 53% *ide*). For the others TR enclosed in R1, BLASTN analysis matched: RS0285 with *Lactobacillus brevis* (90% *cov* / 75% *ide*), RS0360 with *Leuconostoc gelidum* (90% / 73%) and RS0375 with *Lb. brevis* (59% *cov* / 75% *ide*).

*Genetic study of genomic region R2 (RS0975-RS1105)*

The R2 of *O. oeni* enclosed 28 ORFs (Figure 3B), four of them codifying for HP, another five for TR and six sequences potentially associated to fitness (Table 3). Among

these latter sequences, three PTS sugar transporters (phosphotransferase system) were harboured in more than 84% of the strains studied. The gene RS0980, annotated as 1,4-beta-N-acetylmuramidase and related to cell wall biogenesis, is harboured by less strains (48 strains).

ORF RS1025, one of the four annotated threonine dehydrogenase (*trhD*) in PSU-1 genome, is also located in R2. A phylogenetic analysis of these four *trhD* sequences was carried out to know if they were copies of the same gene present in different parts of the genome. This analysis revealed that the two protein sequences encoded by RS1025 and RS8155 showed some similarity, with a 30% amino acid identity, while the others are not phylogenetically correlated therefore they could not derive from gene duplication (Figure S1). The BLASTX analysis of RS1025 of *O. oeni* with other LAB species matched different species of *Lactobacillus* (99% *cov* / 65% *ide*).

No similarities were found between the others TRs of R2 and other LAB species, being the highest hit for RS1065 with *Oenococcus kitaharae*. This sequence is widespread among the *O. oeni* genomes studied (54 strains) and no bias of GC% (37%) was detected in the whole R2.

*Genetic study of genomic region R3 (RS1595-RS1740)*

This region of *O. oeni* included 37 ORFs (Figure 3C), which harboured various genes dedicated to sugar transport and metabolism, being three of them PTS transporters. Also, six HP, four pseudogenes and four T are annotated. These latter genetic mobile elements sandwich several genes (Table 3). The genomic island (GI) acquired by an HGT described by Bon et al. (2009) is located between the transposase genes RS1660 and RS1705. It is worth noting that among the ten current ORFs in this GI, six were used as a genetic markers for their oenological potential (Renouf et al. 2008). Moreover, this GI between RS1665 to RS1700 matches with several strains of *Lactobacillus plantarum* (BLASTN analysis 100% *cov* / 99% *ide*), among other *Lactobacillus* species.

The stress related genes enclosed in this GI are a thioredoxin (*trx*) found in PSU-1, a DNA binding protein gene (*dpsA*), a copper chaperone and a Crp/Fnr family TR (cAMP receptor protein / fumarate-nitrate-reductase). The genomes from strains IOEB_L40_4, IOEB_L65_2, IOEB_0608, IOEB_1491, S19, S22 and S23 harboured this GI in different structural genetic composition (Figure 5). Surprisingly, the mentioned

genomes do not harbour the gene RS1660 annotated as transposase. Other strains (IOEB_S450, IOEB_B16, IOEB_0205, AWRIB548 and AWRIB422) are harbouring this gene with a high similarity with that of PSU-1. Also, all of them, except AWRIB422, present another copy of this gene (96% *cov* / 73% *ide*). It is worth noting that strain IOEB_9304 harbours part of this gene (75% *cov* / 98% *ide*). Other strains presented the entire flanking transposases, but not the genes enclosed between them.

The distribution of the selected genes of this GI was assessed for wine isolates using PCR detection with specific primers (Table S2 in the supplementary data). In this case, the seven strains harbouring *trx* (Table 1), according the previous study (Margalef-Català et al. 2016b), were selected for the PCR analysis. Single gene and intergenic PCRs were performed to identify the prevalence of those genes and the complete composition of the GI. While the presence of most of the single genes could be assessed by PCR, the intergenic fragments were not obtained for all the strains analysed even with the presence of the flanked genes (Figure 6). Taking together all the results from PCR reactions, only strain 1P1 presented the same synteny of genes that in PSU-1. Moreover, the BLASTN analysis of transposase RS1660 (100% *cov* / 99% *ide*) revealed the highest identities with species of the genera *Lactobacillus, Pediococcus*, *Enterococcus* and *Lactococcus.* Also, several species of *Lactobacillus*, *Weissella*, *Pediococcus*, *Tetragenococcus* (in a plasmid), *Enterococcus* and *Leuconostoc* harboured the RS1705 DNA sequence (92% *cov* / 95% *ide*).

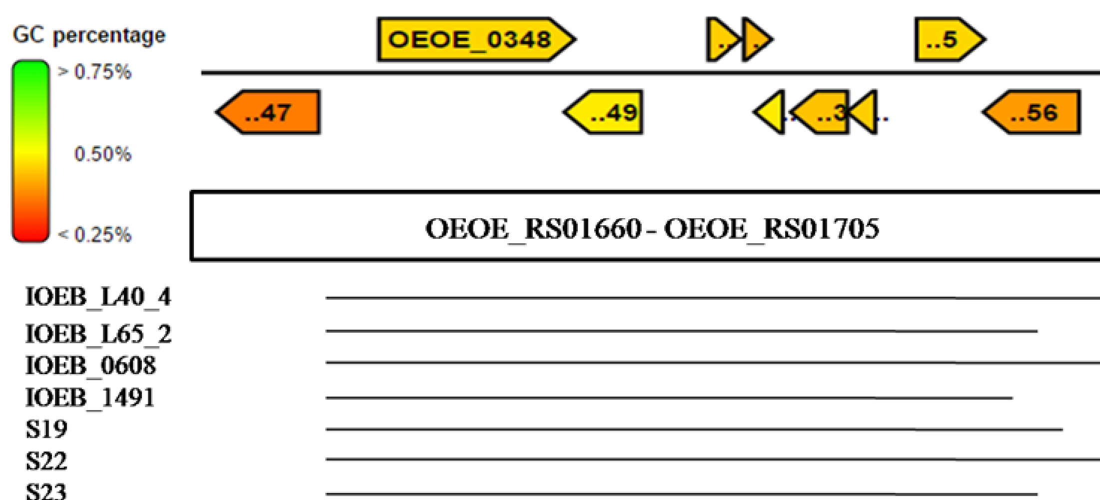


**Figure 4** Schema of BLASTN analysis of seven *O. oeni* strain genomes containing the GI described by Bon et al. (2009) and found in region R3 of this study, compared with PSU-1 genome.

The up-stream region of the HGT reported by Bon et al. (2009), RS1615–RS1655, is conserved among 40% of the strains studied. BLASTN analysis from Stefanelli (2014) revealed that this portion was not conserved in other microorganisms, but only single genes were present in some lactobacilli. Because of the massive sequencing in the recent years, we could find a match of this DNA region with *Lactobacillus farciminis* CNCM-I-3699-R (96% *cov* / 82% *ide*) and *Lactobacillus heilongjiangensis* DSM 28069 (92% *cov* / 85% *ide*). Regarding the transposases (RS1600-RS1605), we found that their DNA sequence matched exactly (100% *cov* / 99% *ide*) with seven different species of *Lactobacillus* and one *Pediococcus*. The analysis of the downstream region (RS1710-RS1740) of the already described GI revealed gene fragments only present in a 10% of strains studied. That suggests that the GI could be bigger for some strains of *O. oeni*, despite the BLASTN analysis of RS1710-RS1740 did not match completely with other microorganisms. No other information is available for this downstream region, because of its poor annotation in which there are only one metal transporter CorA, four pseudo-genes and one HP.

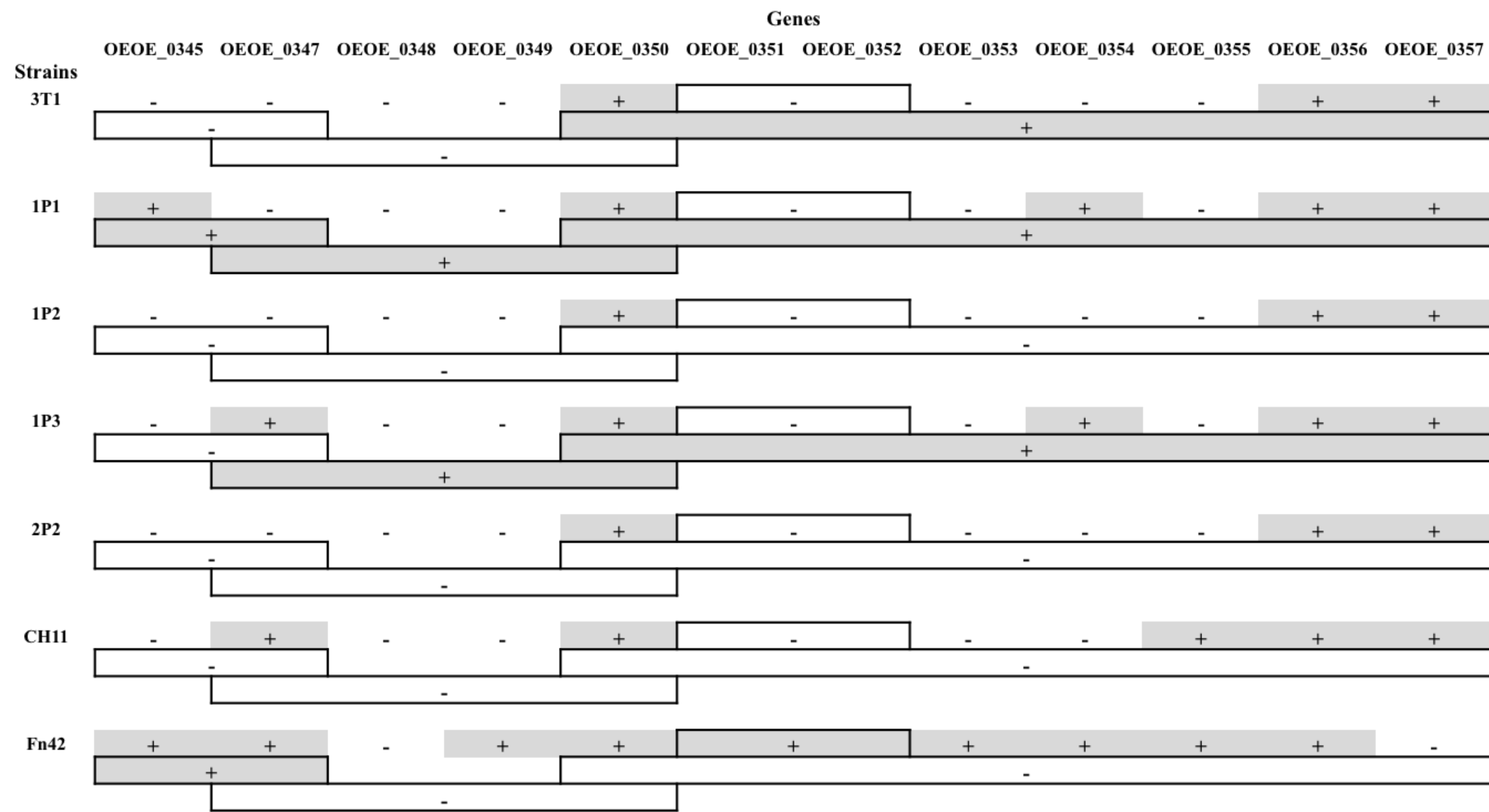


**Figure 5** Single gene and intergenic PCR detection of genes containing the GI described by Bon et al. (2009) and found in the region R3 of this study, using some *O. oeni* strains enclosing the thioredoxin gene OEOE_0350, compared with PSU-1 strain genome. Intergenic PCR are signalled with rectangles. Positive amplifications are signalled with "+".

*Genetic study of genomic region R4 (RS2360-RS2410)*

The R4 of *O. oeni* comprised 13 ORF's (Figure 3D) and this region was selected by Stefanelli (2014) for analysis because the two TR harboured (RS2375 and RS2390) were poorly widespread among their studied genomes. However, both TR presented the same pattern of distribution and were not associated with known genomic regions of flexibility. The resulted plasticity region in which these two regulators were included was reported from RS2365 to partially RS2400, for a total of 4123 bp. This region corresponds exactly to the part of R4 most poorly widespread in our *in silico* analysis, since it is missed in 66% of genomes (Figure 7 and Table S3). Also, this complete region is not found in other LAB species.

Apart from the two regulators enclosed, only one reductase (RS2365) and one MFS transporter (RS2400) are defined in R4. It is worth noting that this latter gene is conserved among *Lactobacillus* species (98% *cov* / 71% *ide*). For the other ORFs no biological role is known since several genes are annotated as HP or pseudo-genes. However, as pointed out already by Stefanelli (2014), downstream this region there is a gene coding for tRNA (RS2410) which corresponds to the flanked gene of R4 in this study.

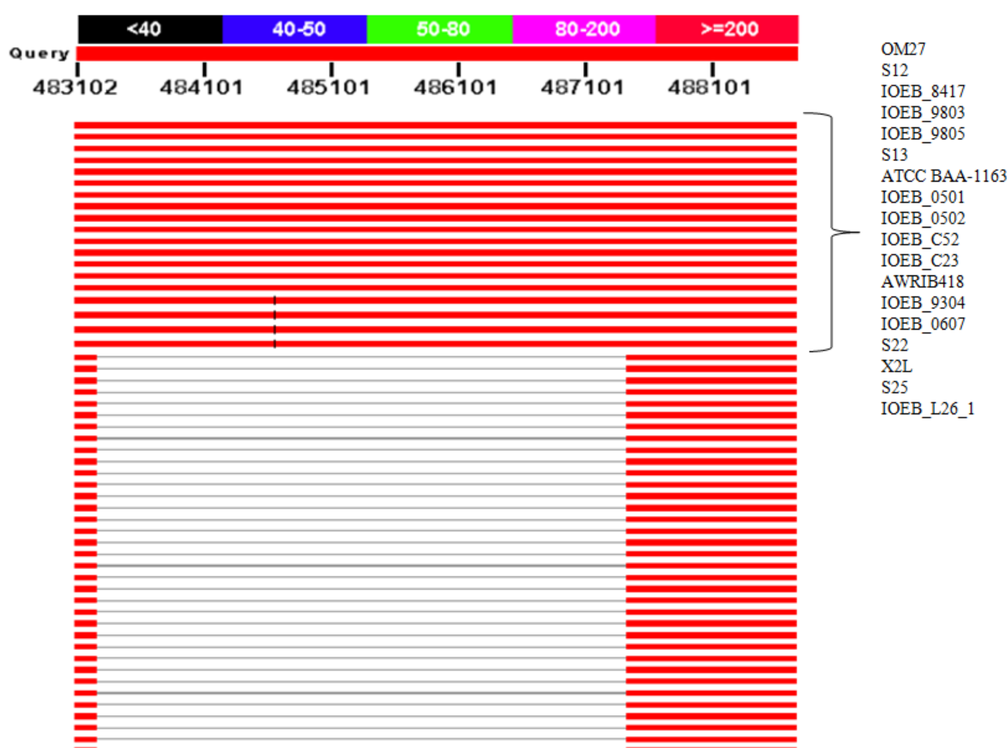


**Figure 6** BLASTN analysis of genomic region R4 in *O. oeni* genomes.

*Genetic study of genomic region R5 (RS3535-RS3585)*

Among the 10 ORFs enclosed in R5 (Figure 3E), there is a specific fragment flanked by two transposases, a pair in each side. Also the GC content of this fragment is higher (43%) than the regular GC one of *O. oeni* strains. The genes harboured between the transposases are two, RS3560, encoding for a threonine dehydrogenase (*trhD*), and RS3565 codifying for an HP. In order to study this genomic portion, the analysis was extended to the flanking annotated genes, from the gene RS3535 (methyltransferase) to RS3585 (NADH-flavin reductase). The BLASTN analysis of R5 (Figure 8) among all the genomes of *O. oeni* in NCBI (Table S1 in the supplementary data) concluded the same variation of this region as found by Stefanelli (2014), and there was no correlation with the source of strains. The genetic structure was variable among *O. oeni* strains. It is worth noting that this region is characterized by the presence of the four transposases that show 100% identity for the two couples (RS3550-RS3555 and RS3570-RS3575): this could have determined, in some strains, difficulties in genome assembly thus affecting the results of the *in silico* analysis, as reported for strain DSM 20252$^T$. Unlike what emerged from the BLASTN analysis, this strain presented the same genetic structure of PSU-1, since both the intergenic PCRs provided amplification (Stefanelli, 2014). The putative GI (RS3550–RS3575) was used in a BLASTN analysis and resulted to be harboured by strains of species *Lactobacillus casei* (Figure 8). This match was not found before (Stefanelli, 2014), and it is the hint, together with the bias of GC content (43%), that this is a GI more widespread than the one already reported for R3.

PCR primers designed for the genes RS3535, RS3560 and RS3585 (Table S2 in the supplementary data) were used to evaluate the distribution of this R5 in other wine isolates (Table 1). The screening was performed with both kind of amplifications for the three single genes. Four different patterns of gene composition could be established (Table 1): ten strains were harbouring all three genes, 17 were lacking the sandwiched gene (RS3560), three strains were lacking all three genes, and one strain enclosed only RS3560. As seen, no correlation between the presence of these genes and good oenological characteristics, such as finishing MLF in WLM with 12% and 14% ethanol, could be established.

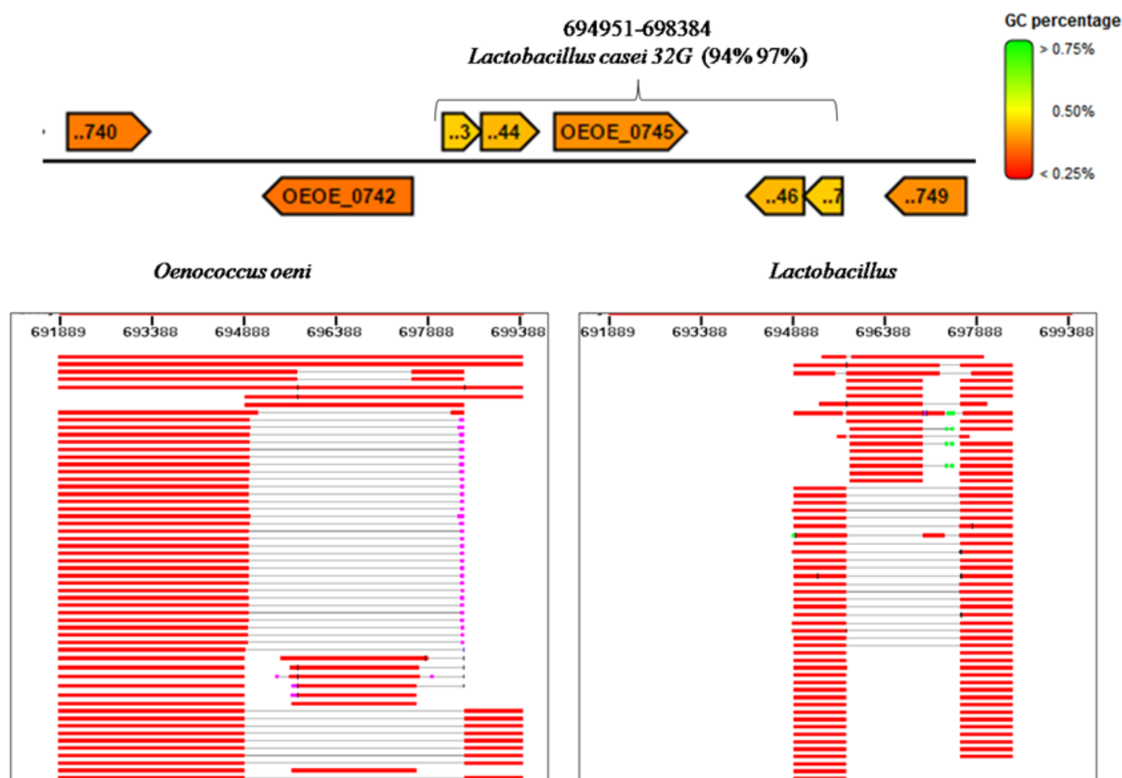


**Figure 7** BLASTN analysis of *O. oeni* and *Lactobacillus* genomes containing the genomic region R5 or part of it.

*Genetic study of genomic region R6 (RS4000-RS4050)*

This region of *O. oeni* harbouring 11 ORF's is the most widespread among the regions selected in this study (Figures 2 and 3F). The lowest presence in this region is found for the HP codified for RS4005, present in 35 genomes. The most prevalent function in R6 is amino acid metabolism and transport. Three peptide ABC transporters are enclosed and three more are present downstream R6. All of them are codified by the leading DNA strand in replication. RS4030 codifies for an enzyme that act on sulphur containing amino acids and its prevalence was studied through other LAB species due to its oenological relevance (Mtshali, 2011). In our study this gene appears in all the genomes available, so probably it should be part of the core genome of *O. oeni*. The RS4035 codifies for a tRNA, but it is not linked with any bacteriophage, such those found by Borneman et al. (2012).

*Genetic study of genomic region R7 (RS8360-RS8475)*

This last region located in the fourth quadrant of the *O. oeni* PSU-1 genome (Figure 1) harboured 25 ORFs. The genes enclosed in this region showed a very high variable presence among the genomes analysed (Figure 3G). There were two HP, two pseudogenes and one transposase annotated. Different authors have described and studied this region in different parts: RS8320-RS8365 (Borneman et al., 2012), RS8370-RS8415 (Dimopoulou et al., 2012) and RS8415-RS8485 (Athané et al. 2008).

Most of the genes harboured in R7 are poorly spread among *O. oeni* genomes (Table S3). Interestingly, RS8370 corresponds to the marker M3 found by Renouf et al. (2008) in the 75% of the studied strains, but our *in silico* analysis identified this gene only in 12 strains (21% of genomes studied). Its annotation as polysaccharide biosynthesis export protein suggests the possibility of a good oenological trait.

The gene RS8440, annotated as a ferritin (*dpsA*), was found in 12 genomes of *O. oeni*. It is worth noting near it the presence of a transposase-annotated (RS8450), which is a mobile element also present in *Leuconostoc mesenteroides*, *Pediococcus damnosus* and several species of *Lactobacillus* (100% *cov* / 99% *ide*). Moreover the BLASTN of the genomic fragment enclosed between the ferritin and the transposase (location 1671913-1674160) in different LAB species revealed the lack of RS8445 (HP). On the other hand, the ferritin is shared among *Lactobacillus* species, presenting *Lactobacillus fermentum* the highest identity (100% *cov* / 99% *ide*).

## Discussion

The genomic composition of good oenological starters is important to unveil possible markers and targets for the selection of *O. oeni* strains to overcome the stress conditions and carry on MLF in wine. In this study, we analysed the distribution of selected sequences among all the 57 genomes of *O. oeni* published in database. We identified seven genomic regions starting from a subset of 106 genes related to stress. Some of these candidate genomic regions harboured genes contributing to resistance to wine conditions, which could be added to the complex mechanisms so far described in *O. oeni*. Even though some regions had been identified previously, the current exhaustive

information given for each gene in this study can open doors to new genetic markers and enhance knowledge on this industrially relevant species.

In R1, the biggest region reported in this study, we have detected several genes related to oxidoreductase activity that are not totally widespread in the pan-genome of *O. oeni*. The enzymes related to redox reactions are very important for *O. oeni* because of its lack of superoxide mutase and catalase. Recent transcriptomic data have identified six of the HP genes of R1 affected under wine-like conditions (Margalef-Català et al., 2016a), together with other genes or proteins from RS0325 (a stress protein), RS0355 (a short-chain dehydrogenase), and RS0320 (a 2,5-diketo-D-gluconic acid reductase). Moreover, part of this R1 (RS0275-RS0405), poorly widespread among genomes analysed, was already reported (Bon et al., 2009), and certain harboured genes were used as genetic oenological markers (Renouf et al., 2008). Overall, it confirms the relevance of R1 as a fragment containing possible target genes with stress response under wine conditions.

The genomic region R2 was widespread among genomes analysed and as far as we are concerned, it has not been studied yet. We could identify 11 genes from R2 inhibited under wine conditions by DNA microarrays (Margalef-Català et al., 2016a; Olguín et al., 2015). Among them, RS0980 and RS1015 are related to cell wall biosynthesis. The former was repressed after the shock of 12% ethanol (Olguín et al., 2015) while the latter was down-regulated during acclimation to WLM (Margalef-Català et al., 2016a). On the other hand, several genes of *O. oeni* which encode enzymes involved in cell wall biosynthesis and degradation have been reported strongly induced by ethanol acclimation (8% and 12%, v/v) in WLM (Costantini et al., 2015). Cell-wall renewal and membrane modifications already described (Chu-Ky et al., 2005; Grandvalet et al., 2008) are important to counteract ethanol effects.

A well-described route for threonine catabolism in both prokaryotes and eukaryotes is the conversion of threonine to 2-amino-3-ketobutyrate by threonine dehydrogenase (TrhD) (Marcus and Dekker, 1993). Based on the annotated genomes, the first reaction in this pathway - conversion of threonine to 2-amino-3-ketobutyrate - may be present in *O. oeni*. Moreover, Borneman et al. (2012) detected the presence of nonsense mutations in homoserine kinase gene involved in the pathway synthesis of threonine in specific strains (PSU-1 and AWRIB304). The four different gene sequences of *trhD* found in

PSU-1 genome and different expression detected for the *trhD* (RS1895) under ethanol stress (Olguín et al., 2015) and *trhD* (RS3560) harboured in R5 (Stefanelli 2014), may indicate the importance of this enzyme. However, no transcriptomic data is available for the specific *trhD* gene (RS1025) harboured in R2.

We agree with Stefanelli (2014) that the HGT in R3 belongs to a wider flexibility region. As well as the down-stream genomic region, this HGT is poorly widespread, thus this GI could be redefined until the gene RS1710, as in *Lactobacillus* species. The variable genomic composition of this GI found for *O. oeni* genomes in database was also seen for other strains isolated from different vineyards but not related to the MLF performance of the strains. Thus, these different genomic structures suggest different HGT events, which generated this plasticity region in these *O. oeni* strains.

Genes as thioredoxin, DNA binding protein from starved cells (*dps*), copper chaperone and a positive transcriptional factor are harboured in R3. This thioredoxin is present only in few strains genomes from database, including PSU-1. The activation of this gene has been reported under wine stress conditions (Margalef-Català et al., 2016b). Two genes encoding for Dps have been annotated in *O. oeni* PSU-1: RS1690 for *dpsB* and RS8440 for *dpsA*. This latter gene was found in another flexibility genomic region by Athané et al. (2008) and in R7 of the present study under the current annotation of ferritin. Athané et al. (2008) also suggested that Dps may also regulate gene expression by modulating DNA structure. The last remarkable gene of this GI codifies for a DNA-binding protein that predominantly functions as positive transcription factor. This gene codifies for a Crp/Fnr-like regulator, a class of transcriptional factor, which responds to a broad spectrum of intracellular and exogenous signals, including cyclic AMP, anoxia, redox state, oxidative stress, and temperature (Körner et al., 2003).

There is no clear proof of the genomic region R4 being the result of a genomic transfer from other LAB species because of the lack of similarity found by the BLASTN analysis. However, this region presents a lower GC content (32%) in comparison to that reported for oenococcal genomes (38%) and, as pointed out already by Stefanelli (2014), downstream this region there is a gene coding for tRNA (RS2410). The presence of structural RNAs can be associated with GIs (Dobrindt et al., 2004; Howard et al., 2013; Hsiao et al., 2003) because they have been linked to bacteriophage

attachment sites (Borneman et al., 2012), and the different GC content may indicate a foreign origin.

The new GI harboured in the genomic region R5 would come from *Lactobacillus* spp.. Being more present among the available *O. oeni* genomes, it seems that this transfer is not as recent as the GI of R3. The *in silico* analysis has also revealed the different genetic compositions of this short GI. Further study is needed to understand how this threonine dehydrogenase gene sandwiched between two pairs of transposases could be beneficial for *O. oeni* in wine. For the time being, the transcriptomic data available (Stefanelli 2014) revealed that this gene (RS3560) is inhibited under ethanol stress and at 42ºC.

The genomic region R6 has most of its genes widespread among the different genomes. However, it is the first time that this region has been pointed out. The most notable aspect of the harboured genes is that most of them have been affected under wine like conditions (Table 3). Among them, several genes codifying for ABC transporters have been inhibited under these conditions and specifically, the codifying peptide ABC transporter substrate-binding protein from RS4065, harboured downstream the R6, was down-regulated in presence of 12% ethanol, as well (Olguín et al., 2015).

On the other hand, the genomic region R7 has been previously related to EPS production, genetic oenological markers and fitness gene container (Athané et al., 2008; Borneman et al., 2012; Dimopoulou et al., 2012; Renouf et al., 2008). It seems clear after our BLAST analysis that part of R7 was acquired from other close LAB species. As pointed out by Athané et al. (2008) and Borneman et al. (2012), the up-stream fragment of R7, RS8320-RS8365 was present in half of the strains and was shown to contain evidence of two independent HGT events (separated by ~65 kb) involving IS element that appear to have been horizontally transferred from *Lactobacillus* spp. The gene RS8365, present in 26 *O. oeni* genomes, is annotated as a glucosaminidase and it was inhibited in the transcriptomic study of Olguín et al. (2015) in cells grown with 12% of ethanol. Glucosaminidase is an enzymatic activity involved in peptidoglycan recycling (Vollmer et al., 2008) and autolysis (Delcour et al., 1999; Govindasamy-Lucey et al., 2000; Rashid et al., 1995). Olguín et al. (2015) associated the inhibition of this activity to the prevention of cell wall weakening as part of the response to ethanol stress.

Athané et al. (2008) studied the plasticity of R7 from RS8415-RS8485 due to IS elements and high GC content. They found that RS8440 (*dpsA*) was a mobile gene in *O. oeni* and about 40% of the tested strains harboured the corresponding sequence. The presence of mobile sequences in the vicinity (RS8450, annotated as transposase) strongly suggested a foreign origin of the locus, possibly through a transposition event. The protein encoded by RS8440 has been detected more abundant during the first hours of acclimation to WLM in *O. oeni* PSU-1 strain (Margalef-Català et al., 2016a). This is in agreement with the fact that ferritin-like proteins protect *E. coli* from the deleterious effects of wine (Nair and Finkel, 2004).

Our new approach using genome analysis based on stress-related genes, integrating elements from literature and "omics" data, provided another view of the current pan-genome of *O. oeni*. The results suggested that the presence/absence of specific genes is not sufficient to characterize the different oenological properties of strains, but it is useful to target relevant genomic fragments important in adaptation and resistance to the ecological niche. As pointed out by Athané et al. (2008), strains harbouring certain genes may exhibit different gene activities because of variations in the expression level and/or in the stability of the protein. Our data, however, is consistent with recent developments in the study of *O. oeni* genomes since three out of seven detected regions coincide with what has been previously reported. As conclusive perspective, it can be said that comparative genomic, transcriptomic and proteomic techniques appear as the future logical approach in order to identify and further characterize the molecular mechanisms involved in *O. oeni* resistance to wine conditions.

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## Supplementary data

**Table S1** Strains of *O. oeni* whose genomes were used for BLASTN analysis, available in NCBI.

| Strain | Genome size (Mb) | G+C content (%) | No. of genes |
|---|---|---|---|
| PSU-1 | 1.78 | 37.9 | 1799 |
| ATCC BAA-1163 | 1.75 | 37.9 | 1766 |
| AWRIB429 | 1.93 | 37.9 | 1986 |
| AWRIB304 | 1.85 | 37.9 | 1869 |
| AWRIB318 | 1.81 | 38 | 1810 |
| AWRIB419 | 1.79 | 37.8 | 1809 |
| AWRIB418 | 1.84 | 37.8 | 1841 |
| AWRIB422 | 1.81 | 37.9 | 1832 |
| AWRIB548 | 1.84 | 38 | 1862 |
| AWRIB553 | 1.76 | 37.8 | 1765 |
| AWRIB576 | 1.88 | 38 | 1895 |
| AWRIB568 | 1.87 | 38 | 1896 |
| AWRIB202 | 1.84 | 37.9 | 1857 |
| DSM 20252 | 1.74 | 37.8 | 1748 |
| OM27 | 1.79 | 37.9 | 1816 |
| OT3 | 1.77 | 37.8 | 1787 |
| IOEB_0205 | 1.80 | 38 | 1815 |
| IOEB_0501 | 1.83 | 37.9 | 1829 |
| OT4 | 1.77 | 37.9 | 1789 |
| OT5 | 1.77 | 37.9 | 1782 |
| OM22 | 1.86 | 38 | 1913 |
| OT25 | 1.83 | 37.9 | 1853 |
| IOEB_0502 | 1.82 | 37.9 | 1817 |
| IOEB_0608 | 1.81 | 38 | 1827 |
| IOEB_0607 | 1.82 | 37.8 | 1799 |
| IOEB_9304 | 1.83 | 37.7 | 1816 |
| IOEB_8417 | 1.84 | 37.8 | 1848 |
| IOEB_9517 | 1.74 | 37.9 | 1768 |
| IOEB_9803 | 1.83 | 37.8 | 1847 |
| IOEB_9805 | 1.84 | 37.8 | 1857 |
| IOEB_S277 | 1.74 | 37.8 | 1752 |
| IOEB_B16 | 1.79 | 38 | 1814 |
| S13 | 1.81 | 37.8 | 1823 |
| S22 | 1.81 | 37.8 | 1834 |
| S25 | 1.74 | 37.9 | 1744 |
| S28 | 1.84 | 37.9 | 1860 |
| IOEB_B10 | 1.78 | 37.8 | 1791 |
| IOEB_CiNe | 1.79 | 37.9 | 1809 |
| S11 | 1.83 | 38 | 1844 |
| IOEB_C23 | 1.84 | 37.8 | 1874 |
| IOEB_L65_2 | 1.78 | 37.8 | 1781 |
| S14 | 1.73 | 37.9 | 1747 |
| IOEB_C28 | 1.80 | 37.9 | 1804 |
| IOEB_L40_4 | 1.73 | 37.9 | 1740 |
| IOEB_S436a | 1.76 | 37.8 | 1780 |
| IOEB_C52 | 1.90 | 37.6 | 1898 |
| IOEB_1491 | 1.77 | 37.9 | 1790 |
| S19 | 1.81 | 37.8 | 1834 |
| IOEB_VF | 1.78 | 37.8 | 1803 |
| IOEB_L18_3 | 1.74 | 37.8 | 1743 |
| IOEB_L26_1 | 1.79 | 37.8 | 1796 |
| IOEB_S450 | 1.76 | 37.8 | 1782 |
| S12 | 1.81 | 37.8 | 1808 |
| S15 | 1.74 | 37.8 | 1734 |
| S23 | 1.81 | 38 | 1806 |
| S161 | 1.79 | 37.8 | 1795 |
| X2L | 1.81 | 37.9 | 1840 |

**Table S2** Primers used for detection of some genes harboured in genomic regions R3 and R5 of *O. oeni*.

| **Target** | **Primer** | | | **Reference** |
|---|---|---|---|---|
| **Annotation** | **Name** | **Sequence (5'-3')** | **Amplicon length (bp)** | |
| OEOE_RS01655 | 0345-F | CGTCAAGACTCATCACAACGT | 391‡ | Stefanelli (2014) |
| acetyltransferase | 0345-R | TTCTTTCAACATGGCCGTGC | | |
| OEOE_RS01660 | Tsp1-F | CAAAGGGCGAAAGAAAACAC | 395‡ | Stefanelli (2014) |
| Transposase | Tsp1-R | GGGCCACTTTTCCAACTGTA | | |
| OEOE_RS01665 | M1-F | GAAGCTCAAGATACCATCC | 650* | Renouf et al. (2008) |
| haloacid dehalogenase | M1-R | CGACTTGTGCACAGATTCC | | |
| OEOE_RS01670 | M13-F | CTAACGCATTCCTGAAGAAC | 602* | Renouf et al. (2008) |
| glycerol uptake permease | M13-R | CCCAACTATATTCCCAGTGA | | |
| OEOE_RS01675 | M12-F | GTTTCTGAAGACCCGCTTA | 300* | Renouf et al. (2008) |
| Thioredoxin | M12-R | TGATGCCCCCTTCGTAAT | | |
| OEOE_RS01690 | M2-F | TTGGTTAATTCAGCCGTTGT | 500* | Renouf et al. (2008) |
| DNA-binding protein | M2-R | ATTGATCACGATGTCCCAAC | | |
| OEOE_RS01695 | M10-F | CCTCCTACTTAACCTTGACG | 420* | Renouf et al. (2008) |
| copper chaperone | M10-R | AGTCCCACCTCCTGAATAAA | | |
| OEOE_RS01700 | M5-F | TGGCAAACGTCTCAATCAAC | 380* | Renouf et al. (2008) |
| Crp/Fnr family transcriptional regulator | M5-R | AGCTTACGGCTGATGCTTT | | |
| OEOE_RS01705 | Tsp2-F | TCCTCGTGGGCATAGTAAGG | 369‡ | Stefanelli (2014) |
| transposase | Tsp2-R | GGTCGCTGGTTAATTTGGAG | | |
| OEOE_RS01710 | 0357-F | AATATCAACGTCCGGTCCAC | 364‡ | Stefanelli (2014) |
| metal transporter CorA | 0357-R | ATCGACATTGATTCCCCAAA | | |
| OEOE_RS03535 | 0740-F | ATTGTTGCAGGGGTTTGTGG | 345‡ | Stefanelli (2014) |
| methyltransferase | 0740-R | TACTGTAGCTGAAACGCCGA | | |
| OEOE_RS03560 | 0745-F | ATATTCCTGATCCGGCACCA | 419‡ | Stefanelli (2014) |
| threonine dehydrogenase | 0745-R | AGCCCCTAAATCAAGCGGAA | | |
| OEOE_RS03585 | 0749-F | ATGATGGTCAGTTCCGGTGT | 342‡ | Stefanelli (2014) |
| NADH-flavin reductase | 0749-R | TTAATCATCGGCGCAACTGG | | |

**Table S3** Complete information of all the genes harboured in the seven identified flexible genomic regions found in *O. oeni*.

| Gene code | New annotation | Old annotation | No. of strains harbouring | Current gene code | Start position | End position | Orientation |
|---|---|---|---|---|---|---|---|
| **R1** | | | | | | | |
| OEOE_0049 | MFS transporter | arabinose efflux permease | 57 | OEOE_RS00225 | 45914 | 47101 | minus |
| OEOE_0050 | NAD(P)-dependent oxidoreductase | dehydrogenase or related protein | 42 | OEOE_RS00230 | 47289 | 48311 | plus |
| OEOE_0051 | D-aminopeptidase | L-aminopeptidase/D-esterase | 42 | OEOE_RS00235 | 48403 | 49512 | plus |
| OEOE_0052 | | Hypothetical protein | 41 | discontinued | 49967 | 50218 | plus |
| OEOE_0053 | hypothetical protein | Hypothetical protein | 29 | OEOE_RS00240 | 50342 | 50761 | plus |
| OEOE_0054 | hypothetical protein | Hypothetical protein | 42 | OEOE_RS00245 | 50821 | 51126 | minus |
| OEOE_0055 | membrane protein | Hypothetical protein | 42 | OEOE_RS00250 | 51156 | 51395 | minus |
| OEOE_0056 | membrane protein | Hypothetical protein | 42 | OEOE_RS00255 | 51501 | 51758 | minus |
| OEOE_0057 | transcriptional regulator | Hypothetical protein | 42 | OEOE_RS00260 | 51840 | 52409 | minus |
| OEOE_0058 | discontinued | Hypothetical protein | 42 | OEOE_RS00265 | 52450 | 52623 | minus |
| OEOE_0059 | hypothetical protein | Hypothetical protein | 42 | OEOE_RS00270 | 52634 | 53194 | minus |
| OEOE_0060 | alpha/beta hydrolase | alpha/beta fold family hydrolase | 41 | OEOE_RS00275 | 53547 | 54074 | plus |
| OEOE_0061 | hypothetical protein | Hypothetical protein | 38 | OEOE_RS00280 | 54505 | 54876 | plus |
| OEOE_0062 | transcriptional regulator | transcriptional regulator | 27 | OEOE_RS00285 | 55123 | 56484 | minus |
| OEOE_0063 | hypothetical protein | Hypothetical protein | 27 | OEOE_RS00290 | 56802 | 57041 | plus |
| OEOE_0064 | phytoene synthase | phytoene/squalene synthetase | 27 | OEOE_RS00295 | 57439 | 58068 | minus |
| OEOE_0065 | pseudo | pseudo | 27 | OEOE_RS00300 | 58308 | 59827 | minus |
| OEOE_0066 | | Hypothetical protein | 27 | discontinued | 59986 | 60102 | plus |
| OEOE_0067 | oxidoreductase | Short-chain alcohol dehydrogenase | 27 | OEOE_RS00305 | 60419 | 61309 | plus |
| OEOE_0068 | hypothetical protein | Hypothetical protein | 26 | OEOE_RS00310 | 61655 | 61972 | plus |
| OEOE_0069 | membrane protein | Hypothetical protein | 26 | OEOE_RS00315 | 62422 | 62676 | plus |
| OEOE_0070 | 2,5-diketo-D-gluconic acid reductase | aldo/keto reductase related protein | 46 | OEOE_RS00320 | 62925 | 63776 | minus |
| OEOE_0071 | general stress protein | exopolysaccharide biosynthesis protein | 26 | OEOE_RS00325 | 63867 | 64895 | minus |
| OEOE_0072 | pseudo | pseudo | 25 | OEOE_RS00330 | 64924 | 66311 | minus |
| OEOE_0073 | | Hypothetical protein | 26 | discontinued | 66506 | 66625 | plus |
| OEOE_0074 | hypothetical protein | Hypothetical protein | 29 | OEOE_RS00335 | 67273 | 67692 | minus |
| OEOE_0075 | MFS transporter | arabinose efflux permease | 57 | OEOE_RS00340 | 68016 | 69209 | minus |
| OEOE_0076 | MFS transporter | arabinose efflux permease | 39 | OEOE_RS00345 | 69494 | 70657 | plus |
| OEOE_0077 | diacetyl reductase [(S)-acetoin forming] | 3-oxoacyl-ACP reductase | 41 | OEOE_RS00350 | 70772 | 71554 | plus |
| OEOE_0078 | short-chain dehydrogenase | short chain dehydrogenase | 40 | OEOE_RS00355 | 71784 | 72542 | minus |
| OEOE_0079 | transcriptional regulator | transcriptional regulator | 42 | OEOE_RS00360 | 72839 | 73201 | plus |
| OEOE_0080 | serine acetyltransferase | galactoside O-acetyltransferase | 41 | OEOE_RS00365 | 73470 | 74003 | minus |
| OEOE_0081 | NADPH:quinone reductase | NADPH:quinone reductase or related Zn-dependent oxidoreductase | 41 | OEOE_RS00370 | 74025 | 74927 | minus |
| OEOE_0082 | MarR family transcriptional regulator | MarR family transcriptional regulator | 42 | OEOE_RS00375 | 75029 | 75481 | minus |
| OEOE_0083 | | Hypothetical protein | 42 | discontinued | 75683 | 75823 | minus |
| OEOE_0084 | | Hypothetical protein | 50 | discontinued | 75918 | 76064 | minus |
| OEOE_0085 | hypothetical protein | Hypothetical protein | 42 | OEOE_RS00380 | 76428 | 76817 | plus |
| OEOE_0086 | hypothetical protein | Hypothetical protein | 41 | OEOE_RS00385 | 77947 | 78183 | plus |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| OEOE_0087 | | pseudo | 41 | discontinued | 78737 | 78886 | minus |
| OEOE_0088 | | phosphoglycerate mutase | 41 | discontinued | 78883 | 79523 | minus |
| | pseudo | | | OEOE_RS00390 | 79090 | 79623 | minus |
| OEOE_0089 | hypothetical protein | Hypothetical protein | 50 | OEOE_RS00395 | 79943 | 80143 | minus |
| | discontinued | Hypothetical protein | | OEOE_RS00400 | | | |
| OEOE_0090 | | pseudo | 10 | discontinued | 80351 | 81095 | minus |
| OEOE_0091 | | pseudo | 51 | discontinued | 81179 | 82441 | minus |
| | Hypothetical protein | | | OEOE_RS00405 | 81179 | 81625 | minus |
| | Hypothetical protein | | | OEOE_RS00410 | 81670 | 82125 | minus |
| | Hypothetical protein | | | OEOE_RS00415 | 82100 | 82441 | minus |
| OEOE_0092 | serine hydrolase | Beta-lactamase class C related penicillin binding protein | 51 | OEOE_RS00420 | 82607 | 83566 | plus |
| **R2** | | | | | | | |
| OEOE_0205 | multidrug ABC transporter ATPase | ABC-type multidrug transport system, ATPase component | 57 | OEOE_RS00975 | 192334 | 193041 | plus |
| OEOE_0206 | 1,4-beta-N-acetylmuramidase | Lyzozyme M1 (1,4-beta-N-acetylmuramidase) | 48 | OEOE_RS00980 | 193219 | 195228 | plus |
| OEOE_0207 | acetyltransferase | acetyltransferase | 48 | OEOE_RS00985 | 195379 | 195942 | plus |
| OEOE_0208 | hydrolase | alpha/beta fold family hydrolase | 48 | OEOE_RS00990 | 196359 | 197300 | plus |
| OEOE_0209 | AraC family transcriptional regulator | AraC family transcriptional regulator | 44 | OEOE_RS00995 | 197377 | 198036 | minus |
| OEOE_0210 | hypothetical protein | double-stranded beta-helix-like protein | 44 | OEOE_RS01000 | 198033 | 198347 | minus |
| OEOE_0211 | hypothetical protein | pseudo | 42 | OEOE_RS01005 | 198961 | 199950 | plus |
| OEOE_0212 | | Hypothetical protein | 44 | discontinued | 200008 | 201201 | plus |
| OEOE_0213 | | pseudo | 44 | discontinued | 201297 | 201983 | plus |
| | pseudo | | | OEOE_RS01010 | 200008 | 201983 | plus |
| OEOE_0214 | D-alanyl-D-alanine dipeptidase | D-alanyl-D-alanine dipeptidase | 48 | OEOE_RS01015 | 202372 | 203106 | minus |
| OEOE_0215 | TetR family transcriptional regulator | transcriptional regulator | 48 | OEOE_RS01020 | 203281 | 203856 | minus |
| OEOE_0216 | | Hypothetical protein | 36 | discontinued | 203937 | 204026 | minus |
| OEOE_0217 | threonine dehydrogenase | threonine dehydrogenase or related Zn-dependent dehydrogenase | 50 | OEOE_RS01025 | 204071 | 205090 | plus |
| OEOE_0218 | glycosyltransferase | glycosyltransferase-like protein | 42 | OEOE_RS01030 | 205318 | 206310 | plus |
| OEOE_0219 | membrane protein | Hypothetical protein | 30 | OEOE_RS01035 | 206303 | 208333 | plus |
| OEOE_0220 | hypothetical protein | Hypothetical protein | 56 | OEOE_RS01040 | 208462 | 208728 | plus |
| OEOE_0221 | PTS sugar transporter subunit IIA | cellobiose phosphotransferase system IIC component | 54 | OEOE_RS01045 | 208972 | 210324 | minus |
| OEOE_0222 | PTS cellobiose transporter subunit IIA | cellobiose-specific PTS system IIA component | 50 | OEOE_RS01050 | 210499 | 210822 | minus |
| OEOE_0223 | PTS sugar transporter | cellobiose-specific PTS system IIB component | 48 | OEOE_RS01055 | 210824 | 211132 | minus |
| OEOE_0224 | 6-phospho-beta-glucosidase | 6-phospho-beta-glucosidase | 53 | OEOE_RS01060 | 211150 | 212592 | minus |
| OEOE_0225 | transcriptional regulator | transcriptional regulator | 54 | OEOE_RS01065 | 212848 | 213576 | plus |
| OEOE_0226 | GntR family transcriptional regulator | GntR family transcriptional regulator | 56 | OEOE_RS01070 | 213672 | 214760 | plus |
| OEOE_0227 | ATPase | L-ribulokinase (putative) | 56 | OEOE_RS01075 | 214985 | 216583 | plus |
| OEOE_0228 | araD pseudo | pseudo | 58 | OEOE_RS01080 | 216583 | 217311 | plus |
| OEOE_0229 | pseudo | pseudo | 57 | OEOE_RS01085 | 217344 | 218767 | plus |
| | hypothetical protein | | | OEOE_RS01090 | 218820 | 219032 | plus |
| OEOE_0230 | pseudo | pseudo | 50 | OEOE_RS01095 | 218820 | 220062 | plus |
| OEOE_0231 | aldose 1-epimerase | aldose 1-epimerase | 40 | OEOE_RS01100 | 220329 | 221354 | plus |
| OEOE_0232 | transcriptional antiterminator | transcriptional antiterminator | 54 | OEOE_RS01105 | 221568 | 223058 | plus |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **R3** | | | | | | | |
| OEOE_0333 | aspartate aminotransferase | aminotransferase | 57 | OEOE_RS01595 | 325107 | 326321 | plus |
| OEOE_0334 | transposase | transposase | 37 | OEOE_RS01600 | 326469 | 326789 | plus |
| OEOE_0335 | transposase | transposase | 31 | OEOE_RS01605 | 326786 | 327256 | plus |
| OEOE_0336 | hypothetical protein | Hypothetical protein | 1 | OEOE_RS01610 | 327495 | 328091 | minus |
| OEOE_0337 | hypothetical protein | pseudo | 27 | OEOE_RS01615 | 328558 | 328788 | plus |
| OEOE_0338 | PTS sugar transporter | cellobiose-specific PTS system IIB component | 27 | OEOE_RS01620 | 329032 | 329340 | plus |
| OEOE_0339 | PTS cellobiose transporter subunit IIA | cellobiose-specific PTS system IIA component | 27 | OEOE_RS01625 | 329456 | 329791 | plus |
| OEOE_0340 | 6-phospho-beta-glucosidase | Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase | 25 | OEOE_RS01630 | 329798 | 331243 | plus |
| OEOE_0341 | 6-phospho-beta-glucosidase | Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase | 23 | OEOE_RS01635 | 331260 | 332717 | plus |
| OEOE_0342 | RpiR family transcriptional regulator | pseudo | 27 | OEOE_RS01640 | 332858 | 333635 | plus |
| OEOE_0343 | PTS fructose transporter subunit IIC | cellobiose-specific PTS system IIC component | 27 | OEOE_RS01645 | 333774 | 335075 | plus |
| OEOE_0344 | hypothetical protein | Hypothetical protein | 27 | OEOE_RS01650 | 335100 | 336185 | plus |
| OEOE_0345 | acetyltransferase | acetyltransferase | 26 | OEOE_RS01655 | 336239 | 337402 | plus |
| OEOE_0346 | | pseudo | 22 | discontinued | 337547 | 337717 | plus |
| OEOE_0347 | | transposase | 6 | discontinued | 337764 | 338756 | minus |
| | transposase | | | OEOE_RS01660 | 337764 | 338177 | minus |
| OEOE_0348 | haloacid dehalogenase | cation transport ATPase | 7 | OEOE_RS01665 | 339094 | 340998 | plus |
| OEOE_0349 | glycerol uptake permease | glycerol uptake facilitator or related permease (major Intrinsic protein family) | 7 | OEOE_RS01670 | 341152 | 341904 | minus |
| OEOE_0350 | thioredoxin | thioredoxin | 8 | OEOE_RS01675 | 342289 | 342609 | plus |
| OEOE_0351 | hypothetical protein | nickel resistance determinant | 8 | OEOE_RS01680 | 342633 | 342902 | plus |
| OEOE_0352 | hypothetical protein | Hypothetical protein | 8 | OEOE_RS01685 | 343002 | 343205 | minus |
| OEOE_0353 | DNA-binding protein | DNA-binding ferritin-like protein (oxidative damage protectant) | 8 | OEOE_RS01690 | 343334 | 343888 | minus |
| OEOE_0354 | | copper chaperone | 7 | discontinued | 343901 | 344074 | minus |
| | copper chaperone | | | OEOE_RS01695 | 343901 | 344122 | minus |
| OEOE_0355 | Crp/Fnr family transcriptional regulator | Crp-like transcriptional regulator | 7 | OEOE_RS01700 | 344323 | 344988 | plus |
| OEOE_0356 | transposase | IS30 family transposase | 22 | OEOE_RS01705 | 345210 | 346139 | minus |
| OEOE_0357 | metal transporter CorA | Mg2+ and Co2+ transporter | 52 | OEOE_RS01710 | 346461 | 347411 | plus |
| OEOE_0358 | | pseudo | 13 | discontinued | 347542 | 347877 | minus |
| | pseudo | | | OEOE_RS01715 | 347542 | 347979 | minus |
| OEOE_0359 | hypothetical protein | Hypothetical protein | 11 | OEOE_RS01720 | 347925 | 348518 | plus |
| OEOE_0360 | | pseudo | 6 | discontinued | 348515 | 348886 | minus |
| | pseudo | | | OEOE_RS01725 | 348602 | 349083 | minus |
| OEOE_0361 | | pseudo | 17 | discontinued | 348868 | 349968 | minus |
| | pseudo | | | OEOE_RS01730 | 349108 | 350052 | minus |
| OEOE_0362 | | pseudo | 24 | discontinued | 350282 | 350398 | minus |
| | pseudo | | | OEOE_RS01735 | 350059 | 350398 | minus |
| OEOE_0363 | membrane protein | membrane protein | 57 | OEOE_RS01740 | 350875 | 351894 | plus |
| **R4** | | | | | | | |
| OEOE_0494 | hypothetical protein | Hypothetical protein | 57 | OEOE_RS02360 | 482923 | 483105 | plus |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| OEOE_0495 | NADPH:quinone reductase | NADPH:quinone reductase or related Zn-dependent oxidoreductase | 19 | OEOE_RS02365 | 483102 | 483509 | minus |
| OEOE_0496 | | pseudo | 19 | discontinued | 483496 | 484072 | minus |
| | hypothetical protein | | | OEOE_RS02370 | 483755 | 484072 | minus |
| OEOE_0497 | ArsR family transcriptional regulator | transcriptional regulator | 19 | OEOE_RS02375 | 484202 | 484657 | plus |
| OEOE_0498 | | Hypothetical protein | 19 | discontinued | 484668 | 484847 | minus |
| OEOE_0499 | | Hypothetical protein | 19 | discontinued | 484892 | 485116 | minus |
| | pseudo | | | OEOE_RS02380 | 484668 | 485131 | minus |
| OEOE_0500 | pseudo | pseudo | 19 | OEOE_RS02385 | 485162 | 486066 | minus |
| OEOE_0501 | transcriptional regulator | transcriptional regulator | 19 | OEOE_RS02390 | 486063 | 486482 | minus |
| OEOE_0502 | hypothetical protein | Hypothetical protein | 19 | OEOE_RS02395 | 486639 | 486908 | plus |
| OEOE_0503 | MFS transporter | major facilitator superfamily permease | 19 | OEOE_RS02400 | 487023 | 488729 | minus |
| OEOE_0504 | | transcriptional regulator AcrR | 57 | discontinued | 489022 | 489363 | plus |
| | pseudo | | | OEOE_RS02405 | 488828 | 489363 | plus |
| OEOE_0505 | | miscRNA | 57 | discontinued | 489667 | 490016 | minus |
| OEOE_0506 | tRNA | tRNA | 54 | OEOE_RS02410 | 490141 | 490212 | plus |
| **R5** | | | | | | | |
| OEOE_0740 | methyltransferase | Mg2+ transporter | 57 | OEOE_RS03535 | 691889 | 692566 | plus |
| OEOE_0741 | pseudo | pseudo | 57 | OEOE_RS03540 | 692735 | 693468 | minus |
| OEOE_0742 | hypothetical protein | hypothetical protein | 56 | OEOE_RS03545 | 693643 | 694866 | minus |
| OEOE_0743 | transposase | transposase | 37 | OEOE_RS03550 | 694951 | 695271 | plus |
| OEOE_0744 | transposase | transposase | 31 | OEOE_RS03555 | 695268 | 695738 | plus |
| OEOE_0745 | threonine dehydrogenase | threonine dehydrogenase or related Zn-dependent dehydrogenase | 43 | OEOE_RS03560 | 695866 | 696948 | plus |
| | hypothetical protein | | | OEOE_RS03565 | 697066 | 697302 | minus |
| OEOE_0746 | transposase | transposase | 31 | OEOE_RS03570 | 697597 | 698067 | minus |
| OEOE_0747 | transposase | transposase | 37 | OEOE_RS03575 | 698064 | 698384 | minus |
| OEOE_0748 | hypothetical protein | pseudo | 37 | OEOE_RS03580 | 698383 | 698616 | plus |
| OEOE_0749 | NADH-flavin reductase | putative NADH-flavin reductase | 57 | OEOE_RS03585 | 698735 | 699391 | minus |
| **R6** | | | | | | | |
| OEOE_0832 | hypothetical protein | hypothetical protein | 57 | OEOE_RS04000 | 777346 | 777756 | minus |
| OEOE_0833 | hypothetical protein | hypothetical protein | 35 | OEOE_RS04005 | 777845 | 778078 | minus |
| OEOE_0834 | acetyltransferase | acetyltransferase | 57 | OEOE_RS04010 | 778113 | 778673 | plus |
| OEOE_0835 | membrane protein | hypothetical protein | 57 | OEOE_RS04015 | 778802 | 779755 | plus |
| OEOE_0836 | haloacid dehalogenase | HAD superfamily hydrolase | 57 | OEOE_RS04020 | 779850 | 780671 | plus |
| OEOE_0837 | hypothetical protein | hypothetical protein | 57 | OEOE_RS04025 | 780672 | 781418 | minus |
| OEOE_0838 | S-adenosylmethionine synthase | S-adenosylmethionine synthetase | 57 | OEOE_RS04030 | 781617 | 782783 | plus |
| OEOE_0839 | leucine--tRNA ligase | leucyl-tRNA synthetase | 57 | OEOE_RS04035 | 783009 | 785441 | plus |
| OEOE_0840 | peptide ABC transporter substrate-binding protein | peptide ABC transporter substrate-binding protein | 49 | OEOE_RS04040 | 785709 | 787376 | plus |
| OEOE_0841 | peptide ABC transporter substrate-binding protein | peptide ABC transporter substrate-binding protein | 44 | OEOE_RS04045 | 787517 | 789181 | plus |
| OEOE_0842 | peptide ABC transporter permease | peptide ABC transporter permease | 57 | OEOE_RS04050 | 789256 | 790179 | plus |

| **R7** | | | | | | | |
|---|---|---|---|---|---|---|---|
| OEOE_1733 | RNA polymerase I and III, subunit | RNA polymerase I and III, subunit | 57 | OEOE_RS08360 | 1653787 | 1654434 | minus |
| OEOE_1734 | glucosaminidase | N-acetylmuramidase | 26 | OEOE_RS08365 | 1654695 | 1655321 | minus |
| OEOE_1735 | polysaccharide biosynthesis export protein | PST family polysaccharide transporter | 12 | OEOE_RS08370 | 1655666 | 1657105 | minus |
| OEOE_1736 | glycosyl transferase | glycosyltransferase | 12 | OEOE_RS08375 | 1657106 | 1657915 | minus |
| OEOE_1737 | UDP-glucose 6-dehydrogenase | UDP-glucose 6-dehydrogenase | 57 | OEOE_RS08380 | 1657975 | 1659141 | minus |
| OEOE_1738 | glycosyl transferase | glycosyltransferase-like protein | 14 | OEOE_RS08385 | 1659314 | 1660252 | minus |
| OEOE_1739 | hypothetical protein | hypothetical protein | 14 | OEOE_RS08390 | 1660393 | 1661760 | minus |
| OEOE_1740 | capsular polysaccharide biosynthesis protein | capsular polysaccharide biosynthesis protein | 14 | OEOE_RS08395 | 1661760 | 1662368 | minus |
| OEOE_1741 | UDP-galactopyranose mutase | UDP-galactopyranose mutase | 56 | OEOE_RS08400 | 1662506 | 1663681 | minus |
| OEOE_1742 | glycosyl transferase | glycosyltransferase | 14 | OEOE_RS08405 | 1663822 | 1664742 | minus |
| OEOE_1743 | exopolysaccharide biosynthesis protein | lipopolysaccharide biosynthesis glycosyltransferase | 46 | OEOE_RS08410 | 1664790 | 1665542 | minus |
| OEOE_1744 | multidrug MFS transporter | lipopolysaccharide synthesis sugar transferase | 57 | OEOE_RS08415 | 1665542 | 1666171 | minus |
| OEOE_1745 | membrane protein | GtcA family membrane protein | 57 | OEOE_RS08420 | 1666278 | 1666778 | plus |
| OEOE_1746 | methionyl-tRNA synthetase | methionyl-tRNA synthetase | 57 | OEOE_RS08425 | 1666779 | 1668806 | minus |
| OEOE_1747 | amino acid transporter | gamma-aminobutyrate permease or related permease | 57 | OEOE_RS08430 | 1668880 | 1670250 | plus |
| OEOE_1748 | HD family phosphohydrolase | HD superfamily hydrolase | 57 | OEOE_RS08435 | 1670348 | 1671007 | plus |
| OEOE_1749 | | pseudo | 24 | discontinued | 1671515 | 1671631 | plus |
| OEOE_1750 | ferritin | DNA-binding ferritin-like protein (oxidative damage protectant) | 20 | OEOE_RS08440 | 1671913 | 1672395 | minus |
| OEOE_1751 | hypothetical protein | hypothetical protein | 19 | OEOE_RS08445 | 1672513 | 1672743 | plus |
| OEOE_1752 | | pseudo | 45 | discontinued | 1672919 | 1673638 | minus |
| | transposase | | | OEOE_RS08450 | 1672919 | 1674160 | minus |
| OEOE_1753 | | restriction endonuclease | 57 | discontinued | 1674291 | 1674938 | minus |
| | restriction endonuclease | | | OEOE_RS08455 | 1674291 | 1675175 | minus |
| OEOE_1754 | histidine phosphatase | phosphoglycerate mutase | 54 | OEOE_RS08460 | 1675296 | 1675865 | minus |
| OEOE_1755 | | hypothetical protein | 45 | discontinued | 1676054 | 1676368 | minus |
| | pseudo | | | OEOE_RS08465 | 1675966 | 1676431 | minus |
| OEOE_1756 | pseudo | pseudo | 54 | OEOE_RS08470 | 1676915 | 1677685 | plus |
| OEOE_1757 | serine O-acetyltransferase | serine O-acetyltransferase | 57 | OEOE_RS08475 | 1677907 | 1678404 | minus |

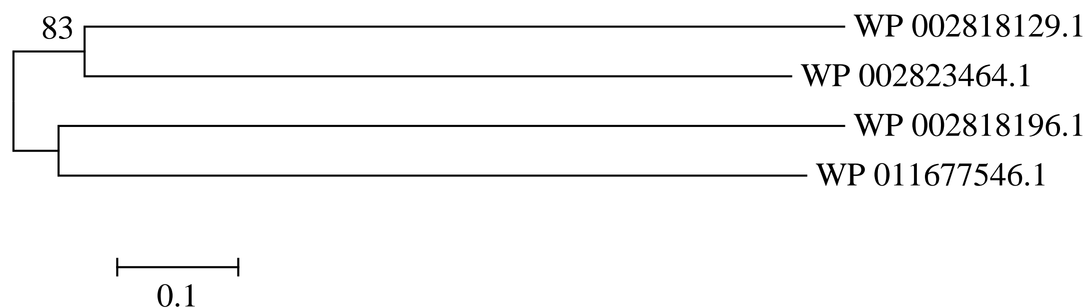


**Figure S1** Phylogenetic tree based on four TrdH protein sequences obtained from *O. oeni* PSU-1. The corresponding gene codes are RS1025 for protein sequence WP_002818129.1, RS8155 for WP_002823464.1, RS1895 for WP_002818196.1, and RS3560 for WP_011677546.1 The tree was inferred using the Poisson model together with neighbour-joining. Bootstrap values (1000 replicates) are shown as a percentage at the nodes. The scale bar represents the number of substitutions per site. The tree was constructed using MEGA v5.0 software.

# CHAPTER II

## Transcriptomic and proteomic analysis of *Oenococcus oeni* adaptation to wine stress conditions

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UNIVERSITAT ROVIRA I VIRGILI
OENOCOCCUS OENI RESPONSE TO WINE STRESS: ROLE OF GLUTATHIONE AND THIOREDOXIN SYSTEMS
Maria del Mar Margalef Català



## Abstract

*Oenococcus oeni*, the main lactic acid bacteria responsible for malolactic fermentation in wine, has to adapt to stressful conditions, such as low pH and high ethanol content. In this study, the changes in the transcriptome and the proteome of *O. oeni* PSU-1 during the adaptation period before MLF start have been studied. DNA microarrays were used for the transcriptomic analysis and two complementary proteomic techniques, 2-D DIGE and iTRAQ labeling were used to analyze the proteomic response. One of the most influenced functions in PSU-1 due to inoculation into wine-like medium (WLM) was translation, showing the over-expression of certain ribosomal genes and the corresponding proteins. Amino acid metabolism and transport was also altered and several peptidases were up regulated both at gene and protein level. Certain proteins involved in glutamine and glutamate metabolism showed an increased abundance revealing the key role of nitrogen uptake under stressful conditions. A strong transcriptional inhibition of carbohydrate metabolism related genes was observed. On the other hand, the transcriptional up-regulation of malate transport and citrate consumption was indicative of the use of L-malate and citrate associated to stress response and as an alternative energy source to sugar metabolism. Regarding the stress mechanisms, our results support the relevance of the thioredoxin and glutathione systems in the adaptation of *O. oeni* to wine related stress. Genes and proteins related to cell wall showed also significant changes indicating the relevance of the cell envelop as protective barrier to environmental stress. The differences found between transcriptomic and proteomic data suggested the relevance of post-transcriptional mechanisms and the complexity of the stress response in *O. oeni* adaptation. Further research should deepen into the metabolisms mostly altered due to wine conditions to elucidate the role of each mechanism in the *O. oeni* ability to develop MLF.

## Keywords

*Oenococcus oeni* - malolactic fermentation - transcriptomic – proteomic - stress - wine

## Introduction

Malolactic fermentation (MLF) occurs in wine spontaneously and, alternatively, can be induced inoculating selected strains of lactic acid bacterium (LAB), mainly *Oenococcus oeni*, usually after alcoholic fermentation (Betteridge et al., 2015). The wide range of physiological characteristics and the ability to cope with several environmental stresses make *O. oeni* the main responsible for MLF. This process consists in the conversion of L-malate to L-(+)-lactate and $CO_2$ and is required in wine, mainly from red grape varieties, because it confers positive sensory traits and improves wine's microbiological stability (Lonvaud-Funel, 1999; Mills et al., 2005).

Wine is a harsh environment for *O. oeni* due to its physicochemical characteristics, such as low pH, ethanol and $SO_2$ content, which can negatively affect bacterial survival and consequently MLF development. In contrast to the diversity of stress response mechanisms described in *Bacillus subtilis* (Hecker and Völker, 1998; Hecker et al., 1996), the model organism for Gram-positive bacteria, no gene encoding an alternative sigma factor or any other known regulator of stress response, such as HrcA, could be identified in *O. oeni*. Grandvalet et al. (2005) described in *O. oeni* the CtsR as the regulator for most of molecular chaperone genes. Different studies have characterized some of the stress response genes in *O. oeni*, such as *clp*, *grpE*, *groES*, *hsp18*, *hdc, ftsH*, *omrA*, *cfa*, *atpB*, and *trxA*, among others (Beltramo et al., 2004, 2006; Bourdineaud, 2006; Bourdineaud et al., 2003, 2004; Guzzo et al., 2000; Jobin et al., 1997; Olguín et al., 2009, 2010; Spano and Massa, 2006). These studies revealed that *O. oeni* has developed cellular mechanisms that make it more resistant to adverse conditions than other LAB species (Beltramo et al., 2006). The knowledge of the stress response machinery of this bacterium is key to understand the ability of adaptation to wine environment of each strain and select the best starter culture.

Thanks to the publication over the recent years of the genomes of different *O. oeni* strains in the National Center for Biotechnology Information (NCBI), nowadays it is possible the global study of stress response by “omics” technologies. There are two published studies of *O. oeni* combining transcriptomic and proteomic analysis, applying DNA microarrays and 2DE or 2D-DIGE: Olguín et al. (2015) studied the effect of ethanol addition during growth (after 1h), and Costantini et al. (2015) studied wine like media adaptation during 24h. The first proteomic study was from Silveira et al. (2004)

and showed that both ethanol stress and adaptation significantly changed the protein profiles of *O. oeni* cells. Later, Cecconi et al. (2009) performed a proteomic study using 2DE examining *O. oeni* adaptation to wine conditions. Other authors have studied oenological starters to determine their proteomic profile (Cafaro et al., 2014; Napoli et al., 2014).

In this work, we combined a transcriptomic and proteomic approach to elucidate the changes involved in the adaptation of *O. oeni* PSU-1 to wine-like conditions, evaluating the period between the inoculation and the beginning of MLF. The transcriptomic analysis was developed using DNA microarrays designed for PSU-1 strain and the results obtained were validated by real-time qPCR. For the proteomic study two complementary techniques were employed: 2D-DIGE and iTRAQ labeling. 2D-DIGE technique (Unlü et al., 1997) relies on a pre-electrophoretic labeling, allowing sample multiplexing in the same gel. This gel-dependent technique has been used in several studies with other LAB species (Genovese et al., 2013; Koponen et al., 2012; Mehmeti et al., 2011). However, the variable reproducibility of this technique along with the difficult automation and detection of low abundance and membrane hydrophobic proteins have led to a wide variety of off-gel methodologies for protein quantification. In order to complement DIGE analysis, in this work it was used a tandem mass spectrometry (MS/MS) coupled with isobaric tags for relative and absolute quantification (iTRAQ) (Ross et al., 2004) labeling to enable the identification and quantification of differentially expressed proteins in specific times. The combination of liquid chromatography (LC) and electrospray ionization MS/MS analysis is an emerging powerful methodology enabling quantification and comparison of protein levels directly from samples with greater efficiency and accuracy. This is the first proteomic analysis using this gel-free technique with *O. oeni*.

## Materials and Methods

### *Growth conditions and MLF monitoring*

The strain used in this study was *O. oeni* PSU-1, the only in its species with the genome fully annotated (Mills et al., 2005). Stock cultures (kept frozen at -80ºC) were grown in MRS broth medium (De Man et al., 1960) supplemented with 4 g/L L-malic acid and 5

g/L fructose at pH 5.0 at 28ºC in a 10% $CO_2$ atmosphere. Cells were collected at the end of the exponential phase ($OD_{600nm}$ = 1.4 - 1.6) and inoculated into the medium. Then, cells were harvested at the end of the exponential phase and inoculated (2% v/v) into 5 L screw-cap bottles of wine-like medium (WLM). WLM was prepared following (Bordas et al., 2015) containing 12% ethanol (v/v) at pH 3.4. The bottles were incubated at 20ºC. The assays were run in triplicate. Measurements of L-malic acid consumption were performed with the multianalyser Miura One (I.S.E. S.r.l, Guidonia, Italy) and the enzymatic kit ready to use (TDI SL, Barcelona, Spain) in order to determine the beginning and evolution of MLF.

*RNA and protein extraction*

After the inoculation into WLM, samples at different times (0, 0.5, 1, 2, 4, 6 and 8 h) were taken for RNA and protein extraction. For RNA extraction, 20 mL were collected from WLM, or 0.3 mL from MRS culture used for inoculation (0 h). Samples were centrifuged at 10,000 x *g* for 5 min at 4°C, supernatant was removed and pellet was washed with 10 mM Tris-HCl prepared with diethylpyrocarbonate-treated water (DEPC), and then frozen in liquid nitrogen and kept at -80°C until RNA extraction. High Pure RNA Isolation Kit (Roche, Mannheim, Germany) was used for RNA extraction following the instructions of the manufacturer with some modifications, such as lysis with lysozyme dissolved in 10 mM Tris-HCl buffer DEPC, at 50 mg/mL during 30 min at 37ºC. RNA was treated with Turbo DNA-free (Life Technologies, USA). Total acid nucleic concentrations were calculated using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany).

For protein extract preparation, 800 mL of WLM, or 35 mL from MRS culture used for inoculation (0 h), were centrifuged at 5,000 x *g* rpm for 15 min. Supernatant was removed and pellet was washed twice with 10 mM Tris-HCl buffer at pH 8, frozen in liquid nitrogen and kept at -80°C until protein extraction. Cell pellets were then resuspended to a final $OD_{600}$ = 30 in a solution of 0.1 M Tris-HCl at pH 7.5, mixed with protease inhibitor cocktail from Roche. Cells were disrupted using One-shot disruptor (Constant Systems Ltd.) at 5ºC, applying twice a 2.7 kbar pressure. Protein suspension was centrifuged at 4,500 x *g* for 15 min at 4ºC to remove cell debris and the supernatant was frozen in liquid nitrogen until protein analysis.

*Transcriptomic analysis*

*DNA microarray description, labeling and hybridization*

Microarrays (090324_*Oenococcus oeni* expression 4-plex array), based on PSU-1 genome, were developed by Roche NimbleGen (Madison, WI, USA) and samples were analyzed at the Functional Genomics Core of the Institute for Research in Biomedicine (IRB, Barcelona, Spain) as described by Olguín et al. (2015). The results were submitted to GEO (Gene Expression Omnibus Database, NCBI) under accession number GSE85137.

*Microarray results validation by real-time qPCR*

Nucleotide sequences of *O. oeni* strain PSU-1 (NC_008528) were obtained from the NCBI. Several genes were selected for real-time qPCR validation of the microarray data. The primers used for these analyses are shown in Table 1. Some genes were selected due to their involvement in stress response according to previous studies (Beltramo et al., 2006; Bordas et al., 2013; Jobin et al., 1999b; Olguín et al., 2009; Margalef-Català et al., 2016) and others were randomly selected with the sole objective of validating the methodology. Reverse transcription, real-time qPCR and primer design were performed according to Olguín et al. (2009). The Primer Express® Software was used to select primer sequences and analyze secondary structures and dimer formation. The absence of chromosomal DNA contamination was confirmed by qPCR. For the normalization of qPCR data (Cafaro et al., 2014; Sumby et al., 2012; Vandesompele et al., 2002) four genes (*ldhD*, *dpoIII*, *gyrA* and *gyrB*) were evaluated as internal controls, using the primers described in Table 1. Of these, *ldhD* and *gyrA* genes showed the lowest variation under the experimental conditions used (data not shown) and were chosen as internal controls. The amplification efficiency was calculated as in Olguín et al. (2015).

**Table 1** Gene descriptions and the corresponding primer sequences used for validation of microarray results by real-time qPCR. RNA samples were taken at signaled times, where maximum over- or under-expression had been observed in microarray assay.

| Gene symbol and old tag (OEOE_) | Sequence (5'- 3') | Amplicon length (bp) | Reference | Microarray[a] | qPCR[b] |
|---|---|---|---|---|---|
| **1 h** | | | | | |
| RS04745/0988<br>diacylglycerol kinase | Fw-TTGGGTCGGCATTTACTTTC<br>Rv-CCAACCGTAACCCATAACCA | 57 | This work | -1.05 | 0.71 |
| RS06455/1342<br>PTS sugar transporter subunit IIA | Fw-TGGTCGGAAATCAAGAAAGC<br>Rv-TCGGAAACTCCGTAATCGAC | 104 | This work | -1.61 | -0.40 |
| RS02005/0417<br>citrate lyase | Fw-GCACGTGAACTGCTGAAAAA<br>Rv-TGAGTGTTCCGATTCCACAA | 94 | This work | 1 | 3.28 |
| RS02030/0422<br>citrate lyase (*citE*) | Fw-CCGCACGATGATGTTTGTTCC<br>Rv-GCTCAAAGAAACGGCATCTTCC | 108 | Olguín et al. (2009) | 1.49 | 3.69 |
| RS01385/0289<br>heat-shock protein Hsp20 (*hsp18*) | Fw-CGGTATCAGGAGTTTTGAGTTC<br>Rv-CGTAGTAACTGCGGGAGTAATTC | 102 | Beltramo et al. (2006) | -0.42 | 1.13 |
| RS02715/0570<br>ATP dependent Clp protease proteolytic subunit (*clpp*) | Fw-CGGTACCAAAGGCAAGCGTTTTAT<br>Rv-CTCTTCCGAGTCTTCAAAAGTTGAT | 131 | Beltramo et al. (2006) | 0.41 | 0.15 |
| RS05660/1176<br>cyclopropane-fatty-acyl-phospholipid synthase (*cfa*) | Fw-TGGTATTACATTGAGCGAGGAG<br>Rv-CGTCTTTGAGATCACGATAATCC | 113 | Beltramo et al. (2006) | -1.11 | -0.17 |
| **2 h** | | | | | |
| RS01480/0310<br>phosphoglycerate mutase | Fw-CCGAAACCGCACAAAAGTAT<br>Rv-CTTCGTGACCCAAAAGTGGT | 87 | This work | 1.22 | 3.66 |
| RS04220/0881<br>Acyl carrier protein phosphodiesterase | Fw-GATCTCCCGAAGGATCAACA<br>Rv-AAAATTCATCCAGCCATTCG | 61 | This work | 1.51 | 3.90 |
| RS06410/1332<br>2-dehydro-3-deoxyphosphooctonate aldolase | Fw-CCAAAATCGACCCAATTACG<br>Rv-TCCCTCATCTCGATCAGACC | 106 | This work | 1.05 | 4.03 |
| **4 h** | | | | | |
| RS04710/0981<br>peptidase | Fw-GAATTGGCTCCCGACACTAA<br>Rv-TGACGATCCTTTGGAGCAAT | 71 | This work | 1.15 | 3.72 |
| **8 h** | | | | | |
| RS00900/0189<br>disulfide bond formation protein | Fw-GCTGTTGGTGTTTCGGTTTT<br>Rv-GCTCCAGGCAAAGTTTGAAG | 83 | This work | 1.00 | 3.60 |
| RS01290/0269<br>phosphonate ABC transporter ATP-binding protein | Fw-TTTTCAGGATCCGAAGATGG<br>Rv-GCAACAAATTTTCGGCAACT | 59 | This work | 2.65 | 4.38 |
| RS02980/0624<br>cobalt ABC transporter | Fw-ACTTTGGCTCCTCTGGTTGA<br>Rv-CAGCATTCATCGGTTTGCTA | 100 | This work | 1.24 | 2.77 |
| RS04600/0959<br>Xaa-Pro aminopeptidase | Fw-GTGGAAGTGGTGAAGGGATG<br>Rv-GGTCGACTCCATTTGGAAGA | 108 | This work | 1.93 | 3.82 |
| RS05245/1092<br>oligoendopeptidase F | Fw-CGGCAAATACTGGCAAAGAT<br>Rv-TGGACCCCATATGGAAATGT | 55 | This work | 1.90 | 3.82 |
| RS06245/1296<br>branched-chain amino acid aminotransferase | Fw-TTTCCCAGAAGACCGTTTTG<br>Rv-AAGTTGCACCGGAACCATAC | 89 | This work | 3.02 | 5.71 |
| RS07835/1625<br>Thiol-disulfide isomerase *trxA2* | Fw-TGGCAGTCTTTGAAACCTGA<br>Rv-CCAAGGGTCGCAATTTAATG | 105 | Margalef-Català et al. (2016) | -1.07 | 0.44 |
| RS08215/1702<br>Thioredoxin *trxA3* | Fw-GCCACTTGGTGTACCCCTTGT<br>Rv-TCCATTTGCCGTTTCCTGGTTT | 120 | Margalef-Català et al. (2016) | -0.81 | 0.92 |
| RS02695/0566<br>Thioredoxin reductase *trxB* | Fw-ATGCCAGCTCAACTCGTTTT<br>Rv-GTCGCTCCGCTAGCAACTAT | 139 | Margalef-Català et al. (2016) | 1.23 | 3.45 |
| RS00770/0163<br>Ferredoxin-NADP reductase *fdr* | Fw-AGCGAAGTTGCCGATAAAGA<br>Rv-TATCACGCCGATGAATCAAA | 115 | Margalef-Català et al. (2016) | 1.35 | 3.54 |
| RS05740/1191<br>glutathione reductase (*gshR*) | Fw-GGCATTATCACCGAGCTGTT<br>Rv-TCCCGAAGAAGCAAAGAAGA | 106 | Bordas et al. (2013) | -0.98 | -0.76 |
| **qPCR control genes** | | | | | |
| RS01985/0413<br>D-lactate dehydrogenase (*ldhD*) | Fw-GCCGCAGTAAAGAACTTGATG<br>Rv-TGCCGACAACACCAACTGTTT | 102 | Desroche et al. (2005) | | |

| | | | |
|---|---|---|---|
| RS04805/1000 DNA polymerase III subunit alpha (*dpoIII*) | Fw-AATTCGCACGGATTGTTTTC Rv-GCGAACCAGCATAGGTCAAT | 103 | Stefanelli (2014) |
| RS04780/0995 DNA primase (*dna G*) | Fw-TGTGGACGGAGTGGCAATGT Rv-CAGTATTTTCTGTATATTTACTATCG | 127 | Desroche et al. (2005) Margalef-Català et al. (2016) |
| RS00030/0006 DNA gyrase subunitA (*gyrA*) | Fw-CGCCCGACAAACCGCATAAA Rv-CAAGGACTCATAGATTGCCGAA | 95 | Desroche et al. (2005) |
| RS00025/0005 DNA gyrase subunitB (*gyrB*) | Fw-GAGGATGTCCGAGAAGGAATTA Rv-ACCTGCTGGGCATCTGTATTG | 107 | Desroche et al. (2005) Margalef-Català et al. (2016) |

[a]Microarray and [b]RT-qPCR fold changes between: t= 0h and t in which there is the maximum expression or inhibition after the inoculation.

*Proteomic analysis*

*2D-DIGE*

Protein extracts were analyzed in the Center for Omic Sciences from *Servei de Recursos Científics i Tècnics* of University Rovira i Virgili (Reus, Spain). Proteins were precipitated using TCA/Acetone, and the pellet was resuspended in 200 µL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-base) at final pH 8.5. The samples were quantified using Bradford and stored at −20°C. 50 µg of each protein sample was minimally labeled with 400 pmol of either Cy3 or Cy5 (N-hydroxy succinimidyl ester-derivatives of the cyanine dyes). To facilitate image matching and cross-gel normalization, an internal standard was made pooling all samples and labeling with Cy2 at the same ratio (50µg:400pmols). Hence, two samples and the internal standard could be run in the same gel and quantified on multiple 2-DE. Labeling reactions were performed on ice and darkness during 30 min and quenched using an excess of free L-lysine.

Isoelectrofocusing (IEF) was carried out using 24 cm Immobiline Dry-strips (pH interval 4–7, nonlinear, GE Healthcare), and sample was loaded by two rehydration steps (passively for 5 h at 20ºC, and actively at 50V during 12 h in an Ettan IPGphor 3 system from GE Healthcare). IEF migration program started focusing 500V for 7 h, ramping until 1KV during 4 h and ramping again until 10KV during 3h, and finally a step maintained at 10KV to reach 70KVh. Strips were then equilibrated for 15 min in a 50 mM Tris–HCl (pH 8.8) 6 M urea, 30% glycerol and 2% SDS buffer, adding first 1% DTT, and in a second time supplemented with 4% iodoacetamine (Görg et al., 2004).

*Imaging and Data Processing*

Gels were scanned using a PharosFX™ Plus Molecular Imager and analyzed using Progenesis Same Spots Analysis Software v4.5 (Totallab). Spots displaying a ≥1 average-fold increase or decrease in abundance with a p-value <0.05 were selected for protein identification. Features detected from non-protein sources (e.g. dust particles and dirty backgrounds) were filtered out. Picking analysis was carried loading 720 µg of the internal standard mix without labeling in a 2DE gel as described above. Gels were stained with Coomassie blue G250 and imaged using Pharos FXTM Plus Molecular Imager from BioRad using Quantity One version 4.6.9 software.

*In gel-trypsin digestion and MS-Based protein identification*

Spots of interest were automatically excised from 2-DE gels using the ExquestTM Spot Cutter with the PDQuestTM Advanced 2D Analysis Software V8.0.1 both from Bio-Rad. Excised spots were de-hydrated by extensive washings with 25mM ammonium bicarbonate and acetonitrile. All gel pieces were incubated with 15ng/µL sequencing-grade trypsin in 50 mM ammonium bicarbonate at pH 7.9 overnight at 37ºC. Following digestion, the peptides were desalted using C18 zip-tip (Millipore), and eluted with 75% ACN + 0.1% Trifluoroacetic acid.

Peptides were spotted onto an HTP BigAnchor 384 (Bruker) target using α-cyano-4-hydroxy-cinnamic acid as matrix and were analyzed on a MALDI TOF/TOF (Ultraflextreme, Bruker Daltonics, Bremen, Germany) instrument operated in the positive ion mode. All mass spectra were calibrated externally with the Peptide Calibration Standard I from Bruker. The analyzed mass range was 600 - 3500 Da. MS and MS/MS analyses were performed automatically. For MS analysis, 3100 satisfactory shots were accumulated by recording 100-shot steps at 20 random positions using fuzzy control laser attenuation between 40% and 100% at initial and maximal power respectively. For MS/MS 3100 satisfactory shots were accumulated by recording 100-shot steps and 4000 for the fragment ion spectra.

MS and tandem MS/MS spectra were searched by Protein Scape v: 3.0.0 446 using MASCOT (Matrix Science Inc., MA, 2.4.0) against NCBInr database (46742655 entries) restricted to Bacteria (Eubacteria). The search parameters were set to: MS accuracy 20 ppm, MS/MS accuracy 0.5 Da, two missed cleavage by trypsin allowed,

carbamidomethylation of cysteine as fixed modification and oxidation of methionine and N-terminal amino acid conversion of glu and gln to pyroglutamic acid as variable modification. Significant protein hits (for peptide mass fingerprinting $p \leq 0.05$ and for MS/MS at least two peptides with $p \leq 0.05$, and protein scores greater than 30) were considered significant.

*iTRAQ labeling*

*Protein digestion and iTRAQ labeling*

On a SDS-PAGE gel (12% resolving gel and 4% stacking gel) at 20 mA/gel 40 µg total protein per sample were run. The electrophoresis was stopped when the front dye had barely passed from the stacking gel into the resolving gel, and a unique concentrated band was obtained for every sample, which was stained using Coomassie Brilliant Blue G-250, excised, cut into small pieces and stored at 4°C in ultrapure water.

Protein digestion was performed according to Shevchenko et al. (1996) with minor variations as described before. Proteins were reduced using 5 mM tris(2-carboxyethyl)phosphine (TCEP) in 50 mM triethylammonium bicarbonate pH 7.9 during 1 h at 60°C and alkylated with 10 mM methyl methanethiosulfate (MMTS) in the same buffer during 30 min at room temperature. To digest the samples, they were incubated with 15.4 ng/µL sequencing-grade trypsin in 50 mM triethylammonium bicarbonate at pH 7.9 overnight at 37ºC. After digestion, the peptides were extracted from gel by elution in a mixture of 50% acetonitrile 5% formic acid. Tryptic peptides were dried by SpeedVac and re-suspended in 30 µL TEAB 0.5 M at pH 8.5.

iTRAQ-8plex labeling reagents (AB SCIEX) were added to each peptide samples according manufacturer's instructions and incubated at room temperature for 120 min. Mixtures of labeled samples were washed from unreacted reagents using SCX column (Strata® SCX 55 µm, 70 Å, Phenomenex) in 10 mM phosphoric acid, 25% acetonitrile, pH 3 as binding buffer and 5% ammonium hydroxide, 25% acetonitrile for the elution. After the elution, samples were vacuum dried and re-suspended in water for the next step.

*Sample Fractionation and Mass Spectrometry Analysis (LC-MS/MS)*

Pooled peptides were separated in an Agilent 3100 OFFGEL Fractionator (Agilent Technologies, Santa Clara, CA) through 24-well IPG strips (linear gradient from pH 3 to 10) according to the supplier's protocol. After separation, fractions were desalted and concentrated through C18 Sep-Pak column (Waters, Bedford, MA) previously to LC-MS/MS detection.

A nano LC II coupled to an LTQ-Orbitrap Velos Pro mass analyzer, both from Thermo Scientific (Bremen, Germany), was used for peptide analysis. The chromatographic separation was achieved using a nanoLC $C_{18}$ trap column (100 µm I.D.; 2 cm length; 5 µm particle diameter, Thermo Fisher Scientific) coupled to a nanoLC $C_{18}$ analytical column (75 µm I.D.; 15 cm length; 3 µm particle diameter, Nikkyo Technos Co. LTD, Japan) under gradient elution conditions. Ultrapure water with 0.1% HCOOH (solvent A) and acetonitrile with 0.1% HCOOH (solvent B) was the mobile phase and the gradient consisted of 0–5% B during 5 min, 5-35% B 30 min, 35-80% B 15 min and 80-100% B 12 min, and finally is maintained at 100% B during 10 min. A flow rate of 300 nL/min was used to elute peptides for real time ionization on a nanoFlex electrospray ion source from Thermo Fisher Scientific.

MS measurements detected intact peptides in a full scan (m/z 350-2,000), with the Orbitrap at FT-resolution spectrum (R=30,000 FHMW), followed by data dependent MS/MS scan from most intense ten parent ions with a charge state rejection of one. The signal threshold for triggering an MS/MS event was set to 10,000 counts. The low mass cutoff was set to 100 m/z. Dynamic exclusion of 30 s and activation time of 0.1 s was used. For efficient fragmentation and detection of iTRAQ reporter ions, HCD normalized collision energy of 45 was used. All fragment ions were detected in the Orbitrap (R=7,500 FHMW). Internal calibration was performed using the ion signal of (Si(CH3)2O)6H+ at m/z 445.120025 as a lock mass. Maximal ion accumulation time allowed on the LTQ Orbitrap was 1 s for all scan modes; automatic gain control was used to prevent over-filling of the ion trap.

*Database Searches and Quantitative Proteome Analysis*

Tandem mass spectra were extracted and charge state deconvoluted by Proteome Discoverer v1.4.0.288 (Thermo Fisher Scientific). All MS/MS samples were analyzed

using Mascot (v 2.4.1.0) as search engine node. Mascot was set up to search in NCBInr database (46,742,655 entries), following the application of the restriction for Firmicutes taxonomy. Two missed cleavages were allowed for trypsin digestion, and an error of 0.80 Da for fragment ion mass and 10.0 ppm for a parent ion were tolerated. Oxidation of methionine, acetylation of N-termini and ITRAQ 8-plex modifications were specified as variable modifications, whereas methylation of cysteines was set as static modification. The false discovery rate (FDR) and protein probabilities were calculated by Target Decoy PSM Validator working between 0.01 and 0.05 for strict and relaxed, respectively. For proteins identified with only one single peptide meeting these criteria, we required the Mascot score to be at least 30, and visual verification of fragmentation spectra was done. Identified proteins were grouped by the software to minimize redundancy. For quantitative analysis centroided iTRAQ reporter ion signals were computed and only unique peptides were used for relative protein quantification. iTRAQ reporter ion intensities were normalized to sample 113, that were replicated in the two ITRAQ mixtures.

The statistical analysis was performed on Mass Profile Professional software v. 12.6 from Agilent Technologies. To find differential proteins, a paired 1-way ANOVA test was used selecting a $p < 0.05$ and fold change $> 1.5$ as cut-off values. Principal component analysis (PCA) was used to evaluate variations in the mean quantity of spots.

*Bioinformatic tools*

On-line databases like NCBI information of each gene, Computational Biology at Oak Ridge National Laboratory (ORNL; http://compbio.ornl.gov/public/section/), DAVID database (https://david.ncifcrf.gov/), KOBAS 2.0 (KEGG Orthology Based Annotation System; http://kobas.cbi.pku.edu.cn/) (Xie et al., 2011) were used to assess all the Clusters of Orthologous Groups (COGs) described for *O. oeni* genes and proteins and metabolic pathways. We analyzed the expression data of arrays with MEV (Multi Experiment View) cluster software using Quality Threshold Clustering (QTC) tool (Heyer et al., 1999). Pearson correlation and a minimum cluster population of the number of genes representative of 10% were used and a maximum cluster diameter of 0.9. For construction of Venn diagrams, Venny (an interactive tool for comparing lists with Venn's diagrams, http://bioinfogp.cnb.csic.es/tools/venny/index.html) was used.

## Results and discussion

Functional analysis using comparative transcriptomics and proteomics can provide deeper insight into the molecular mechanisms of adaptation of *O. oeni* to wine stress conditions. The aim of this work was to evaluate which genes and proteins were affected during the adaptation period occurring before the start of MLF. The study was performed with the reference strain PSU-1 using wine-like medium (WLM) at pH 3.4 and with 12% of ethanol (v/v). Under these conditions, PSU-1 showed an adaptation period of 8 h from inoculation until the beginning of MLF. Once L-malate consumption started, MLF was successfully finished in 72 hours. The viability of PSU-1 did not decrease during the adaptation period, indicating that there was no detectable cell death of the inoculated population ($6.87 \cdot 10^7 \pm 1.70 \cdot 10^7$ CFU/mL).

*Global analysis of functions affected during adaptation to WLM*

In the transcriptomic analysis 1,611 expressed sequence tag (EST) were detected. Among the EST that were differently expressed during adaptation, 27 were classified as discontinued or pseudo genes in NCBI, and they were not included in the analysis. Among the analyzed genes showing significant changes along the assay, 314 were over-expressed, whereas 308 genes were down-regulated. They can be consulted in Table S1 in the supplementary data. It is worth noting that 52 over-expressed genes and 51 down-regulated genes were annotated as hypothetical proteins (Table S2 in the supplementary data).

These 622 genes differently expressed were classified in Clusters Orthologous Groups (COGs) in order to identify the main biological processes influenced by adaptation to wine conditions (Figure 1A-B). Besides, the Quality Threshold Clustering (QTC) analysis grouped the differentially expressed genes into six transcriptional profiles (Figure 2).

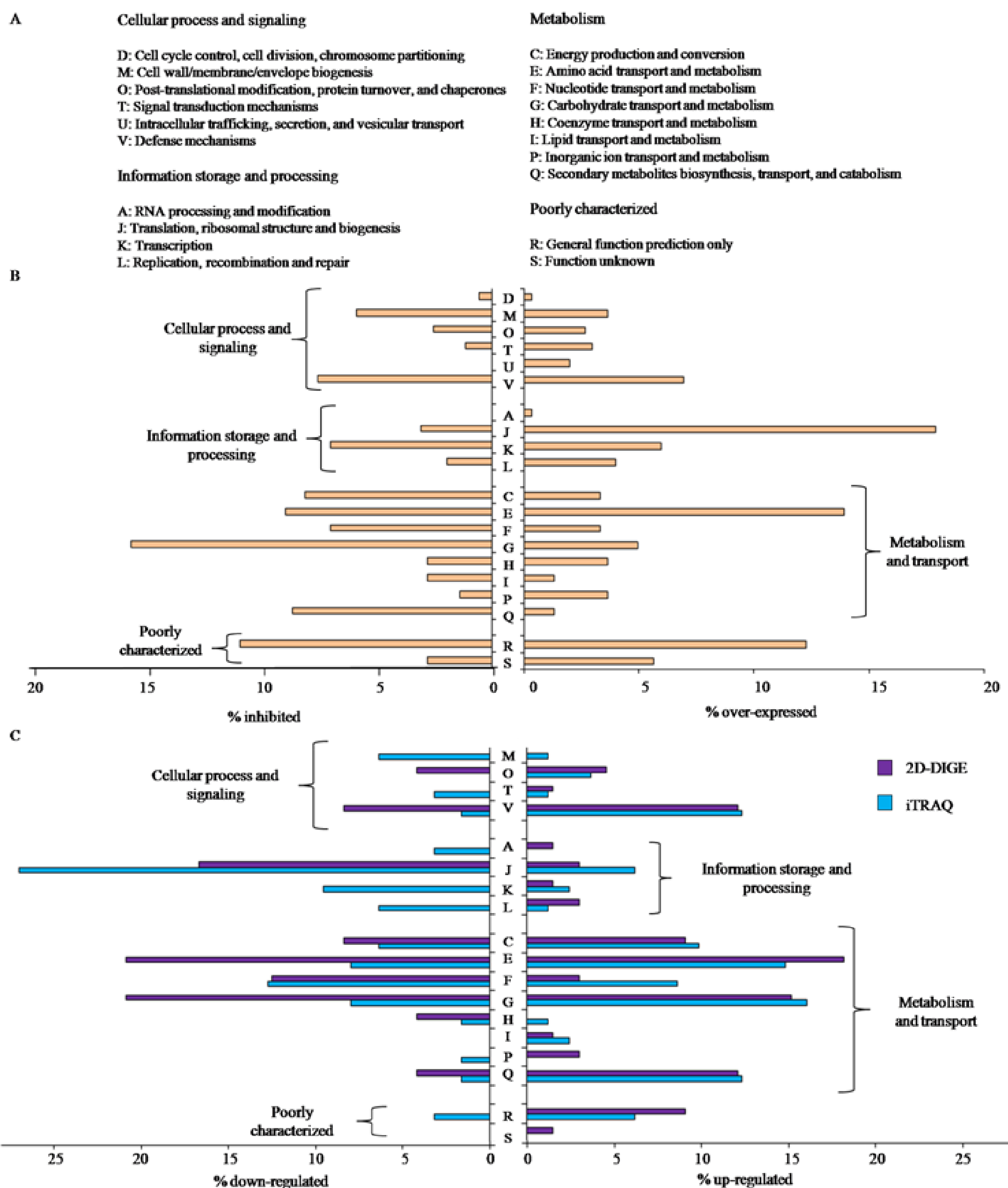


**Figure 1** A) Clusters of Orthologous Groups (COGs) definitions. B) Percentage of genes of each representative COG significantly over or under-expressed according to transcriptomic analysis. C) Percentage of proteins of each representative COG showing significant abundance changes, detected by 2D-DIGE or iTRAQ.

The main functions transcriptionally activated due to inoculation in WLM in *O. oeni* PSU-1 were translation, ribosomal structure and biogenesis (J) and amino acid transport and metabolism (E) (Figure 1B). The QTC analysis (Figure 2) showed two expression profiles, I and III (65.9% and 13.6% of over-expressed genes, respectively), in which

translation (J) was the most represented function. These two profiles are indicative of an adaptive response since gene transcription increases progressively along adaptation process. The profile II (17.5% of over-expressed genes) included mostly genes of amino acid metabolism showing an increase in their transcription level between 0.5 – 1 h which was later decreased. This behavior is indicative of an early-response to WLM stress conditions. Regarding the genes showing an inhibited transcription, carbohydrate metabolism (G) was the main negatively regulated function in PSU-1 during adaptation to WLM (Figure 1B). The genes related to this metabolism mostly showed a constant down-regulation and were present in profile IV (Figure 2), representing 62.2% of total down-regulated genes. Fewer genes were clustered into profile V (15.3%), showing transcriptional repression only at the beginning of the assay (0.5 -1 h). Finally, the profile VI grouped 19.8% of down-regulated genes which had a progressive repression during the adaptation period.

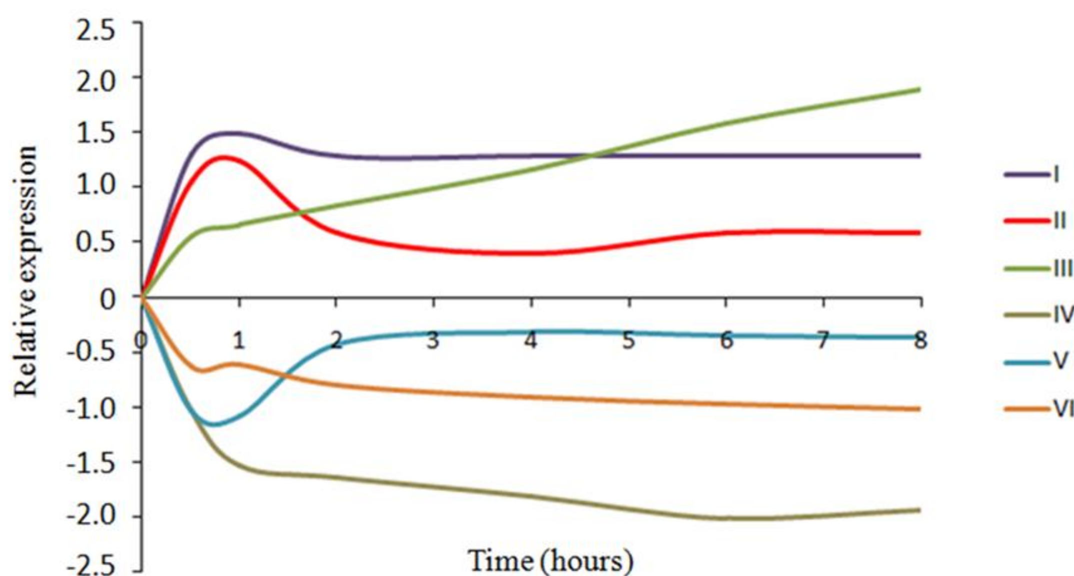


**Figure 2** Representative gene expression profiles according to Quality Threshold Clustering (QTC) based on transcriptomic data. An example of each profile is shown. Profile I: OEOE_RS07930 (UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase); Profile II: OEOE_RS03595 (amino acid ABC transporter substrate-binding protein); Profile III: OEOE_RS05245 (oligoendopeptidase F); Profile IV: OEOE_RS07040 (glycerol-3-phosphate ABC transporter permease); Profile V: OEOE_RS03155 (F0F1 ATP synthase subunit A); Profile VI: OEOE_RS01045 (PTS sugar transporter subunit IIA).

Proteomic analysis of *O. oeni* PSU-1 adaptation to WLM conditions was conducted using two techniques: 2D-DIGE and iTRAQ. The PCA analyses to evaluate the variability among samples clearly indicated the presence of three different protein populations 0 h, 1 h and 6 h (data not shown). The 2D-DIGE results showed between 27-62 protein spots along the assay exhibiting differential abundance with statistical significance ($p \leq 0.05$). The maximum differences were observed comparing the samples from 1 h and 6 h after inoculation vs. 0 h. For this reason, the protein identification was carried out only for these samples and also these samples were analyzed using iTRAQ labeling in order to complement 2D-DIGE results. Using 2D-DIGE, 33 different proteins could be determined which were not found with iTRAQ. On the other hand, the off-gel technique detected 71 proteins exclusively. At 1 h there were detected more proteins up regulated than at 6 h, revealing a fast stress proteomic response of the cell against the new environment. All proteomic identification and COG classification from 2D-DIGE and iTRAQ analysis can be consulted in Table S3 (supplementary data).

A high percentage of the significantly down-regulated proteins (Figure 1C) belonged to the COG associated to translation, ribosomal structure and biogenesis (J). This is in accordance to previous studies that described the lower abundance of proteins involved in protein synthesis during acid stress in *Lactobacillus* species (Heunis et al., 2014; Koponen et al., 2012). On the other hand, some ribosomal proteins of 50S and 30S subunits showed an increased abundance in *O. oeni* PSU-1 after inoculation into WLM. This increase in protein concentration was coincident with an up-regulated gene transcription (Tables 2 and 3). Huang et al. (2011) and Koponen et al. (2012) also described the increased abundance of 50S and/or 30S ribosomal proteins in *Lactobacillus* species as a mechanism of response to acidic stress. Therefore, certain ribosomal proteins involved in the regulation of translation may play a role in stress response as suggested by Dressaire et al. (2010).

A relevant number of proteins related to amino acid and carbohydrate metabolism (E, G) showed significant variations in abundance (Figure 1C) both increasing or decreasing, being some of them in accordance to the transcriptional response (Tables 2 and 3). On the other hand, detected proteins related to defense mechanism (V) and secondary metabolites (Q) showed mostly an increased abundance (Figure 1C).

**Table 2** Selection of genes related with relevant metabolisms or functions, differently regulated after inoculation into WLM from the microarray analysis. Genes which regulation is coincident with proteomic results are grey highlighted. For each gene, time sample with maximum over- or under-expression is bold highlighted.

| Related metabolism | Gene annotation | Gene symbol | Relative expression | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | **0.5 h** | **1 h** | **2 h** | **4 h** | **6 h** | **8 h** |
| **Malate metabolism** | malate dehydrogenase | OEOE_RS02010 | 2.20 | **2.51** | 2.25 | 1.77 | 1.68 | 1.61 |
| | malate permease | OEOE_RS02015 | 2.25 | **2.45** | 2.08 | 1.74 | 1.71 | 1.67 |
| | malate transporter | OEOE_RS06985 | 3.28 | 4.03 | **4.19** | 3.84 | 3.68 | 3.69 |
| **Citrate metabolism** | citrate lyase | OEOE_RS02005 | 0.82 | **1.01** | 0.76 | 0.63 | 0.55 | 0.49 |
| | [citrate [pro-3S]-lyase] ligase | OEOE_RS02020 | 1.85 | **2.22** | 1.77 | 1.23 | 1.20 | 1.17 |
| | citrate lyase ACP | OEOE_RS02025 | 1.68 | **1.96** | 1.88 | 1.44 | 1.24 | 1.45 |
| | citrate lyase | OEOE_RS02030 | 1.25 | **1.50** | 1.25 | 0.95 | 0.91 | 1.07 |
| | citrate lyase subunit alpha | OEOE_RS02035 | 0.73 | **1.13** | 0.93 | 0.59 | 0.54 | 0.64 |
| | acetoin reductase | OEOE_RS03325 | -0.96 | -1.32 | -1.53 | -1.80 | **-1.82** | -2.01 |
| | diacetyl reductase | OEOE_RS07730 | -0.63 | -1.01 | -1.25 | -1.35 | **-1.37** | -1.43 |
| **ATPase activity** | F0F1 ATP synthase subunit A | OEOE_RS03155 | -1.03 | **-1.08** | -0.42 | -0.31 | -0.35 | -0.37 |
| | ATP synthase subunit delta | OEOE_RS03170 | -0.92 | **-1.16** | -0.92 | -0.90 | -0.91 | -0.91 |
| | ATP synthase subunit gamma | OEOE_RS03180 | **-1.28** | -1.25 | -0.97 | -0.93 | -0.84 | -0.83 |
| | F0F1 ATP synthase subunit epsilon | OEOE_RS03190 | -1.35 | -1.26 | -1.45 | -1.49 | -1.56 | **-1.69** |
| **Amino acid transport and metabolism** | 4-aminobutyrate aminotransferase | OEOE_RS01860 | 2.98 | **3.30** | 3.27 | 3.20 | 3.21 | 3.30 |
| | amino acid permease | OEOE_RS01865 | 2.81 | 3.11 | **3.12** | 3.08 | 3.01 | 3.05 |
| | peptide ABC transporter permease | OEOE_RS02110 | 4.02 | 4.40 | **4.48** | 4.19 | 4.00 | 4.07 |
| | spermidine/putrescine import ATP-binding protein PotA | OEOE_RS03010 | 0.69 | 0.61 | 0.99 | **1.27** | 1.21 | 1.20 |
| | spermidine/putrescine ABC transporter permease | OEOE_RS03015 | 0.53 | 0.65 | 0.84 | **1.14** | 1.08 | 1.03 |
| | spermidine/purescine ABC transporter permease | OEOE_RS03020 | 0.68 | 0.78 | 0.97 | **1.32** | 1.28 | 1.31 |
| | carboxypeptidase | OEOE_RS04315 | 1.15 | **1.45** | 1.14 | 1.14 | 1.14 | 1.14 |
| | glutamine synthetase | OEOE_RS04565 | 1.88 | 2.08 | **2.20** | 2.01 | 1.83 | 1.81 |
| | Xaa-Pro aminopeptidase | OEOE_RS04600 | 0.58 | 0.47 | 1.16 | 1.75 | 1.90 | **1.93** |
| | oligoendopeptidase F | OEOE_RS05245 | 0.56 | 0.66 | 0.83 | 1.17 | 1.59 | **1.90** |
| | spermidine/putrescine ABC transporter ATP-binding protein | OEOE_RS07070 | **1.69** | 1.40 | 1.21 | 1.06 | 0.97 | 0.99 |
| | spermidine/putrescine ABC transporter ATP-binding protein | OEOE_RS07075 | **1.66** | 1.16 | 1.01 | 0.92 | 0.91 | 0.86 |
| | spermidine/putrescine ABC transporter permease | OEOE_RS07080 | **1.27** | 0.75 | 0.71 | 0.51 | 0.41 | 0.49 |
| | amino acid permease | OEOE_RS07900 | 2.93 | 3.16 | **3.18** | 2.98 | 2.90 | 2.81 |
| | peptidase M20 | OEOE_RS08295 | 0.89 | 1.14 | 1.08 | 1.35 | 1.49 | **1.53** |
| | aspartate carbamoyltransferase | OEOE_RS01235 | -1.14 | -1.75 | -2.00 | -2.27 | **-2.56** | -2.50 |
| **Carbohydrate transport and metabolism** | mannose-6-phosphate isomerase | OEOE_RS00125 | -2.56 | -2.56 | -2.56 | -2.56 | -2.58 | **-2.81** |
| | phosphoglyceromutase | OEOE_RS00565 | -1.37 | **-1.60** | -1.51 | -1.40 | -1.37 | -1.42 |
| | sugar phosphate isomerase | OEOE_RS00595 | -1.71 | -1.98 | **-2.05** | -1.72 | -1.58 | -1.71 |
| | 6-phospho-β-glucosidase | OEOE_RS01060 | -1.04 | -1.23 | -1.58 | -1.81 | **-2.02** | -1.94 |
| | PTS fructose transporter subunit IIA | OEOE_RS01110 | -1.85 | -2.20 | -2.60 | **-2.90** | -2.69 | -2.79 |
| | PTS mannose transporter subunit IIAB | OEOE_RS02230 | -0.73 | -1.02 | -1.31 | **-1.38** | -1.06 | -1.00 |
| | PTS mannose transporter subunit IID | OEOE_RS02240 | -0.76 | -1.08 | **-1.43** | -1.43 | -1.23 | -0.97 |
| | phosphocarrier protein HPr | OEOE_RS03075 | -2.19 | -2.16 | -2.20 | **-2.33** | -2.25 | -2.32 |
| | lactate dehydrogenase | OEOE_RS05695 | -1.20 | -0.93 | -1.10 | -1.22 | -1.14 | **-1.37** |
| | PTS sugar transporter | OEOE_RS05805 | -2.37 | -2.45 | -2.68 | -2.86 | -2.87 | **-2.90** |
| | glycerol-3-phosphate ABC transporter ATP-binding protein | OEOE_RS07030 | -1.42 | -1.92 | -2.10 | -2.34 | -2.51 | **-2.54** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | glycerol-3-phosphate ABC transporter permease | OEOE_RS07035 | -0.90 | -1.36 | -1.53 | -1.65 | -1.72 | **-1.76** |
| | glycerol-3-phosphate ABC transporter permease | OEOE_RS07040 | -1.03 | -1.54 | -1.64 | -1.81 | **-2.01** | -1.93 |
| | glycerol-3-phosphate ABC transporter substrate-binding protein | OEOE_RS07045 | -1.16 | -1.47 | -1.86 | -2.13 | -2.20 | **-2.22** |
| | UDP-phosphate galactose phosphotransferase | OEOE_RS07255 | -1.98 | -1.96 | -1.96 | **-2.01** | -1.94 | -1.96 |
| | ribokinase | OEOE_RS07775 | -1.53 | -1.87 | **-2.32** | -2.18 | -1.64 | -1.57 |
| | D-ribose pyranase | OEOE_RS07780 | -1.27 | -1.82 | **-2.09** | -2.08 | -1.59 | -1.33 |
| | sugar:proton symporter | OEOE_RS07785 | -1.28 | -1.75 | **-2.22** | -2.19 | -1.73 | -1.56 |
| | enolase | OEOE_RS07960 | -0.76 | -0.76 | -0.75 | -1.06 | -1.07 | **-1.12** |
| | sugar phosphate isomerase | OEOE_RS08055 | -1.35 | -1.33 | -1.79 | -2.11 | -2.02 | **-2.24** |
| | fructokinase | OEOE_RS08245 | -1.42 | -2.08 | -2.15 | -2.28 | -2.40 | **-2.46** |
| **Lipid transport and metabolism** | tannase | OEOE_RS05040 | 1.88 | 2.26 | **2.72** | 2.22 | 1.89 | 1.90 |
| | cyclopropane-fatty-acyl-phospholipid synthase | OEOE_RS05660 | -0.90 | **-1.11** | -0.98 | -0.90 | -0.86 | -0.83 |
| | glycerophosphoryl diester phosphodiesterase | OEOE_RS07050 | -1.49 | -1.64 | -2.08 | -2.51 | **-2.57** | -2.56 |
| **Cell wall/membrane/envelope biogenesis** | glucosamine--fructose-6-phosphate aminotransferase | OEOE_RS03035 | 2.94 | 3.14 | 3.65 | 3.92 | **3.99** | 3.97 |
| | D-alanyl-D-alanine carboxypeptidase | OEOE_RS03435 | 5.53 | 5.83 | **6.01** | 5.82 | 5.61 | 5.64 |
| | peptidoglycan interpeptide bridge formation protein | OEOE_RS06965 | 0.95 | 1.87 | **2.52** | 2.39 | 1.85 | 1.70 |
| | sortase | OEOE_RS06970 | 1.46 | 2.00 | **2.48** | 2.27 | 1.94 | 1.91 |
| | peptidoglycan interpeptide bridge formation protein | OEOE_RS06975 | 1.55 | 2.27 | **2.77** | 2.52 | 2.24 | 2.19 |
| | glycosyl transferase | OEOE_RS07820 | 1.78 | 1.90 | 2.86 | **3.39** | 3.35 | 3.08 |
| | D-alanyl-D-alanine carboxypeptidase | OEOE_RS07530 | -0.96 | -1.45 | -1.49 | -1.60 | **-1.75** | -1.70 |
| **Translation, ribosomal structure and biogenesis** | serine--tRNA ligase | OEOE_RS02120 | 1.44 | 1.93 | **2.07** | 1.93 | 1.77 | 1.72 |
| | elongation factor 3 | OEOE_RS02460 | 1.48 | 1.97 | **2.28** | 1.95 | 1.83 | 1.82 |
| | 30S ribosomal protein S10 | OEOE_RS02840 | 1.88 | 1.82 | 1.88 | 1.92 | **1.93** | 1.90 |
| | 30S ribosomal protein S8 | OEOE_RS02910 | 2.07 | 2.04 | 2.35 | **2.57** | 2.45 | 2.42 |
| | 50S ribosomal protein L15 | OEOE_RS02935 | 1.00 | 1.18 | 1.41 | **1.70** | 1.64 | 1.63 |
| | 50S ribosomal protein L17 | OEOE_RS02970 | 0.56 | 0.93 | 1.41 | 1.75 | **1.83** | 1.75 |
| | 50S ribosomal protein L32 | OEOE_RS03680 | -0.14 | 0.59 | 0.78 | 0.87 | 0.88 | **1.07** |
| | acetyltransferase | OEOE_RS04010 | 2.37 | 2.70 | 2.99 | **3.18** | 3.07 | 2.77 |
| | 30S ribosomal protein S20 | OEOE_RS06185 | 0.29 | 0.84 | 1.07 | 1.29 | **1.32** | 0.83 |
| | 50S ribosomal protein L7/L12 | OEOE_RS06825 | 0.63 | 1.16 | 1.71 | **2.00** | 1.74 | 1.46 |
| **Stress response** | ferredoxin--NADP reductase | OEOE_RS00770 | 0.93 | 1.14 | 1.24 | 1.35 | **1.39** | 1.35 |
| | multidrug ABC transporter ATP-binding protein | OEOE_RS02115 | 3.69 | 4.43 | **4.75** | 4.45 | 4.26 | 4.08 |
| | thioredoxin reductase | OEOE_RS02695 | 1.06 | **1.66** | 1.60 | 1.23 | 1.20 | 1.23 |
| | N-acetylmuramoyl-L-alanine amidase | OEOE_RS02805 | 1.85 | 2.06 | **2.14** | 2.01 | 1.79 | 1.71 |
| | acetyl esterase | OEOE_RS03440 | 3.23 | 3.72 | **4.21** | 4.03 | 3.69 | 3.61 |
| | multidrug ABC transporter ATPase | OEOE_RS03445 | 1.72 | 1.69 | 2.02 | **2.16** | 1.93 | 2.02 |
| | multidrug ABC transporter permease | OEOE_RS03450 | 2.15 | 2.01 | 2.34 | **2.48** | 2.35 | 2.18 |
| | multidrug ABC transporter permease | OEOE_RS03640 | 0.88 | **1.23** | 1.10 | 1.03 | 1.06 | 1.17 |
| | multidrug MFS transporter | OEOE_RS04200 | 2.39 | 2.77 | 3.14 | 3.51 | 3.73 | **3.75** |
| | molecular chaperone DnaJ | OEOE_RS06305 | 1.56 | **1.76** | 1.61 | 1.36 | 1.32 | 1.10 |
| | molecular chaperone DnaK | OEOE_RS06310 | 1.03 | 1.35 | **1.38** | 1.04 | 1.03 | 0.95 |
| | protein GrpE | OEOE_RS06315 | 0.93 | 1.41 | **1.59** | 1.11 | 1.02 | 0.94 |
| | multidrug ABC transporter ATP-binding protein | OEOE_RS07890 | **1.07** | 0.93 | 0.72 | 0.69 | 0.59 | 0.65 |
| | peptidylprolyl isomerase | OEOE_RS07905 | 2.23 | **2.74** | 2.62 | 2.30 | 2.10 | 1.97 |
| | multidrug ABC transporter ATP-binding protein | OEOE_RS08260 | 1.48 | 1.54 | 1.68 | **1.80** | 1.47 | 1.05 |
| | multidrug ABC transporter ATP-binding protein | OEOE_RS08265 | 1.01 | 1.04 | 1.25 | **1.40** | 1.10 | 0.76 |
| | general stress protein | OEOE_RS00325 | -2.18 | -2.10 | -2.46 | -2.54 | **-2.60** | -2.58 |
| | glutaredoxin | OEOE_RS00645 | **-1.04** | -0.44 | -0.27 | -0.64 | -0.82 | -0.86 |
| | heat-shock protein Hsp20 | OEOE_RS01385 | -0.92 | -0.43 | -0.55 | -0.89 | -0.88 | **-1.13** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | glutathione reductase | OEOE_RS05740 | -0.63 | -0.78 | -0.83 | -0.96 | **-1.00** | -0.98 |
| | cold-shock protein | OEOE_RS06620 | -1.75 | -1.14 | -1.30 | -1.49 | -1.73 | **-1.81** |
| | thiol-disulfide isomerase | OEOE_RS07835 | -1.17 | **-1.32** | -1.18 | -1.03 | -1.01 | -1.07 |
| | thioredoxin | OEOE_RS08215 | **-1.10** | -0.71 | -0.71 | -0.70 | -0.68 | -0.81 |
| **Nucleotide transport and metabolism** | adenylate kinase | OEOE_RS02945 | 1.21 | 1.08 | 1.22 | 1.36 | **1.44** | 1.22 |
| | deoxyadenosine kinase | OEOE_RS04085 | -1.23 | **-1.25** | -1.08 | -1.07 | -1.13 | -1.24 |
| **Coenzyme transport and metabolism** | thiamine pyrophosphokinase | OEOE_RS03790 | -0.93 | **-1.07** | -1.05 | -0.96 | -0.93 | -0.84 |
| | pyridoxal biosynthesis protein | OEOE_RS04980 | -0.89 | -1.10 | -1.22 | -1.19 | **-1.23** | -1.15 |

**Table 3** Selection of relevant proteins detected by 2D-DIGE and iTRAQ analysis differently regulated after WLM inoculation at 1 and 6 h. Proteins which regulation is coincident with transcriptomic results are grey highlighted.

| Related metabolism | Protein annotation | Gene symbol | Fold change | | | | Theoretical Mr (KDa) | Pi |
|---|---|---|---|---|---|---|---|---|
| | | | DIGE | | iTRAQ | | DIGE / iTRAQ | DIGE / iTRAQ |
| | | | 1h | 6h | 1h | 6h | | |
| **Malate metabolism** | malate dehydrogenase | OEOE_RS02010 | - | - | -1 | -0.8 | 41.4 | |
| **Citrate metabolism** | acetoin reductase | OEOE_RS03325 | - | - | -1.7 | -0.9 | 27.4 | |
| | diacetyl reductase | OEOE_RS07730 | ND | 1.237 | - | - | 27.4 | 5.26 |
| | | | ND | 1.626 | - | - | 27.4 | 5 |
| **ATPase activity** | F0F1 ATP synthase subunit alpha | OEOE_RS03175 | - | - | -1.3 | 0.7 | 56.7 | |
| **Amino acid transport and metabolism** | aspartate carbamoyltransferase | OEOE_RS01235 | -1.31 | 1.53 (2) | - | - | 35 / 38.9 | 6.11 |
| | aminopeptidase C | OEOE_RS02220 | ND | -1.1 | - | - | 50.5 | 5.2 |
| | dipeptidase | OEOE_RS02735 | - | - | 1.1 | -0.8 | 41.2 | |
| | glutamine synthetase | OEOE_RS04565 | - | - | 0.6 | 1 | 49.9 | |
| | peptidase M20 | OEOE_RS04760 | 1.3 | 1.36 | 1.3 | 0.7 | 44.1 / 42.1 | 4.4 |
| | glutamine amidotransferase | OEOE_RS04955 | - | - | -1 | 0.7 | 27.4 | |
| | aminopeptidase N | OEOE_RS05080 | ND | -2.14 | 0.9 | -1.1 | 95.1 | 5.1 |
| | succinate-semialdehyde dehdyrogenase | OEOE_RS06260 | - | - | 1.6 | 1.1 | 51.5 | |
| | S-ribosylhomocysteine lyase | OEOE_RS07535 | 1.51 (3) | 1.57 (2) | 1.8 | 1.5 | 17.7 | 5.3 |
| | peptidase C69 | OEOE_RS08595 | - | - | 1.9 | 0.9 | 53.5 | |
| **Carbohydrate transport and metabolism** | phosphoglycerate mutase | OEOE_RS00565 | -1.57 | -1.53 | - | - | 27.1 | 5.4 |
| | aldehyde dehydrogenase | OEOE_RS01550 | 1.21 (3) | 1.59 (2) | - | - | 52.5 | 4.9 |
| | lactate dehydrogenase | OEOE_RS01985 | - | - | 1.5 | 1.1 | 36.5 | |
| | PTS mannose transporter subunit IIAB | OEOE_RS02230 | -0.73 | -1.02 | -1.31 | -1.38 | -1.06 | -1.00 |
| | PTS mannose transporter subunit EIIAB | OEOE_RS02230 | - | - | -2.4 | -0.7 | 35.6 | |
| | HPr kinase/phosphorylase | OEOE_RS02680 | 1.6 | 1.9 | - | - | 35.4 | 5.3 |
| | phosphocarrier protein HPr | OEOE_RS03075 | - | - | -0.7 | 2.3 | 9.0 | |
| | phosphoenol pyruvate-protein phosphotransferase | OEOE_RS03095 | -1.2 | ND | - | - | 63.2 | 5 |
| | galactose mutarotase | OEOE_RS04920 | - | - | 0.7 | -1.1 | 34.1 | |
| | D-lactate dehydrogenase | OEOE_RS05695 | -1.3 | ND | - | - | 36.5 | 5.74 |
| | UDP-glucose 4-epimerase | OEOE_RS06755 | - | - | 1.1 | 0.7 | 36.9 | |
| | enolase | OEOE_RS07960 | 1.54 | 1.387 | 0.7 | 1.8 | 48.4 / 47.3 | 4.6 |
| | fructokinase | OEOE_RS08245 | -1.2 | ND | - | - | 32.1 | 6.44 |
| **Lipid transport and metabolism** | ACP S-malonyltransferase | OEOE_RS07670 | - | - | -0.8 | 1.1 | 33.6 | |
| | 2-nitropropane dioxygenase | OEOE_RS07675 | - | - | -1.5 | 0.7 | 33.5 | |
| **Cell wall/membrane/envelope biogenesis** | glucosamine--fructose-6-phosphate aminotransferase | OEOE_RS03035 | - | - | 1.7 | 1.1 | 66.2 | |
| | rod shape-determining protein MreB | OEOE_RS03200 | - | - | -1.2 | -1.1 | 40.1 | |
| | UDP-N-acetylmuramate--L-alanine ligase | OEOE_RS06110 | - | - | 0.6 | -1.6 | 48.1 | |
| | peptidoglycan interpeptide bridge formation protein | OEOE_RS06975 | - | - | -1.1 | -1 | 39.4 | |
| | D-alanyl-D-alanine carboxypeptidase | OEOE_RS07530 | - | - | -0.8 | -1.5 | 31.0 | |
| | UDP-N-acetylglucosamine 1-carboxyvinyltransferase | OEOE_RS08605 | - | - | 0.7 | 1 | 45.7 | |
| **Translation, ribosomal structure and biogenesis** | threonyl-tRNA synthase | OEOE_RS02215 | -2.02 | -3.3 | -0.7 | -1.1 | 76.3 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 30S ribosomal protein S8 | OEOE_RS02910 | - | - | -1.5 | 1 | 14.6 | |
| | 50S ribosomal protein L15 | OEOE_RS02935 | - | - | 0.7 | 2.3 | 16.5 | |
| | 50S ribosomal protein L17 | OEOE_RS02970 | - | - | -0.7 | 1.2 | 14.9 | |
| | 50S ribosomal protein L32 | OEOE_RS03680 | - | - | 0.7 | 1.2 | 6.7 | |
| | elongation factor Tu | OEOE_RS03795 | -1.3 | -1.6 | -1.6 | -0.9 | 43.6 | 4.9 |
| | elongation factor Ts | OEOE_RS04685 | - | - | -2.3 | -0.9 | 31.8 | |
| | 30S ribosomal protein S20 | OEOE_RS06185 | - | - | 0.7 | 1 | 9.8 | |
| | elongation factor G (fusA) | OEOE_RS06335 | -1.67 | 1.015 | - | - | 77.9 | |
| | valyl-tRNA synthase | OEOE_RS06700 | - | - | 0.8 | -1.9 | 104.6 | |
| | 50S ribosomal protein L7/L12 (rplL) | OEOE_RS06825 | ND | 1.99 | - | - | 12.2 | 4.20 |
| | methionyl-tRNA synthetase | OEOE_RS08425 | - | - | -0.7 | -1.1 | 77.0 | |
| **Stress response** | glutathione reductase | OEOE_RS05740 | - | - | 2 | 1 | 48.6 | |
| | cold-shock protein | OEOE_RS06620 | 1.28 | ND | - | - | 7.4 | 4.7 |
| | molecular chaperone GroEL | OEOE_RS06725 | 1.1 | 1.35 (2) | - | - | 57.5 | 5.02 |
| | Co-chaperonin GroES (HSP10) | OEOE_RS06730 | 1.67 | 1.82 (2) | - | - | 9.7 | 4.7 |
| | thiol reductase thioredoxin | OEOE_RS07835 | - | - | -0.6 | 1.4 | 12.6 | |
| | thiol reductase thioredoxin | OEOE_RS08215 | - | - | -0.7 | 1.3 | 11.5 | |
| | DNA-binding ferritin-like protein | OEOE_RS08440 | 1.64 | 1.78 | - | - | 18.3 | 4.4 |
| **Nucleotide transport and metabolism** | adenylate kinase | OEOE_RS02945 | - | - | -0.9 | 1.1 | 20.7 | |
| | deoxynucleoside kinase | OEOE_RS04085 | -2.4 | -2.7 | - | - | 25.8 | 5.6 |
| **Coenzyme transport and metabolism** | thiamine pyrophosphokinase | OEOE_RS03790 | -2.1 | ND | - | - | 25.4 | 4.7 |
| | pyridoxal biosynthesis lyase PdxS | OEOE_RS04980 | - | - | -2.5 | 1 | 31.4 | |

UNIVERSITAT ROVIRA I VIRGILI
OENOCOCCUS OENI RESPONSE TO WINE STRESS: ROLE OF GLUTATHIONE AND THIOREDOXIN SYSTEMS
Maria del Mar Margalef Català



*Main metabolisms modified by wine-like conditions*

*Malate and citrate metabolism*

Three out of the five malate related genes annotated in *O. oeni* PSU-1 genome were over-expressed: one of the permeases (*mleP*), the transporter OEOE_RS06985 -which had a four-fold expression at 1 h after the inoculation-, and the malate dehydrogenase (*mae*) (Table 2). The observed transcriptional activation of malate transporters under wine-related conditions were in accordance with previous studies (Labarre et al., 1996) and were indicative of the induction of MLF as a part of stress response. In Augagneur et al. (2007) a significant increase was observed in the abundance of mRNA encoding MleP derived from cells incubated in presence of L-malate at pH 4.5 and 3.2. Similarly, *mleP* was over-expressed in the microarray performed by Costantini et al. (2015) due to adaptation (1 day) to ethanol 8% and 12%.

In this work it was detected the over-expression of the citrate lyase operon, observing the highest expression 1h after the inoculation into WLM (Table 2). The transcriptional activation of *O. oeni* citrate lyase in response to ethanol stress has been previously reported by Olguín et al. (2009). The transcriptional up-regulation of malate transport and citrate consumption could be indicative of the use of L-malate and citrate associated to stress response and as an alternative energy source to sugar metabolism. Significant changes were also observed for genes involved in diacetyl utilization. Diacetyl is the main aromatic compound associated to MLF and is derived from citrate consumption. Diacetyl reductase showed transcriptional inhibition, while its protein abundance increased 6 h after inoculation into WLM. On the other hand, acetoin reductase was inhibited both at gene and protein level. Diacetyl and acetoin reductases are involved in two reactions of transformation of diacetyl, first into acetoin and then into 2,3-butanediol as the final product. These two reactions involve the oxidation of NAD(P)H and would participate in the maintenance of the cofactor redox balance. Diacetyl metabolism has been described as strain-dependent (Bartowsky and Henschke, 2004). In the studied conditions with PSU-1 strain, diacetyl and acetoin reductases would be initially inhibited, however proteomic data showed the increase in abundance of diacetyl reductase towards the beginning of MLF, which could be correlated to the activation of citrate consumption and the consequent production of diacetyl.

*ATPase activity*

ATPase activity has been associated to MLF (Salema et al., 1996). Cox and Henick-Kling (1989) proposed a chemiosmotic mechanism where energy is produced by the efflux of L-lactate from L-malate degradation. Fortier et al. (2003) described the increase of $F_0F_1$-ATPase β subunit mRNA in response to low pH. However, in this work several genes codifying for other ATPase subunits (α, δ, γ, and Ɛ) were down-regulated before the beginning of MLF (Table 2). Although in the proteomic study the subunit α was initially down-regulated (1 h), its abundance increased at 6 h. This could indicate that when cells are longer acclimated to WLM (6 h after inoculation), and closer to the start of L-malate consumption, ATPase activity is increased.

*Amino acid transport and metabolism*

It is worth to note the activation of several genes related with peptidase activity and amino acid transport (Table 2). Also, five peptidases were identified in the proteomic analysis, but their abundance varies depending on the protein and the analyzed time. Liu et al. (2010) reported that many of the peptidases seem to be essential for bacterial growth or survival as they are encoded in all LAB genomes, such as PepC, PepN, and PepM, and proline peptidases PepX and PepQ. Currently in PSU-1 genome there are annotated 29 peptidases. Also, related to nitrogen uptake, three permeases involved in nitrogen compounds transport are strongly over-expressed (Table 2) after the inoculation into WLM, reaching the maximum activation at 2 h. Since peptides account for the largest proportion of total nitrogen in wine (Feuillat et al., 1998), these results suggest the relevance of wine nitrogen composition and the ability of *O. oeni* to cope with its environment as reported by Manca de Nadra et al. (1999) and Ritt et al. (2008).

Some genes and proteins related to glutamine and glutamate synthesis, involved in the assimilation and re-distribution of nitrogen within the cell, were up-regulated revealing the key role of nitrogen uptake for *O. oeni* in a poor nutrient media, such as WLM. Glutamine synthetase was up-regulated both at gene and protein level. The 4-aminobutyrate aminotransferase gene (OEOE_RS01860), which transforms GABA into succinate semialdehyde and L-glutamate, was threefold over-expressed during the 8 h of *O. oeni* PSU-1 adaptation to WLM. GABA can be assimilated as a nitrogen and/or carbon source in bacteria such as *Escherichia coli* (Bartsch et al., 1990) and

*Corynebacterium glutamicum* (Zhao et al., 2012), but no information is available about LAB in this respect.

Six genes involved in the transport of spermidine/putrescine were over-expressed (Table 2). The uptake of these two polyamines has been associated with an energy-producing state/membrane potential of the cell in *E. coli* (Kashiwagi et al., 1997). Both putrescine and spermidine protect against oxidative stress (Tkachenko et al., 2001). Olguín et al. (2015) reported that this protective mechanism may also be a target of ethanol damage in an ethanol shock, which would inhibit the uptake of these polyamines. In this case, the adaptation to conditions of WLM resulted in an over-expression of six out of the eight transporters of these polyamines annotated in PSU-1 genome.

*Carbohydrate transport and metabolism*

Microarray data revealed that sugar transport was repressed (Table 2) in response to WLM conditions. In particular, glycerol-3-phosphate ATP-binding cassette ABC transporters and mannose phosphotransferase transporters (PTS) were down-regulated. This inhibition is probably due to the lower availability of sugars in WLM with respect to rich growth medium in which inocula were prepared. A strong transcriptional inhibition of sugar metabolism and transport in response to ethanol was also observed by Olguín et al. (2015).

Enolase, among others, were strongly up-regulated at low pH compared with the optimal growth at pH 6.8 (Lee et al., 2008). In this assay, the enolase protein increased in abundance 1 h after inoculation. On the contrary, the transcriptomic data reported in this work and by Costantini et al. (2015) show the inhibition of enolase gene under wine-like conditions. This suggests that enolase, might be up-regulated at translational level in response to stress. This protein, besides being involved in sugar fermentation, has been related to host tissue adhesion in probiotic bacteria including *Lactobacillus plantarum* (Castaldo et al., 2009).

*Lipid transport and metabolism*

The regulation at gene and protein level of lipid metabolism was scarce and, in most of the cases, was down-regulated. For instance, the gene *cfa* (OEOE_RS05660) which is involved in the conversion of monounsaturated fatty acids to cyclopropane fatty acids (CFAs) was inhibited at 1 h. The increase in *cfa* transcription was observed in acid- and

ethanol-grown cells (Grandvalet et al., 2008; Olguín et al., 2010) after a longer period of stress exposure than in this work, once MLF had started.

*Cell wall/membrane/envelope biogenesis*

Several genes and proteins related to cell envelope biogenesis were over-expressed. One of the genes annotated as D-alanyl-D-alanine carboxypeptidase (OEOE_RS03435) was six-fold over-expressed at 2 h and its sharp over-expression started 0.5 h after the inoculation into WLM. This gene and OEOE_RS06975 (for peptidoglycan interpeptide bridge formation protein) were also over-expressed in transcriptomic analysis by Costantini et al. (2015), both after adaptation with 12% of ethanol. However, in the present study, another D-alanyl-D-alanine carboxypeptidase (OEOE_RS07530) showed an opposite behavior, being down-regulated both at gene and protein level. Peptidoglycan (PG) is an essential component of the bacterial cell envelope, required for cell shape and stability (Vollmer et al., 2008). A supply of D-amino acids is essential for peptidoglycan synthesis; moreover, D-Ala is the main constituent of wall teichoic acids and lipoteichoic acids, which are polyanionic polymers exclusively found in Gram-positive bacteria (Wecke et al., 2009). Another activated function, both at gene and protein level, was glucosamine:fructose-6-phosphate aminotransferase, which is also involved in cell wall biosynthesis. Cecconi et al. (2009) found a major concentration of glucosamine 6-phosphate aminotransferase in *O. oeni* cells acclimated to ethanol than in not acclimated cells. These results point out the specificity and relevance of some enzymes involved in cell envelope protection against stress challenge.

Three proteins related to cell wall biogenesis were down-regulated. Among them, the rod shape-determining protein (MreB), with an actin-like role, was less abundant at 1 h and 6 h. These results correlate with the reported information for proteins MreB1 and B2 determining cell shape from *Lactobacillus plantarum* 423 which were less abundant in acid-stressed cells (Heunis et al., 2014) and the transcriptional inhibition due to ethanol of other rod shape-determining proteins, such as MreB, in *O. oeni* (Olguín et al., 2015). Also, microarray analysis revealed the inhibition of three genes (OEOE_RS07265, OEOE_RS03340 and OEOE_RS07010) involved in cell-wall biogenesis, encoding one of them a capsular polysaccharide biosynthesis protein, gene which was described as well by Dimopoulou et al. (2012) as *wzd*. All these down-

regulated functions would be targets of the damage caused by factors such as ethanol and low pH.

*Translation, ribosomal structure and biogenesis*

One of the categories showing more significant changes in *O. oeni* PSU-1 transcriptome and proteome due to WLM conditions was translation related functions. It is worth noting that several 30S and 50S ribosomal genes were over-expressed like their correspondent proteins. Several ribosomal proteins were up-regulated in *Lactobacillus rhamnosus* under acidic stress (Koponen et al., 2012). Moreover, in *O. oeni* in agreement with these observations, Cecconi et al. (2009) reported that adaptation in half strength wine-like medium correlates with the up regulation of some transcription/translation proteins as elongation factor Ts and ribosomal protein 30S. A gene codifying for a ribosomal protein was differentially over expressed between 0 h and 1 h after ethanol addition in *O. oeni* PSU-1 (Olguín et al., 2015). As well in samples of *O. oeni* adapted to 12% ethanol, a ribosomal protein was up-regulated (Costantini et al., 2015). Studies with *Lactococcus lactis* suggest that the regulation of translation has a major role in stress response (Dressaire et al., 2010). According to our results this would also happen in *O. oeni*.

*Stress response*

As expected, it was observed the activation of chaperon function in response to WLM stress conditions.  Some genes, such as *grpE*, *dnaJ* and *dnaK*, and proteins, like GroEL and GroES (Hsp10), showed up-regulation in PSU-1 after inoculation into WLM (Tables 2 and 3). The latter chaperone, Hsp10, is conserved along LAB (Sugimoto et al., 2008). However, Hsp20, the most characterized stress protein in *O. oeni* (Guzzo et al., 2000, 1997), showed transcriptional inhibition in our assay and no changes in protein concentration. This is in accordance with Costantini et al. (2015), that described the transcriptional activation of *hsp20* only in mild ethanol stress (8%) but the under-expression of this gene with 12% ethanol, as found in our work. A cold shock protein (OEOE_RS06620) showed increased abundance 1 h after *O. oeni* PSU-1 inoculation, but its gene expression was inhibited along the assay. This protein could play a role in the early response to wine-related stress but not in the long term adaptation process.

Our data revealed that wine-like conditions caused an increase of proteins involved in oxidative stress protection, related to thioredoxin and glutathione systems. Two out of the three thioredoxins (*trxA*) annotated for PSU-1, OEOE_RS07835 and OEOE_RS08215, were up-regulated 6 h after inoculation. Also thioredoxin reductase (*trxB*), OEOE_RS02695, and a ferredoxin reductase gene (*fdr*: OEOE_RS00770), annotated in NCBI as *trxB* until February 2015, were activated under wine stress conditions. Glutathione reductase (GshR) was significantly more abundant in *O. oeni* PSU-1 after inoculation into WLM. However, transcriptomic data revealed that some of these genes were inhibited; indicating that translational regulation of these functions would be prevalent under the studied conditions. Our results support the relevance of the thioredoxin and glutathione systems in the adaptation of *O. oeni* to wine related stress. There are few studies regarding thioredoxin in *O. oeni* (Jobin et al., 1999a; Guzzo et al., 2000; Margalef-Català et al., 2016), thus the role of this mechanism and glutathione system against wine stress is quite unknown.

Among the genes over-expressed related to defense mechanism there were eight multidrug transport genes. ABC transporters are a major part of the efflux systems involved in the transport of harmful-compounds and cell detoxification (Leverrier et al., 2004).

*Evaluation of omic data correlation*

*Real-time qPCR validation*

In order to validate the results obtained from the microarray analysis, real-time qPCR was performed with the same RNA from the original microarray experiment. Twenty-two genes, some related to stress response, were selected, taking the RNA sample of time where maximum over- or under- expression had been observed in microarray (Table 1). There was a general accordance between microarray and real-time qPCR data for all the genes tested. Of the 22 genes, 17 were clearly correlated using both techniques. For *hsp18* gene higher values by qPCR were obtained than for microarray data. Finally the four remaining genes (diacylglycerol kinase, PTS sugar, *cfa* and *trxA2*) displayed lower numerical values by qPCR, indicating no significant changes using this technique, while with microarray they were slightly inhibited. Overall, the correlation between real-time qPCR and microarray was good, suggesting that the microarray gene

expression measurements were valid. Moreover the validation of two thioredoxins (*trxA2* and *trxA3*) was useful for the proteomic identification.

*Integration of transcriptomic and proteomic analysis*

It has been largely reported that the correspondence of transcriptomic and proteomic data is low due to the numerous and complex regulatory mechanisms involved in gene transcription and protein synthesis (Dressaire et al., 2010; Haider and Pal 2013). In this work, 19 genes presented a correlation with proteomic results (Tables 2 and 3). The most relevant, in terms of understanding *O. oeni* stress response, have already been discussed in the text. Venn diagram shown in Figure 3 shows the number of coincident modifications of genes and proteins at different analyzed times versus time zero.

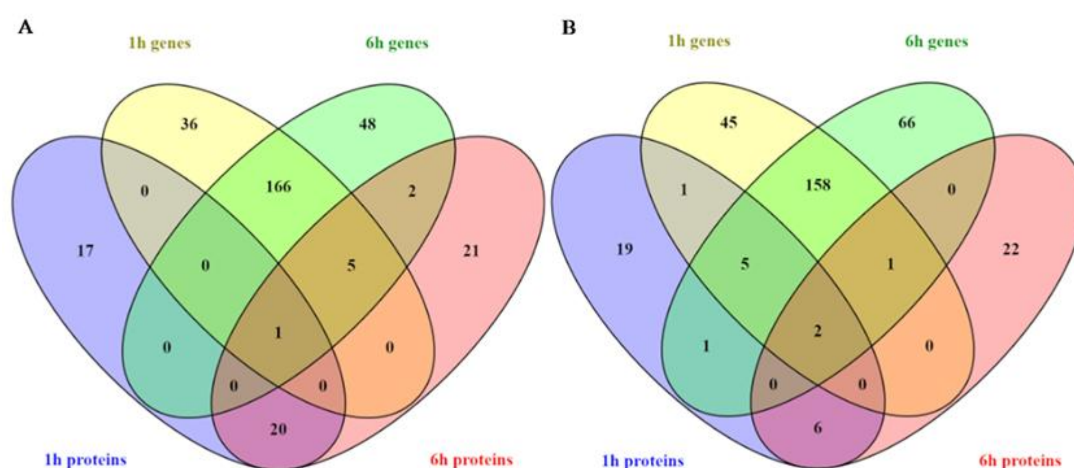


**Figure 3** Venn diagram of the number of proteins and genes showing significant changes in abundance and expression, respectively, according to transcriptomic and proteomic analysis 1 h and 6 h after the inoculation of *O. oeni* PSU-1 into WLM. A) over-expressed genes and up-regulated proteins, B) under-expressed genes and down-regulated protein. The color of diagram petals match with the colored legends in the figure.

It is worth to note the highest number of coincidences was observed for genes up/down regulated (166 and 158, respectively) 1 h and 6 h after inoculation in WLM. This indicates that most of the transcriptional changes were sustained along the 8 h assay, before MLF start. However, some genes were only modified at one of the analyzed times, indicating its specific role in early (1 h) or adaptive (6 h) response, respectively. Regarding protein changes, the observed pattern was different and many proteins showed modifications only at one of the two analyzed times. However, some proteins

maintained the up or down regulation along the assay. Altogether, the data reported illustrates the complexity of *O. oeni* cell regulation and the difficulty of finding specific marker genes and/or proteins associated to stress response.

## Conclusions

The combined transcriptomic and proteomic study was useful to identify the metabolisms mostly altered due to wine-like conditions. The use of two complementary proteomic techniques allowed the detection of a major number of proteins influenced by stress factors. Our results revealed the relevance of translation regulation and nitrogen uptake as key metabolisms involved in the adaptation of *O. oeni* PSU-1 to wine related stress. Cell wall biosynthesis and redox maintenance mechanisms seem to play also a relevant role in the protection of *O. oeni* against cell damage. Finally, sugar metabolism is inhibited in contrast to the transcriptional activation of L-malate transport and citrate consumption before the beginning of MLF.

Most of the molecular modifications occurring during *O. oeni* adaptation to wine will depend on the strain and/or fermentation conditions. However, the *omic* analysis allows the identification of the most relevant functions affected by wine-related stress, on which should be focused future research.

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## Supplementary data

**Table S1** Relative expression of genes affected during acclimation to WLM, grouped by Clusters of Orthologous Groups (COGs). Time samples with over- or under-expression are highlighted (green and yellow, respectively).

| COGs | Old locus tag | Gene symbol | Gene annotation | Relative expression in function of time (h) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 0.5 | 1 | 2 | 4 | 6 | 8 |
| **C: Energy production and conversion** | | | | | | | | | |
| C | OEOE_0238 | OEOE_RS01135 | carbonic anhydrase | -1.37 | -1.39 | -1.80 | -2.07 | -2.15 | -2.08 |
| C | OEOE_0419 | OEOE_RS02015 | malate permease | 2.25 | 2.45 | 2.08 | 1.74 | 1.71 | 1.67 |
| C | OEOE_0423 | OEOE_RS02035 | citrate lyase subunit alpha | 0.73 | 1.13 | 0.93 | 0.59 | 0.54 | 0.64 |
| C | OEOE_0441 | OEOE_RS02125 | phosphosulfolactate synthase | 1.01 | 1.62 | 2.22 | 1.99 | 1.73 | 1.66 |
| C | OEOE_0516 | OEOE_RS02455 | NADH-dependent flavin oxidoreductase | -0.71 | -0.61 | -0.83 | -1.10 | -1.05 | -1.07 |
| C | OEOE_0550 | OEOE_RS02615 | ABC transporter permease | 1.57 | 1.86 | 2.17 | 2.27 | 2.08 | 2.00 |
| C | OEOE_0551 | OEOE_RS02620 | sulfonate ABC transporter ATP-binding protein | 1.70 | 1.70 | 1.71 | 1.82 | 1.78 | 1.62 |
| C | OEOE_0659 | OEOE_RS03155 | F0F1 ATP synthase subunit A | -1.03 | -1.08 | -0.42 | -0.31 | -0.35 | -0.37 |
| C | OEOE_0662 | OEOE_RS03170 | ATP synthase subunit delta | -0.92 | -1.16 | -0.92 | -0.90 | -0.91 | -0.91 |
| C | OEOE_0664 | OEOE_RS03180 | ATP synthase subunit gamma | -1.28 | -1.25 | -0.97 | -0.93 | -0.84 | -0.83 |
| C | OEOE_0666 | OEOE_RS03190 | F0F1 ATP synthase subunit epsilon | -1.35 | -1.26 | -1.45 | -1.49 | -1.56 | -1.69 |
| C | OEOE_0749 | OEOE_RS03585 | NADH-flavin reductase | -0.71 | -0.73 | -0.98 | -1.01 | -0.97 | -0.84 |
| C | OEOE_0829 | OEOE_RS03985 | energy-coupling factor transporter ATP-binding protein EcfA2 | -0.23 | -0.34 | -0.78 | -0.93 | -1.01 | -0.97 |
| C | OEOE_1046 | OEOE_RS05025 | NADH:flavin oxidoreductase | -1.09 | -1.54 | -1.76 | -1.70 | -1.70 | -1.51 |
| C | OEOE_1047 | OEOE_RS05030 | NADH:flavin oxidoreductase | -1.13 | -1.44 | -1.67 | -1.78 | -1.93 | -1.76 |
| C | OEOE_1087 | OEOE_RS05225 | energy-coupling factor transporter ATP-binding protein EcfA3 | -0.79 | -1.02 | -1.16 | -0.87 | -0.87 | -0.82 |
| C | OEOE_1110 | OEOE_RS05335 | Fe-S cluster formation protein, NifU | -0.37 | -0.25 | -0.76 | -0.95 | -1.04 | -1.01 |
| C | OEOE_1187 | OEOE_RS05720 | ABC transporter ATPase | -1.24 | -1.04 | -1.29 | -1.32 | -1.29 | -1.24 |
| C | OEOE_1290 | OEOE_RS06215 | NADPH:quinone reductase | -0.98 | -0.96 | -0.95 | -1.12 | -0.98 | -1.16 |
| C | OEOE_1446 | OEOE_RS06985 | malate transporter | 3.28 | 4.03 | 4.19 | 3.84 | 3.68 | 3.69 |
| C | OEOE_1838 | OEOE_RS08875 | pyridine nucleotide-disulfide oxidoreductase | -1.11 | -1.37 | -1.39 | -1.34 | -1.29 | -1.29 |
| **D: Cell cycle control, cell division, chromosome partitioning** | | | | | | | | | |
| D | OEOE_0247 | OEOE_RS01180 | GTP-binding protein | -1.20 | -1.11 | -1.17 | -1.35 | -1.34 | -1.43 |
| D | OEOE_1150 | OEOE_RS05535 | cell division protein FtsL | -1.07 | -0.87 | -0.76 | -0.66 | -0.48 | -0.42 |
| D | OEOE_1261 | OEOE_RS06080 | cell division protein | 0.42 | 0.58 | 0.79 | 1.03 | 1.06 | 1.10 |
| **E: Amino acid transport and metabolism** | | | | | | | | | |
| E | OEOE_0017 | OEOE_RS00080 | peptidase | -1.00 | -1.23 | -0.78 | -0.63 | -0.68 | -0.67 |
| E | OEOE_0149 | OEOE_RS00695 | chorismate mutase | 2.34 | 2.33 | 2.16 | 1.91 | 2.16 | 2.29 |
| E | OEOE_0239 | OEOE_RS01140 | O-acetylhomoserine aminocarboxypropyltransferase | -0.74 | -1.13 | -1.04 | -1.08 | -1.34 | -1.25 |
| E | OEOE_0267 | OEOE_RS01280 | peptide ABC transporter substrate-binding protein | 0.85 | 1.71 | 1.39 | 1.04 | 1.21 | 1.94 |
| E | OEOE_0268 | OEOE_RS01285 | branched-chain amino acid ABC transporter permease | 0.91 | 2.11 | 1.86 | 1.48 | 1.79 | 2.64 |
| E | OEOE_0269 | OEOE_RS01290 | phosphonate ABC transporter ATP-binding protein | 0.47 | 1.89 | 1.67 | 1.43 | 1.74 | 2.65 |
| E | OEOE_0270 | OEOE_RS01295 | peptide ABC transporter ATPase | 0.54 | 0.56 | 0.38 | 0.37 | 0.75 | 1.23 |
| E | OEOE_0287 | OEOE_RS01375 | D-alanine--poly(phosphoribitol) ligase | -2.24 | -2.07 | -2.01 | -1.87 | -1.93 | -1.95 |
| E | OEOE_0388 | OEOE_RS01865 | amino acid permease | 2.81 | 3.11 | 3.12 | 3.08 | 3.01 | 3.05 |
| E | OEOE_0438 | OEOE_RS02110 | peptide ABC transporter permease | 4.02 | 4.40 | 4.48 | 4.19 | 4.00 | 4.07 |
| E | OEOE_0443 | OEOE_RS02135 | branched-chain amino acid transporter II carrier protein | 1.11 | 1.39 | 1.64 | 1.64 | 1.78 | 1.95 |
| E | OEOE_0508 | OEOE_RS02415 | peptide methionine sulfoxide reductase | -1.26 | -1.25 | -0.81 | -0.56 | -0.55 | -0.54 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| E | OEOE_0559 | OEOE_RS02660 | phosphate ABC transporter ATP-binding protein | 0.80 | 0.78 | 0.64 | 0.98 | 1.09 | 1.04 |
| E | OEOE_0570 | OEOE_RS02715 | ATP-dependent Clp protease proteolytic subunit | 0.14 | 0.41 | 0.47 | 0.05 | 0.10 | 0.02 |
| E | OEOE_0630 | OEOE_RS03010 | spermidine/putrescine import ATP-binding protein PotA | 0.69 | 0.61 | 0.99 | 1.27 | 1.21 | 1.20 |
| E | OEOE_0631 | OEOE_RS03015 | spermidine/putrescine ABC transporter permease | 0.53 | 0.65 | 0.84 | 1.14 | 1.08 | 1.03 |
| E | OEOE_0632 | OEOE_RS03020 | spermidine/purescine ABC transporter permease | 0.68 | 0.78 | 0.97 | 1.32 | 1.28 | 1.31 |
| E | OEOE_0751 | OEOE_RS03595 | amino acid ABC transporter substrate-binding protein | 1.06 | 1.25 | 0.58 | 0.41 | 0.58 | 0.58 |
| E | OEOE_0840 | OEOE_RS04040 | peptide ABC transporter substrate-binding protein | -0.57 | -0.91 | -1.16 | -1.22 | -1.15 | -1.15 |
| E | OEOE_0841 | OEOE_RS04045 | peptide ABC transporter substrate-binding protein | -1.51 | -1.62 | -1.98 | -2.12 | -2.10 | -2.04 |
| E | OEOE_0951 | OEOE_RS04560 | glutamine synthetase | 1.73 | 2.21 | 2.47 | 2.23 | 1.97 | 1.78 |
| E | OEOE_0959 | OEOE_RS04600 | Xaa-Pro aminopeptidase | 0.58 | 0.47 | 1.16 | 1.75 | 1.90 | 1.93 |
| E | OEOE_1055 | OEOE_RS05065 | ABC transporter permease | 1.25 | 0.86 | 0.62 | 0.62 | 0.58 | 0.56 |
| E | OEOE_1056 | OEOE_RS05070 | methionine import ATP-binding protein MetN 1 | 1.11 | 0.73 | 0.64 | 0.64 | 0.64 | 0.64 |
| E | OEOE_1092 | OEOE_RS05245 | oligoendopeptidase F | 0.56 | 0.66 | 0.83 | 1.17 | 1.59 | 1.90 |
| E | OEOE_1191 | OEOE_RS05740 | glutathione reductase | -0.63 | -0.78 | -0.83 | -0.96 | -1.00 | -0.98 |
| E | OEOE_1299 | OEOE_RS06260 | succinate-semialdehyde dehydrogenase | -1.07 | -1.14 | -1.40 | -1.39 | -1.24 | -1.20 |
| E | OEOE_1427 | OEOE_RS06890 | ABC transporter ATP-binding protein | -0.95 | -0.81 | -0.91 | -1.00 | -0.91 | -1.03 |
| E | OEOE_1464 | OEOE_RS07070 | spermidine/putrescine ABC transporter ATP-binding protein | 1.69 | 1.40 | 1.21 | 1.06 | 0.97 | 0.99 |
| E | OEOE_1465 | OEOE_RS07075 | spermidine/putrescine ABC transporter ATP-binding protein | 1.66 | 1.16 | 1.01 | 0.92 | 0.91 | 0.86 |
| E | OEOE_1466 | OEOE_RS07080 | spermidine/putrescine ABC transporter permease | 1.27 | 0.75 | 0.71 | 0.51 | 0.41 | 0.49 |
| E | OEOE_1538 | OEOE_RS07415 | peptide methionine sulfoxide reductase | -0.51 | -0.63 | -1.00 | -1.07 | -0.95 | -1.12 |
| E | OEOE_1562 | OEOE_RS07535 | S-ribosylhomocysteine lyase | -1.21 | -1.39 | -1.32 | -1.47 | -1.55 | -1.62 |
| E | OEOE_1615 | OEOE_RS07790 | peptide ABC transporter substrate-binding protein | -0.94 | -1.16 | -1.38 | -1.29 | -1.13 | -1.06 |
| E | OEOE_1616 | OEOE_RS07795 | peptide ABC transporter permease | -1.22 | -1.57 | -1.41 | -1.00 | -0.67 | -0.47 |
| E | OEOE_1638 | OEOE_RS07900 | amino acid permease | 2.93 | 3.16 | 3.18 | 2.98 | 2.90 | 2.81 |
| E | OEOE_1665 | OEOE_RS08035 | amino acid ABC transporter substrate-binding protein | 1.44 | 1.58 | 1.58 | 1.58 | 1.75 | 1.93 |
| E | OEOE_1667 | OEOE_RS08045 | amino acid ABC transporter permease | 1.28 | 1.24 | 1.19 | 1.28 | 1.53 | 1.51 |
| E | OEOE_1705 | OEOE_RS08230 | glyoxalase | -0.49 | -0.75 | -0.93 | -1.00 | -0.93 | -1.03 |
| E | OEOE_1717 | OEOE_RS08285 | ABC transporter substrate-binding protein | 0.83 | 0.85 | 0.93 | 1.34 | 1.56 | 1.56 |
| E | OEOE_1719 | OEOE_RS08295 | peptidase M20 | 0.89 | 1.14 | 1.08 | 1.35 | 1.49 | 1.53 |
| E | OEOE_1806 | OEOE_RS08705 | glutamine ABC transporter substrate-binding protein | 1.92 | 2.14 | 2.22 | 2.20 | 2.12 | 2.09 |
| **F. Nucleotide transport and metabolism** | | | | | | | | | |
| F | OEOE_0259 | OEOE_RS01240 | dihydroorotase | -1.13 | -1.26 | -1.67 | -2.01 | -2.16 | -2.06 |
| F | OEOE_0262 | OEOE_RS01255 | orotidine 5'-phosphate decarboxylase | -0.62 | -0.82 | -1.07 | -1.44 | -1.63 | -1.64 |
| F | OEOE_0263 | OEOE_RS01260 | orotate phosphoribosyltransferase | -0.57 | -0.77 | -1.04 | -1.53 | -1.62 | -1.61 |
| F | OEOE_0264 | OEOE_RS01265 | dihydroorotate dehydrogenase | -0.63 | -0.70 | -1.05 | -1.42 | -1.54 | -1.51 |
| F | OEOE_0316 | OEOE_RS01510 | deoxyuridine 5'-triphosphate nucleotidohydrolase | -1.15 | -1.28 | -1.15 | -1.26 | -1.42 | -1.31 |
| F | OEOE_0320 | OEOE_RS01530 | nucleoside-triphosphate diphosphatase | 0.83 | 1.21 | 1.09 | 0.82 | 0.83 | 0.82 |
| F | OEOE_0376 | OEOE_RS01805 | uracil transporter | -0.90 | -1.09 | -0.64 | -0.21 | -0.30 | -0.45 |
| F | OEOE_0849 | OEOE_RS04085 | deoxyadenosine kinase | -1.23 | -1.25 | -1.08 | -1.07 | -1.13 | -1.24 |
| F | OEOE_0880 | OEOE_RS04215 | ribonucleoside-diphosphate reductase | -1.00 | -0.91 | -1.00 | -1.00 | -1.01 | -1.30 |
| F | OEOE_0969 | OEOE_RS04650 | nucleoside 2-deoxyribosyltransferase | 1.14 | 1.04 | 1.01 | 0.94 | 0.93 | 1.00 |
| F | OEOE_0981 | OEOE_RS04710 | nucleoside 2-deoxyribosyltransferase | 0.76 | 0.69 | 0.83 | 1.15 | 1.14 | 1.01 |
| F | OEOE_1033 | OEOE_RS04965 | uridine/cytidine kinase | -1.17 | -1.34 | -1.46 | -1.32 | -1.36 | -1.41 |
| F | OEOE_1069 | OEOE_RS05140 | adenine phosphoribosyltransferase | -1.32 | -1.32 | -1.41 | -1.42 | -1.51 | -1.43 |
| F | OEOE_1123 | OEOE_RS05400 | GMP synthase | -0.32 | -0.58 | -1.01 | -1.08 | -1.08 | -1.02 |
| F | OEOE_1413 | OEOE_RS06810 | deaminase | 1.11 | 1.55 | 1.80 | 2.09 | 1.76 | 1.56 |
| F | OEOE_1543 | OEOE_RS07440 | purine operon repressor | -1.10 | -1.30 | -1.42 | -1.55 | -1.62 | -1.50 |
| F | OEOE_1579 | OEOE_RS07615 | uracil phosphoribosyltransferase | -1.81 | -2.02 | -1.91 | -1.82 | -1.82 | -1.88 |
| **G: Carbohydrate transport and metabolism** | | | | | | | | | |
| G | OEOE_0026 | OEOE_RS00125 | mannose-6-phosphate isomerase | -2.56 | -2.56 | -2.56 | -2.56 | -2.58 | -2.81 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| G | OEOE_0077 | OEOE_RS00350 | diacetyl reductase [(S)-acetoin forming] | -1.31 | -1.59 | -1.80 | -1.93 | -1.90 | -1.81 |
| G | OEOE_0128 | OEOE_RS00595 | sugar phosphate isomerase | -1.71 | -1.98 | -2.05 | -1.72 | -1.58 | -1.71 |
| G | OEOE_0221 | OEOE_RS01045 | PTS sugar transporter subunit IIA | -0.63 | -0.61 | -0.79 | -0.90 | -0.96 | -1.01 |
| G | OEOE_0222 | OEOE_RS01050 | PTS cellobiose transporter subunit IIA | -0.39 | -0.70 | -0.86 | -0.97 | -1.06 | -0.86 |
| G | OEOE_0224 | OEOE_RS01060 | 6-phospho-β-glucosidase | -1.04 | -1.23 | -1.58 | -1.81 | -2.02 | -1.94 |
| G | OEOE_0233 | OEOE_RS01110 | PTS fructose transporter subunit IIA | -1.85 | -2.20 | -2.60 | -2.90 | -2.69 | -2.79 |
| G | OEOE_0234 | OEOE_RS01115 | PTS sugar transporter subunit IIA | -1.05 | -1.35 | -1.74 | -1.81 | -1.68 | -1.89 |
| G | OEOE_0235 | OEOE_RS01120 | PTS galactitol transporter subunit IIC | -0.44 | -0.23 | -0.70 | -1.05 | -1.13 | -1.02 |
| G | OEOE_0236 | OEOE_RS01125 | PTS galactitol transporter subunit IIB | -0.40 | -0.12 | -0.62 | -0.98 | -1.08 | -1.24 |
| G | OEOE_0253 | OEOE_RS01210 | sugar ABC transporter substrate-binding protein | 1.11 | 0.93 | 0.75 | 0.71 | 0.67 | 0.75 |
| G | OEOE_0254 | OEOE_RS01215 | sugar ABC transporter permease | 1.51 | 1.38 | 1.11 | 1.10 | 1.10 | 1.11 |
| G | OEOE_0255 | OEOE_RS01220 | sugar ABC transporter permease | 1.33 | 1.23 | 1.06 | 1.08 | 1.25 | 1.43 |
| G | OEOE_0303 | OEOE_RS01445 | peptidase S24 | -0.68 | -0.78 | -0.88 | -0.98 | -1.01 | -1.08 |
| G | OEOE_0324 | OEOE_RS01550 | aldehyde dehydrogenase | -0.48 | -0.72 | -1.13 | -1.35 | -1.39 | -1.43 |
| G | OEOE_0379 | OEOE_RS01820 | PTS mannose transporter subunit IID | 2.10 | 2.31 | 2.69 | 2.38 | 2.18 | 1.90 |
| G | OEOE_0380 | OEOE_RS01825 | PTS fructose transporter subunit IIC | 2.15 | 2.34 | 2.31 | 2.15 | 2.06 | 2.07 |
| G | OEOE_0381 | OEOE_RS01830 | PTS fructose transporter subunit IID | 1.84 | 2.20 | 2.22 | 1.97 | 1.79 | 1.70 |
| G | OEOE_0382 | OEOE_RS01835 | PTS sugar transporter subunit IIA | 1.38 | 1.90 | 1.90 | 1.61 | 1.49 | 1.44 |
| G | OEOE_0413 | OEOE_RS01985 | lactate dehydrogenase | -1.18 | -1.41 | -1.65 | -1.95 | -1.90 | -1.75 |
| G | OEOE_0464 | OEOE_RS02230 | PTS mannose transporter subunit IIAB | -0.73 | -1.02 | -1.31 | -1.38 | -1.06 | -1.00 |
| G | OEOE_0465 | OEOE_RS02235 | PTS alpha-glucoside transporter subunit IIBC | -0.48 | -0.83 | -0.97 | -1.12 | -0.85 | -0.81 |
| G | OEOE_0466 | OEOE_RS02240 | PTS mannose transporter subunit IID | -0.76 | -1.08 | -1.43 | -1.43 | -1.23 | -0.97 |
| G | OEOE_0643 | OEOE_RS03075 | phosphocarrier protein HPr | -2.19 | -2.16 | -2.20 | -2.33 | -2.25 | -2.32 |
| G | OEOE_0644 | OEOE_RS03080 | glucosamine-6-phosphate deaminase | -1.27 | -1.16 | -1.51 | -1.86 | -1.95 | -1.79 |
| G | OEOE_1182 | OEOE_RS05695 | lactate dehydrogenase | -1.20 | -0.93 | -1.10 | -1.22 | -1.14 | -1.37 |
| G | OEOE_1204 | OEOE_RS05805 | PTS sugar transporter | -2.37 | -2.45 | -2.68 | -2.86 | -2.87 | -2.90 |
| G | OEOE_1341 | OEOE_RS06450 | PTS β-glucoside transporter subunit IIABC | -0.94 | -1.26 | -1.40 | -1.23 | -0.89 | -0.56 |
| G | OEOE_1342 | OEOE_RS06455 | PTS sugar transporter subunit IIA | -1.31 | -1.61 | -1.41 | -0.92 | -0.50 | -0.39 |
| G | OEOE_1456 | OEOE_RS07030 | glycerol-3-phosphate ABC transporter ATP-binding protein | -1.42 | -1.92 | -2.10 | -2.34 | -2.51 | -2.54 |
| G | OEOE_1457 | OEOE_RS07035 | glycerol-3-phosphate ABC transporter permease | -0.90 | -1.36 | -1.53 | -1.65 | -1.72 | -1.76 |
| G | OEOE_1458 | OEOE_RS07040 | glycerol-3-phosphate ABC transporter permease | -1.03 | -1.54 | -1.64 | -1.81 | -2.01 | -1.93 |
| G | OEOE_1459 | OEOE_RS07045 | glycerol-3-phosphate ABC transporter substrate-binding protein | -1.16 | -1.47 | -1.86 | -2.13 | -2.20 | -2.22 |
| G | OEOE_1504 | OEOE_RS07255 | UDP-phosphate galactose phosphotransferase | -1.98 | -1.96 | -1.96 | -2.01 | -1.94 | -1.96 |
| G | OEOE_1532 | OEOE_RS07385 | fructosamine kinase | 0.31 | 0.11 | 0.48 | 0.87 | 0.83 | 1.05 |
| G | OEOE_1602 | OEOE_RS07730 | diacetyl reductase | -0.63 | -1.01 | -1.25 | -1.35 | -1.37 | -1.43 |
| G | OEOE_1609 | OEOE_RS07760 | sugar ABC transporter permease | -0.38 | -0.52 | -1.03 | -1.23 | -1.22 | -1.21 |
| G | OEOE_1612 | OEOE_RS07775 | ribokinase | -1.53 | -1.87 | -2.32 | -2.18 | -1.64 | -1.57 |
| G | OEOE_1613 | OEOE_RS07780 | D-ribose pyranase | -1.27 | -1.82 | -2.09 | -2.08 | -1.59 | -1.33 |
| G | OEOE_1614 | OEOE_RS07785 | sugar:proton symporter | -1.28 | -1.75 | -2.22 | -2.19 | -1.73 | -1.56 |
| G | OEOE_1651 | OEOE_RS07965 | sugar ABC transporter ATP-binding protein | -0.89 | -0.80 | -0.88 | -1.06 | -1.06 | -1.04 |
| G | OEOE_1669 | OEOE_RS08055 | sugar phosphate isomerase | -1.35 | -1.33 | -1.79 | -2.11 | -2.02 | -2.24 |
| G | OEOE_1708 | OEOE_RS08245 | fructokinase | -1.42 | -2.08 | -2.15 | -2.28 | -2.40 | -2.46 |
| G | OEOE_1714 | OEOE_RS08275 | glucose transporter | 0.62 | 0.81 | 0.82 | 1.17 | 1.31 | 1.30 |
| **H: Coenzyme Metabolism** | | | | | | | | | |
| H | OEOE_0327 | OEOE_RS01565 | lipoate-protein ligase A | -1.07 | -1.21 | -1.27 | -1.53 | -1.43 | -1.24 |
| H | OEOE_0676 | OEOE_RS03240 | dephospho-CoA kinase | -0.88 | -1.23 | -1.38 | -1.43 | -1.26 | -1.23 |
| H | OEOE_0779 | OEOE_RS03730 | 5-formyltetrahydrofolate cyclo-ligase | 0.66 | 1.13 | 1.35 | 1.08 | 0.88 | 0.90 |
| H | OEOE_0791 | OEOE_RS03790 | thiamine pyrophosphokinase | -0.93 | -1.07 | -1.05 | -0.96 | -0.93 | -0.84 |
| H | OEOE_1036 | OEOE_RS04980 | pyridoxal biosynthesis protein | -0.89 | -1.10 | -1.22 | -1.19 | -1.23 | -1.15 |
| H | OEOE_1473 | OEOE_RS07110 | 6-pyruvoyl-tetrahydropterin synthase | 1.22 | 1.00 | 0.59 | 0.57 | 0.60 | 0.59 |
| H | OEOE_1597 | OEOE_RS07705 | biotin transporter | 0.41 | 0.93 | 0.94 | 1.04 | 0.94 | 0.85 |
| H | OEOE_1642 | OEOE_RS07920 | holo-ACP synthase | 2.36 | 2.55 | 2.69 | 2.94 | 2.85 | 2.58 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| H | OEOE_1834 | OEOE_RS08855 | 1,4-dihydroxy-2-naphthoate octaprenyltransferase | 1.03 | 1.04 | 0.97 | 0.99 | 0.97 | 0.85 |
| **I: Lipid transport and metabolism** | | | | | | | | | |
| I | OEOE_0145 | OEOE_RS00675 | phospholipid phosphatase | -1.52 | -1.50 | -1.25 | -1.04 | -1.13 | -1.27 |
| I | OEOE_0485 | OEOE_RS02315 | phospholipid phosphatase | -1.35 | -1.88 | -1.76 | -1.75 | -1.75 | -1.79 |
| I | OEOE_1015 | OEOE_RS04875 | lysophospholipase | 0.78 | 0.87 | 1.10 | 1.14 | 1.06 | 1.16 |
| I | OEOE_1049 | OEOE_RS05040 | tannase | 1.88 | 2.26 | 2.72 | 2.22 | 1.89 | 1.90 |
| I | OEOE_1176 | OEOE_RS05660 | cyclopropane-fatty-acyl-phospholipid synthase | -0.90 | -1.11 | -0.98 | -0.90 | -0.86 | -0.83 |
| I | OEOE_1292 | OEOE_RS06225 | esterase | -1.02 | -0.96 | -0.96 | -1.12 | -0.99 | -0.82 |
| I | OEOE_1343 | OEOE_RS06460 | lysophospholipase | -0.32 | -0.43 | -0.93 | -1.22 | -1.18 | -1.14 |
| I | OEOE_1366 | OEOE_RS06570 | acyl-CoA synthetase | -1.33 | -1.62 | -2.00 | -1.78 | -1.70 | -1.61 |
| I | OEOE_1460 | OEOE_RS07050 | glycerophosphoryl diester phosphodiesterase | -1.49 | -1.64 | -2.08 | -2.51 | -2.57 | -2.56 |
| I | OEOE_1675 | OEOE_RS08085 | lipid kinase | 1.42 | 1.44 | 1.51 | 1.38 | 1.29 | 1.37 |
| **J: Translation, ribosomal structure and biogenesis** | | | | | | | | | |
| J | OEOE_0014 | OEOE_RS00065 | 50S ribosomal protein L9 | 0.01 | 0.53 | 1.04 | 1.12 | 0.79 | 0.52 |
| J | OEOE_0019 | OEOE_RS00090 | D-aminoacyl-tRNA deacylase | -1.33 | -1.53 | -1.57 | -1.47 | -1.41 | -1.26 |
| J | OEOE_0176 | OEOE_RS00835 | pseudouridylate synthase | 1.06 | 1.19 | 1.00 | 0.89 | 0.94 | 0.88 |
| J | OEOE_0321 | OEOE_RS01535 | glutamate--tRNA ligase | 0.82 | 1.33 | 1.26 | 1.06 | 1.06 | 1.10 |
| J | OEOE_0429 | OEOE_RS02065 | ribosome maturation factor RimP | 0.93 | 1.00 | 1.41 | 1.79 | 1.83 | 1.74 |
| J | OEOE_0432 | OEOE_RS02080 | 50S ribosomal protein L7ae | 1.06 | 0.95 | 1.06 | 1.18 | 1.23 | 1.28 |
| J | OEOE_0433 | OEOE_RS02085 | translation initiation factor IF-2 | 0.68 | 0.63 | 0.81 | 1.02 | 1.08 | 1.01 |
| J | OEOE_0434 | OEOE_RS02090 | ribosome-binding factor A | 0.48 | 0.54 | 0.83 | 1.07 | 1.14 | 1.02 |
| J | OEOE_0440 | OEOE_RS02120 | serine--tRNA ligase | 1.44 | 1.93 | 2.07 | 1.93 | 1.77 | 1.72 |
| J | OEOE_0517 | OEOE_RS02460 | elongation factor 3 | 1.48 | 1.97 | 2.28 | 1.95 | 1.83 | 1.82 |
| J | OEOE_0581 | OEOE_RS02770 | elongation factor 4 | 0.80 | 1.09 | 0.89 | 0.74 | 0.60 | 0.69 |
| J | OEOE_0589 | OEOE_RS02810 | histidyl-tRNA synthetase | 0.69 | 0.65 | 0.84 | 0.94 | 0.93 | 1.08 |
| J | OEOE_0594 | OEOE_RS02840 | 30S ribosomal protein S10 | 1.88 | 1.82 | 1.88 | 1.92 | 1.93 | 1.90 |
| J | OEOE_0595 | OEOE_RS02845 | 50S ribosomal protein L3 | 1.73 | 1.63 | 1.68 | 1.70 | 1.66 | 1.66 |
| J | OEOE_0596 | OEOE_RS02850 | 50S ribosomal protein L4 | 1.20 | 1.18 | 1.19 | 1.16 | 1.18 | 1.16 |
| J | OEOE_0598 | OEOE_RS02860 | 50S ribosomal protein L2 | 1.38 | 1.39 | 1.29 | 1.29 | 1.29 | 1.29 |
| J | OEOE_0599 | OEOE_RS02865 | 30S ribosomal protein S19 | 1.50 | 1.47 | 1.61 | 1.52 | 1.53 | 1.48 |
| J | OEOE_0600 | OEOE_RS02870 | 50S ribosomal protein L22 | 1.44 | 1.40 | 1.40 | 1.41 | 1.40 | 1.37 |
| J | OEOE_0601 | OEOE_RS02875 | 30S ribosomal protein S3 | 1.66 | 1.66 | 1.74 | 1.73 | 1.66 | 1.66 |
| J | OEOE_0602 | OEOE_RS02880 | 50S ribosomal protein L16 | 1.44 | 1.41 | 1.41 | 1.46 | 1.49 | 1.36 |
| J | OEOE_0603 | OEOE_RS02885 | 50S ribosomal protein L29 | 1.66 | 1.69 | 1.69 | 1.75 | 1.79 | 1.70 |
| J | OEOE_0604 | OEOE_RS02890 | 30S ribosomal protein S17 | 1.52 | 1.43 | 1.53 | 1.61 | 1.56 | 1.64 |
| J | OEOE_0605 | OEOE_RS02895 | 50S ribosomal protein L14 | 1.00 | 1.00 | 1.03 | 1.05 | 1.13 | 1.06 |
| J | OEOE_0606 | OEOE_RS02900 | 50S ribosomal protein L24 | 1.40 | 1.39 | 1.44 | 1.51 | 1.55 | 1.42 |
| J | OEOE_0607 | OEOE_RS02905 | 50S ribosomal protein L5 | 1.50 | 1.50 | 1.66 | 1.79 | 1.74 | 1.62 |
| J | OEOE_0608 | OEOE_RS02910 | 30S ribosomal protein S8 | 2.07 | 2.04 | 2.35 | 2.57 | 2.45 | 2.42 |
| J | OEOE_0609 | OEOE_RS02915 | 50S ribosomal protein L6 | 1.27 | 1.29 | 1.41 | 1.43 | 1.46 | 1.32 |
| J | OEOE_0610 | OEOE_RS02920 | 50S ribosomal protein L18 | 1.53 | 1.56 | 1.76 | 1.91 | 1.90 | 1.77 |
| J | OEOE_0611 | OEOE_RS02925 | 30S ribosomal protein S5 | 0.98 | 0.97 | 1.05 | 1.11 | 1.04 | 1.05 |
| J | OEOE_0612 | OEOE_RS02930 | 50S ribosomal protein L30 | 0.99 | 0.98 | 1.04 | 1.17 | 1.19 | 1.20 |
| J | OEOE_0613 | OEOE_RS02935 | 50S ribosomal protein L15 | 1.00 | 1.18 | 1.41 | 1.70 | 1.64 | 1.63 |
| J | OEOE_0619 | OEOE_RS02960 | 30S ribosomal protein S11 | 0.64 | 0.82 | 0.95 | 1.09 | 1.13 | 1.10 |
| J | OEOE_0621 | OEOE_RS02970 | 50S ribosomal protein L17 | 0.56 | 0.93 | 1.41 | 1.75 | 1.83 | 1.75 |
| J | OEOE_0629 | OEOE_RS03005 | 30S ribosomal protein S9 | 0.48 | 0.45 | 0.91 | 1.28 | 1.24 | 1.02 |
| J | OEOE_0739 | OEOE_RS03530 | 2'-5' RNA ligase | -1.26 | -1.35 | -1.35 | -1.47 | -1.39 | -1.69 |
| J | OEOE_0770 | OEOE_RS03680 | 50S ribosomal protein L32 | -0.14 | 0.59 | 0.78 | 0.87 | 0.88 | 1.07 |
| J | OEOE_0798 | OEOE_RS03825 | 30S ribosomal protein S16 | -1.55 | -1.20 | -1.26 | -1.24 | -1.26 | -1.37 |
| J | OEOE_0800 | OEOE_RS03835 | ribosome maturation factor RimM | -0.93 | -1.10 | -0.86 | -0.94 | -0.94 | -0.93 |

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|---|---|---|---|---|---|---|---|---|---|
| J | OEOE_0834 | OEOE_RS04010 | acetyltransferase | 2.37 | 2.70 | 2.99 | 3.18 | 3.07 | 2.77 |
| J | OEOE_0899 | OEOE_RS04310 | arginine--tRNA ligase | 1.78 | 2.04 | 1.78 | 1.78 | 1.76 | 1.79 |
| J | OEOE_0917 | OEOE_RS04400 | phenylalanine--tRNA ligase subunit alpha | -1.23 | -1.31 | -1.03 | -0.78 | -0.70 | -0.45 |
| J | OEOE_0957 | OEOE_RS04590 | 50S ribosomal protein L21 | 0.52 | 0.76 | 1.06 | 1.20 | 1.20 | 1.18 |
| J | OEOE_0994 | OEOE_RS04775 | glycine--tRNA ligase subunit β | -1.04 | -0.79 | -0.40 | -0.16 | 0.08 | 0.05 |
| J | OEOE_1079 | OEOE_RS05185 | RNA pseudouridine synthase | 0.73 | 0.82 | 0.87 | 0.87 | 0.90 | 1.02 |
| J | OEOE_1151 | OEOE_RS05540 | ribosomal RNA small subunit methyltransferase H | -1.32 | -1.30 | -0.82 | -0.61 | -0.52 | -0.24 |
| J | OEOE_1158 | OEOE_RS05575 | pseudouridine synthase | 0.70 | 1.02 | 0.98 | 0.87 | 0.88 | 0.95 |
| J | OEOE_1228 | OEOE_RS05915 | RNA-binding protein | -1.73 | -1.31 | -1.73 | -1.81 | -1.93 | -1.98 |
| J | OEOE_1267 | OEOE_RS06100 | peptide chain release factor 3 | 1.07 | 0.84 | 0.75 | 0.75 | 0.73 | 0.71 |
| J | OEOE_1278 | OEOE_RS06155 | 50S ribosomal protein L20 | 0.73 | 0.82 | 0.92 | 1.06 | 1.18 | 1.11 |
| J | OEOE_1279 | OEOE_RS06160 | 50S ribosomal protein L35 | 0.92 | 0.97 | 1.37 | 1.65 | 1.73 | 1.62 |
| J | OEOE_1283 | OEOE_RS06180 | 30S ribosomal protein S15 | 0.32 | 1.02 | 1.14 | 1.33 | 1.28 | 1.00 |
| J | OEOE_1284 | OEOE_RS06185 | 30S ribosomal protein S20 | 0.29 | 0.84 | 1.07 | 1.29 | 1.32 | 0.83 |
| J | OEOE_1289 | OEOE_RS06210 | queuine tRNA-ribosyltransferase | 1.50 | 1.74 | 1.92 | 1.86 | 1.84 | 1.83 |
| J | OEOE_1360 | OEOE_RS06545 | raiA ribosome-associated inhibitor A | -1.35 | -1.40 | -1.06 | -1.11 | -1.27 | -1.47 |
| J | OEOE_1414 | OEOE_RS06815 | 16S RNA G1207 methylase RsmC | 0.61 | 1.05 | 1.24 | 1.40 | 1.16 | 1.05 |
| J | OEOE_1416 | OEOE_RS06825 | 50S ribosomal protein L7/L12 | 0.63 | 1.16 | 1.71 | 2.00 | 1.74 | 1.46 |
| J | OEOE_1417 | OEOE_RS06830 | 50S ribosomal protein L10 | 0.87 | 1.20 | 1.27 | 1.56 | 1.48 | 1.30 |
| J | OEOE_1418 | OEOE_RS06835 | 50S ribosomal protein L1 | 0.58 | 1.23 | 1.31 | 1.52 | 1.37 | 1.23 |
| J | OEOE_1419 | OEOE_RS06840 | 50S ribosomal protein L11 | 0.59 | 0.98 | 1.05 | 1.14 | 1.14 | 0.94 |
| J | OEOE_1549 | OEOE_RS07470 | ribosomal RNA small subunit methyltransferase A | 0.11 | 0.57 | 0.97 | 1.00 | 1.07 | 1.14 |
| J | OEOE_1559 | OEOE_RS07520 | cysteine--tRNA ligase | -0.32 | -0.54 | -0.81 | -0.98 | -1.03 | -1.04 |
| J | OEOE_1674 | OEOE_RS08080 | RNA methyltransferase | 0.99 | 1.09 | 1.02 | 0.95 | 0.88 | 0.90 |
| J | OEOE_1813 | OEOE_RS08745 | prolyl-tRNA synthetase | -1.03 | -1.03 | -1.08 | -1.03 | -1.07 | -1.11 |
| J | OEOE_1863 | OEOE_RS08995 | ribonuclease P protein component | 0.38 | 0.90 | 1.14 | 0.85 | 0.82 | 0.64 |
| **K: Transcription** | | | | | | | | | |
| K | OEOE_0047 | OEOE_RS00215 | XRE family transcriptional regulator | 1.15 | 1.12 | 1.21 | 1.04 | 0.95 | 0.95 |
| K | OEOE_0099 | OEOE_RS00455 | XRE family transcriptional regulator | -1.17 | -1.24 | -1.42 | -1.38 | -1.39 | -1.55 |
| K | OEOE_0179 | OEOE_RS00850 | TetR family transcriptional regulator | 0.80 | 1.16 | 1.30 | 0.90 | 0.69 | 0.70 |
| K | OEOE_0195 | OEOE_RS00930 | XRE family transcriptional regulator | -2.45 | -2.43 | -2.04 | -2.05 | -2.24 | -2.05 |
| K | OEOE_0209 | OEOE_RS00995 | AraC family transcriptional regulator | -1.87 | -2.17 | -1.91 | -1.87 | -1.86 | -1.85 |
| K | OEOE_0215 | OEOE_RS01020 | TetR family transcriptional regulator | -1.50 | -1.49 | -1.45 | -1.49 | -1.57 | -1.63 |
| K | OEOE_0225 | OEOE_RS01065 | transcriptional regulator | -2.34 | -2.52 | -2.45 | -2.34 | -2.47 | -2.28 |
| K | OEOE_0232 | OEOE_RS01105 | transcriptional antiterminator | -2.81 | -3.13 | -2.90 | -2.68 | -2.43 | -2.84 |
| K | OEOE_0245 | OEOE_RS01170 | TetR family transcriptional regulator | -2.55 | -2.84 | -2.68 | -2.59 | -2.50 | -2.55 |
| K | OEOE_0302 | OEOE_RS01440 | LacI family transcriptional regulator | -0.82 | -0.72 | -0.86 | -0.89 | -1.03 | -0.97 |
| K | OEOE_0417 | OEOE_RS02005 | citrate lyase | 0.82 | 1.01 | 0.76 | 0.63 | 0.55 | 0.49 |
| K | OEOE_0430 | OEOE_RS02070 | transcription termination factor NusA | 1.27 | 1.20 | 1.41 | 1.59 | 1.59 | 1.69 |
| K | OEOE_0435 | OEOE_RS02095 | transcriptional regulator | 1.13 | 1.40 | 1.36 | 1.35 | 1.36 | 1.41 |
| K | OEOE_0540 | OEOE_RS02565 | MarR family transcriptional regulator | -0.28 | -0.45 | -1.01 | -1.02 | -0.84 | -1.19 |
| K | OEOE_0569 | OEOE_RS02710 | sporulation protein | 0.89 | 1.23 | 1.15 | 0.97 | 0.97 | 0.97 |
| K | OEOE_0704 | OEOE_RS03375 | ArsR family transcriptional regulator | 0.97 | 1.32 | 1.91 | 1.76 | 1.47 | 1.35 |
| K | OEOE_0718 | OEOE_RS03430 | transcriptional regulator | 2.09 | 2.68 | 2.49 | 2.01 | 1.78 | 1.61 |
| K | OEOE_0724 | OEOE_RS03455 | transcriptional regulator | 0.66 | 0.70 | 0.94 | 1.08 | 0.89 | 0.89 |
| K | OEOE_0728 | OEOE_RS03475 | MarR family transcriptional regulator | 1.07 | 0.37 | 0.58 | 0.57 | 0.60 | 0.61 |
| K | OEOE_0733 | OEOE_RS03500 | TetR family transcriptional regulator | -1.12 | -0.68 | -0.17 | -0.41 | -0.47 | -0.65 |
| K | OEOE_0793 | OEOE_RS03800 | XRE family transcriptional regulator | 1.87 | 2.01 | 2.13 | 2.04 | 2.09 | 2.13 |
| K | OEOE_0808 | OEOE_RS03880 | LacI family transcriptional regulator | -0.76 | -1.12 | -0.96 | -0.93 | -0.93 | -1.08 |
| K | OEOE_0926 | OEOE_RS04440 | ArsR family transcriptional regulator | 0.64 | 0.66 | 1.34 | 1.62 | 1.42 | 1.55 |
| K | OEOE_0996 | OEOE_RS04785 | RNA polymerase sigma factor RpoD | 0.58 | 0.83 | 1.03 | 1.23 | 1.18 | 0.98 |
| K | OEOE_1010 | OEOE_RS04850 | transcriptional regulator | -0.96 | -1.03 | -1.12 | -1.17 | -1.17 | -1.27 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| K | OEOE_1045 | OEOE_RS05020 | LysR family transcriptional regulator | -1.19 | -1.51 | -1.85 | -1.75 | -1.52 | -1.50 |
| K | OEOE_1152 | OEOE_RS05545 | transcriptional regulator MraZ | -1.71 | -1.57 | -1.27 | -1.00 | -0.88 | -0.66 |
| K | OEOE_1238 | OEOE_RS05965 | transcription termination factor NusB | -1.18 | -1.18 | -0.56 | -0.43 | -0.53 | -0.11 |
| K | OEOE_1297 | OEOE_RS06250 | MarR family transcriptional regulator | -0.48 | -0.38 | -0.31 | -0.70 | -0.52 | -1.04 |
| K | OEOE_1346 | OEOE_RS06470 | ArsR family transcriptional regulator | -1.04 | -1.52 | -1.52 | -1.82 | -1.56 | -1.89 |
| K | OEOE_1376 | OEOE_RS06620 | cold-shock protein | -1.75 | -1.14 | -1.30 | -1.49 | -1.73 | -1.81 |
| K | OEOE_1450 | OEOE_RS07005 | cell filamentation protein Fic | -0.52 | -0.86 | -1.03 | -1.07 | -0.88 | -0.94 |
| K | OEOE_1455 | OEOE_RS07025 | AraC family transcriptional regulator | -1.48 | -1.52 | -1.59 | -1.62 | -1.71 | -1.60 |
| K | OEOE_1467 | OEOE_RS07085 | GntR family transcriptional regulator | -1.01 | -1.11 | -1.16 | -1.21 | -0.92 | -0.89 |
| K | OEOE_1572 | OEOE_RS07585 | AraC family transcriptional regulator | -1.53 | -1.68 | -1.79 | -1.69 | -1.60 | -1.60 |
| K | OEOE_1575 | OEOE_RS07600 | AraC family transcriptional regulator | -1.12 | -1.31 | -1.50 | -1.40 | -1.30 | -1.28 |
| K | OEOE_1685 | OEOE_RS08130 | TetR family transcriptional regulator | 0.77 | 0.96 | 1.18 | 1.32 | 1.25 | 1.21 |
| K | OEOE_1733 | OEOE_RS08360 | RNA polymerase I and III, subunit | 0.35 | 0.22 | 0.30 | 0.65 | 0.98 | 1.00 |
| K | OEOE_1765 | OEOE_RS08510 | MarR family transcriptional regulator | -2.63 | -2.44 | -2.26 | -2.39 | -2.46 | -2.36 |
| **L: Replication, recombination and repair** | | | | | | | | | |
| L | OEOE_0308 | OEOE_RS01470 | ATP-dependent helicase/deoxyribonuclease subunit B | 0.35 | 0.95 | 1.02 | 0.52 | 0.37 | 0.35 |
| L | OEOE_0309 | OEOE_RS01475 | ATP-dependent helicase/nuclease subunit A | 0.43 | 0.75 | 1.23 | 1.07 | 0.87 | 0.77 |
| L | OEOE_0318 | OEOE_RS01520 | DNA repair protein RadA | 1.02 | 1.38 | 1.19 | 1.01 | 0.87 | 0.93 |
| L | OEOE_0795 | OEOE_RS03810 | DNA polymerase III subunit alpha | 0.44 | 0.73 | 0.89 | 1.03 | 1.09 | 1.25 |
| L | OEOE_0799 | OEOE_RS03830 | methylated DNA-protein cysteine methyltransferase | -1.29 | -1.54 | -0.92 | -1.16 | -1.23 | -1.11 |
| L | OEOE_0990 | OEOE_RS04755 | DNA repair protein RecO | -1.40 | -1.40 | -1.31 | -1.18 | -1.31 | -1.44 |
| L | OEOE_0995 | OEOE_RS04780 | DNA primase | 0.82 | 1.17 | 1.31 | 1.63 | 1.53 | 1.47 |
| L | OEOE_1020 | OEOE_RS04900 | DNA-binding protein | 0.83 | 1.01 | 1.00 | 0.96 | 0.99 | 1.05 |
| L | OEOE_1021 | OEOE_RS04905 | DNA topoisomerase I | 0.97 | 0.88 | 0.77 | 1.35 | 1.58 | 1.64 |
| L | OEOE_1023 | OEOE_RS04915 | tyrosine recombinase XerC | -1.19 | -1.26 | -1.03 | -0.68 | -0.51 | -0.40 |
| L | OEOE_1094 | OEOE_RS05255 | Holliday junction DNA helicase RecU | -1.09 | -1.22 | -0.91 | -0.77 | -0.55 | -0.68 |
| L | OEOE_1096 | OEOE_RS05265 | DNA replication protein DnaD | -1.10 | -1.20 | -1.20 | -1.12 | -1.11 | -1.12 |
| L | OEOE_1099 | OEOE_RS05280 | DNA helicase | -0.92 | -1.12 | -0.97 | -0.81 | -0.88 | -0.78 |
| L | OEOE_1106 | OEOE_RS05315 | ATPase AAA | 0.84 | 0.56 | 0.90 | 1.03 | 0.99 | 0.98 |
| L | OEOE_1382 | OEOE_RS06650 | protein RecA | 0.92 | 0.84 | 0.95 | 1.18 | 1.21 | 1.28 |
| L | OEOE_1440 | OEOE_RS06955 | ATP-dependent DNA helicase | 1.22 | 1.52 | 1.52 | 1.63 | 1.47 | 1.31 |
| L | OEOE_1525 | OEOE_RS07355 | DNA replication initiation protein | 0.65 | 0.81 | 1.18 | 1.05 | 0.81 | 0.88 |
| **M: Cell wall/membrane/envelope biogenesis** | | | | | | | | | |
| M | OEOE_0121 | OEOE_RS00560 | D-alanyl-D-alanine carboxypeptidase | -1.41 | -1.41 | -1.53 | -1.41 | -1.41 | -1.45 |
| M | OEOE_0197 | OEOE_RS00940 | hemolysin D | 2.23 | 1.79 | 1.60 | 1.55 | 1.53 | 1.47 |
| M | OEOE_0218 | OEOE_RS01030 | glycosyltransferase | -1.17 | -1.38 | -1.31 | -1.39 | -1.49 | -1.51 |
| M | OEOE_0284 | OEOE_RS01360 | D-Ala-teichoic acid biosynthesis protein | -1.03 | -1.36 | -1.30 | -1.30 | -1.40 | -1.59 |
| M | OEOE_0286 | OEOE_RS01370 | D-alanyl-lipoteichoic acid biosynthesis protein DltB | -1.93 | -1.81 | -1.81 | -1.81 | -1.92 | -1.82 |
| M | OEOE_0288 | OEOE_RS01380 | D-alanyl-lipoteichoic acid biosynthesis protein DltD | -1.60 | -1.55 | -1.61 | -1.66 | -1.66 | -1.71 |
| M | OEOE_0696 | OEOE_RS03340 | aggregation promoting factor surface protein | -2.57 | -2.92 | -2.81 | -2.61 | -2.48 | -2.29 |
| M | OEOE_0719 | OEOE_RS03435 | D-alanyl-D-alanine carboxypeptidase | 5.53 | 5.83 | 6.01 | 5.82 | 5.61 | 5.64 |
| M | OEOE_0850 | OEOE_RS04090 | large-conductance mechanosensitive channel | -1.73 | -1.73 | -1.79 | -1.78 | -1.69 | -1.99 |
| M | OEOE_0882 | OEOE_RS04225 | peptidoglycan interpeptide bridge formation protein | -1.09 | -1.22 | -1.09 | -1.09 | -1.12 | -1.09 |
| M | OEOE_0988 | OEOE_RS04745 | diacylglycerol kinase | -0.87 | -1.05 | -0.08 | 0.46 | 0.22 | 0.22 |
| M | OEOE_1199 | OEOE_RS05780 | peptidoglycan-binding protein | -1.16 | -1.56 | -1.79 | -1.80 | -1.59 | -1.43 |
| M | OEOE_1332 | OEOE_RS06410 | 2-dehydro-3-deoxyphosphooctonate aldolase | 0.39 | 0.82 | 1.05 | 0.53 | 0.30 | 0.23 |
| M | OEOE_1430 | OEOE_RS06905 | peptidoglycan-binding protein | -0.67 | -1.02 | -1.31 | -1.42 | -1.27 | -1.31 |
| M | OEOE_1442 | OEOE_RS06965 | peptidoglycan interpeptide bridge formation protein | 0.95 | 1.87 | 2.52 | 2.39 | 1.85 | 1.70 |
| M | OEOE_1443 | OEOE_RS06970 | sortase | 1.46 | 2.00 | 2.48 | 2.27 | 1.94 | 1.91 |
| M | OEOE_1444 | OEOE_RS06975 | peptidoglycan interpeptide bridge formation protein | 1.55 | 2.27 | 2.77 | 2.52 | 2.24 | 2.19 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| M | OEOE_1451 | OEOE_RS07010 | glycosyl transferase | -2.14 | -2.39 | -2.08 | -2.03 | -2.02 | -1.73 |
| M | OEOE_1452 | OEOE_RS07015 | glycosyl transferase | -0.98 | -1.08 | -1.07 | -0.84 | -0.69 | -0.47 |
| M | OEOE_1502 | OEOE_RS07245 | glycosyl transferase family 1 | -1.15 | -1.24 | -1.25 | -1.26 | -1.38 | -1.36 |
| M | OEOE_1503 | OEOE_RS07250 | glycosyl transferase | -1.78 | -1.78 | -1.78 | -1.83 | -1.80 | -1.83 |
| M | OEOE_1506 | OEOE_RS07265 | capsular polysaccharide biosynthesis protein | -2.39 | -2.18 | -1.99 | -1.88 | -1.89 | -1.71 |
| M | OEOE_1561 | OEOE_RS07530 | D-alanyl-D-alanine carboxypeptidase | -0.96 | -1.45 | -1.49 | -1.60 | -1.75 | -1.70 |
| M | OEOE_1568 | OEOE_RS07565 | capsular polysaccharide biosynthesis protein | -1.35 | -1.37 | -1.42 | -1.35 | -1.38 | -1.35 |
| M | OEOE_1621 | OEOE_RS07820 | glycosyl transferase | 1.78 | 1.90 | 2.86 | 3.39 | 3.35 | 3.08 |
| M | OEOE_1666 | OEOE_RS08040 | polar amino acid ABC transporter ATPase | 1.04 | 1.07 | 1.02 | 1.07 | 1.31 | 1.45 |
| M | OEOE_1689 | OEOE_RS08150 | macrolide ABC transporter ATP-binding protein | 1.11 | 1.45 | 1.84 | 1.80 | 1.47 | 1.35 |
| M | OEOE_1839 | OEOE_RS08880 | peptidoglycan-binding protein | -1.45 | -1.48 | -1.90 | -1.66 | -1.11 | -0.85 |
| **O: Post-translational modification, protein turnover, and chaperones** | | | | | | | | | |
| O | OEOE_0139 | OEOE_RS00645 | glutaredoxin | -1.04 | -0.44 | -0.27 | -0.64 | -0.82 | -0.86 |
| O | OEOE_0189 | OEOE_RS00900 | disulfide bond formation protein | 0.34 | 0.64 | 0.80 | 0.94 | 0.98 | 1.00 |
| O | OEOE_0289 | OEOE_RS01385 | heat-shock protein Hsp20 | -0.92 | -0.43 | -0.55 | -0.89 | -0.88 | -1.13 |
| O | OEOE_1115 | OEOE_RS05360 | anaerobic ribonucleoside-triphosphate reductase activating protein | -0.42 | -0.83 | -0.84 | -0.91 | -1.03 | -1.02 |
| O | OEOE_1308 | OEOE_RS06305 | molecular chaperone DnaJ | 1.56 | 1.76 | 1.61 | 1.36 | 1.32 | 1.10 |
| O | OEOE_1309 | OEOE_RS06310 | molecular chaperone DnaK | 1.03 | 1.35 | 1.38 | 1.04 | 1.03 | 0.95 |
| O | OEOE_1310 | OEOE_RS06315 | protein GrpE | 0.93 | 1.41 | 1.59 | 1.11 | 1.02 | 0.94 |
| O | OEOE_1431 | OEOE_RS06910 | trypsin | 0.95 | 1.32 | 1.45 | 1.06 | 0.79 | 0.74 |
| O | OEOE_1554 | OEOE_RS07495 | methionine sulfoxide reductase B | 1.01 | 0.96 | 0.98 | 0.90 | 0.89 | 0.83 |
| O | OEOE_1625 | OEOE_RS07835 | thiol-disulfide isomerase | -1.17 | -1.32 | -1.18 | -1.03 | -1.01 | -1.07 |
| O | OEOE_1630 | OEOE_RS07860 | peptidase M13 | -0.66 | -0.88 | -1.04 | -1.06 | -1.09 | -0.93 |
| O | OEOE_1639 | OEOE_RS07905 | peptidylprolyl isomerase | 2.23 | 2.74 | 2.62 | 2.30 | 2.10 | 1.97 |
| O | OEOE_1702 | OEOE_RS08215 | thioredoxin | -1.10 | -0.71 | -0.71 | -0.70 | -0.68 | -0.81 |
| O | OEOE_1852 | OEOE_RS08940 | osmotically inducible protein C | 1.27 | 0.95 | 0.89 | 0.90 | 0.94 | 1.03 |
| **P: Inorganic ion transport and metabolism** | | | | | | | | | |
| P | OEOE_0172 | OEOE_RS00815 | cobalt ABC transporter permease | 1.03 | 0.95 | 0.66 | 0.76 | 0.76 | 0.74 |
| P | OEOE_0173 | OEOE_RS00820 | cobalt ABC transporter | 1.21 | 1.14 | 0.84 | 0.89 | 0.89 | 0.93 |
| P | OEOE_0246 | OEOE_RS01175 | manganese transporter | -0.61 | -1.12 | -1.29 | -1.37 | -1.37 | -1.30 |
| P | OEOE_0305 | OEOE_RS01455 | sodium:solute symporter | -1.27 | -1.33 | -1.21 | -1.41 | -1.41 | -1.51 |
| P | OEOE_0462 | OEOE_RS02225 | potassium transporter Kef | -2.19 | -2.17 | -1.29 | -0.77 | -0.67 | -0.71 |
| P | OEOE_0623 | OEOE_RS02975 | cobalt ABC transporter | 0.27 | 0.26 | 0.56 | 1.08 | 1.16 | 1.16 |
| P | OEOE_0624 | OEOE_RS02980 | cobalt ABC transporter | 0.35 | 0.42 | 0.64 | 1.10 | 1.23 | 1.24 |
| P | OEOE_0625 | OEOE_RS02985 | cobalt ABC transporter permease | 0.75 | 0.84 | 1.03 | 1.30 | 1.52 | 1.51 |
| P | OEOE_0706 | OEOE_RS03385 | chloride channel protein | 0.64 | 0.56 | 0.88 | 1.07 | 0.93 | 0.97 |
| P | OEOE_0819 | OEOE_RS03935 | MFS transporter | 1.44 | 1.67 | 2.02 | 2.34 | 2.27 | 2.13 |
| P | OEOE_1188 | OEOE_RS05725 | metal ABC transporter substrate-binding protein | -1.11 | -1.33 | -1.48 | -1.72 | -1.64 | -1.70 |
| P | OEOE_1363 | OEOE_RS06560 | ATPase | 1.66 | 1.66 | 1.92 | 1.98 | 2.02 | 2.05 |
| P | OEOE_1533 | OEOE_RS07390 | sodium ABC transporter permease | 1.28 | 1.06 | 0.93 | 1.08 | 1.14 | 1.23 |
| P | OEOE_1534 | OEOE_RS07395 | sodium ABC transporter ATP-binding protein | 1.22 | 0.85 | 0.99 | 1.10 | 1.20 | 1.30 |
| P | OEOE_1540 | OEOE_RS07425 | cobalt ABC transporter ATPase | -0.28 | -0.35 | -0.84 | -1.10 | -1.12 | -1.12 |
| P | OEOE_1679 | OEOE_RS08100 | ammonia permease | 1.28 | 1.61 | 1.57 | 1.46 | 1.35 | 1.33 |
| **Q: Secondary metabolites biosynthesis, transport, and catabolism** | | | | | | | | | |
| Q | OEOE_0064 | OEOE_RS00295 | phytoene synthase | 1.25 | 0.82 | 0.84 | 0.98 | 0.84 | 0.83 |
| Q | OEOE_0804 | OEOE_RS03860 | 4-oxalocrotonate tautomerase | 1.56 | 1.14 | 1.38 | 2.01 | 2.16 | 2.19 |
| **R: General function prediction only** | | | | | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| R | OEOE_0025 | OEOE_RS00120 | 2-hydroxyacid dehydrogenase | -0.55 | -0.85 | -1.07 | -1.12 | -1.24 | -1.20 |
| R | OEOE_0036 | OEOE_RS00165 | oxidoreductase ion channel protein IolS | -1.10 | -1.36 | -1.56 | -1.65 | -1.65 | -1.72 |
| R | OEOE_0043 | OEOE_RS00200 | ketosteroid isomerase | -1.05 | -0.92 | -1.13 | -1.21 | -1.05 | -1.10 |
| R | OEOE_0044 | OEOE_RS00205 | oxidoreductase | -0.99 | -0.58 | -0.99 | -1.08 | -1.15 | -1.09 |
| R | OEOE_0070 | OEOE_RS00320 | 2,5-diketo-D-gluconic acid reductase | -0.94 | -1.09 | -1.09 | -1.20 | -1.14 | -1.16 |
| R | OEOE_0071 | OEOE_RS00325 | general stress protein | -2.18 | -2.10 | -2.46 | -2.54 | -2.60 | -2.58 |
| R | OEOE_0076 | OEOE_RS00345 | MFS transporter | 1.04 | 0.72 | 0.67 | 0.69 | 0.69 | 0.50 |
| R | OEOE_0097 | OEOE_RS00445 | MFS transporter | 1.10 | 1.03 | 0.75 | 0.71 | 0.70 | 0.75 |
| R | OEOE_0103 | OEOE_RS00475 | phosphoesterase | 1.56 | 1.30 | 1.15 | 1.28 | 1.26 | 1.30 |
| R | OEOE_0129 | OEOE_RS00600 | gluconate:proton symporter | -1.16 | -1.09 | -1.43 | -1.59 | -1.64 | -1.35 |
| R | OEOE_0137 | OEOE_RS00635 | hemolysin | 1.40 | 1.55 | 1.63 | 1.33 | 1.27 | 1.17 |
| R | OEOE_0148 | OEOE_RS00690 | MFS transporter | 1.91 | 1.98 | 2.18 | 1.96 | 2.00 | 1.96 |
| R | OEOE_0168 | OEOE_RS00795 | acylphosphatase | -1.31 | -1.18 | -1.04 | -0.90 | -0.88 | -0.97 |
| R | OEOE_0174 | OEOE_RS00825 | transporter | 1.01 | 0.79 | 0.70 | 0.75 | 0.76 | 0.81 |
| R | OEOE_0175 | OEOE_RS00830 | transporter | 1.44 | 1.28 | 1.27 | 1.17 | 1.09 | 1.03 |
| R | OEOE_0196 | OEOE_RS00935 | transporter | 1.54 | 1.05 | 1.05 | 1.05 | 1.05 | 1.05 |
| R | OEOE_0241 | OEOE_RS01150 | alpha/β hydrolase | -0.72 | -0.53 | -0.76 | -0.93 | -1.07 | -1.10 |
| R | OEOE_0242 | OEOE_RS01155 | 3-β-hydroxysteroid dehydrogenase | -0.84 | -1.07 | -1.38 | -1.48 | -1.50 | -1.52 |
| R | OEOE_0243 | OEOE_RS01160 | oxidoreductase ion channel protein IolS | -1.16 | -1.03 | -1.07 | -1.21 | -1.07 | -1.09 |
| R | OEOE_0244 | OEOE_RS01165 | DNA-binding protein | -1.71 | -1.67 | -1.96 | -2.01 | -1.99 | -2.00 |
| R | OEOE_0265 | OEOE_RS01270 | MFS transporter | -0.83 | -1.11 | -1.17 | -1.28 | -1.49 | -1.41 |
| R | OEOE_0290 | OEOE_RS01390 | MFS transporter permease | 1.56 | 1.51 | 1.53 | 1.59 | 1.53 | 1.79 |
| R | OEOE_0345 | OEOE_RS01655 | acetyltransferase | -1.27 | -1.02 | -1.29 | -1.40 | -1.27 | -1.27 |
| R | OEOE_0384 | OEOE_RS01845 | MFS transporter permease | 0.76 | 1.11 | 1.24 | 1.33 | 1.27 | 1.34 |
| R | OEOE_0396 | OEOE_RS01905 | carbonyl reductase | 0.72 | 0.86 | 0.75 | 0.89 | 1.05 | 1.20 |
| R | OEOE_0408 | OEOE_RS01960 | oxidoreductase | -1.05 | -1.06 | -1.21 | -1.25 | -1.14 | -1.18 |
| R | OEOE_0416 | OEOE_RS02000 | glucosaminidase | 1.09 | 1.07 | 0.90 | 0.79 | 0.70 | 0.80 |
| R | OEOE_0490 | OEOE_RS02340 | FMN-binding protein | -0.74 | -0.67 | -0.66 | -0.90 | -0.91 | -1.02 |
| R | OEOE_0503 | OEOE_RS02400 | MFS transporter | 1.46 | 1.20 | 1.06 | 1.22 | 1.21 | 1.23 |
| R | OEOE_0693 | OEOE_RS03325 | acetoin reductase | -0.96 | -1.32 | -1.53 | -1.80 | -1.82 | -2.01 |
| R | OEOE_0705 | OEOE_RS03380 | MFS transporter | 1.30 | 1.42 | 1.76 | 1.73 | 1.63 | 1.63 |
| R | OEOE_0757 | OEOE_RS03625 | short-chain dehydrogenase | -0.57 | -0.67 | -0.95 | -1.12 | -1.11 | -1.16 |
| R | OEOE_0784 | OEOE_RS03755 | metallophosphatase | -1.07 | -1.14 | -1.18 | -1.16 | -1.13 | -1.13 |
| R | OEOE_0863 | OEOE_RS04145 | acyltransferase | 1.03 | 1.90 | 2.28 | 1.87 | 1.73 | 1.70 |
| R | OEOE_0868 | OEOE_RS04165 | acetyltransferase | 1.32 | 1.29 | 1.57 | 1.48 | 1.43 | 1.37 |
| R | OEOE_0869 | OEOE_RS04170 | MFS transporter | 1.24 | 1.14 | 1.24 | 1.47 | 1.31 | 1.39 |
| R | OEOE_0881 | OEOE_RS04220 | ACP phosphodiesterase | 0.58 | 1.35 | 1.51 | 0.58 | 0.31 | 0.01 |
| R | OEOE_0886 | OEOE_RS04245 | oxalate:formate antiporter | -1.39 | -1.93 | -2.06 | -2.23 | -2.27 | -2.33 |
| R | OEOE_0896 | OEOE_RS04295 | adenylyltransferase | -0.44 | -0.66 | -0.81 | -1.06 | -0.94 | -0.90 |
| R | OEOE_0927 | OEOE_RS04445 | acetyltransferase | 1.20 | 1.07 | 1.56 | 1.88 | 1.77 | 1.83 |
| R | OEOE_0938 | OEOE_RS04495 | short-chain dehydrogenase | -0.99 | -1.48 | -1.51 | -1.38 | -1.40 | -1.45 |
| R | OEOE_0989 | OEOE_RS04750 | GTPase Era | -1.23 | -1.50 | -1.19 | -0.95 | -1.10 | -1.11 |
| R | OEOE_0997 | OEOE_RS04790 | SAM-dependent methyltransferase | 0.99 | 1.05 | 1.31 | 1.56 | 1.73 | 1.55 |
| R | OEOE_1018 | OEOE_RS04890 | GTPase | 0.50 | 0.84 | 1.07 | 0.95 | 0.90 | 0.88 |
| R | OEOE_1080 | OEOE_RS05190 | pore-forming protein | 0.77 | 0.95 | 1.13 | 1.21 | 1.39 | 1.45 |
| R | OEOE_1202 | OEOE_RS05795 | general stress protein | -0.74 | -1.00 | -1.03 | -0.91 | -0.82 | -0.86 |
| R | OEOE_1260 | OEOE_RS06075 | SAM-dependent methyltransferase | 0.88 | 1.05 | 1.16 | 1.29 | 1.40 | 1.37 |
| R | OEOE_1322 | OEOE_RS06370 | amidophosphoribosyltransferase | 1.14 | 0.71 | 0.78 | 0.84 | 0.96 | 0.81 |
| R | OEOE_1347 | OEOE_RS06475 | acetyltransferase | -1.08 | -1.22 | -1.14 | -1.23 | -1.19 | -1.12 |
| R | OEOE_1364 | OEOE_RS06565 | acyltransferase | -1.53 | -1.90 | -1.91 | -1.57 | -1.42 | -1.18 |
| R | OEOE_1412 | OEOE_RS06805 | haloacid dehalogenase | -0.89 | -0.91 | -1.03 | -1.00 | -1.20 | -1.35 |
| R | OEOE_1424 | OEOE_RS06865 | competence protein ComX | 1.67 | 1.81 | 1.76 | 1.62 | 1.64 | 1.52 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| R | OEOE_1474 | OEOE_RS07115 | glycosyl transferase | 1.19 | 0.77 | 0.60 | 0.62 | 0.62 | 0.59 |
| R | OEOE_1514 | OEOE_RS07295 | glycosyl transferase | 0.99 | 0.87 | 0.77 | 0.94 | 1.01 | 1.13 |
| R | OEOE_1536 | OEOE_RS07405 | MFS transporter | 0.76 | 0.62 | 0.74 | 1.12 | 1.31 | 1.44 |
| R | OEOE_1560 | OEOE_RS07525 | 17-β-hydroxysteroid dehydrogenase | -0.45 | -0.85 | -1.01 | -1.12 | -1.21 | -1.13 |
| R | OEOE_1607 | OEOE_RS07750 | heme ABC transporter ATP-binding protein | -0.42 | -0.85 | -1.14 | -1.36 | -1.37 | -1.37 |
| R | OEOE_1608 | OEOE_RS07755 | ABC transporter permease | -0.29 | -0.52 | -1.00 | -1.25 | -1.23 | -1.24 |
| R | OEOE_1748 | OEOE_RS08435 | HD family phosphohydrolase | -1.22 | -0.86 | -1.05 | -0.90 | -0.78 | -0.88 |
| R | OEOE_1782 | OEOE_RS08590 | aldo/keto reductase | -1.16 | -1.31 | -1.60 | -1.87 | -1.89 | -1.88 |
| R | OEOE_1820 | OEOE_RS08780 | acetyltransferase | 0.87 | 0.81 | 0.89 | 0.98 | 1.12 | 1.03 |
| R | OEOE_1841 | OEOE_RS08890 | CAAX amino protease | 1.02 | 0.81 | 0.73 | 0.72 | 0.68 | 0.65 |
| R | OEOE_1843 | OEOE_RS08900 | permease | 1.57 | 1.39 | 1.39 | 1.48 | 1.44 | 1.63 |
| R | OEOE_1851 | OEOE_RS08935 | O-acetyltransferase | 1.04 | 0.81 | 0.73 | 0.81 | 0.84 | 0.81 |
| R | OEOE_1858 | OEOE_RS08970 | 2,5-diketo-D-gluconic acid reductase | -0.81 | -0.61 | -0.97 | -1.22 | -1.28 | -1.20 |
| R | OEOE_1859 | OEOE_RS08975 | oxidoreductase | 1.15 | 1.47 | 1.37 | 1.14 | 1.15 | 1.04 |
| **S: Function unknown** | | | | | | | | | |
| S | OEOE_0069 | OEOE_RS00315 | membrane protein | -1.66 | -1.64 | -1.84 | -1.95 | -2.01 | -2.19 |
| S | OEOE_0406 | OEOE_RS01950 | membrane protein | 1.70 | 1.87 | 1.62 | 1.48 | 1.54 | 1.50 |
| S | OEOE_0483 | OEOE_RS02305 | membrane protein | -2.20 | -2.54 | -2.49 | -2.61 | -2.59 | -2.99 |
| S | OEOE_0484 | OEOE_RS02310 | cytochrome O ubiquinol oxidase | -2.05 | -2.42 | -2.40 | -2.59 | -2.40 | -2.69 |
| S | OEOE_0547 | OEOE_RS02595 | amidase | 1.16 | 1.10 | 1.23 | 1.08 | 1.15 | 1.03 |
| S | OEOE_0634 | OEOE_RS03030 | membrane protein | 1.44 | 1.24 | 1.37 | 1.84 | 1.75 | 1.83 |
| S | OEOE_0639 | OEOE_RS03055 | membrane protein | -1.49 | -1.56 | -1.63 | -1.57 | -1.51 | -1.51 |
| S | OEOE_0650 | OEOE_RS03110 | membrane protein | 1.59 | 1.71 | 1.72 | 1.67 | 1.72 | 1.67 |
| S | OEOE_0713 | OEOE_RS03410 | membrane protein | 2.72 | 3.21 | 3.72 | 3.61 | 3.47 | 3.40 |
| S | OEOE_0753 | OEOE_RS03605 | membrane protein | 1.82 | 2.06 | 2.07 | 2.14 | 2.18 | 2.15 |
| S | OEOE_0835 | OEOE_RS04015 | membrane protein | 1.72 | 1.42 | 1.72 | 2.02 | 1.83 | 1.81 |
| S | OEOE_0930 | OEOE_RS04465 | membrane protein | 2.28 | 2.77 | 3.32 | 3.10 | 2.70 | 2.56 |
| S | OEOE_0933 | OEOE_RS04470 | 5'-3'-deoxyribonucleotidase | 1.24 | 1.52 | 1.81 | 2.38 | 2.94 | 3.25 |
| S | OEOE_1035 | OEOE_RS04975 | membrane protein | -1.30 | -1.71 | -1.80 | -1.76 | -1.73 | -1.88 |
| S | OEOE_1109 | OEOE_RS05330 | membrane protein | -1.23 | -0.96 | -0.66 | -0.46 | -0.49 | -0.52 |
| S | OEOE_1229 | OEOE_RS05920 | cytochrome O ubiquinol oxidase | 0.46 | 0.45 | 0.67 | 0.90 | 0.94 | 1.06 |
| S | OEOE_1354 | OEOE_RS06515 | membrane protein | -1.33 | -1.55 | -1.28 | -1.07 | -0.89 | -1.14 |
| S | OEOE_1367 | OEOE_RS06575 | membrane protein | -0.40 | -0.92 | -1.20 | -0.99 | -0.92 | -1.08 |
| S | OEOE_1472 | OEOE_RS07105 | membrane protein | 0.96 | 1.07 | 0.62 | 0.55 | 0.60 | 0.70 |
| S | OEOE_1567 | OEOE_RS07560 | membrane protein | 1.49 | 1.31 | 1.08 | 1.04 | 0.98 | 1.07 |
| S | OEOE_1606 | OEOE_RS07745 | membrane protein | -0.98 | -1.08 | -1.46 | -1.74 | -1.69 | -1.74 |
| S | OEOE_1620 | OEOE_RS07815 | membrane protein | -0.36 | 0.06 | 0.54 | 0.97 | 1.00 | 0.54 |
| S | OEOE_1829 | OEOE_RS08830 | glycosyl transferase | 1.38 | 1.07 | 0.96 | 1.07 | 1.07 | 1.12 |
| **T: Signal transduction mechanisms** | | | | | | | | | |
| T | OEOE_0120 | OEOE_RS00555 | histidine kinase | -1.07 | -1.18 | -1.28 | -1.14 | -1.17 | -1.14 |
| T | OEOE_0143 | OEOE_RS00665 | histidine kinase | 0.82 | 0.93 | 1.02 | 1.13 | 1.01 | 0.96 |
| T | OEOE_0420 | OEOE_RS02020 | [citrate [pro-3S]-lyase] ligase | 1.85 | 2.22 | 1.77 | 1.23 | 1.20 | 1.17 |
| T | OEOE_0421 | OEOE_RS02025 | citrate lyase ACP | 1.68 | 1.96 | 1.88 | 1.44 | 1.24 | 1.45 |
| T | OEOE_0422 | OEOE_RS02030 | citrate lyase | 1.25 | 1.50 | 1.25 | 0.95 | 0.91 | 1.07 |
| T | OEOE_0489 | OEOE_RS02335 | histidine kinase | -0.75 | -0.84 | -1.22 | -1.32 | -1.24 | -1.23 |
| **U: Intracellular trafficking, secretion, and vesicular transport** | | | | | | | | | |
| U | OEOE_0614 | OEOE_RS02940 | preprotein translocase subunit SecY | 1.20 | 1.05 | 1.32 | 1.62 | 1.56 | 1.50 |
| U | OEOE_0794 | OEOE_RS03805 | type VI secretion protein ImpB | 0.63 | 0.98 | 1.01 | 1.06 | 1.13 | 1.31 |
| U | OEOE_1254 | OEOE_RS06045 | type II secretion protein | 1.21 | 0.82 | 0.61 | 0.60 | 0.61 | 0.49 |
| U | OEOE_1255 | OEOE_RS06050 | competence protein ComGC | 1.08 | 0.67 | 0.45 | 0.44 | 0.46 | 0.17 |
| U | OEOE_1256 | OEOE_RS06055 | type II secretion system protein F | 1.35 | 0.80 | 0.66 | 0.56 | 0.58 | 0.47 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| U | OEOE_1862 | OEOE_RS08990 | membrane protein | 1.18 | 1.52 | 1.70 | 1.68 | 1.59 | 1.53 |
| **V: Defense mechanisms** | | | | | | | | | |
| V | OEOE_0439 | OEOE_RS02115 | multidrug ABC transporter ATP-binding protein | 3.69 | 4.43 | 4.75 | 4.45 | 4.26 | 4.08 |
| V | OEOE_0512 | OEOE_RS02435 | diguanylate cyclase | 1.23 | 1.22 | 0.99 | 1.08 | 1.04 | 1.15 |
| V | OEOE_0588 | OEOE_RS02805 | N-acetylmuramoyl-L-alanine amidase | 1.85 | 2.06 | 2.14 | 2.01 | 1.79 | 1.71 |
| V | OEOE_0720 | OEOE_RS03440 | acetyl esterase | 3.23 | 3.72 | 4.21 | 4.03 | 3.69 | 3.61 |
| V | OEOE_0722 | OEOE_RS03445 | multidrug ABC transporter ATPase | 1.72 | 1.69 | 2.02 | 2.16 | 1.93 | 2.02 |
| V | OEOE_0723 | OEOE_RS03450 | multidrug ABC transporter permease | 2.15 | 2.01 | 2.34 | 2.48 | 2.35 | 2.18 |
| V | OEOE_0737 | OEOE_RS03520 | multidrug ABC transporter permease | -0.79 | -0.88 | -0.96 | -0.98 | -1.06 | -1.01 |
| V | OEOE_0761 | OEOE_RS03640 | multidrug ABC transporter permease | 0.88 | 1.23 | 1.10 | 1.03 | 1.06 | 1.17 |
| V | OEOE_0877 | OEOE_RS04200 | multidrug MFS transporter | 2.39 | 2.77 | 3.14 | 3.51 | 3.73 | 3.75 |
| V | OEOE_1345 | OEOE_RS06465 | multidrug ABC transporter permease | -0.50 | -0.52 | -0.99 | -1.21 | -1.29 | -0.94 |
| V | OEOE_1636 | OEOE_RS07890 | multidrug ABC transporter ATP-binding protein | 1.07 | 0.93 | 0.72 | 0.69 | 0.59 | 0.65 |
| V | OEOE_1711 | OEOE_RS08260 | multidrug ABC transporter ATP-binding protein | 1.48 | 1.54 | 1.68 | 1.80 | 1.47 | 1.05 |
| V | OEOE_1712 | OEOE_RS08265 | multidrug ABC transporter ATP-binding protein | 1.01 | 1.04 | 1.25 | 1.40 | 1.10 | 0.76 |
| **Multifunctional** | | | | | | | | | |
| A J K F | OEOE_1643 | OEOE_RS07925 | DEAD/DEAH box helicase | 2.15 | 2.46 | 2.37 | 2.47 | 2.47 | 2.37 |
| C H | OEOE_0769 | OEOE_RS03675 | formate--tetrahydrofolate ligase | 1.02 | 1.45 | 1.27 | 1.25 | 1.30 | 1.32 |
| C O | OEOE_0414 | OEOE_RS01990 | cysteine ABC transporter ATP-binding protein | -0.62 | -0.86 | -1.01 | -1.16 | -1.23 | -1.25 |
| C R | OEOE_1358 | OEOE_RS06535 | NADPH:quinone reductase | -0.74 | -1.14 | -1.03 | -0.99 | -0.94 | -0.99 |
| E F | OEOE_0260 | OEOE_RS01245 | carbamoyl phosphate synthase small subunit | -1.08 | -1.62 | -1.82 | -2.07 | -2.28 | -2.24 |
| E C G T | OEOE_0952 | OEOE_RS04565 | glutamine synthetase | 1.88 | 2.08 | 2.20 | 2.01 | 1.83 | 1.81 |
| E F | OEOE_0261 | OEOE_RS01250 | carbamoyl-phosphate synthase large chain | -0.66 | -0.89 | -1.18 | -1.54 | -1.69 | -1.78 |
| E G | OEOE_0387 | OEOE_RS01860 | 4-aminobutyrate aminotransferase | 2.98 | 3.30 | 3.27 | 3.20 | 3.21 | 3.30 |
| E G | OEOE_0638 | OEOE_RS03050 | phosphoglycerate kinase | -1.12 | -1.25 | -1.39 | -1.29 | -1.22 | -1.22 |
| E G V | OEOE_0635 | OEOE_RS03035 | glucosamine--fructose-6-phosphate aminotransferase | 2.94 | 3.14 | 3.65 | 3.92 | 3.99 | 3.97 |
| E H Q V | OEOE_1296 | OEOE_RS06245 | branched-chain amino acid aminotransferase | 0.95 | 1.42 | 2.04 | 2.25 | 2.61 | 3.02 |
| E M S | OEOE_1644 | OEOE_RS07930 | UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase | 1.30 | 1.50 | 1.29 | 1.29 | 1.29 | 1.29 |
| E Q | OEOE_0766 | OEOE_RS03660 | cystathionine β-lyase | 0.82 | 1.15 | 0.81 | 0.69 | 0.74 | 0.77 |
| E Q | OEOE_0895 | OEOE_RS04290 | aspartate aminotransferase | 1.42 | 1.89 | 1.99 | 2.06 | 1.85 | 2.02 |
| E Q | OEOE_1048 | OEOE_RS05035 | aspartate racemase | -0.61 | -0.81 | -1.08 | -1.10 | -1.13 | -0.99 |
| E R | OEOE_0394 | OEOE_RS01895 | threonine dehydrogenase | -0.99 | -1.29 | -1.15 | -0.99 | -0.96 | -0.87 |
| E R | OEOE_1707 | OEOE_RS08240 | alcohol dehydrogenase | -0.41 | -0.77 | -1.02 | -1.14 | -1.27 | -1.30 |
| E R | OEOE_1780 | OEOE_RS08580 | alcohol dehydrogenase | -0.66 | -1.31 | -1.40 | -1.50 | -1.64 | -1.68 |
| E V C G | OEOE_0122 | OEOE_RS00565 | phosphoglyceromutase | -1.37 | -1.60 | -1.51 | -1.40 | -1.37 | -1.42 |
| E V Q | OEOE_0152 | OEOE_RS00715 | 3-phosphoshikimate 1-carboxyvinyltransferase | 1.71 | 1.92 | 1.62 | 1.49 | 1.64 | 1.95 |
| E V Q | OEOE_0153 | OEOE_RS00720 | shikimate kinase | 2.12 | 2.16 | 2.08 | 1.92 | 2.08 | 2.26 |
| E V Q | OEOE_0939 | OEOE_RS04500 | argininosuccinate synthase | -0.20 | -0.58 | -0.82 | -0.94 | -1.10 | -1.01 |
| E V Q | OEOE_1546 | OEOE_RS07455 | homoserine dehydrogenase | 1.40 | 1.52 | 1.58 | 1.65 | 1.59 | 1.74 |
| E V Q C G | OEOE_0310 | OEOE_RS01480 | phosphoglycerate mutase | 0.51 | 0.73 | 1.22 | 1.11 | 0.77 | 0.82 |
| E V Q C G | OEOE_1611 | OEOE_RS07770 | ribose 5-phosphate isomerase | -0.87 | -0.94 | -1.09 | -1.37 | -1.27 | -1.16 |
| E V Q C G | OEOE_1652 | OEOE_RS07970 | triosephosphate isomerase | -1.34 | -1.12 | -1.25 | -1.55 | -1.51 | -1.67 |
| E V Q G C O | OEOE_1650 | OEOE_RS07960 | enolase | -0.76 | -0.76 | -0.75 | -1.06 | -1.07 | -1.12 |
| F E | OEOE_0163 | OEOE_RS00770 | ferredoxin--NADP reductase | 0.93 | 1.14 | 1.24 | 1.35 | 1.39 | 1.35 |
| F E | OEOE_0258 | OEOE_RS01235 | aspartate carbamoyltransferase | -1.14 | -1.75 | -2.00 | -2.27 | -2.56 | -2.50 |
| F E | OEOE_0566 | OEOE_RS02695 | thioredoxin reductase | 1.06 | 1.66 | 1.60 | 1.23 | 1.20 | 1.23 |
| F E V Q | OEOE_1125 | OEOE_RS05410 | adenylosuccinate lyase | -0.42 | -0.88 | -0.98 | -1.16 | -1.06 | -0.94 |
| F G R | OEOE_0929 | OEOE_RS04460 | HIT family hydrolases | 2.67 | 3.22 | 3.98 | 3.81 | 3.44 | 3.28 |
| F H | OEOE_1469 | OEOE_RS07095 | allantoin permease | -0.95 | -0.85 | -1.12 | -1.24 | -1.14 | -1.01 |
| F Q | OEOE_0537 | OEOE_RS02555 | xanthine phosphoribosyltransferase | -1.08 | -1.09 | -0.82 | -0.28 | -0.07 | -0.03 |
| F V Q | OEOE_0615 | OEOE_RS02945 | adenylate kinase | 1.21 | 1.08 | 1.22 | 1.36 | 1.44 | 1.22 |
| G C T | OEOE_0418 | OEOE_RS02010 | malate dehydrogenase | 2.20 | 2.51 | 2.25 | 1.77 | 1.68 | 1.61 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| G I | OEOE_1781 | OEOE_RS08585 | alpha-galactosidase | -1.21 | -1.24 | -1.58 | -1.92 | -2.08 | -2.06 |
| G Q | OEOE_0249 | OEOE_RS01190 | mannose-6-phosphate isomerase | -1.06 | -1.41 | -1.55 | -1.41 | -1.24 | -1.26 |
| G T | OEOE_0544 | OEOE_RS02580 | hydrolase | 1.73 | 1.89 | 1.49 | 1.32 | 1.22 | 1.29 |
| G V | OEOE_1542 | OEOE_RS07435 | bifunctional protein GlmU | -0.61 | -1.02 | -1.03 | -1.15 | -1.16 | -1.10 |
| G V Q C | OEOE_0920 | OEOE_RS04415 | glucokinase | -0.95 | -1.18 | -1.20 | -0.95 | -0.80 | -0.57 |
| H R | OEOE_0700 | OEOE_RS03355 | alpha/β hydrolase | -1.62 | -2.02 | -1.82 | -1.54 | -1.30 | -1.34 |
| H R | OEOE_0836 | OEOE_RS04020 | haloacid dehalogenase | 1.37 | 1.20 | 1.27 | 1.42 | 1.43 | 1.34 |
| H R | OEOE_1196 | OEOE_RS05765 | haloacid dehalogenase | 0.54 | 0.65 | 0.78 | 0.88 | 0.77 | 1.05 |
| H R | OEOE_1845 | OEOE_RS08910 | haloacid dehalogenase | 1.31 | 1.34 | 1.03 | 0.98 | 1.04 | 1.04 |
| I H | OEOE_1586 | OEOE_RS07650 | 3-hydroxyacyl-ACP dehydratase | 0.33 | 0.28 | 0.70 | 0.91 | 0.94 | 1.03 |
| J O | OEOE_0717 | OEOE_RS03425 | spermidine N1-acetyltransferase | 0.36 | 1.00 | 1.28 | 1.22 | 1.00 | 1.00 |
| L F | OEOE_1663 | OEOE_RS08025 | DNA polymerase III subunit alpha | 1.18 | 1.21 | 0.89 | 0.80 | 0.89 | 0.89 |
| L O | OEOE_1192 | OEOE_RS05745 | ATPase AAA | -1.08 | -1.15 | -1.21 | -1.20 | -1.20 | -1.08 |
| M G | OEOE_1649 | OEOE_RS07955 | saccharopine dehydrogenase | -0.71 | -0.75 | -0.96 | -1.03 | -1.12 | -1.17 |
| M G I | OEOE_1044 | OEOE_RS05015 | β-galactosidase | -0.96 | -1.45 | -1.54 | -1.45 | -1.33 | -1.27 |
| M V | OEOE_0900 | OEOE_RS04315 | carboxypeptidase | 1.16 | 1.45 | 1.15 | 1.15 | 1.15 | 1.15 |
| Q F | OEOE_1127 | OEOE_RS05420 | inosine-5-monophosphate dehydrogenase | -0.74 | -1.14 | -1.14 | -1.27 | -1.27 | -1.31 |
| Q G I V | OEOE_1248 | OEOE_RS06015 | alcohol dehydrogenase | -0.51 | -0.76 | -1.09 | -1.46 | -1.61 | -1.58 |
| Q G I V | OEOE_1330 | OEOE_RS06400 | alcohol dehydrogenase | -0.64 | -1.02 | -1.06 | -1.10 | -1.24 | -1.30 |
| Q H | OEOE_0279 | OEOE_RS01335 | dihydroxynaphthoic acid synthetase | -0.60 | -0.91 | -0.93 | -0.92 | -0.98 | -1.15 |
| Q H | OEOE_0280 | OEOE_RS01340 | acyl-CoA synthetase | -0.85 | -0.98 | -1.12 | -1.24 | -1.25 | -1.34 |
| Q V | OEOE_1100 | OEOE_RS05285 | kinase | -1.13 | -1.09 | -0.88 | -0.83 | -0.84 | -0.84 |
| Q V | OEOE_1445 | OEOE_RS06980 | dTDP-4-dehydrorhamnose reductase | 2.21 | 2.97 | 3.26 | 3.05 | 2.78 | 2.71 |
| Q V | OEOE_1447 | OEOE_RS06990 | dTDP-glucose 4,6-dehydratase | -0.72 | -0.89 | -1.06 | -1.35 | -1.38 | -1.23 |
| Q V | OEOE_1448 | OEOE_RS06995 | dTDP-4-dehydrorhamnose 3,5-epimerase | -0.55 | -0.72 | -0.88 | -1.09 | -1.18 | -1.12 |
| Q V | OEOE_1449 | OEOE_RS07000 | glucose-1-phosphate thymidylyltransferase | -0.62 | -0.95 | -1.09 | -1.36 | -1.32 | -1.25 |
| T K | OEOE_0142 | OEOE_RS00660 | transcriptional regulator | 1.18 | 1.67 | 1.99 | 2.17 | 1.93 | 1.67 |
| T K | OEOE_0488 | OEOE_RS02330 | PhoB family transcriptional regulator | -0.77 | -1.03 | -1.18 | -1.21 | -1.14 | -1.23 |
| T S | OEOE_0214 | OEOE_RS01015 | D-alanyl-D-alanine dipeptidase | -0.75 | -0.46 | -0.81 | -1.02 | -1.33 | -0.99 |
| V Q C E G | OEOE_0135 | OEOE_RS00625 | glucose-6-phosphate 1-dehydrogenase | -0.53 | -0.86 | -1.02 | -1.17 | -1.26 | -1.31 |
| V Q C E G | OEOE_0892 | OEOE_RS04275 | 6-phosphogluconate dehydrogenase | -0.60 | -0.73 | -1.07 | -1.38 | -1.43 | -1.44 |
| V Q C E G | OEOE_1523 | OEOE_RS07345 | 6-phosphogluconate dehydrogenase | -0.79 | -1.25 | -1.35 | -1.57 | -1.76 | -1.73 |
| V Q C G | OEOE_0328 | OEOE_RS01570 | pyruvate dehydrogenase E1 subunit alpha | -0.58 | -0.90 | -0.93 | -1.22 | -1.20 | -0.90 |
| V Q C G | OEOE_0329 | OEOE_RS01575 | 2-oxoisovalerate dehydrogenase subunit β | -0.41 | -0.59 | -0.70 | -1.08 | -1.03 | -0.75 |
| V Q C G | OEOE_0330 | OEOE_RS01580 | dihydrolipoamide acetyltransferase | -0.61 | -0.60 | -0.85 | -1.25 | -1.15 | -0.97 |
| V Q C G E | OEOE_0331 | OEOE_RS01585 | dihydrolipoamide dehydrogenase | -0.66 | -0.61 | -0.84 | -1.16 | -1.11 | -0.90 |
| V Q F | OEOE_1136 | OEOE_RS05465 | phosphoribosylaminoimidazole-succinocarboxamide synthase | -0.19 | -0.68 | -0.96 | -1.09 | -1.25 | -1.44 |
| V Q F | OEOE_1137 | OEOE_RS05470 | phosphoribosylaminoimidazole carboxylase | -0.13 | -0.60 | -0.67 | -0.89 | -1.02 | -0.97 |
| V Q F | OEOE_1138 | OEOE_RS05475 | N5-carboxyaminoimidazole ribonucleotide mutase | -1.03 | -1.33 | -1.42 | -1.61 | -1.64 | -1.66 |
| V Q G | OEOE_0301 | OEOE_RS01435 | aldose 1-epimerase | -1.52 | -1.02 | -1.55 | -1.77 | -1.76 | -1.85 |
| V Q H F | OEOE_1129 | OEOE_RS05430 | bifunctional purine biosynthesis protein PurH | -0.16 | -0.37 | -0.70 | -1.03 | -1.10 | -1.07 |
| V Q H F | OEOE_1130 | OEOE_RS05435 | phosphoribosylglycinamide formyltransferase | -0.11 | -0.26 | -0.62 | -0.95 | -1.05 | -1.05 |
| V T | OEOE_0164 | OEOE_RS00775 | diguanylate cyclase | 0.96 | 1.20 | 1.44 | 1.51 | 1.60 | 1.56 |

**Table S2** Over-expressed and under-expressed genes annotated as hypothetical proteins, during acclimation to WLM. Time samples with over- or under-expression are grey highlighted.

| | Relative expression in function of time (h) | | | | | |
|---|---|---|---|---|---|---|
| | **0.5** | **1** | **2** | **4** | **6** | **8** |
| **Over-expressed genes** | | | | | | |
| OEOE_0013 | 1.26 | 1.39 | 1.60 | 1.60 | 1.42 | 1.39 |
| OEOE_0063 | 1.31 | 1.03 | 1.23 | 1.38 | 1.23 | 1.25 |
| OEOE_0085 | 0.91 | 1.04 | 0.91 | 0.89 | 1.00 | 0.91 |
| OEOE_0086 | 1.10 | 0.89 | 0.89 | 0.89 | 0.89 | 0.88 |
| OEOE_0151 | 1.38 | 1.69 | 1.63 | 1.57 | 1.69 | 2.11 |
| OEOE_0177 | 0.95 | 1.41 | 1.17 | 0.89 | 0.71 | 0.77 |
| OEOE_0178 | 1.44 | 1.78 | 2.09 | 1.86 | 1.56 | 1.42 |
| OEOE_0180 | 0.60 | 1.16 | 0.75 | 0.40 | 0.25 | 0.21 |
| OEOE_0250 | 1.41 | 0.83 | 0.70 | 0.82 | 0.86 | 0.84 |
| OEOE_0275 | 1.25 | 1.19 | 1.42 | 1.44 | 1.77 | 2.41 |
| OEOE_0386 | 2.71 | 3.31 | 3.34 | 3.39 | 3.39 | 3.43 |
| OEOE_0389 | 1.93 | 1.93 | 2.01 | 2.06 | 1.76 | 2.04 |
| OEOE_0392 | 1.88 | 1.76 | 1.95 | 1.76 | 1.76 | 1.76 |
| OEOE_0399 | 1.17 | 0.94 | 0.78 | 0.83 | 0.79 | 0.83 |
| OEOE_0402 | 0.74 | 1.22 | 1.36 | 1.23 | 1.10 | 1.10 |
| OEOE_0431 | 1.43 | 1.32 | 1.43 | 1.49 | 1.50 | 1.64 |
| OEOE_0502 | 1.16 | 0.81 | 0.79 | 0.81 | 0.81 | 0.81 |
| OEOE_0509 | 1.66 | 2.15 | 2.48 | 2.74 | 2.49 | 2.66 |
| OEOE_0546 | 1.29 | 1.14 | 1.31 | 1.20 | 1.20 | 1.20 |
| OEOE_0568 | 0.84 | 1.08 | 1.04 | 0.97 | 0.88 | 0.98 |
| OEOE_0651 | 0.81 | 1.09 | 1.14 | 1.24 | 1.28 | 1.15 |
| OEOE_0691 | 0.74 | 1.59 | 2.50 | 1.91 | 1.36 | 1.12 |
| OEOE_0692 | 0.05 | 0.76 | 1.42 | 0.98 | 0.58 | 0.35 |
| OEOE_0715 | 2.12 | 2.50 | 2.79 | 2.68 | 2.57 | 2.50 |
| OEOE_0760 | 1.23 | 1.78 | 1.68 | 1.49 | 1.51 | 1.65 |
| OEOE_0817 | 1.74 | 1.99 | 2.59 | 2.70 | 2.69 | 2.71 |
| OEOE_0818 | 0.16 | 0.11 | 0.81 | 1.18 | 1.23 | 1.19 |
| OEOE_0837 | 1.39 | 1.32 | 1.21 | 1.37 | 1.36 | 1.29 |
| OEOE_0874 | 1.19 | 1.21 | 2.00 | 2.26 | 2.48 | 2.17 |
| OEOE_0876 | 2.73 | 3.10 | 3.33 | 3.69 | 3.82 | 3.78 |
| OEOE_0925 | 0.49 | 0.50 | 1.04 | 1.44 | 1.38 | 1.22 |
| OEOE_0944 | 1.04 | 1.00 | 1.04 | 0.98 | 0.98 | 0.97 |
| OEOE_0945 | 0.95 | 1.02 | 0.81 | 0.70 | 0.59 | 0.57 |
| OEOE_0961 | 2.85 | 2.59 | 2.37 | 2.57 | 2.75 | 2.79 |
| OEOE_0971 | 1.11 | 0.98 | 0.91 | 0.91 | 0.91 | 0.78 |
| OEOE_1057 | 1.16 | 0.41 | 0.69 | 0.76 | 0.83 | 0.85 |
| OEOE_1161 | 1.58 | 1.36 | 1.09 | 0.95 | 0.99 | 0.94 |
| OEOE_1362 | 0.30 | 0.23 | 0.68 | 0.87 | 1.04 | 1.02 |
| OEOE_1490 | -0.28 | -0.28 | -0.29 | 0.48 | 0.84 | 1.07 |
| OEOE_1521 | 1.28 | 1.09 | 1.08 | 1.24 | 1.29 | 1.39 |
| OEOE_1522 | 1.14 | 0.83 | 0.75 | 0.88 | 0.91 | 0.93 |
| OEOE_1526 | 0.62 | 1.14 | 1.02 | 0.90 | 0.83 | 0.76 |
| OEOE_1548 | 0.71 | 1.24 | 1.23 | 1.20 | 1.22 | 0.98 |
| OEOE_1683 | 0.49 | 0.12 | 0.40 | 1.20 | 1.95 | 2.42 |
| OEOE_1684 | 1.12 | 1.41 | 1.73 | 1.71 | 1.77 | 1.66 |
| OEOE_1718 | 1.51 | 1.44 | 1.41 | 1.67 | 1.79 | 1.82 |
| OEOE_1789 | 0.96 | 0.96 | 0.96 | 1.06 | 1.08 | 0.94 |
| OEOE_1810 | 1.21 | 1.33 | 1.54 | 2.00 | 2.07 | 2.09 |
| OEOE_1817 | 1.02 | 0.75 | 0.61 | 0.58 | 0.63 | 0.63 |
| OEOE_1844 | 1.40 | 1.33 | 1.37 | 1.38 | 1.38 | 1.47 |
| OEOE_1850 | 1.29 | 0.90 | 0.90 | 0.90 | 0.82 | 0.92 |
| OEOE_1856 | 1.26 | 1.37 | 1.39 | 1.23 | 1.30 | 1.16 |

| | **0.5** | **1** | **2** | **4** | **6** | **8** |
|---|---|---|---|---|---|---|
| **Under-expressed genes** | | | | | | |
| OEOE_0053 | -1.54 | -1.47 | -1.26 | -0.94 | -0.79 | -1.03 |
| OEOE_0068 | -1.54 | -1.72 | -1.91 | -1.98 | -1.70 | -2.21 |
| OEOE_0074 | -1.53 | -1.46 | -1.28 | -0.95 | -0.78 | -1.02 |
| OEOE_0098 | -2.12 | -2.24 | -2.17 | -2.12 | -2.12 | -2.12 |
| OEOE_0118 | -1.03 | -1.26 | -1.07 | -1.02 | -1.02 | -1.01 |
| OEOE_0127 | -2.05 | -2.16 | -2.40 | -1.92 | -1.64 | -1.74 |
| OEOE_0210 | -2.51 | -2.69 | -2.27 | -2.18 | -1.96 | -2.25 |
| OEOE_0240 | -2.04 | -2.27 | -2.53 | -2.74 | -2.77 | -2.92 |
| OEOE_0315 | -2.15 | -2.00 | -2.52 | -2.49 | -2.42 | -2.28 |
| OEOE_0352 | -1.36 | -1.41 | -1.30 | -1.30 | -1.32 | -1.33 |
| OEOE_0359 | -0.82 | -1.00 | -1.07 | -0.92 | -0.92 | -0.92 |
| OEOE_0520 | -1.14 | -1.08 | -0.76 | -0.64 | -0.63 | -0.67 |
| OEOE_0527 | -0.66 | -0.96 | -1.26 | -1.45 | -1.51 | -1.56 |
| OEOE_0637 | -1.73 | -1.62 | -1.14 | -0.74 | -0.67 | -0.69 |
| OEOE_0708 | -3.71 | -3.42 | -3.53 | -3.19 | -3.21 | -3.35 |
| OEOE_0709 | -1.25 | -1.03 | -0.93 | -0.89 | -0.82 | -0.87 |
| OEOE_0729 | -0.72 | -1.03 | -0.98 | -0.97 | -0.90 | -0.90 |
| OEOE_0822 | -1.12 | -1.24 | -1.08 | -0.94 | -1.01 | -1.27 |
| OEOE_0823 | -1.08 | -1.12 | -0.80 | -0.74 | -0.82 | -0.83 |
| OEOE_0832 | -0.60 | -0.94 | -0.97 | -0.92 | -1.01 | -0.96 |
| OEOE_0855 | -1.75 | -1.53 | -1.54 | -1.64 | -1.79 | -1.71 |
| OEOE_0856 | -1.79 | -1.75 | -1.40 | -1.49 | -1.50 | -1.51 |
| OEOE_0858 | -0.90 | -0.82 | -0.81 | -1.04 | -0.95 | -1.19 |
| OEOE_0862 | -1.68 | -1.81 | -1.77 | -1.68 | -1.79 | -1.68 |
| OEOE_0871 | -1.08 | -0.21 | 0.21 | -0.02 | -0.21 | -0.34 |
| OEOE_0872 | -1.22 | -1.17 | -1.07 | -1.07 | -1.11 | -1.11 |
| OEOE_1022 | -1.79 | -1.74 | -1.47 | -0.92 | -0.62 | -0.54 |
| OEOE_1107 | -1.53 | -1.41 | -1.33 | -1.10 | -0.99 | -1.03 |
| OEOE_1122 | -0.76 | -0.82 | -0.97 | -1.03 | -1.04 | -1.06 |
| OEOE_1153 | -1.35 | -1.35 | -1.42 | -1.27 | -1.38 | -1.35 |
| OEOE_1198 | -0.38 | -0.57 | -1.02 | -0.66 | -0.50 | -0.28 |
| OEOE_1200 | -1.85 | -2.16 | -2.08 | -1.98 | -1.75 | -1.58 |
| OEOE_1203 | -1.72 | -1.42 | -1.20 | -1.10 | -1.05 | -1.29 |
| OEOE_1291 | -1.07 | -0.98 | -0.89 | -0.90 | -0.89 | -0.85 |
| OEOE_1302 | -1.35 | -1.45 | -1.44 | -1.48 | -1.44 | -1.49 |
| OEOE_1304 | -0.77 | -1.00 | -0.87 | -0.90 | -0.95 | -0.87 |
| OEOE_1305 | -1.34 | -1.31 | -1.43 | -1.61 | -1.48 | -1.60 |
| OEOE_1320 | -1.10 | -1.17 | -0.84 | -0.35 | -0.30 | -0.29 |
| OEOE_1329 | -1.15 | -1.43 | -1.43 | -1.44 | -1.49 | -1.67 |
| OEOE_1349 | -0.74 | -1.00 | -0.99 | -1.02 | -1.04 | -1.24 |
| OEOE_1350 | -1.47 | -1.82 | -1.82 | -1.99 | -2.12 | -1.99 |
| OEOE_1351 | -1.77 | -2.11 | -2.24 | -2.31 | -2.30 | -2.37 |
| OEOE_1356 | -2.56 | -2.45 | -2.81 | -2.63 | -2.59 | -2.56 |
| OEOE_1406 | -1.70 | -1.61 | -1.61 | -1.65 | -1.68 | -1.58 |
| OEOE_1453 | -1.32 | -1.05 | -0.88 | -1.13 | -1.10 | -1.20 |
| OEOE_1511 | -0.60 | -0.89 | -1.23 | -0.92 | -0.84 | -0.89 |
| OEOE_1535 | -1.74 | -1.54 | -1.29 | -1.02 | -0.95 | -0.73 |
| OEOE_1626 | -2.15 | -2.17 | -1.52 | -1.37 | -1.52 | -1.50 |
| OEOE_1654 | -0.78 | -1.02 | -1.09 | -1.06 | -1.04 | -0.99 |
| OEOE_1706 | -0.69 | -1.17 | -1.49 | -1.66 | -1.74 | -1.79 |
| OEOE_1751 | -0.77 | -0.72 | -0.95 | -1.05 | -1.04 | -0.92 |

**Table S3** Proteomic identification and COG classification from 2D-DIGE and iTRAQ analysis.

| COGs classification | | | | Fold change | | | | Theoretical Mr (KDa) | Pi |
|---|---|---|---|---|---|---|---|---|---|
| Old locus tag | Gene symbol | Short name | Protein annotation | DIGE | | iTRAQ | | DIGE/iTRAQ | |
| | | | | 1h | 6h | 1h | 6h | | |
| **RNA processing and modification ( A )** | | | | | | | | | |
| OEOE_1287 | OEOE_RS06200 | | metal-dependent hydrolase | 1.3 | 1.4 | - | - | 25.2 | 4.9 |
| **Energy production and conversion ( C )** | | | | | | | | | |
| OEOE_0663 | OEOE_RS03175 | atpA | F0F1 ATP synthase subunit alpha | - | - | -1.3 | 0.7 | 56.7 | |
| **Amino acid transport and metabolism ( E )** | | | | | | | | | |
| OEOE_0461 | OEOE_RS02220 | pepC | aminopeptidase C | ND | -1.1 | - | - | 50.5 | 5.2 |
| OEOE_0574 | OEOE_RS02735 | | dipeptidase | - | - | 1.1 | -0.8 | 41.2 | |
| OEOE_0991 | OEOE_RS04760 | | peptidase M20 | 1.3 | 1.4 | 1.3 | 0.7 | 44.1 / 42.1 | 4.4 |
| OEOE_1031 | OEOE_RS04955 | | glutamine amidotransferase | - | - | -1 | 0.7 | 27.4 | |
| OEOE_1058 | OEOE_RS05080 | pepN | aminopeptidase N | ND | -2.1 | 0.9 | -1.1 | 95.1 | 5.1 |
| OEOE_1191 | OEOE_RS05740 | | glutathione reductase | - | - | 2 | 1 | 48.6 | |
| OEOE_1299 | OEOE_RS06260 | | succinate-semialdehyde dehdyrogenase | - | - | 1.6 | 1.1 | 51.5 | |
| OEOE_1562 | OEOE_RS07535 | luxS | S-ribosylhomocysteine lyase | 1.5 (3) | 1.6 (2) | 1.8 | 1.5 | 17.7 | 5.3 |
| OEOE_1783 | OEOE_RS08595 | | peptidase C69 | - | - | 1.9 | 0.9 | 53.5 | |
| **Nucleotide transport and metabolism ( F )** | | | | | | | | | |
| OEOE_0519 | OEOE_RS02470 | | ADP-ribose pyrophosphatase | - | - | 0.8 | 1.2 | 19.1 | |
| OEOE_0849 | OEOE_RS04085 | | deoxynucleoside kinase | -2.4 | -2.7 | - | - | 25.8 | 5.6 |
| | | | | -1.5 | -1.6 | - | - | 24.7 | 4.9 |
| OEOE_1116 | OEOE_RS05365 | | ribonucleoside triphosphate reductase | - | - | 1 | -1.7 | 82.8 | |
| OEOE_1598 | OEOE_RS07710 | tdk | thymidine kinase | - | - | 0.7 | -1.1 | 21.9 | |
| OEOE_1786 | OEOE_RS08610 | pyrG | CTP synthetase | - | - | 1.6 | -0.8 | 60.1 | |
| **Carbohydrate transport and metabolism ( G )** | | | | | | | | | |
| OEOE_0324 | OEOE_RS01550 | | aldehyde dehydrogenase | 1.2 (3) | 1.6 (2) | - | - | 52.5 | 4.9 |
| OEOE_0413 | OEOE_RS01985 | | lactate dehydrogenase | - | - | 1.5 | 1.1 | 36.5 | |
| OEOE_0464 | OEOE_RS02230 | | PTS mannose transporter subunit EIIAB | - | - | -2.4 | -0.7 | 35.6 | |
| OEOE_0563 | OEOE_RS02680 | hprK | HPr kinase/phosphorylase | 1.6 | 1.9 | - | - | 35.4 | 5.3 |
| OEOE_0643 | OEOE_RS03075 | HPr | phosphocarrier protein | - | - | -0.7 | 2.3 | 9.0 | |
| OEOE_0647 | OEOE_RS03095 | | phosphoenol pyruvate-protein phosphotransferase | -1.2 | ND | - | - | 63.2 | 5 |
| OEOE_1024 | OEOE_RS04920 | | galactose mutarotase | - | - | 0.7 | -1.1 | 34.1 | |
| OEOE_1182 | OEOE_RS05695 | ldhD | D-lactate dehydrogenase | -1.3 | ND | - | - | 36.5 | 5.7 |
| OEOE_1402 | OEOE_RS06755 | | UDP-glucose 4-epimerase | - | - | 1.1 | 0.7 | 36.9 | |
| OEOE_1602 | OEOE_RS07730 | | diacetyl reductase | ND | 1.2 | - | - | 27.4 | 5.3 |
| | | | | ND | 1.6 | - | - | 27.4 | 5 |
| OEOE_1708 | OEOE_RS08245 | | fructokinase | -1.2 | ND | - | - | 32.1 | 6.4 |
| **Coenzyme transport and metabolism ( H )** | | | | | | | | | |
| OEOE_0791 | OEOE_RS03790 | | thiamine pyrophosphokinase | -2.1 | ND | - | - | 25.4 | 4.7 |
| OEOE_1036 | OEOE_RS04980 | pdxS | pyridoxal biosynthesis lyase | - | - | -2.5 | 1 | 31.4 | |
| **Lipid transport and metabolism ( I )** | | | | | | | | | |
| OEOE_1590 | OEOE_RS07670 | | ACP S-malonyltransferase | - | - | -0.8 | 1.1 | 33.6 | |
| OEOE_1591 | OEOE_RS07675 | | 2-nitropropane dioxygenase | - | - | -1.5 | 0.7 | 33.5 | |
| **Translation, ribosomal structure and biogenesis ( J )** | | | | | | | | | |
| ND | OEOE_RS03845 | rplS | 50S ribosomal protein L19 | - | - | -1.3 | -0.7 | 14.6 | 4.7 |
| ND | OEOE_RS06715 | rpsD | 30S ribosomal protein S4 | - | - | -0.8 | -2.5 | 23.3 | 5.4 |
| OEOE_0010 | OEOE_RS00045 | rpsF | 30S ribosomal protein S6 | - | - | -1 | 0.8 | 16.3 | 5.8 |
| OEOE_0433 | OEOE_RS02085 | infB | translation initiation factor IF-2 | - | - | -1.1 | -1.1 | 90.5 | 4.2 |
| OEOE_0460 | OEOE_RS02215 | thrS | threonyl-tRNA synthase | -2.0 | -3.3 | -0.7 | -1.1 | 76.3 | |
| OEOE_0596 | OEOE_RS02850 | rplD | 50S ribosomal protein L4 | - | - | -0.7 | -1.4 | 22.4 | |
| OEOE_0600 | OEOE_RS02870 | rplV | 50S ribosomal protein L22 | - | - | -1 | 0.7 | 13.1 | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| OEOE_0601 | OEOE_RS02875 | rpsC | 30S ribosomal protein S3 | - | - | -1.3 | -0.7 | 29.9 | |
| OEOE_0607 | OEOE_RS02905 | rplE | 50S ribosomal protein L5 | - | - | -1.5 | -0.8 | 20.0 | |
| OEOE_0608 | OEOE_RS02910 | rpsH | 30S ribosomal protein S8 | - | - | -1.5 | 1 | 14.6 | |
| OEOE_0613 | OEOE_RS02935 | rplO | 50S ribosomal protein L15 | - | - | 0.7 | 2.3 | 16.5 | |
| OEOE_0621 | OEOE_RS02970 | rplQ | 50S ribosomal protein L17 | - | - | -0.7 | 1.2 | 14.9 | |
| OEOE_0770 | OEOE_RS03680 | rpmF | 50S ribosomal protein L32 | - | - | 0.7 | 1.2 | 6.7 | |
| OEOE_0792 | OEOE_RS03795 | tuf | elongation factor Tu | -1.3 | -1.6 | -1.6 | -0.9 | 43.6 | 4.9 |
| OEOE_0976 | OEOE_RS04685 | tsf | elongation factor Ts | - | - | -2.3 | -0.9 | 31.8 | |
| OEOE_0978 | OEOE_RS04695 | frr | ribosome recycling factor | -1.6 | ND | - | - | 20.4 | |
| OEOE_1030 | OEOE_RS04950 | rpsN | 30S ribosomal protein S14 | - | - | -1.7 | -0.7 | 10.0 | |
| OEOE_1284 | OEOE_RS06185 | rpsT | 30S ribosomal protein S20 | - | - | 0.7 | 1 | 9.8 | |
| OEOE_1314 | OEOE_RS06335 | fusA | elongation factor G | -1.7 | 1 | - | - | 77.9 | |
| OEOE_1392 | OEOE_RS06700 | valS | valyl-tRNA synthase | - | - | 0.8 | -1.9 | 104.6 | |
| OEOE_1416 | OEOE_RS06825 | rplL | 50S ribosomal protein L7/L12 | ND | 2.0 | - | - | 12.2 | 4.2 |
| OEOE_1746 | OEOE_RS08425 | metG | methionyl-tRNA synthetase | - | - | -0.7 | -1.1 | 77.0 | |
| OEOE_1784 | OEOE_RS08600 | rpmE2 | 50S ribosomal protein L31 type B, partial | - | - | -0.9 | 2.1 | 6.5 | |
| **Transcription ( K )** | | | | | | | | | |
| OEOE_0561 | OEOE_RS02670 | | PhoU family transcriptional regulator | - | - | 0.7 | -1.1 | 25.9 | |
| OEOE_1376 | OEOE_RS06620 | | cold-shock protein | 1.3 | ND | - | - | 7.4 | 4.7 |
| **Replication, recombination and repair ( L )** | | | | | | | | | |
| OEOE_0011 | OEOE_RS00050 | | single-stranded DNA-binding protein | ND | 1.4 | - | - | 20 | 4.6 |
| OEOE_0372 | OEOE_RS01785 | uvrB | excinuclease ABC subunit B | - | - | 0.7 | -1.8 | 76.6 | |
| OEOE_0426 | OEOE_RS02050 | pcrA | ATP-dependent DNA helicase | - | - | 0.7 | -1 | 85.0 | |
| OEOE_1072 | OEOE_RS05155 | obg | GTPase CgtA | - | - | 0.7 | -1.1 | 48.2 | |
| OEOE_1382 | OEOE_RS06650 | recA | protein RecA | - | - | -0.7 | -1.1 | 43.5 | |
| **Cell wall/membrane/envelope biogenesis ( M )** | | | | | | | | | |
| OEOE_0668 | OEOE_RS03200 | mreB | rod shape-determining protein | - | - | -1.2 | -1.1 | 40.1 | |
| OEOE_1444 | OEOE_RS06975 | | peptidoglycan interpeptide bridge formation protein | - | - | -1.1 | -1 | 39.4 | |
| OEOE_1561 | OEOE_RS07530 | | D-alanyl-D-alanine carboxypeptidase | - | - | -0.8 | -1.5 | 31.0 | |
| **Post-translational modification, protein turnover, and chaperones ( O )** | | | | | | | | | |
| OEOE_1114 | OEOE_RS05355 | sufC | Fe-S cluster assembly ABC-type transport system,. ATPase component | -1.9 | -2.1 | - | - | 28.2 | 5.7 |
| OEOE_1396 | OEOE_RS06725 | groL | molecular chaperone GroEL | 1.1 | 1.4 (2) | - | - | 57.5 | 5.0 |
| OEOE_1397 | OEOE_RS06730 | groS | Co-chaperonin GroES (HSP10) | 1.7 | 1.8 (2) | - | - | 9.7 | 4.7 |
| OEOE_1625 | OEOE_RS07835 | | thiol reductase thioredoxin | - | - | -0.6 | 1.4 | 12.6 | |
| OEOE_1702 | OEOE_RS08215 | | thiol reductase thioredoxin | - | - | -0.7 | 1.3 | 11.5 | |
| **Inorganic ion transport and metabolism ( P )** | | | | | | | | | |
| OEOE_1111 | OEOE_RS05340 | | Fe-S cluster assembly protein SufB | - | - | 0.7 | -1.1 | 51.7 | |
| OEOE_1705 | OEOE_RS08230 | | glyoxalase | 1.3 | 1.4 | - | - | 18.5 | 6.1 |
| OEOE_1750 | OEOE_RS08440 | | DNA-binding ferritin-like protein (oxidative damage protectant) | 1.6 | 1.8 | - | - | 18.3 | 4.4 |
| **General function prediction only ( R )** | | | | | | | | | |
| OEOE_0070 | OEOE_RS00320 | ara1 | 2,5-diketo-D-gluconic acid reductase | 1.3 | ND | 1.3 | 1.1 | 31.7 | 5.5 |
| OEOE_0156 | OEOE_RS00735 | nnrE | NAD(P)H-hydrate epimerase | - | - | -0.7 | -1.1 | 22.5 | |
| OEOE_0518 | OEOE_RS02465 | | 2,5-diketo-D-gluconic acid reductase | ND | 1.4 | - | - | 32 | 5.3 |
| OEOE_0693 | OEOE_RS03325 | | acetoin reductase | - | - | -1.7 | -0.9 | 27.4 | |
| OEOE_0904 | OEOE_RS04335 | | 3'-5' exoribonuclease | - | - | 1.1 | -0.7 | 36.3 | |
| OEOE_0936 | OEOE_RS04485 | pox | pyruvate oxidase | - | - | 1.7 | -0.8 | 64.0 | |
| OEOE_1336 | OEOE_RS06430 | | 1,3-propanediol dehydrogenase | ND | 1.4 | -0.7 | 2.4 | 42.1 / 37.6 | 5.2 |
| OEOE_1782 | OEOE_RS08590 | | aldo/keto reductase | 1.3 | 1.5 (2) | 1.3 | 0.7 | 35.9 | 5.2 |
| **Function unknown ( S )** | | | | | | | | | |
| OEOE_0683 | OEOE_RS03275 | | hypothetical protein | 1.3 | ND | - | - | 18 | 4.6 |
| **Signal transduction mechanisms ( T )** | | | | | | | | | |
| OEOE_0807 | OEOE_RS03875 | | GTP-binding protein | ND | 2.6 | -0.8 | -1.1 | 68.2 | 5.1 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Multifunctional** | | | | | | | | | |
| OEOE_0002 | OEOE_RS00010 | | DNA polymerase III subunit β | ND | 1.3 | 1.5 | 0.9 | 40.7 | 5.15 |
| OEOE_0122 | OEOE_RS00565 | | phosphoglycerate mutase | -1.6 | -1.5 | - | - | 27.1 | 5.4 |
| OEOE_0159 | OEOE_RS00750 | | ribose-5-phosphate isomerase A | 1.1 | 1.4 | - | - | 25.2 | 5 |
| OEOE_0160 | OEOE_RS00755 | prs | ribose-phosphate pyrophosphokinase | - | - | -1.5 | -0.9 | 36.9 | |
| OEOE_0258 | OEOE_RS01235 | pyrB | aspartate carbamoyltransferase | -1.3 | 1.5 (2) | - | - | 35 / 38.9 | 6.11 |
| OEOE_0404 | OEOE_RS01940 | gapA | glyceraldehyde-3-phosphate dehydrogenase | 1.2 | ND | 1.1 | 0.8 | 37.1 | 5.85 |
| OEOE_0418 | OEOE_RS02010 | mae | malate dehydrogenase | - | - | -1 | -0.8 | 41.4 | |
| OEOE_0527 | OEOE_RS02510 | | zinc-dependent alcohol dehydrogenase | 1.5 (2) | 1.5 (4) | 1.5 | 1 | 37.5 | 4.9 |
| " | " | | " " | 1.2 | 1.3 | | | 37.5 | 5.24 |
| OEOE_0564 | OEOE_RS02685 | gpsA | glycerol-3-phosphate dehydrogenase | - | - | 1.8 | 0.7 | 36.5 | |
| OEOE_0615 | OEOE_RS02945 | | adenylate kinase | - | - | -0.9 | 1.1 | 20.7 | |
| OEOE_0620 | OEOE_RS02965 | rpoA | DNA-directed RNA polymerase subunit alpha | - | - | 0.9 | 1.3 | 34.5 | |
| OEOE_0635 | OEOE_RS03035 | glmS | glucosamine--fructose-6-phosphate aminotransferase | - | - | 1.7 | 1.1 | 66.2 | |
| OEOE_0636 | OEOE_RS03040 | pgi | glucose-6-phosphate isomerase | - | - | 1.9 | -0.8 | 48.6 | |
| OEOE_0774 | OEOE_RS03705 | dapA | 4-hydroxy-tetrahydrodipicolinate synthase | ND | 1.3 | - | - | 31.9 | 5.7 |
| OEOE_0790 | OEOE_RS03785 | | ribulose-phosphate 3-epimerase | ND | 1.5 | - | - | 24 | 5.1 |
| OEOE_0892 | OEOE_RS04275 | | phosphogluconate dehydrogenase (NADP(+)-dependent. decarboxylating) | 1.2 (2) | 1.3 | 1.8 | 1 | 53 | 5.3 |
| OEOE_0920 | OEOE_RS04415 | glk | glucokinase | - | - | 1.6 | 0.8 | 34.3 | |
| OEOE_0952 | OEOE_RS04565 | glnA | glutamine synthetase | - | - | 0.6 | 1 | 49.9 | |
| OEOE_1002 | OEOE_RS04810 | pyk | pyruvate kinase | ND | -1.4 | - | - | 52.2 | 6.3 |
| OEOE_1249 | OEOE_RS06020 | ackA | acetate kinase | - | - | 1.9 | 0.7 | 43.9 | |
| OEOE_1269 | OEOE_RS06110 | murC | UDP-N-acetylmuramate--L-alanine ligase | - | - | 0.6 | -1.6 | 48.1 | |
| OEOE_1285 | OEOE_RS06190 | | DEAD/DEAH box helicase | - | - | -0.7 | -1 | 50.4 | |
| OEOE_1374 | OEOE_RS06610 | rpoC | DNA-directed RNA polymerase subunit β' | - | - | -0.8 | -1.5 | 137.0 | |
| OEOE_1375 | OEOE_RS06615 | rpoB | DNA-directed RNA polymerase subunit β | - | - | -0.6 | -1.5 | 133.0 | |
| OEOE_1421 | OEOE_RS06850 | | gluconokinase | - | - | 1 | -0.8 | 57.3 | |
| OEOE_1435 | OEOE_RS06930 | pta | phosphate acetyltransferase | - | - | -1.3 | -0.6 | 35.3 | |
| OEOE_1523 | OEOE_RS07345 | gnd | 6-phosphogluconate dehydrogenase | 1.12 | ND | - | - | 32.8 | 4.94 |
| OEOE_1641 | OEOE_RS07915 | | alanine racemase | - | - | 1.4 | -0.6 | 43.0 | |
| OEOE_1643 | OEOE_RS07925 | | DEAD/DEAH box helicase | - | - | -1.8 | -0.8 | 56.9 | |
| OEOE_1650 | OEOE_RS07960 | eno | enolase | 1.5 | 1.5 | 0.7 | 1.8 | 48.4 / 47.3 | 4.6 |
| " | " | | " | | 1.3 | | | 48.4 | 4.8 |
| OEOE_1707 | OEOE_RS08240 | | Threonine dehydrogenase-like Zn-dependent dehydrogenase | 1.5 | 1.6 | - | - | 36.1 | 5.6 |
| OEOE_1780 | OEOE_RS08580 | | alcohol dehydrogenase | ND | 1.6 | - | - | 36.4 | 4.9 |
| " | " | | " | | 1.6 | | | 37.6 | 5 |
| OEOE_1785 | OEOE_RS08605 | murA | UDP-N-acetylglucosamine 1-carboxyvinyltransferase | - | - | 0.7 | 1 | 45.7 | |
| OEOE_1788 | OEOE_RS08615 | rpoE | DNA-directed RNA polymerase subunit delta | - | - | -1 | 1.3 | 21.9 | |

# CHAPTER III

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## Protective role of glutathione addition against wine-related stress in *Oenococcus oeni*

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## Highlights

- Glutathione (GSH) is taken up by *Oenococcus oeni* in a strain-dependent manner.
- GSH addition increases the CFA content of the *O. oeni* membrane at the exponential phase.
- Cells grown with GSH showed an improved survival against ethanol shock (14% v/v).
- GSH addition increases *O. oeni* biomass during the adaptation to wine stress conditions.
- GSH addition could be useful for the adaptation of MLF starter cultures.

## Abstract

*Oenococcus oeni* is the main species responsible for the malolactic fermentation (MLF) of wine due to its ability to survive in this environment. Some wine-related stress factors, such as ethanol and low pH, may alter the cell redox balance of *O. oeni*. For the first time, the ability to uptake glutathione (GSH), an almost universal tripeptide with antioxidant properties, has been associated to the improvement of stress response in *O. oeni*. Despite the inability of *O. oeni* to synthesize GSH, this bacterium can capture it from the media. The ability of 30 *O. oeni* strains to uptake GSH was assessed in this study. Although all of the strains tested were able to import GSH, substantial variability among them was detected. To assess the physiological function of GSH, three strains with different GSH-import capacities were selected. Significant changes in membrane fatty acids composition were observed due to GSH addition. The most relevant was the increase of cyclopropane fatty acids in cell membrane, in both the exponential and the stationary phases. Cells grown with GSH showed an improved survival against ethanol shock (14% v/v). GSH addition also increased biomass production during the adaptation to wine stress conditions (pH 4, pH 3.4 and 6% ethanol). The results suggest that GSH enrichment could improve the resistance to stress to *O. oeni,* which could be useful for the adaptation of MLF starter cultures.

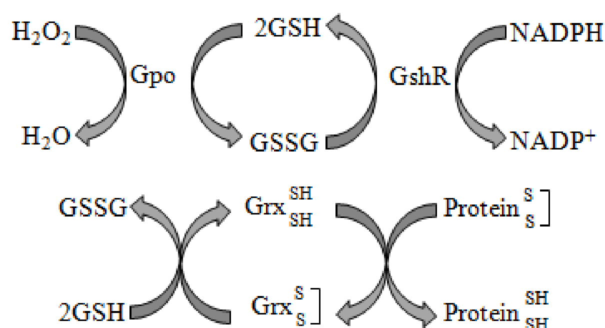
## Keywords

*Oenococcus oeni* – Wine conditions - Glutathione – Stress - Fatty acids - Anisotropy

## Introduction

*Oenococcus oeni*, the species of lactic acid bacteria (LAB) best adapted to wine, is mostly used to induce malolactic fermentation (MLF) (Lonvaud-Funel, 1999). Despite the difficulty of developing *O. oeni* in wine, its ability to better withstand various types of stress compared with other LAB species makes this bacterium an interesting model for study (Liu, 2002). The main niche of this Gram-positive bacterium is wine, which is characterized by several stress factors that are lethal to most food-borne bacteria ($SO_2$ content, temperature, low pH and the presence of ethanol). To survive, *O. oeni* utilizes multiple mechanisms associated with the stress response and adaptation. Among these mechanisms, the consumption of L-malate and citrate generate a proton membrane potential associated with ATP synthesis that improves cell survival (Cox & Henick-Kling, 1989; Ramos et al., 1994). The adjustment of membrane fluidity by the modification of its composition has also been described as a key mechanisms of cell protection in *O. oeni* (Chu-Ky et al., 2005; Silveira et al., 2003). Another well-studied adaptive mechanism is the synthesis of stress proteins, including chaperones, proteases and heat-shock proteins (Beltramo et al., 2004; Guzzo et al., 2000).

Some proteomic studies associate the increase of glutathione reductase in *O. oeni* to the acclimation to ethanol (Cecconi et al., 2009; Silveira et al., 2004). However, there are no published studies about the role of glutathione in stress adaptation in this bacterium. Glutathione (GSH) is an almost universal non-proteic tripeptide (Glu-Cys-Gly), which in its reduced form, acts as an antioxidant through the thiol group of cysteine, which neutralizes reactive oxygen species (ROS) and can also reduce protein sulfur bridges (Masip et al., 2006). The main functions of GSH in the cell are represented below.



As can be seen, glutathione peroxidase (Gpo) catalyzes the reaction of reduced GSH and peroxide to generate glutathione disulfide (GSSG) and $H_2O$. GSSG can be reduced to GSH by glutathione reductase (GshR) using NADPH as a cofactor. GSH is also involved in the reduction of oxidized proteins. In this case, glutaredoxin (Grx) reduces protein sulfur bridges and is recycled to its reduced form by GSH.

Intracellular GSH has been found in various LAB, including *Lactococcus* and *Lactobacillus* species (Lee et al., 2010; Li et al., 2003). This compound has been shown to play an important role in the response to a multitude of stresses in different LAB species (Kim et al., 2012; Smid and Hugenholtz, 2010; J. Zhang et al., 2007). According to J. Zhang et al. (2010), *Lb. sanfranciscensis* cells grown with GSH display higher resistance to cold and freeze-thawing stresses, presumably by maintaining the cell-membrane structure and regulating the redox status. Moreover, an enhancement in the growth of other LAB, such as *Leuconostoc mesenteroides*, *Lb. salivarius* and *Lc. lactis*, due to the presence of GSH, has been observed by some authors (Kim et al., 2012; Lee et al., 2010; Li et al., 2003). A proteomic analysis of *Lc. lactis* cells grown with or without GSH revealed that glycolysis is protected in the presence of this antioxidant, explaining why *Lc. lactis* cells that import GSH are resistant to various stresses (Y. Zhang et al., 2010). The unique entire analysis of a GSH system in LAB was assessed by Kullisaar et al. (2010) in *Lc. fermentum* ME-3. Pophaly et al. (2012) found that *O. oeni* does not have the genes to synthesize nor degrade GSH, however they described the presence of several genes related to GSH metabolism in *O. oeni*, including glutaredoxin, glutathione peroxidase and glutathione reductase and a putative GSH transporter. The recent release of new genomes (Jara & Romero, 2015, Capozzi et al., 2014; Lamontanara et al., 2014) confirms the presence of these genes in *O. oeni*.

The aim of this study was to evaluate for the first time the physiological effect of GSH addition on *O. oeni*. In order to achieve this objective it was evaluated the capacity of GSH import of different strains, the changes due to GSH addition in fatty acids composition of the cell membrane and the derived modification of its fluidity, and the protective effect of this antioxidant on the adaptation to wine-related stress conditions.

## Materials and Methods

*Bacterial strains and culture conditions*

A collection of 30 *O. oeni* strains was used in this work (Table 1): $217^T$ (type strain), PSU-1 (the unique *O. oeni* with a fully annotated genome), nineteen strains from the URV collection (most of which were isolated from red grapes or wines from southern Catalonia) and nine different commercial strains.

For the screening of GSH uptake capacity, cells were grown in MRS broth medium (De Man et al., 1960) supplemented with 4 g/L L-malic acid and 5 g/L fructose at pH 5.0 at 28ºC in a $CO_2$ (10%) incubator.

To test the effect of GSH addition on stress response and membrane modification a medium similar in composition to MRS was used. This medium, here called PC-MRS (Peptone Casaminoacids MRS), contained bacteriological peptone (AppliChem Panreac, Germany) 20 g/L, Bacto™Casamino Acids (BD Biosciences, San José, CA, USA) 5 g/L, dextrose 20 g/L, Tween®80 (Sigma-Aldrich, Saint Louis, MO, USA) 1mL/L, ammonium citrate 2 g/L, sodium acetate 5 g/L, magnesium sulfate 0.1 g/L, manganese sulfate 0.05 g/L, dipotassium phosphate 2 g/L, L-malic acid 4 g/L and fructose 5 g/L. PC-MRS has the advantage with respect to MRS of showing no precipitation at low pH (3.4).

*Detection of intracellular glutathione in a collection of* O. oeni *strains*

The cultures were grown in 100 mL flasks containing MRS broth with 5 mM GSH and without added glutathione until the maximal $OD_{600}$ was reached. Assays were run in triplicate. A volume of 30 mL per duplicate from each condition was harvested by centrifugation (7600 x *g*) for 5 min at 4ºC, and cell pellets were washed with phosphate-buffered saline (1x PBS) . All the wet cell pellets were weighted, and one of the pellets was used for the analysis of GSSG adding 2VP (2-vynil pyridine at 50 mM dissolved in 50% HCl) as a thiol–scavenging reagent (Griffith, 1980; Winther & Thorpe, 2014). The concentration of 2VP (10 µL of scavenger reagent per 100 mg of pellet) was selected after testing three different concentrations (10, 20 and 50 mM). The other pellet was used to analyze the total glutathione (GSH+GSSG).

The samples were frozen in liquid nitrogen and kept at -80ºC until extraction. The cell extracts were prepared following the instructions provided by the manufacturer of the

Glutathione Assay Kit (Sigma Catalogue No. CS0260), with some modifications. Briefly, the sample was first deproteinized with 5% 5-sulfosalicylic acid solution (SSA) and 0.1-mm glass beads were added before the suspension was frozen, thawed at 37ºC and vortexed 15 s. This process was repeated three times. Finally, centrifugation was employed to remove the precipitated protein, and the extracts were kept at -80ºC. The oxidized and total intracellular GSH contents were quantified using the fluorescence protocol described by White et al. (2003). The fluorescence intensity was measured (485 nm excitation / 520 nm emission) with a spectrophotometer PolarStar Omega instrument (BMG Labtech, Ortenberg, Germany) and the reduced GSH concentrations were expressed as nmols per g of wet pellet.

*Analysis of membrane fatty acids*

The membrane fatty acid (FA) composition of the bacteria was determined through gas chromatography, as described by Rozès et al. (1993), with some modifications of the volumes. Briefly, cells were harvested at the exponential phase and stationary phase after growth in PC-MRS with or without GSH (5 mM). Concentrated cells (optical density of approximately 10-20) were washed twice in sterile distilled water. Methylation and extraction were performed by adding 500 µL of sodium methoxide (1 M in methanol) with heptadecanoic acid as internal standard (10 µL of 1.5 mg/mL C17) and shaking for 1 min. Fatty acid methyl esters were extracted using 100 µL of hexane and shaking for 30 s. After centrifugation (2400 x *g* for 5 min), the upper phase was removed and stored in an airtight glass bottle until analysis.

Analytical GC was performed according to the protocol described by Borrull et al. (2015). The relative amounts of the detected FA were calculated from their respective chromatographic peak areas after normalization with the internal standard area (C17). The results were expressed as molar percentages of the total identified fatty acids. The analyses were conducted in triplicate.

**Table 2** Strains used in this work.

| Strains | Source [a] | Isolated from, place (reference) [b] |
|---|---|---|
| PSU-1 (=ATCC BAA-331) | ATCC | Red wine, Pennsylvania State University, US |
| 217$^T$ (=ATCC 23279) | CECT | Red wine, Bordeaux, F |
| 2Pw1 | URV | Carignan grape, Priorat, S (Franquès, J., unpublished data) |
| 2Pw2 | URV | “ |
| 2Pw3 | URV | Grenache grape, Priorat, S (Franquès, J., unpublished data) |
| 2Pw4 | URV | Carignan grape, Priorat, S (Franquès, J., unpublished data) |
| 1Pw13 | URV | Carignan wine, Priorat, S (Franquès, J., unpublished data) |
| 2Pw15 | URV | Grenache wine, Priorat, S (Franquès, J., unpublished data) |
| Vi2FB1 | URV | “ |
| 2T1 | URV | Grenache wine, Tarragona, S (Bordas et al., 2013) |
| 2T2 | URV | “ |
| 3T1 | URV | “ |
| 1P1 | URV | Grenache wine, Priorat, S (Bordas et al. 2013) |
| 1P2 | URV | “ |
| 1P3 | URV | “ |
| 2P2 | URV | Cabernet Sauvignon wine, Priorat, S (Bordas et al. 2013) |
| 3P2 | URV | “ |
| Mf3 (=CECT 7441) | URV | Grenache wine, Tarragona, S (Reguant and Bordons 2003) |
| Mf6 (=CECT 7440) | URV | Tempranillo wine, Tarragona, S (Reguant and Bordons 2003) |
| Fn42 | URV | Monastrell wine, Priorat, S (Masqué and Bordons 1996) |
| UB51 | URV | Tempranillo wine, Conca de Barberà, S (Masqué and Bordons 1996) |
| Enoferm Alfa® (Alfa) | LA | Not specified |
| Lalvin VP41® (VP41) | LA | “ |
| Viniferm OE 104 (Agv) | AG | “ |
| Viniflora® Oenos (VO) | CH | “ |
| Viniflora® CH11 (CH11) | CH | “ |
| Viniflora® CH16 (CH16) | CH | “ |
| Lactoenos 450 PreAc® (Lo450) | LAF | “ |
| Microoenos MBR B1 (MO) | LAF | “ |
| Vitilactic® F (VL) | MV | “ |

[a] ATCC: American Type Culture Collection; CECT: Colección Española de Cultivos Tipo, València, Spain; URV: Universitat Rovira i Virgili, Tarragona, Spain; LA: Lallemand Inc., Montreal, Canada; AG: Agrovin, Alcázar de San Juan, Spain; CH: Chr. Hansen A/S, Hørsholm, Denmark. LAF: Laffort, Bordeaux, France; MV: Martin Vialatte, Épernay, France.

[b] US: United States of America; F: France; S: Spain.

*Fluorescence anisotropy measurements*

Cells grown in PC-MRS with or without GSH (5 mM) were harvested at the exponential phase (three days of culture) and old stationary phase (eight days of culture) by centrifugation (6300 x *g* for 10 min) and washed once in saline solution (9 g/L) at pH 5. The cell pellet was resuspended in saline solution, adjusted to an $OD_{600}$ of 0.6 and immediately used for fluorescence anisotropy measurements. The analyses were conducted in quadruplicate.

Membrane fluidity was determined continuously through fluorescence anisotropy measurements of intact whole cells using hydrophobic 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes, Eugene, OR, USA) as a probe, following Tourdot-Maréchal et al. (2000). The determination of fluorescence anisotropy was optimized as follows: 5 µL of 1.5 mM DPH solution was added to 2.5 mL of cell suspension (final probe concentration of 3.0 µM). The samples were then placed in the stirred and thermostated cuvette holder of the spectrofluorometer (FluoroLog-3, JobinYvon, Inc., NJ, USA) in a T format. The temperature of the samples was measured using a thermometer immersed in the analyzed suspension. To ensure probe stabilization for optimal anisotropy determination, the cells were incubated with the DPH probe for 15 min prior to shocking. The labelled cells were then directly stressed during the measurement.

The stresses used were a shock of ethanol at 14% v/v and a shock of $H_2O_2$ (5 mM). The viability before and after the shock was monitored by counting CFU/mL in MRS agar plates. The analysis was conducted in triplicate.

*RNA extraction*

The total RNA was extracted from cells harvested by centrifugation, frozen in liquid nitrogen and kept at −80 °C. The extractions were performed using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany). The RNA was treated using the Turbo DNA-free (Life Technologies, USA) and the total acid nucleic concentrations were calculated using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany).

*Real-time quantitative PCR*

GSH+ (5 mM) and GSH- cultures collected in the exponential phase were used to measure the expression of *cfa*, the gene (OEOE_RS05660) which encodes the

cyclopropane fatty acyl phospholipid synthase involved in the cyclization of unsaturated fatty acids (UFA). Reverse transcription and real-time qPCR were performed according to Olguín et al. (2009). Absence of chromosomal DNA contamination was confirmed by real-time PCR. The genes used as internal controls were *dnaG* (DNA primase) and *gyrA* (DNA gyrase) using the primers described by Desroche et al. (2005), with the exception of the *dnaG* reverse primer that was designed for this work (5'-CAG TAT TTT CTG TAT ATT TAC TAT CG-3') in order to improve the amplification in PSU-1. The primers used for *cfa* (forward 5'-TGG TAT TAC ATT GAG CGA GGA G-3' and reverse 5'-CGT CTT TGA GAT CAC GAT AAT CC-3') were those described by Beltramo et al. (2006). The efficiency of amplification was calculated using the formula $E = [10^{(1/-s)} - 1] \times 100$, where $s$ is the slope of the standard curve with several dilutions of cDNA (Beltramo et al., 2006). The threshold value was automatically determined by the instrument. Amplification efficiency was calculated from raw data using LinRegPCR software (Ruijter et al., 2009; Tuomi et al., 2010). The relative expression value was calculated using the Ct values of *dnaG* and *gyrA*. The cultures were carried out in triplicate independent assays and the real-time PCR analysis of each sample was conducted in triplicate.

*Effect of GSH addition on* O. oeni *growth*

The effect of GSH addition on pre-adaptation to wine conditions was evaluated using three selected strains: PSU-1, $217^T$ and 3P2 (selected as explained in Section 3.1). Cells were grown in supplemented MRS, collected at the end of the exponential phase ($OD_{600}$ = 1.6) and inoculated (0.1%) into PC-MRS under different stress conditions: pH 5.0 as the control, pH 4.0, pH 3.4, 6% (v/v) and 12% ethanol. For each assayed stress condition, different concentrations of added GSH were evaluated: 0, 1 and 5 mM. The growth was quantified by measuring the maximum optical density ($OD_{max}$) at 600 nm in a spectrophotometer Polarstar Omega (BMG Labtech).

*Statistics*

The data were statistically processed using the XLSTAT software package from Excel. The differences between the various conditions were determined by analysis of variance (one-way factor ANOVA, Tukey test).

## Results

*GSH uptake ability of* O. oeni *strains*

Although *O. oeni* cannot synthesize glutathione (GSH) due to a lack of the required enzymes, the genome of PSU-1 (NC_008528.1) contains several annotated genes related to this system: three glutaredoxin-like genes (OEOE_RS00645, OEOE_RS00435 and OEOE_RS04215) that utilize GSH as a reductant cofactor; a glutathione reductase (OEOE_RS05740), which recycles the GSSG; a glutathione peroxidase (OEOE_RS04255), which uses GSH to reduce ROS; and one putative transporter CydCD composed of two subunits (OEOE_RS01995 and OEOE_RS01990) (Pophaly et al., 2012).

In this work, it was determined that several *O. oeni* strains had the capability to import GSH from the growth medium. It was also observed that the growth of these strains was improved due to the addition of 1 mM GSH in the medium (data not shown). Several concentrations of added GSH (0.25 - 10 mM) were assayed with PSU-1, observing a maximum positive effect on growth with 5 mM GSH.

This concentration of 5 mM was chosen to carry out the screening of 30 *O. oeni* strains (listed in Table 1) for their ability to uptake GSH from the growth medium. Glutathione, either in the reduced or the oxidized form, was only detected in cells grown with added GSH. In these cells, glutathione was mainly detected in the reduced form (GSH), while oxidized glutathione (GSSG) was in very low concentrations or even not detected. The values of intracellular reduced GSH found are represented in Figure 1. The average GSH uptake was 117.6 nmol/g wet cells, although a wide variability was noted among the different strains. The maximum value was obtained for 3P2 strain, isolated from a Priorat wine (Spain), followed by Alfa®, UB51 and 1P1. It is worth to note that six out of nine of the commercial strains tested exhibited values greater than the average (Figure 1). Based on the screening results, three strains were selected for subsequent assays: 3P2 that exhibits a high GSH import capacity, PSU-1 as a medium one and $217^T$ as a representative of poor import ability.

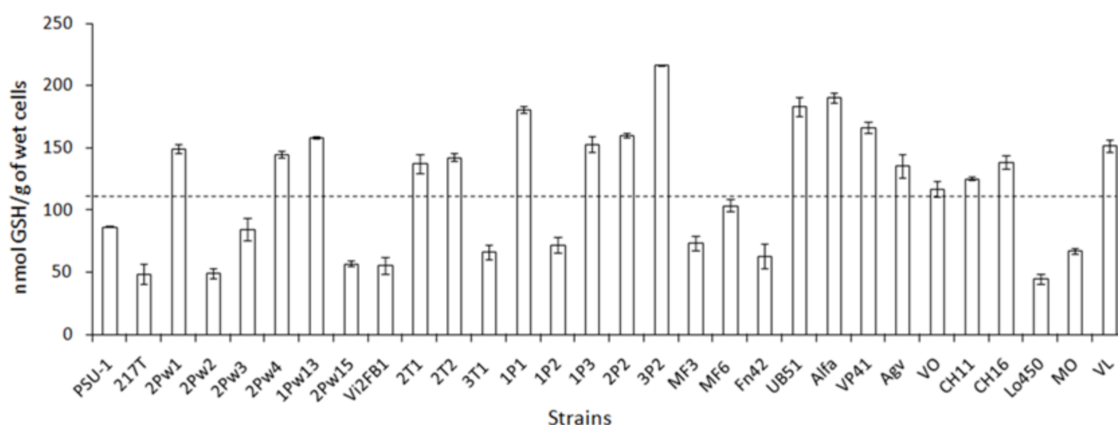



**Figure 1** Content of intracellular reduced GSH in 30 strains of *O. oeni*, expressed as nmol GSH/g wet cells. The dotted line represents the mean of all of the values, and the error bars represent the SD (n = 2). Strains are listed in Table 1.

*Effect of GSH on membrane fatty acids composition*

The changes in the cell membrane fatty acid (FA) content by effect of 5 mM GSH addition were evaluated. The assays were performed using *O. oeni* strains PSU-1, 3P2 and $217^T$ in two cellular phases: exponential and stationary.

The six major components constituting up to 90% of the detected cellular FA pool were myristic acid (C14), palmitic acid (C16:0), oleic acid (C18:1, 9-c), *cis*-vaccenic acid (C18:1 n-7), lactobacillic acid (cycC19:0 n-7) and dihydrosterculic acid (cycC19:0 n-9). The latter two FAs belong to cyclopropane FA (CFA). The molar percentage of the three types of FAs analyzed in the *O .oeni* strains are presented in Table 2. GSH addition produced an increase in CFA molar percentage in all the strains, at both exponential and stationary phases, but the other types of FA changed in a different manner depending on the strain. Both PSU-1 and 3P2 suffered a significant decrease in SFA molar percentage due to GSH addition in the two studied growth phases. However, $217^T$ showed this SFA decrease only at the exponential phase and a very significant decrease in UFA percentage both at exponential and stationary phases, not observed for the other strains. For all the cultures (GSH+ and GSH-) at the stationary phase, it was observed an increase in CFA and a decrease in UFA molar percentage with respect to the same cultures at the exponential phase.

**Table 2** Molar percentage of membrane fatty acid (FA) types and values of the relative expression (RE) of *cfa* at the exponential phase (GSH+5 mM versus GSH-) in three *O. oeni* strains. FA types are unsaturated (UFA), saturated (SFA), and cyclopropane FA (CFA). The statistical analysis was performed by one-way ANOVA ($p < 0.05$; $n = 3 \pm SD$).

| | | **Culture conditions** | | | | |
|---|---|---|---|---|---|---|
| | | **Exponential** | | | **Stationary** | |
| | | **GSH-** | **GSH+5mM** | **RE *cfa* (*gyrA*/*dnaG*)** | **GSH-** | **GSH+5mM** |
| **3P2** | **UFA** | 17.10 ± 0.18 | 17.97 ± 0.33 | | 13.21 ± 0.62 | 13.26 ± 0.16 |
| | **SFA** | 33.41 ± 0.84 | 24.58 ± 0.17* | | 33.49 ± 0.85 | 23.87 ± 0.18* |
| | **UFA/SFA** | 0.51 | 0.73 | | 0.39 | 0.56 |
| | **CFA** | 49.83 ± 0.44 | 57.52 ± 0.19* | 1.7/1.1 | 53.81 ± 0.96 | 62.82 ± 0.07* |
| **PSU-1** | **UFA** | 11.81 ± 0.11 | 12.93 ± 0.25 | | 9.08 ± 0.08 | 9.60 ± 0.24 |
| | **SFA** | 35.09 ± 0.34 | 31.59 ± 0.41* | | 36.25 ± 0.64 | 32.21 ± 0.05* |
| | **UFA/SFA** | 0.32 | 0.40 | | 0.25 | 0.30 |
| | **CFA** | 53.09 ± 0.40 | 55.49 ± 0.59* | 3.0/9.0 | 54.63 ± 0.63 | 58.29 ± 0.17* |
| **217$^T$** | **UFA** | 29.59 ± 1.33 | 17.14 ± 0.22* | | 17.46 ± 1.82 | 11.75 ± 0.07* |
| | **SFA** | 38.76 ± 0.55 | 34.64 ± 0.33* | | 38.36 ± 0.75 | 35.37 ± 1.05 |
| | **UFA/SFA** | 0.76 | 0.49 | | 0.46 | 0.33 |
| | **CFA** | 33.74 ± 0.46 | 48.64 ± 0.58* | 5.7/13.9 | 44.18 ± 1.21 | 51.26 ± 0.58* |

(*) Significant differences due to GSH addition for each FA, strain and cellular phase tested.

Regarding CFA, dihydrosterculic acid and lactobacillic acid were detected under all of the assayed conditions, and these acids represented 39-58% and 3-12% of the total FAs, respectively, depending on the strain (data not shown). The strain 217$^T$ presented the highest values of lactobacillic acid content in both analyzed phases (6.5% in the exponential and 12% in stationary phase). The expression levels of gene *cfa* were measured by real time PCR at the exponential phase comparing GSH- versus GSH+ cultures (Table 2). This gene was over-expressed in PSU-1 and 217$^T$, but no over-expression was detected in 3P2.

*Effect of GSH on cell membrane recovery after stress shock*

The effect of 5 mM GSH addition on the cell membrane recovery after an ethanol 14% (v/v) shock was evaluated in *O. oeni* strains 3P2, PSU-1 and 217$^T$ by measuring the fluorescence anisotropy. The ethanol shock produced a similar effect on the three strains. A drop-off in the initial anisotropy value was observed, indicating an instantaneous increase in membrane fluidity due to ethanol presence. Figure 2A shows

the changes in anisotropy of strain $217^T$, as representative of the behavior observed for all the studied strains. The fluidizing effect induced by ethanol was transitory and the membrane state returned close to its initial state after 30-min shock. However, GSH- cells from exponential phase at the end of the assay measurements presented a percentage of anisotropy above 100%, indicating a slight rigidification of the membrane after the recovery from the ethanol shock. On the contrary, GSH+ cells, both from the exponential and the stationary phase, remained slightly below the initial anisotropy values. This behavior was similar to the observed in GSH- cells from the stationary phase cultures.

The impact of the ethanol shock on cell survival was assessed by determining cell viability 30 min after the ethanol shock (Figure 3). All GSH+ cultures showed a higher cell survival after the ethanol shock in comparison to GSH- cultures.

To compare the stressful effect of ethanol with that of a reference oxidant compound, similar assays were performed adding $H_2O_2$ (5 mM). These assays were carried out with cells from the exponential phase, since the most relevant changes with ethanol were observed in this period of growth. The results (Figure 2B) showed a similar decrease in anisotropy for GSH- cultures after the shock to those observed for ethanol, being the anisotropy drop-off significantly minor in GSH+ cultures. Similar to ethanol assays, no differences were observed among strains. The recovery of the original membrane state after $H_2O_2$ shock was much faster than with ethanol. The cell viability was not significantly affected by $H_2O_2$ shock (data not shown).

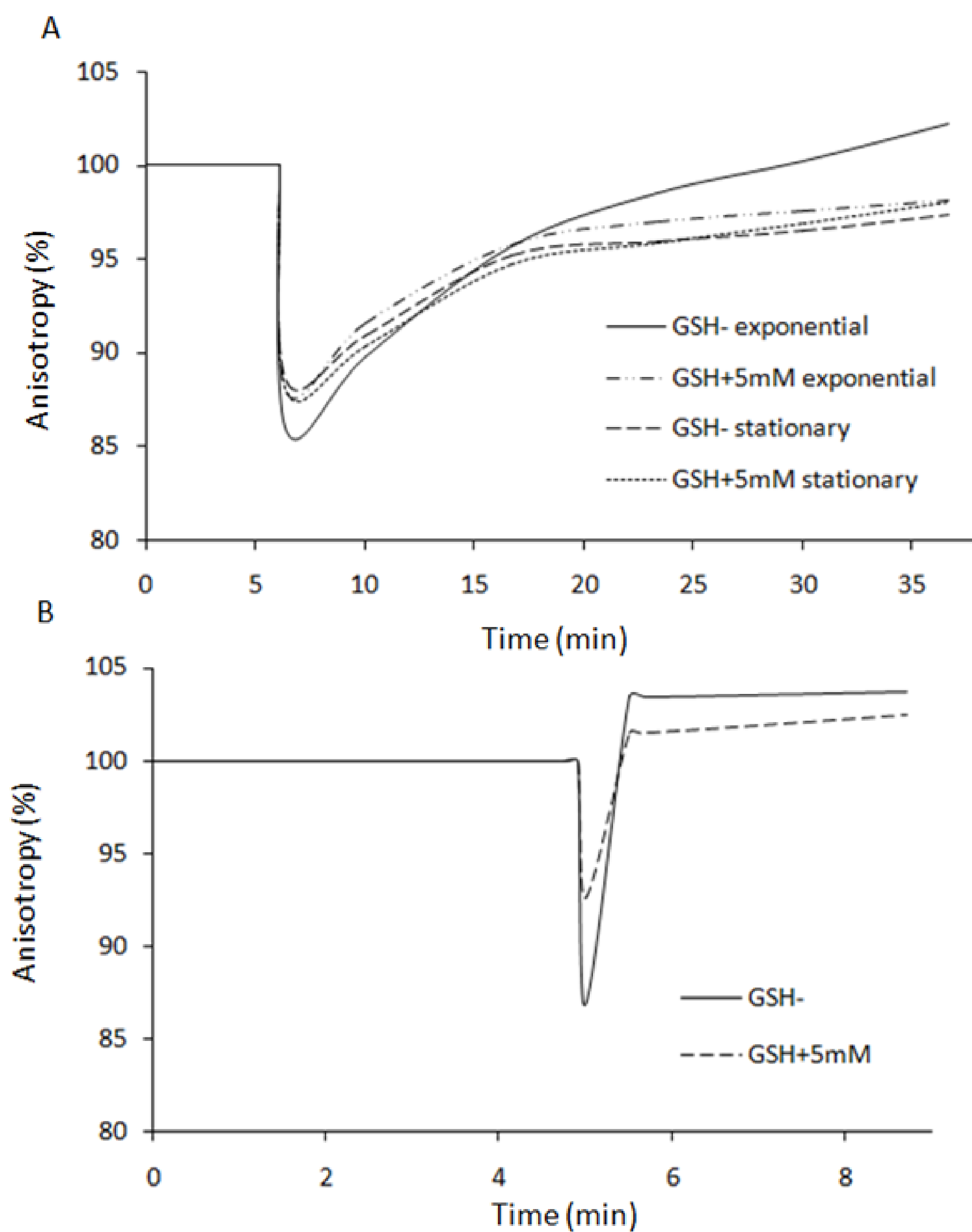



**Figure 2** Evolution of the membrane fluidity (% anisotropy values) in the *O. oeni* 217$^T$ strain: (A) after ethanol shock (14% v/v) in different growth phases, and (B) after peroxide shock (5 mM $H_2O_2$) in the exponential phase.

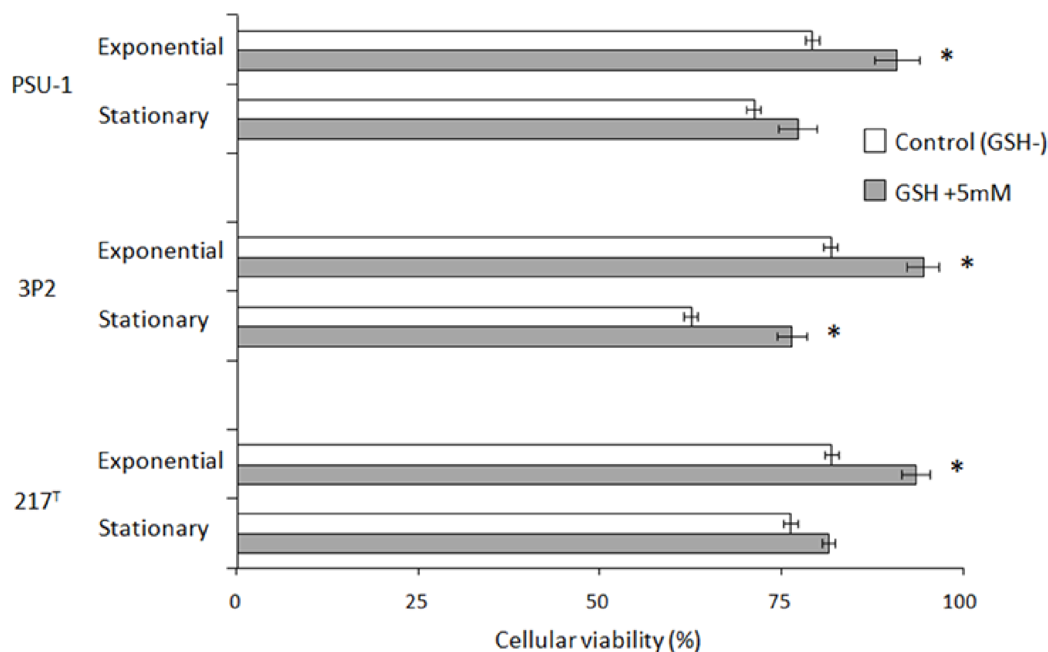



**Figure 3** Cellular viability of three *O. oeni* strains in the exponential and stationary phases after 14% v/v ethanol shock. The viability percentages were calculated as CFU after / CFU before shock, and the error bars represent the SEM (n = 3). * indicates significant differences between GSH+ and GSH- cells (p value < 0.05).

*Effect of GSH on* O. oeni *growth in adaptation to wine conditions*

The potential use of GSH to improve the preparation and adaptation of *O. oeni* strains to be used as MLF starter cultures was evaluated. The three selected strains (PSU-1, 3P2 and $217^T$) were cultivated in PC-MRS medium under different conditions of pH (5.0, 4.0 and 3.4) and ethanol (6% and 12%). Under these five conditions, two different concentrations (1 and 5 mM) of reduced GSH were tested (Figure 4).

It can be observed an increase in $OD_{max}$ in GSH+1mM cultures (Figure 4A) with respect to GSH- cultures under all the tested conditions, with the exception of the strongest inhibitory conditions (12% ethanol and pH 3.4). This increase was statistically significant at pH 5 and pH 4 in all the strains. In the presence of 6% ethanol, only PSU-1 showed a significant increase in $OD_{max}$. Under these conditions, 3P2 grew even better than PSU-1 but there were no differences due to GSH addition.

Faster growth was obtained in the presence of 5 mM of GSH (Figure 4B), even in those cases in which $OD_{max}$ was not significantly different (data not shown). The $OD_{max}$ was

increased over two-fold in GSH+5mM cultures at pH 5 and pH 4 for most strains and at pH 3.4 only for 3P2 strain. The strains PSU-1 and 3P2 also increased significantly their $OD_{max}$ with 6% ethanol. The changes observed in $OD_{max}$ in GSH+5mM cultures were more evident in strains 3P2 and PSU-1 than in $217^T$.

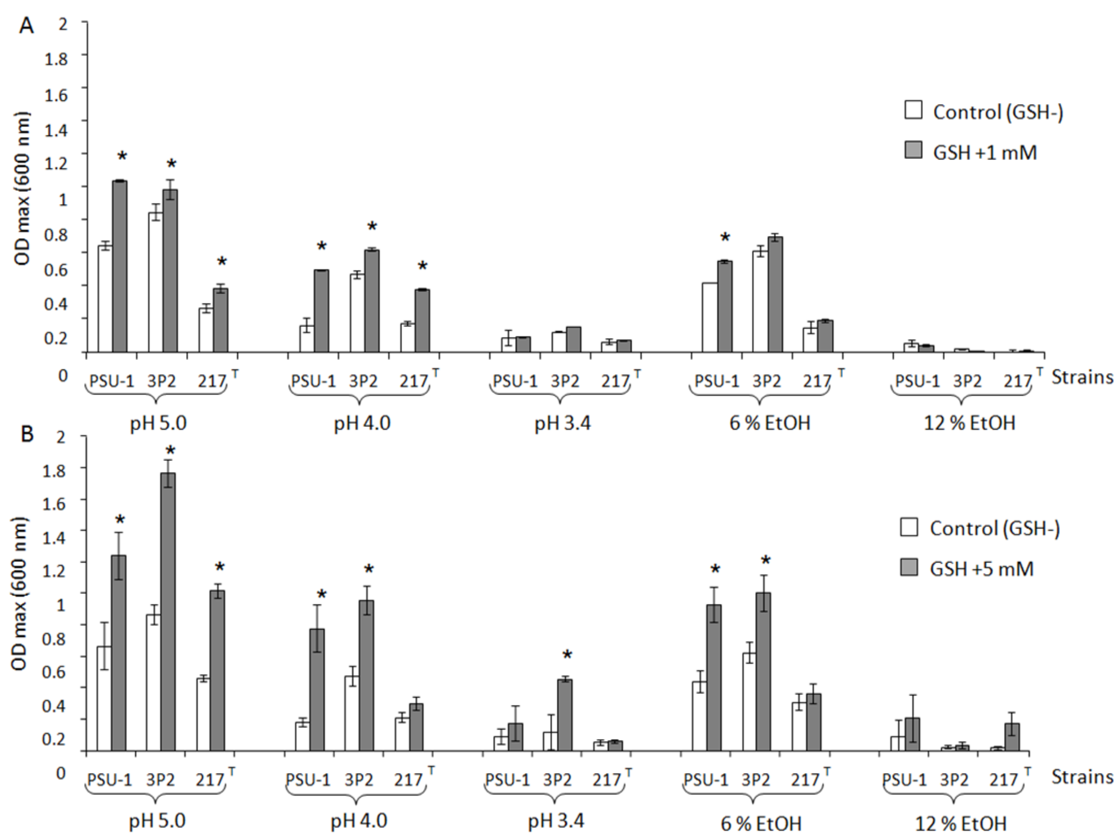



**Figure 4** GSH effect on the growth of three *O. oeni* strains in PC-MRS under wine-like conditions (ethanol content and low pH). The white bars show the $OD_{max}$ values achieved for GSH- cultures, whereas the grey bars show the values obtained for GSH+ cultures, at 1 mM GSH (A) and 5 mM GSH (B). The values are the means of n = 3, and the error bars represent the SD. * indicates significant difference related to the GSH- culture (p value < 0.01).

## Discussion

During MLF, *O. oeni* has to adapt to the adverse conditions of wine. Several molecular mechanisms underlying the stress response of *O. oeni* have been characterized, but little is known regarding the role of GSH in this species. Similar to other Gram-positive bacteria, *O. oeni* cannot synthesize GSH due to a lack of the required genes (Pophaly et al., 2012). However, genomic analyses of PSU-1 revealed the presence of other genes, such as glutaredoxin, glutathione peroxidase and glutathione reductase, which are

involved in GSH use and recycling. It has also been suggested the presence of a membrane transport system, hypothesized as the putative transport protein CydDC (Pophaly et al., 2012). As other LAB species, *O. oeni* published genomes lack γ-glutamyltranspeptidase coding sequences (Pophaly et al., 2012).

Therefore, uptaken GSH cannot be degraded and would be accumulated in the cytoplasm. In this study, the intracellular GSH content of 30 *O. oeni* strains, grown in the presence of 5 mM of this compound, was measured and the results revealed a marked variability in the import capacity among strains. Our analysis showed that some strains presented a four-fold higher import capacity than the other strains. This marked variability in GSH accumulation is in accordance with the differences observed among LAB species and strains (Fernándes & Steele, 1993). Marchand & de Revel (2010) evaluated the GSH content of five Merlot wines before and after MLF and observed that total GSH amount stayed either constant or decreased during MLF. These would be also in accordance with the diverse capability of GSH incorporation observed depending on the *O. oeni* strain.

In this work, three strains were selected as representative models for different degrees of GSH import ability: 3P2, high; PSU-1, moderate; and $217^T$, poor import capacity. Moreover, these strains present a different malolactic activity. Under the same conditions 3P2 performs MLF at a faster rate than the other strains, whereas the $217^T$ strain requires more time and cannot consume all of the available malic acid under harsher conditions (Bordas et al., 2013). However, a correlation between GSH accumulation and MLF rate cannot be established for all of the tested strains.

Because GSH is present in wine at low and very variable concentrations (Kritzinger et al., 2013; Lavigne et al., 2007), the impact of this compound during MLF might be limited. However, GSH could be used to improve the adaptation of MLF starter cultures to wine stress conditions. Until now, there was almost no information concerning the effect of GSH on *O. oeni.* The cytoplasmic membrane plays a key role protecting the cell in response to environmental changes (Šajbidor, 2008; Weber & De Bont 1996). It has been reported that GSH modifies fatty acid (FA) composition of *Lactobacillus sanfranciscensis* cell membrane protecting it from low temperatures (J. Zhang et al., 2010). In this work, significant changes in FA composition were observed in the three *O. oeni* selected strains due to 5 mM GSH addition to growth medium. The strains with

a higher capability of GSH import, 3P2 and PSU-1, showed a significant decrease in SFA molar percentage in GSH+ cultures in both exponential and stationary phases of growth. Notwithstanding, this decrease responded to a change in the relative proportion of the three types of FA and not to a change in SFA concentration, which did not vary. More precisely, UFA and mainly CFA concentrations increased (data not shown) modifying the different FA molar percentages. Because of this, the UFA/SFA ratio was increased in GSH+ cultures with respect to GSH- cultures in 3P2 and PSU-1 but not in $217^T$, the strain with lower GSH import capability. The increase in UFA/SFA due to GSH addition was also described for *Lb. sanfranciscensis* and was associated to the maintenance of membrane fluidity (J. Zhang et al., 2010). It has been described that free radicals can attack directly UFA and initiate lipid peroxidation causing a decrease in membrane fluidity, which alters membrane properties and can disrupt membrane-bound proteins significantly (Cabiscol et al., 2000). In GSH+ cultures of 3P2 and PSU-1, the antioxidant effect of GSH could have accounted for the maintenance of UFA molar percentage, thus the membrane stability.

The three studied strains showed significant increases in CFA molar percentages due to GSH addition in both the exponential and the stationary phase. This increase was also observed in the total amount of CFA (data not shown). In the case of $217^T$, CFA increase could be associated with the decrease of UFA molar percentage in GSH+ cultures, possibly due to UFA cyclization. This was also correlated to the detected over expression of *cfa* in this strain in the exponential phase. In the case of PSU-1 and 3P2, the concentration of UFA increased due to GSH addition (data not shown) although the UFA molar percentage was maintained. This suggests that a part of the UFA pool could have been converted into CFA in these strains. Although the over expression of *cfa* was not detected in 3P2 strain, it has been described a different regulation of this gene depending on the strain (Olguín et al., 2010), suggesting that other post-transcriptional mechanisms could have been involved in the increase of UFA cyclization in 3P2. The increase of CFA molar percentage due to the entrance into the stationary phase, observed here both for GSH+ and GSH- cultures in all strains, has been already described for several LAB (Budin-Verneuil et al., 2005; Grandvalet et al., 2008). In addition, it has been reported that CFAs favor the stress tolerance in some LAB species, including *O. oeni* (Gómez Zavaglia et al., 2000; Grandvalet et al., 2008). Acyl fatty-acid

cyclization reduces membrane fluidity and proton permeability and prevents the penetration of undesirable molecules, helping the cells to adapt to adverse conditions (Chang & Cronan, 1999; Cronan, 2002; Grogan & Cronan, 1997). Our results suggest that GSH intracellular accumulation in the exponential phase of growth leads to an increase in CFA content, maintaining UFA proportion, which would improve cell resistance to environmental changes.

The three evaluated *O. oeni* strains in the exponential phase without added GSH showed a similar behavior in response to 14% ethanol to that previously reported by Chu-Ky et al. (2005) for another *O. oeni* strain under similar conditions. Ethanol shock caused an initial drastic fluidization and, later, a slight rigidification. However, GSH+ cells from the exponential phase showed a lower anisotropy drop-off and maintained lower anisotropy values after ethanol shock compared to GSH- cultures in the three strains. This recovery was similar to that observed for cells harvested at the stationary phase, both GSH+ and GSH-.

Similar results were observed after $H_2O_2$ shock, although in this case the anisotropy drop-off was shorter and cell membranes returned to values close to the initial anisotropy in less time, indicating that ethanol 14% causes more persistent membrane disorganization than 5 mM $H_2O_2$. The anisotropy evolution observed after ethanol and $H_2O_2$ shock was similar for all the strains, despite the differences among these strains in FA composition before the shock, and in the GSH incorporation capability. However, the higher CFA content was a common characteristic in all the culture conditions showing a minor drop-off in the anisotropy values after stress shock. This fact suggest that the increment of CFA, either due to GSH addition or associated to the entrance in the stationary phase (Grandvalet et al., 2008), would counteract the fluidizing effect of ethanol on the membrane. These results indicate that, among other membrane protective agents, such as stress protein Lo18 (Maitre et al., 2014), GSH can contribute to maintain membrane integrity in *O. oeni*. In addition, cells grown with GSH improved their survival after the ethanol shock, mainly in the strain 3P2 from both growth phases. These results indicate that GSH protects *O. oeni* viability against ethanol damage, mainly in strains with higher ability to incorporate GSH. Proteomic studies showed the increased abundance of *O. oeni* GSH reductase in response to ethanol (Margalef-Català et al., 2016; Costantini et al. 2015, Cecconi et al., 2009; Silveira et al., 2004). The

activation of GSH reductase in the presence of ethanol would be indicative of the intracellular use of reduced GSH by *O. oeni*, as our results suggest.

GSH can protect Gram-positive bacteria, such as *Lc. lactis*, against acid, osmotic and oxidative stress (Li et al., 2003; J. Zhang et al., 2007; Y. Zhang et al., 2010). Our results revealed that GSH enhanced biomass production in *O. oeni* strains during adaption to wine stress conditions. The higher growth rate observed in the presence of GSH, even in those cases in which $OD_{max}$ values were not significantly different, suggests that GSH improves *O. oeni* adaptation to stress conditions. At pH 4 and in 6% ethanol, the GSH+ cultures (1 and 5 mM) grew much better than the GSH- cultures, and strain 3P2, which exhibited a higher GSH import capacity, reached the maximum detected $OD_{max}$ in most of the cases. In the presence of 5 mM GSH, the differences between GSH+ and GSH- cultures were more significant, but different results were obtained for $217^T$ strain at pH 4, likely because of the limited ability of this strain for GSH uptake. The improvement of *O. oeni* growth under stress due to GSH incorporation might be related to the protective role of this compound against the oxidation of cell components, such as membrane lipids and membrane associated proteins, already suggested by J. Zhang et al. (2010).

In conclusion, GSH may help to improve the robustness and fitness of *O. oeni* cells against multiple environmental stresses. Future research examining how GSH is transported and how genes and proteins involved in the use of this antioxidant are regulated should improve our understanding. This work represents a step towards the understanding and control of the best pre-inoculation conditions for *O. oeni* strains, which could be useful for the adaptation of MLF starter cultures.

## Acknowledgements

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# CHAPTER IV

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## Genetic and transcriptional study of glutathione metabolism in *Oenococcus oeni*

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## Highlights

- *O. oeni* cannot synthesize GSH but contains genes related to GSH metabolism.
- The addition of GSH modifies the transcription of different GSH related genes.
- The presence of ethanol has a relevant effect on GSH-related gene transcription.
- Transcription of the GSH system depends on the strain capacity of GSH uptake.

## Abstract

Although *Oenococcus oeni* is the main species that is responsible for malolactic fermentation (MLF), harsh wine conditions can limit its performance. Although several mechanisms underlying the response to stress have been studied in this species, little is known regarding the cellular systems that protect against oxidative stress in other bacteria, such as glutathione (GSH). *O. oeni* cannot synthesize GSH but contains several genes related to its utilization. In this study, the relative expression (RE) of the seven genes involved in the GSH redox system found in *O. oeni* PSU-1 (*gshR*, *gpo*, three glutaredoxin-like genes and two subunits of an hypothetical transporter) has been measured. The study was performed using three strains, with each exhibiting a different GSH uptake capacity. The strains were grown in a stress-adaptation medium supplemented with 5 mM GSH and under different adaptation stress conditions (pH 4 and 6% ethanol). The RE showed that only some of these genes, including one for a possible glutaredoxin (OEOE_RS04215) and *cydC* for a subunit of a putative GSH transporter (OEOE_RS1995), responded to the addition of GSH. The presence of ethanol had a relevant effect on gene expression. Among the studied genes, the one for a NrdH-redoxin (OEOE_RS00645) showed a common response to ethanol in the strains, being over-expressed when grown with GSH. In most cases, the transcriptional changes were more evident for the strain with a higher capacity of GSH uptake. Malolactic performance of the three strains after pre-adaptation was evaluated in wine-like medium (12% ethanol and pH 3.4). It was observed that the addition of GSH during pre-adaptation growth had a protective role in the cells exposed to low pH and ethanol, resulting in a quicker MLF.

## Keywords

*Oenococcus oeni* - Malolactic fermentation - Glutathione - Glutaredoxin - NdrH - qPCR

## Introduction

The stress conditions encountered by lactic acid bacteria (LAB) in different niches have led them to evolve to improve their survival capacity under harsh conditions (Mills et al., 2011). LAB have different response systems involved in redox balance that avoid oxidation by ROS (Reactive Oxygen Species) accumulated in the cell. An important compound engaged in oxidative stress protection is glutathione (GSH). This thiol is ubiquitous in eukaryotes and in Gram-negative bacteria. It has been proposed that some Gram-positive organisms possess glutathione synthesis capacity and/or utilization machinery (Fernándes and Steele, 1993). This antioxidant is made-up of three amino acids (Glu-Cys-Gly) and due to the thiol group of cysteine it can reduce ROS. Moreover, GSH has numerous metabolic functions reducing disulphide bonds to cysteine in proteins (Masip et al., 2006). To that end, GSH is oxidized to GSH disulphide (GSSG) by glutaredoxin (Grx) in order to eliminate peroxides or by glutathione peroxidase (Gpo) in order to reduce disulphide bonds. Glutathione reductase (GshR) reduces GSSG into GSH with the use of NAPDH. On the other hand, glutaredoxins utilize the reducing power of GSH to maintain and regulate the cellular redox state and redox-dependent signalling pathways (Lillig et al., 2008). Another GSH-associated function is the glutathione S-transferase (Gst). This large family of enzymes catalyse the transfer of the tripeptide GSH to a xenobiotic substrate for the purpose of detoxification (Vuilleumier and Pagni, 2002). For many organisms the ratio of GSH/GSSG works as a cellular redox hint (Bianucci et al., 2012; Ilyas and Rehman, 2014; Iurlo et al., 2015).

Although *Oenococcus oeni* is the main species responsible for malolactic fermentation (MLF), harsh wine conditions can limit its performance (Versari et al., 1999). Although several mechanisms of response to stress have been studied in this species (Beltramo et al., 2006), little is known regarding the cellular systems that protect against oxidative stress such as GSH. Although *O. oeni* cannot synthesize GSH, it has been previously reported that this bacterium is able to uptake it from the medium (Margalef-Català et al., 2016a). Pophaly et al. (2012) described the presence of several GSH related genes in *O. oeni* and other LAB, according to an *in silico* analysis of the genomes published at that time. However, little is known about the physiological effects of GSH in LAB. In *Lactococcus lactis*, GshR activity has been detected in all of the studied strains, and it

has been shown that GSH protects against oxidative stress during aerobic growth (Li et al., 2003b). The protective role of GSH against acidic stress at pH 2.5 (Zhang et al., 2007) and osmotic stress (Y. Zhang et al., 2010) was also observed in *L. lactis*. In *Lactobacillus sanfranciscensis*, a higher tolerance to oxygen in presence of GSH has been observed and is associated with a higher GshR activity (Jänsch et al., 2007). Regarding *O. oeni*, its metabolism in relation to redox balance has barely been studied. Two proteomic studies have suggested the possible role of GshR in the stress response of *O. oeni* (Silveira et al., 2004; Cecconi et al., 2009). A transcriptional study of the *gshR* gene corroborated the response to wine related stress (Bordas et al., 2015). Therefore, *O. oeni* could use the GSH available in wine. It has been reported that the GSH content in wine depends on the grape variety, but mainly, it depends on the yeast strain responsible for alcoholic fermentation (Lavigne et al., 2007). As in other LAB, GSH could play a protective role against stress in *O. oeni*. This antioxidant compound could be used as an additive to improve the fitness of MLF starter cultures before inoculation. A maximum GSH uptake rate has been observed during exponential growth in *O. oeni* (Margalef-Català et al., 2016a). Therefore, the addition of GSH would be more effective in the preparation of starter cultures than during wine MLF, where cells barely grow.

The main objective of this work was to investigate the genes involved in GSH metabolism in *O. oeni* and to determine whether GSH addition and wine related stress factors can modulate their expression. The potential of GSH addition to improve the preadaptation of *O. oeni* to stress conditions before inoculation was also evaluated. An *in silico* analysis was performed to elucidate the genetic composition associated with GSH utilization in *O. oeni*. The seven GSH related genes found in PSU-1 were analysed by real-time qPCR in three strains under different preadaptation conditions. These strains were chosen due to their differences in GSH uptake capacity (Margalef-Català et al., 2016a). Finally, the malolactic performance of the three strains was evaluated after preadaptation under different stress conditions, with and without added GSH.

## Materials and Methods

*Bacterial strains and culture conditions*

The strains used in this study were as follows: (1) PSU-1 (ATCC BAA-331) whose genome was the first in this genus to be fully sequenced (Mills et al., 2005) and which has a medium capacity to import GSH, (2) the type strain (CECT217=ATCC 23279$^T$), which was named in this study as 217$^T$, and which has a poor GSH import ability, and (3) strain 3P2, which was isolated from a Grenache wine (Bordas et al., 2013) and is the strain with the highest uptake of reduced GSH in a screening of 30 *O. oeni* strains. The stock cultures (kept frozen at -80ºC) were grown in MRSmf broth medium, which consisted of MRS (De Man et al., 1960) supplemented with 5 g/L L-malic acid and 4 g/L fructose at pH 5.0 at a constant 28ºC in a $CO_2$ incubator. Cells were collected at the end of the exponential phase and inoculated (0.1%) into the assay medium as described below. The growth was controlled by measuring the optical density at 600 nm.

*Evaluation of the effect of GSH on gene expression and malolactic fermentation*

The three strains were assayed under different preadaptation conditions using MRS-peptone, which was the same as the MRSmf except the meat extract was substituted by peptone in order to avoid precipitation. The conditions assayed were as follows: pH 5 as the control; pH 5 and 6% ethanol (v/v); pH 4; and pH 4 and 6% ethanol. For each assay condition, there was one culture grown with added GSH (5 mM) and another without GSH. The cells were harvested at the mid-exponential phase. Afterwards, the cells were directly inoculated in flasks containing 50 mL of Wine-Like Medium (WLM) plus 12% (v/v) ethanol, pH 3.4 and maintained at 20ºC. The inoculation and WLM composition were as previously described by Bordas et al. (2013) but diminishing the protein content to 50% and without the addition of L-cysteine in order to increase the harsh conditions of WLM. All of the assays were performed in duplicate and the inoculum growth was monitored by measuring the absorbance ($OD_{600nm}$) and counting plates in modified MRS agar medium. The measurements of L-malic acid consumption were performed with the multianalyser Miura One (TDI SL, Barcelona, Spain) and the enzymatic kit ready to use from the same company in order to determine the end of MLF.

*Gene sequences and primer design*

Nucleotide sequences of *O. oeni* were obtained from the National Center for Biotechnology Information (NCBI). The abbreviations used in this work and the code for the genes from *O. oeni* strain PSU-1 (accession number in NCBI NC_008528) are shown in Table 1. The primer design was performed according to Olguín et al. (2009).

*RNA extraction*

Total RNA was extracted using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) from cells harvested by centrifugation at 4,500 *g* for 15 minutes, washed with cold PBSx1, frozen in liquid nitrogen and kept at −80 °C until analysis. RNA was treated with Turbo DNA-free kit (Life Technologies, USA). The total nucleic acid concentrations were calculated using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany).

*Real-time quantitative PCR*

The reverse transcription and the Real-time qPCR (RT-qPCR) were performed according to Olguín et al. (2009). The absence of chromosomal DNA contamination was confirmed by RT-qPCR. For the selection of the most suitable internal control for qPCR, five housekeeping genes (*dnaG*, *dpoIII*, *gyrA*, *gyrB*, and *ldhD*) were tested. On the basis of primer efficiency and Ct values of different samples, *dnaG* and *gyrA* were chosen for the analysis as they showed the fewest differences between all of the strains in all the samples. The efficiencies of amplifications were calculated using the formula $E = [10^{(1/-s)} - 1] \times 100$, where $s$ is the slope of the standard curve with several dilutions of cDNA (Beltramo et al., 2006). In this study, the threshold value was automatically determined by the instrument. The amplification efficiency was calculated from the raw data using LinRegPCR software (Ruijter et al., 2009; Tuomi et al., 2010). The relative expression value was calculated using the Ct values of *dnaG* and *gyrA* and the final result is the mean of both results. The analysis was made from biologically duplicated independent assays and for each sample technical triplicates were analysed by qPCR.

**Table 3** Gene descriptions and the corresponding primer sequences used in this work.

| Target gene and annotation | Primer | Sequence (5'- 3') | Concentration primer in mix | Amplicon length (bp) | Reference |
|---|---|---|---|---|---|
| OEOE_RS00435 | glut95_F | ATCAAAGCGATCGGCAATAA | 0.25 | 103 | This work |
| glutaredoxin (*rdx1*) | glut95_R | GGTATCCCGACCAAGAATCA | 0.25 | | |
| OEOE_RS00645 | glut139_F | TCGCTCAATTGAAAGCTGAA | 0.25 | 92 | This work |
| glutaredoxin (*rdx*2) | glut139_R | TAAAGCATCGGGACGAAATC | 0.25 | | |
| OEOE_RS04215 | glut80_F | GCAAAAGGTGCTCAACA | 0.25 | 65 | This work |
| ribonucleoside-diphosphate reductase (*rdx3*) | glut80_R | GGCCTAAAACCAGACCA | 0.25 | | |
| OEOE_RS04255 | gper88_F | CAGGAGCGATTGGAAAATCT | 0.25 | 103 | This work |
| glutathione peroxidase (*gpo*) | gper88_R | TTTTCTGGATCGGTCTTTGG | 0.25 | | |
| OEOE_RS01995 | cydC_F | GAGCAACAACGCCTTTCATT | 0.3 | 98 | This work |
| cysteine ABC transporter ATP-binding protein *(cydC)* | cydC_R | CGTTCGGTTAAAGGATCGAG | 0.3 | | |
| OEOE_RS01990 | cydD_F | GAACCAACCGCCCATCTG | 0.15 | 62 | This work |
| cysteine ABC transporter ATP-binding protein (*cydD*) | cydD_R | AGCGGCAGCATCGTTTGT | 0.15 | | |
| OEOE_RS05740 | gsh_F | GGCATTATCACCGAGCTGTT | 0.25 | 106 | Bordas et al. (2015) |
| glutathione reductase *(gshR)* | gsh_R | TCCCGAAGAAGCAAAGAAGA | 0.25 | | |
| OEOE_RS04805 | 1000-F | AATTCGCACGGATTGTTTTC | 0.25 | 103 | Stefanelli (2014) |
| DNA polymerase III subunit alpha (*dpoIII*) | 1000-R | GCGAACCAGCATAGGTCAAT | 0.25 | | |
| OEOE_RS01985 | ldhD-F | GCCGCAGTAAAGAACTTGATG | 0.25 | 102 | Desroche et al. (2005) |
| D-lactate dehydrogenase (*ldhD*) | ldhD-R | TGCCGACAACACCAACTGTTT | 0.25 | | |
| OEOE_RS04780 | dnaG-F | TGTGGACGGAGTGGCAATGT | 0.2 | 127 | Desroche et al. (2005) |
| DNA primase (*dna G*) | dnaG-PSU1-R | CAGTATTTTCTGTATATTTACTATCG | 0.3 | | Margalef-Catala et al. (2016b) |
| OEOE_RS00030 | gyrA-F | CGCCCGACAAACCGCATAAA | 0.3 | 95 | Desroche et al. (2005) |
| DNA gyrase subunit A (*gyrA*) | gyrA-R | CAAGGACTCATAGATTGCCGAA | 0.3 | | |
| OEOE_RS00025 | gyrB-F | GAGGATGTCCGAGAAGGAATTA | 0.2 | 107 | Desroche et al. (2005) |
| DNA gyrase subunit B ( *gyrB*) | gyrB-PSU1-R | ACCTGCTGGGCATCTGTATTG | 0.2 | | Margalef-Catala et al. (2016b) |

### *Bioinformatics tools*

BLAST (Basic Local Alignment Search Tool, NCBI) programs, in particular, BLASTN and BLASTX, were used to evaluate the sequence conservation and the presence or absence of GSH genes and proteins in the different species.

All of the protein sequences used for phylogenetic analysis were obtained from the NCBI database. Each dataset was aligned using Muscle (Edgar, 2004) and was

manually adjusted with Jalview 2.6.1 (Waterhouse et al., 2009). The phylogenetic analyses were performed using the MEGA v6.0 software package (Tamura et al., 2011), and the neighbour-joining method was used for tree reconstruction (Saitou and Nei, 1987). The statistical reliability of phylogenetic tree topology was evaluated by bootstrapping with 1000 replicates (Felsenstein, 1985).

## Results

### *Genomic analysis of the GSH system in* O. oeni

An *in silico* analysis of the available 61 different *O. oeni* genomes (January 2016) revealed the complete absence of the GSH biosynthetic and degradation pathway. However, this analysis showed the presence of other genes related to GSH metabolism in *O. oeni*. Figure 1 shows the putative GSH system in *O. oeni* according to the genes found in the available genomes of this species.

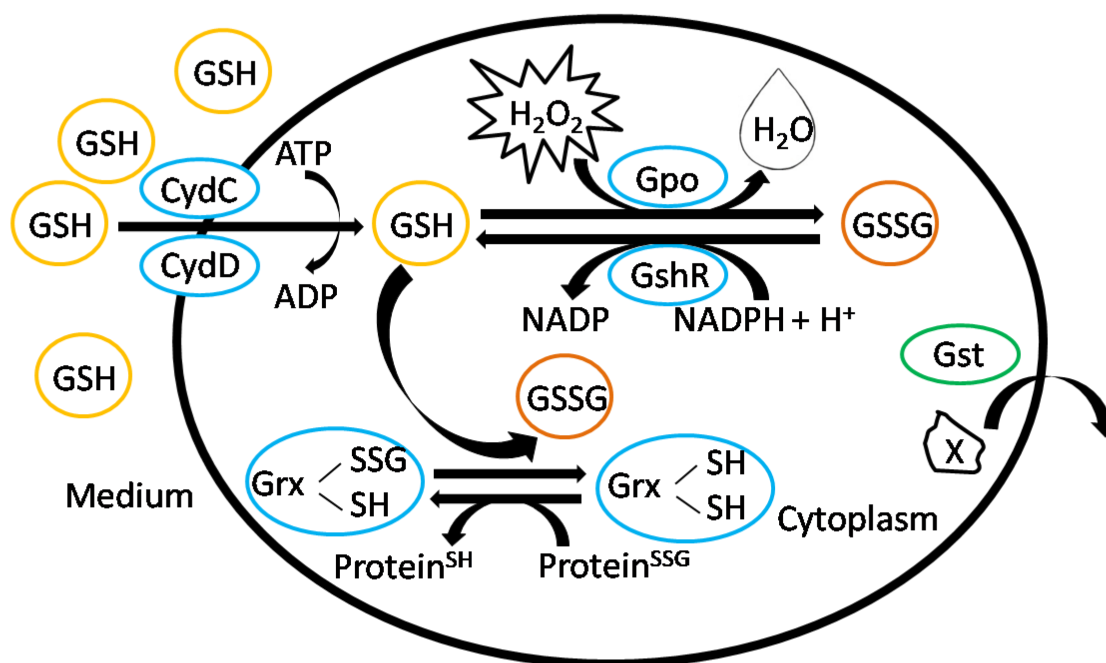


**Figure 1** Schematic diagram showing the putative GSH system and its role in *O. oeni*. Every component is present in some strains from *O. oeni*. The protein abbreviations are: GshR (glutathione reductase), Gpo (glutathione peroxidase), Grx (glutaredoxin), CydC (subunit of transporter CydCD), CydD (subunit of transporter CydCD) and GST (glutathione S-transferase). X is a xenobiotic substrate. Diagram modified from Pophaly et al. (2012).

All of the genes encoding the functions described in Figure 1 are present in all the strains of *O. oeni* annotated in NCBI, except for glutathione S-transferase (*gst*). This GST enzyme catalyses the transfer of the tripeptide GSH to a xenobiotic substrate for the purpose of detoxification, and it is only present in 53% of *O. oeni* strains, none of them being the PSU-1 or the type strain ($217^T$). The GSH system in *O. oeni* contains the gene *gpo* for glutathione peroxidase, which uses GSH for the reduction of hyperoxides (Rotruck et al., 1973) and another, *gshR,* for glutathione reductase (GshR) to recycle GSSG into GSH (Smirnova and Oktyabrsky, 2005). Moreover, one transporter composed of two subunits has been hypothesized by Pophaly et al. (2012), *cydC* and *cydD*. Finally, there are the glutaredoxins (*grx*) which use GSH as a cofactor in order to reduce other compounds.

In Table 2, the three glutaredoxin-like genes annotated in PSU-1 and the information found in NCBI are described. Due to the annotation derived by the automated computational analysis using a gene prediction method (Protein Homology) of the database, certain genes are annotated in a confusing way, and the gene annotation does not always match the protein name. This annotation is the case for the glutaredoxin-like genes found for PSU-1. For this reason, in our study, these genes were named *rdx*, a more general term, as it is not clear that all of them codify for glutaredoxin functions. OEOE_RS00435 (*rdx1*) and OEOE_RS00645 (*rdx2*) are annotated as glutaredoxin but OEOE_RS04215 (*rdx3*) is annotated as a ribonucleoside-diphosphate reductase (RNR). This latter gene was formerly annotated as ribonucleoside-diphosphate reductase class Ib glutaredoxin subunit and renamed as RNR at NCBI database in February 2015. Despite these annotations, the common active site CPXC of a glutaredoxin (Jordan et al., 1997; Røhr et al., 2013) is found only for the latter mentioned protein Rdx3. Conversely, Rdx2 presents the typical motif C[IMV]QC present in NrdH-redoxins and it is, among the three *rdx* genes found in *O. oeni*, the one showing the highest similarity to other LAB protein sequences, mostly annotated as NrdH-redoxins, such those of other *Oenococcus* species, *Fructobacillus* and *Leuconostoc* (Table 2). No common active site has been found for Rdx1 although it is annotated as a glutaredoxin. Instead, it contains the motif SIQS (Figure 2A). However, the motif CLS, which shows a certain similarity to the active site of monothiol Grx in other genera, was found in another position of Rdx1 sequence.

**Table 2** *In silico* analysis of glutaredoxin-like genes and proteins from *O. oeni* PSU-1 using Blastp tools from NCBI against *Lactobacillales* protein sequences (coverage > 93%). A representation of different genera has been taken from each analysis. Analysis made on November 2015.

| Name in this article | Gene/Protein symbol | Gene / Protein annotation | Active site | % *O.oeni* strains[a] | % Protein identity in other LAB spp.[b] | |
|---|---|---|---|---|---|---|
| *rdx1* / Rdx1 | OEOE_RS00435 | Grx / Grx | $C_{19}LS^*$ | 85.2 | 38 | *Lb. sanfranciscensis* (WP_041817636.1) NrdH-redoxin |
| | WP_002818006.1 | | $S_{10}IQS^*$ | | 36 | *O. kitaharae* (WP_007746771.1) NrdH-redoxin |
| | | | | | 36 | *Lb. odoratitofui* (WP_054701968.1) Grx |
| | | | | | 34 | *Lb. paracollinoides* (WP_054711467.1) Grx |
| | | | | | 32 | *S. suis* (WP_044691366.1) NrdH-redoxin |
| *rdx2* / Rdx2 | OEOE_RS00645 | Grx / NrdH-redoxin | $C_{12}VQC$ | 98.4 | 80 | *O. kitaharae* (WP_007744798.1) NrdH-redoxin |
| | WP_002818049.1 | | | | 74 | *O. alcoholitolerans* (KGO32463.1) Grx |
| | | | | | 64 | *L. mesenteroides* (WP_036089595.1) NrdH-redoxin |
| | | | | | 64 | *L. fallax* (WP_010007211.1) NrdH-redoxin |
| | | | | | 63 | *F. tropaeoli* (GAP04633.1) NrdH-redoxin |
| *rdx3* / Rdx3 | OEOE_RS04215 | RNR / NrdH-redoxin | $C_{10}PQC$ | 98.4 | 47 | *O. kitaharae* (WP_007746771.1) NrdH-redoxin |
| | WP_002824237.1 | | | | 46 | *S. suis* (WP_024397333.1) NrdH-redoxin |
| | | | | | 46 | *Lb. mucosae* (WP_039946274.1) NrdH-redoxin |
| | | | | | 42 | *S. castoreus* (WP_027969546.1) NrdH-redoxin |
| | | | | | 40 | *Lc. raffinolactis* (WP_040526073.1) NrdH-redoxin |

Grx: glutaredoxin. RNR: Ribonucleoside-diphosphate reductase. S: *Streptococcus*; Lb: *Lactobacillus*; O: *Oenococcus*; F: *Fructobacillus*; L: *Leuconostoc*; Lc: *Lactococcus*.

[a] % of *Oenococcus oeni* strains sequenced in NCBI containing the referred gene.

[b] Minimum coverage of 80%. *Possible active sites (not assigned by NCBI).

As seen in the protein alignment of Figure 2B, both *Escherichia coli* Grx4 and *Saccharomyces cerevisiae* NP_010383.4, NP_011101.3 and NP_015266.1, described as monothiol Grx (Lillig et al., 2008; Herrero and de la Torre-Ruiz, 2007), contain the active site CGFS. Therefore, in the case of *O. oeni* Rdx1, the possible active site, CLS, would lack one of the intervening amino acid residues. The comparisons at the protein level between Rdx sequences from *O. oeni* and other LAB genera and species (Table 2) revealed that *Oenococcus*, *Lactobacillus, Streptococcus, Leuconostoc*, *Lactococcus* and *Fructobacillus* are the genera presenting the highest identities. Moreover, the identity among the three Rdx proteins was approximately 40% (coverage >83%) in all cases. The alignment of the Rdx proteins found in *O. oeni* with related sequences of the other species and genera presented in Figure 2 helped us to uncover the similarities between other annotated Grx and NrdH-redoxin sequences.

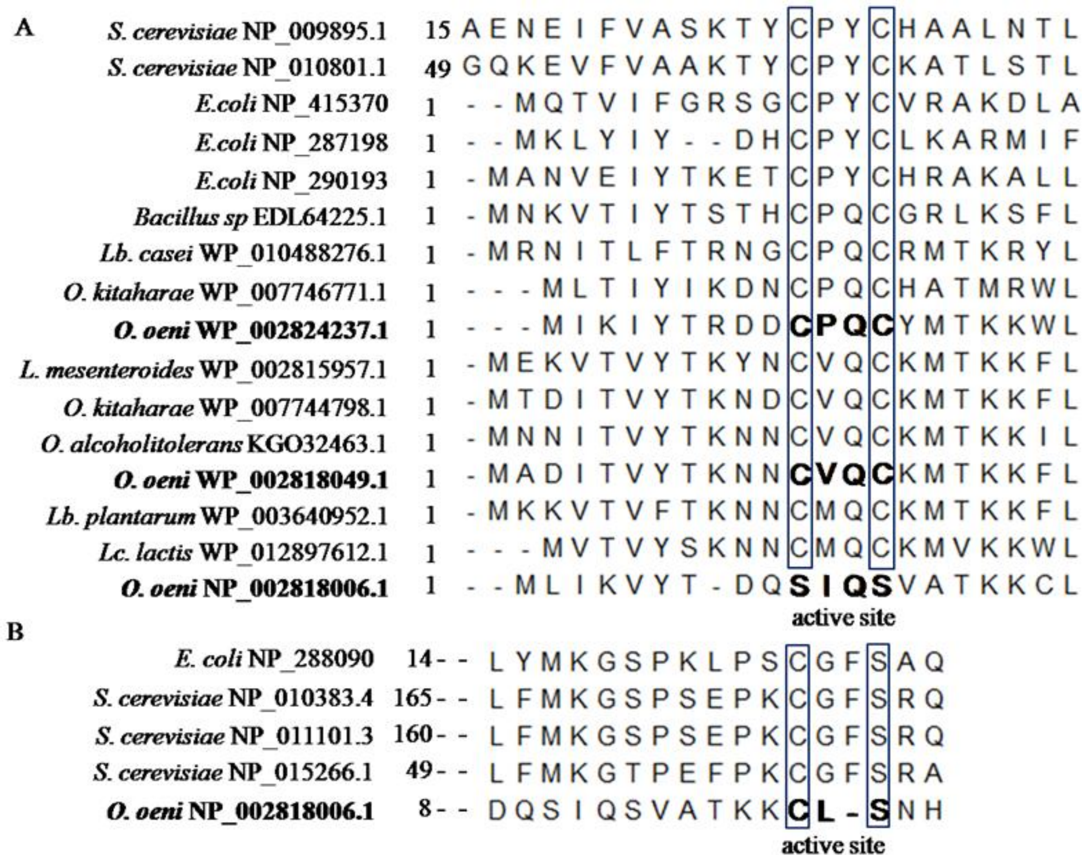


**Figure 2** Alignment of *O. oeni* sequences for the glutaredoxin-like genes included in this study with the sequences of other species and genera indicating the residues involved in the possible active site (A for dithiols Grx and B for monothiol Grx).

Grx: glutaredoxin; S: *Saccharomyces*; Lb: *Lactobacillus*; O: *Oenococcus*; E: *Escherichia*; L: *Leuconostoc*; Lc: *Lactococcus.*

NCBI reference annotation → *E.coli* NP_415370: Grx 1, redox coenzyme for ribonucleotide reductase; *E.coli* NP_287198: Grx 2; *E.coli* NP_290193: Grx; *E.coli* NP_288090: Grx; *S. cerevisiae* NP_009895.1: dithiol Grx1; *S. cerevisiae* NP_010801.1: dithiol Grx2; *S. cerevisiae* NP_010383.4: monothiol Grx3; *S. cerevisiae* NP_011101.3: monothiol Grx4; *S. cerevisiae* NP_015266.1: monothiol Grx5; *Lb. plantarum* WP_003640952.1: NrdH-redoxin; *Lb. casei* WP_010488276.1: NrdH-redoxin; *Lc. lactis* WP_012897612.1: NrdH-redoxin; *L. mesenteroides* WP_002815957.1: NrdH-redoxin; *O. alcoholitolerans* KGO32463.1: Grx; *O. kitaharae* WP_007746771.1: NrdH-redoxin; *O. kitaharae* WP_007744798.1: NrdH-redoxin; *O. oeni* NP_002818006.1: Grx; *O. oeni* NP_002818049.1: NrdH-redoxin; *O. oeni* NP_002824237.1: NrdH-redoxin.

Although the gene *rdx3* is not annotated as a glutaredoxin subunit at present, Rdx3 presents the same active site CPQC as a Grx sequence obtained from *Bacillus* sp. (Røhr et al., 2013), the Gram-positive model, and maintains the residue proline, similar to

other LAB species (Figure 2A). Finally, we could observe that Rdx2 contains the typical NrdH-redoxin active site found in other species (Figure 2A).

Using the protein sequences from Figure 2, we examined their phylogenetic inheritance. The phylogenetic tree (Figure 3) links all of the monothiol protein sequences known from *S. cerevisiae* and *E. coli* together and, in contrast, the dithiol glutaredoxins. The three *O. oeni* Rdx of this study are located in the same large branch of the tree with the sequences from related species and genera, all of them with a dithiol motif. It can be observed that Rdx3 is located close to Rdx1 despite their differences in genetic and protein annotation in the database. Finally, Rdx2, which has the typical NrdH-redoxin active site, is more related to other NrdH-redoxin protein sequences from other LAB species and genera.

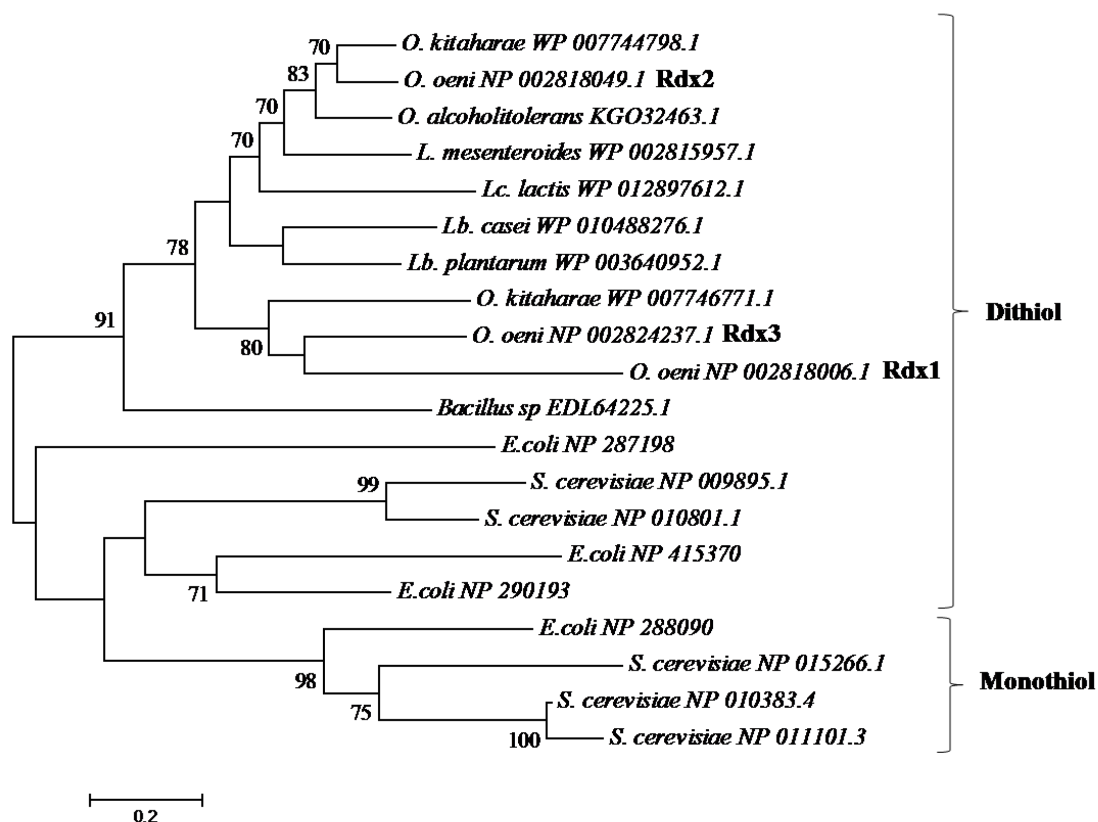


**Figure 3** Phylogenetic tree based on 19 glutaredoxin-like protein sequences obtained from related *O. oeni* species and model microorganisms. The tree was inferred using the Poisson model together with neighbour-joining. Bootstrap values (1000 replicates) are shown as a percentage at the nodes (values below 70% are not shown). The scale bar represents the number of substitutions per site. The tree was constructed using MEGA v5.0 software. See Figure 2 for protein codes access.

*Relative expression of the GSH system*

The relative expression of the seven found genes related to GSH metabolism (Table 1) was studied in three different *O. oeni* strains. The PSU-1 strain was included as a reference because the primers used for RT-qPCR were based on its completely annotated genome. The other two strains were selected according to their ability to uptake GSH from the media. In a previous study it was shown that *O. oeni* strain 3P2 has a high capacity of GSH incorporation while the $217^T$ strain has a low capacity to take up GSH (Margalef-Català et al., 2016a). In that study it was revealed that GSH addition had a positive impact on growth under several stress conditions, such as pH 4 and ethanol 6%, which could be applied to MLF starter adaptation before inoculation. Accordingly, in the current work we studied the relative expression of GSH related genes at pH 4 and ethanol 6% (v/v) using pH 5 and 0% ethanol as the control conditions. Quantitative PCR assays were optimized with regard to primer concentrations (Table 1) and the reaction conditions for each target were optimized in order to assure high amplification specificity and efficiency. As shown in Table 3, under stress conditions, such as pH 4 or/and ethanol 6%, all of the strains could reach a higher maximum optical density at 600 nm ($OD_{max}$) when they were grown with 5 mM of added GSH. In some cases, these differences were more than twice the $OD_{max}$ in the presence of added GSH compared to the absence of GSH, as was the case of PSU-1 for pH5Et6, pH4 and pH4Et6 conditions. The other two strains revealed differences as well, but not as remarkable.

The transcription of all the studied genes could be detected by qPCR in the three strains under all the assayed conditions. In Figures 4, 5 and 6 only the results of those genes that showed significant differences in relative expression (RE) of the compared conditions in each case are presented. As described by Desroche et al. (2005), an over-expression corresponds with a value of RE above two and the down-regulation corresponds to RE values below 0.5. Figure 4 shows the genes that were differentially expressed because of the GSH addition. A transcriptional induction was measured for the subunit transporter *cydC* and *rdx3* genes in strain 3P2 at the condition pH4Et6 (Figure 4C). For PSU-1, a down-regulation was observed for the glutathione peroxidase (*gpo*) gene at pH5, pH4 and pH4Et6 (Figure 4) due to the GSH addition.

**Table 3** Maximal optical density ($OD_{max}$) at 600 nm obtained for the cultures from different conditions of pH and% of ethanol (Et) with 5 mM of added GSH (+) or without GSH (-) during the growth, and the days taken for each strain to carry on the MLF. Values are mean of n = 2.

| Strains | Culture conditions | $OD_{max}$ | Days performing MLF |
|---|---|---|---|
| PSU-1 | pH 5 + | 1.8 | 2 |
| | pH 5 - | 1.8 | 2 |
| | pH 5 Et6 + | 1.7 | 3 |
| | pH 5 Et6 - | 0.6 | 5 |
| | pH 4 + | 1.5 | 3 |
| | pH 4 - | 0.3 | 5 |
| | pH 4 Et6 + | 1.0 | 3 |
| | pH 4 Et6 - | 0.2 | 6 |
| 3P2 | pH 5 + | 1.7 | 3 |
| | pH 5 - | 1.7 | 3 |
| | pH 5 Et6 + | 1.5 | 4 |
| | pH 5 Et6 - | 1.0 | 5 |
| | pH 4 + | 1.7 | 3 |
| | pH 4 - | 1.5 | 4 |
| | pH 4 Et6 + | 0.9 | 5 |
| | pH 4 Et6 - | 0.5 | 5 |
| $217^T$ | pH 5 + | 1.7 | 3 |
| | pH 5 - | 1.7 | 3 |
| | pH 5 Et6 + | 1.4 | 4 |
| | pH 5 Et6 - | 1.0 | 6 |
| | pH 4 + | 1.6 | 3 |
| | pH 4 - | 1.5 | 5 |
| | pH 4 Et6 + | 0.8 | 5 |
| | pH 4 Et6 - | 0.5 | 5 |

Figure 5 shows the effect of pH (between pH5 and pH4). The three *rdx* genes tested were down-regulated (Figure 5BCD) in GSH- cultures in $217^T$. For strain 3P2 and $217^T$ there was a down-regulation of GSH+ for *rdx2*. The gene *gpo* in GSH+ cultures of PSU-1 was over-expressed while for $217^T$, it was down-regulated (Figure 5A). The glutathione reductase (*gshR*) gene was only over-expressed in GSH+ cultures of 3P2 and $217^T$ in the harshest condition (pH4Et6) (Figure 6A). The response to this combined stress was also observed in the increased gene expression of the three *rdx* genes, with all of them being over-expressed in 3P2 GSH+ and only *rdx2* over-expressed for $217^T$ GSH+. Condition pH4Et6, considered to be sub-lethal, represents a significant obstacle to the growth of bacterial cells, as it can be seen by their poor growth in the $OD_{max}$ in Table 3.

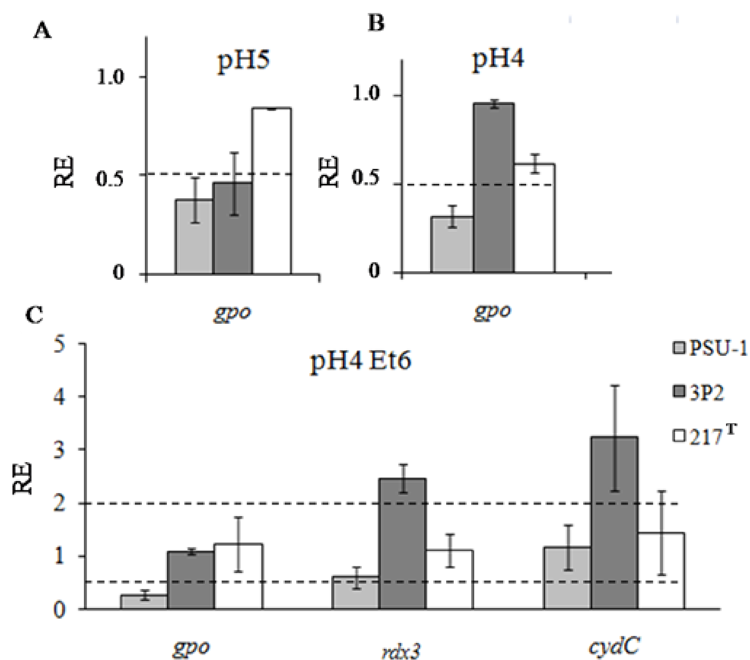


**Figure 4** Effect of the GSH addition on the relative expression (RE) of GSH system genes at pH5, pH4 and pH4Et6 of three *O. oeni* strains. The calibrator condition used was the RE of GSH-. The data shown are mean values between both RE obtained with gene controls *gyrA* and *dnaG* with error bars representing SD values (n = 2).

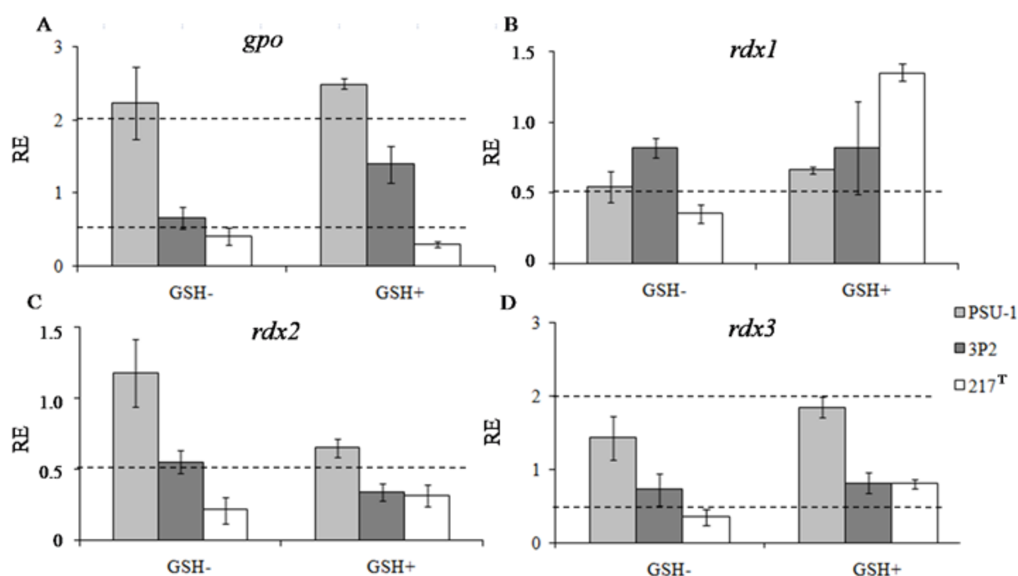


**Figure 5** Effect of pH4 on the relative expression (RE) levels of GSH system genes of three *O. oeni* strains. The calibrator condition used was RE of pH5. The data shown are mean values between both RE obtained with gene controls *gyrA* and *dnaG* with error bars representing SD values (n = 2).

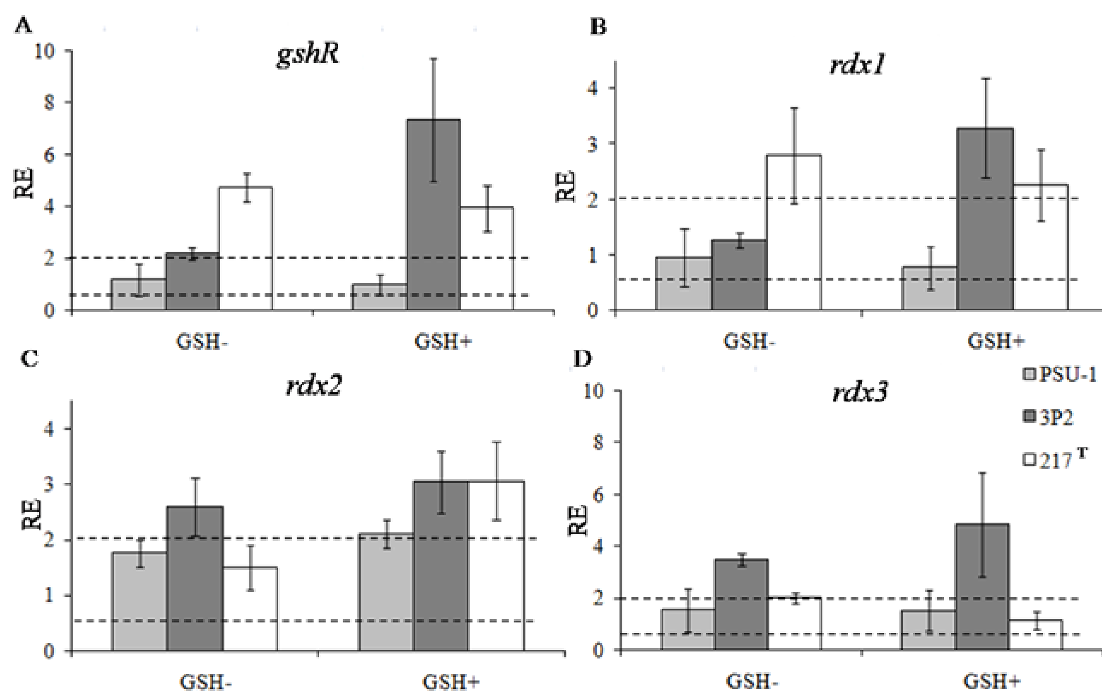


**Figure 6** Effect of 6% ethanol at pH4 on the relative expression (RE) levels system of GSH genes of three *O. oeni* strains. The calibrator condition used was RE of pH4 without ethanol. The data shown are mean values between both RE obtained with gene controls *gyrA* and *dnaG* with error bars representing SD values (n = 2).

### *Malolactic fermentation after preadaptation of the* O. oeni *strains*

After the harvesting of cells at the mid-exponential phase for each preadaptation assay condition, they were inoculated into wine-like media (WLM) at pH 3.4 and 12% ethanol content and maintained at 20ºC to perform MLF. All the inocula could finish the MLF. However, in most of the conditions inoculated with cells previously grown with GSH, MLF was quicker (Table 3). The differences in MLF performance were greater in PSU-1 than in the other strains. For this strain, the MLF was at least two days shorter in the assays in which the inoculum was prepared adding GSH, in the presence of stress (pH4 and/or 6EtOH). However, under the control conditions (pH 5, no ethanol) no differences were observed due to the GSH addition to the inocula. In this case, MLF was the fastest of all of the conditions for all the *O. oeni* strains.

## Discussion

*O. oeni* cannot synthesize GSH because of the lack of GSH synthesis genes. However, in its genome (1.8 Mb), which is relatively small compared to other LAB, such as *Lactobacillus casei* (3.0 Mb), seven GSH pathway genes are annotated: a peroxidase (*gpo*), three glutaredoxin-like genes (named in this study as *rdx*), a reductase (*gshR*) and two subunits for the hypothesized transporter of GSH, *cydC* and *cydD* (Pophaly et al., 2012). All of these genes are present in all the strains annotated in NCBI. It is worth noting that there is a total absence of glutamyl transpeptidase, involved in GSH synthesis and degradation, and the presence of glutathione transferase, *gst*, in half of the strain genomes of *O. oeni*.

The three glutaredoxin-like proteins (Rdx) in PSU-1 are annotated differently in NCBI and also present certain differences in their active site, commonly described for other organisms (Figure 2), such as CXXC for dithiol Grx and CXXS for monothiol Grx (Lillig et al., 2008). Rdx3 has a glutaredoxin motif, CPQC, which is similar to the one present in other species and identical to an annotated glutaredoxin of *Bacillus* sp. Moreover, the gene *rdx3* is the only *rdx* gene tested that presented a transcriptional activation due to the GSH addition in the strain with a higher capacity of GSH uptake, 3P2 (Figure 4). So, despite the current annotation, we suggest that this gene could codify for a glutaredoxin.

Conversely, the *in silico* analysis showed that protein Rdx2 contains an active site similar to that described for NrdH-redoxin, C[VIM]QC (Røhr et al., 2013) found in other species (Table 2). Additionally, the phylogenetic tree and the alignment confirmed the proximity of this sequence to other annotated NrdH-redoxin proteins from different LAB species, including other *Oenococcus* species (Figure 3). NrdH-redoxins are related in amino acid sequence to glutaredoxins but they behave functionally as a thioredoxin. Additionally, they are not reduced by GSH but by thioredoxin reductase and are inactive in the presence of GSH (Jordan et al., 1997). *In vivo* NrdH-redoxin function is still not clear, but its active site is similar to the active site of thioredoxin and glutaredoxin. Therefore, NrdH-redoxin is considered as the functional hydrogen donor for class Ib of ribonucleotide reductases (RNR) (Jordan et al. 1997; Rabinovitch et al., 2010). The RNR are a group of enzymes central to DNA synthesis both during replication and repair. These RNR enzymes are located in the same operon in

*Lactococcus lactis* (nrdHEF) (Jordan et al., 1996, 1997) and in *E. coli* the level of this operon transcription is increased in cells treated with oxidant (Monje-Casas et al., 2001). It is worth noting that the gene *rdx2* is next to several genes annotated as subunits of RNR (OEOE_RS00640, OEOE_RS00650 and OEOE_RS00655), as in *Lactococcus lactis* (Jordan et al., 1996). Additionally, for this gene no difference in expression could be detected due to the GSH addition alone, but the gene responded to ethanol stress (Figure 6C). All of this evidence supports the hypothesis that this gene is codifying for an NrdH-redoxin in PSU-1.

Finally, for Rdx1, although being annotated as Grx, there is no active site prediction in NCBI. We have found two possible motifs, $S_{10}$LQS and $C_{19}$L-S (Figure 2). The former site is located in the amino acid position close to the other Rdx and glutaredoxins from other species, but differs from the typical dithiol Grx active site in its composition because it contains serine instead of cysteine. The latter motif, located in a further position in the sequence, lacks one amino acid when compared to the standard monothiol Grx active site but has the initial cysteine to carry on the sulphur donation. The phylogenetic analysis of the glutaredoxin-like protein sequences indicated that Rdx1 is closer to the other two dithiol Grx found in *O. oeni* PSU-1 than to monothiol Grx described in other microorganisms (Figure 3). Altogether, there is not sufficient information to conclude which would be the active site in Rdx1. Moreover, this protein shows a lower degree of similarity to glutaredoxin-like proteins from other *Oenococcus* and other LAB species (Table 2).

The effect of the GSH addition on expression could be detected for some genes. The down-regulation of *gpo* at pH5 (control condition) in the two strains with higher GSH uptake capacity, 3P2 and PSU-1, could be observed (Figure 4A). The down-regulation of this gene was also observed at pH4 and pH4 Et6 only for PSU-1. The main biological role of glutathione peroxidase (Gpo) is to reduce ROS species using GSH as a cofactor. The down-regulation of this gene due to the GSH addition could indicate a better fitness and less oxidized state of those strains which take up more GSH during growth and therefore have a lower need for Gpo activity. This property is clearer in the control condition in which cells are not exposed to stress. Under stress conditions, the transcriptional response of *gpo* seems to be more variable depending on the strain.

In the strain 3P2, the one showing the highest capacity of GSH uptake, the addition of GSH activated *rdx3* and the *cydC,* subunit of the putative GSH transporter in pH4 Et6 (Figure 4C). This indicates a more active use of GSH in this strain under the most stressful conditions. Despite the fact that a protective role of GSH accumulation in Gram-positive bacteria has been described, little is known concerning the transport mechanisms of GSH in this group of microorganisms (Breidenbach et al., 2013). This mechanism has been characterized only in *Streptococcus mutans,* in which transport is associated with GshT protein and a cysteine ABC importer (Vergauwen et al., 2013). *E. coli* CydDC has been described as a heterodimeric ABC-type transporter that could be involved in either import or export across the cytoplasmic membrane. However, experimental evidence points to its function as a GSH exporter to maintain the redox balance in the cell periplasm (Pittman et al., 2005). The GSH uptake from the medium to the cytoplasm in *E. coli* would be mediated by the ATP-dependent GSH importer YliABCD (Smirnova et al., 2012; Suzuki et al., 2005).

Regarding the effect of low pH, a significant activation was observed for *gpo* in PSU-1, whereas this gene was down-regulated in $217^T$ (Figure 5). As mentioned above, the transcriptional response of *gpo* under stress conditions seems to be strain dependent. The genes *rdx3* and *rdx1* were significantly down-regulated in $217^T$ due to low pH only when grown without the GSH addition, while *rdx2* was down-regulated both with and without GSH added. The presence of ethanol (6% v/v) showed a more significant effect on transcriptional changes than low pH. The glutathione reductase gene, *gshR*, was over-expressed due to the ethanol effect in 3P2 and $217^T$ regardless of the addition of GSH. The activation of *gshR* in these strains is in accordance with previously described results in wine-like medium with 12% ethanol (Bordas et al., 2015). Conversely, the over-expression of *gshR* was not detected in the strain PSU-1. In this way, Su et al. (2015) showed that antioxidant parameters and enzymatic activities of GshR and Gpo were widely dispersed through a collection of 19 *O. oeni* strains, depending on the strain and medium. GshR is the best known enzyme in GSH recycling, catalysing the reduction of glutathione disulphide (GSSG) to the sulfhydryl form glutathione (GSH). Moreover, this protein has been involved in the adaptation to ethanol (8% v/v) (Silveira et al., 2004) and in ethanol-adapted cells (5% v/v) (Cecconi et al., 2009). Silveira et al.

(2004) suggested that GshR played an important role in ethanol adaptation to cope with oxidative damage and maintained the redox balance in *O. oeni*.

The presence of ethanol had a relevant effect on the activation of the three *rdx* genes in 3P2, the strain taking up more GSH, with the exception of only *rdx1* without added GSH (Figure 6). As mentioned above, the functionality and the classification of Rdx1 protein is not clear. Nevertheless, the *rdx1* gene was over-expressed due to the presence of ethanol with the added GSH both for strains 3P2 and $217^T$. This fact suggests that this gene may play a role to combat against the oxidative stress provoked by ethanol. The activation of *rdx2* was detected for the three strains when grown with added GSH and ethanol (Figure 6C). These results indicate that this gene responds to ethanol stress when GSH is available and would confirm its role as NrdH-redoxin, also associated to oxidative stress protection (Monje-Casas et al., 2001). Considering that the three strains evaluated in this study have a different GSH uptake capacity and MLF performance, the *rdx2* activation may be a general response of *O. oeni* strains to ethanol stress when GSH is available. The glutaredoxin-like gene *rdx3* increased its expression due to ethanol only in the strain 3P2, highly active in GSH uptake and that reached even higher *rdx3* expression levels when GSH was added. This could be indicative of GSH usage by this enzyme, which would be in accordance with the hypothesised glutaredoxin identity of this protein.

It had been observed that the addition of GSH could promote the growth of *O. oeni* and slightly accelerate MLF (Rauhut et al., 2004). In our research, some of the inocula coming from the pre-adaptative growth with GSH performed a faster MLF, with certain of them decreasing by half the time. The used wine-like medium presented quite mild conditions, as indicated by shorter fermentation times (2-6 days) than winery MLF times, which can take several weeks (Liu and Gallander, 1983). Consequently, this GHS effect on the pre-adapted inocula could be expected to be more relevant under harsher wine conditions.

In summary, the results of the current study demonstrate that GSH modifies the expression of GSH metabolism genes depending on the GSH uptake capacity of the strain. These changes are more evident under the most stressful conditions, mainly due to the effect of ethanol. Further functional studies would be needed to elucidate the role

of each GSH related gene, however, the current difficulty to obtain stable genetic modifications in *O. oeni* limits this type of research.

To the best of our knowledge, this report describes the first reported genetic and transcriptional study of GSH metabolism in *O. oeni*, illustrating the relevance of this antioxidant in the adaptation of *O. oeni* to wine related stress. The results obtained in this study open the door for future investigations on the redox mechanisms in this species and other related LAB.

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# CHAPTER V

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## Variability in gene content and expression of the thioredoxin system in *Oenococcus oeni*

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UNIVERSITAT ROVIRA I VIRGILI
OENOCOCCUS OENI RESPONSE TO WINE STRESS: ROLE OF GLUTATHIONE AND THIOREDOXIN SYSTEMS
Maria del Mar Margalef Català

## Highlights

- *O.oeni* PSU-1 genome contains three *trxA* genes, a *trxB* gene, and a ferredoxin reductase annotated gene.
- *trxA1* is present in few *O. oeni* strains, and it has an HGT origin from *Lb. plantarum*.
- The expression of the thioredoxin system genes in *O. oeni* is mostly strain-dependent.
- *trxA3* is over-expressed in all strains and plays a crucial role in the ethanol and acidity stress response.

## Abstract

The thioredoxin system protects against oxidative stress through the reversible oxidation of the thioredoxin active center dithiol to a disulphide. The genome of *Oenococcus oeni* PSU-1 contains three thioredoxin genes (*trxA1*, *trxA2*, *trxA3*), one thioredoxin reductase (*trxB*) and one ferredoxin reductase (*fdr*) which, until recently, was annotated as a second thioredoxin reductase. For the first time, the entire thioredoxin system in several *O. oeni* strains isolated from wine has been analysed. Comparisons at the DNA and protein levels have been undertaken between sequences from *O. oeni* and other genera and species, and the genera *Leuconostoc* and *Lactobacillus* were found to present the highest similarities. The gene most frequently absent from a collection of 34 strains and the sequences annotated in the NCBI database was *trxA1*. Moreover, phylogenetic analysis suggested that this gene was horizontally transferred from *Lactobacillus* to *O. oeni*. Strain-dependent expression profiles were determined in rich and in wine-like media. General over-expression was detected after inoculation into wine-like medium, with *trxA3* being the most highly expressed gene. The increased transcriptional levels of the thioredoxin genes are indicative of the crucial role of this system in the *O. oeni* response to wine harsh conditions.

## Keywords

*Oenococcus oeni* - Malolactic Fermentation - Thioredoxin - Stress – qPCR

## Introduction

Thioredoxin (Trx) is a small protein (10-12 kDa) with two cysteine redox-active sites (Holmgren, 1985) that was first discovered in *Escherichia coli* as a natural hydrogen donor for ribonucleotide reductase (Laurent et al., 1964). The catalytic activity of these proteins (containing a -CXXC- motif) and the Trx fold comprised of five β-strands surrounded by four short α-helices is evolutionarily conserved from archaea and bacteria to man (Kumar et al., 2004; Lu and Holmgren, 2014). This protein, together with thioredoxin reductase (TrxR) and the NADPH cofactor, constitute the Trx system, a key antioxidant system in the defence against oxidative stress. Through the Trx system's disulphide reductase activity, this system regulates the protein dithiol/disulphide balance (Mostertz et al., 2008; Lu and Holmgren, 2014).

In addition to the system's conserved role as a high-capacity hydrogen donor system for reductive enzymes, the Trx-TrxR system exhibits other specific functions, including enzymatic reactions (Tsang and Schiff, 1978), $H_2O_2$ reduction (Das and Das, 2000), DNA synthesis (Holmgren, 1989), energy transduction (Lindahl and Florencio, 2003), phage T7 DNA replication (Huber et al., 1987), filamentous phage assembly (Feng et al., 1999), and redox sensing (Kumar et al., 2004).

The diversity of bacterial environments has resulted in the evolution of diverse types of antioxidant systems. The Trx-TrxR system, glutathione-glutathione reductase, and catalase are the major antioxidant systems. While Trx is ubiquitous in bacteria, the glutathione system or catalase are lacking in certain bacteria. Indeed, the glutathione system is absent in some Gram-negative bacteria and in most Gram-positive bacteria, including *Bacillus subtilis*, *Lactococcus lactis* and *Streptococcus aureus* (Scharf et al., 1998; Uziel et al., 2004; Serata et al., 2012; Lu and Holmgren, 2014), while catalase is found in most Gram-negative bacteria.

In *E. coli* the regulation of thioredoxin expression has been studied using different mutants and conditions. The expression of the *trxA* gene has been found to increase during the stationary phase cultures and is inversely proportional to generation time (Lim et al., 2000).

Lactic acid bacteria (LAB) exhibit different patterns of Trx gene expression. Six ORFs for the Trx system have been described in the *Lactobacillus plantarum* WCFS1 genome (NCBI accession PRJNA62911): four genes encoding thioredoxins (*trxA1*, *trxA2*, *trxA3*

and *trxH*), a thioredoxin reductase (*trxB*), and a ferredoxin NAD(P) reductase, which was annotated as thioredoxin reductase until February 2015. These six genes are dispersed throughout the genome and are highly conserved within *Lb. plantarum* strains regardless of ecological niche (Serrano et al., 2007). In contrast, the *Lactobacillus casei* Shirota strain has four thioredoxin genes (*trxA1*, *trxA2*, *trxA3* and *trxA4*) and one putative thioredoxin reductase gene designated *trxB* (Serata et al., 2012). In these studies, the importance of the Trx systems for these bacteria has become clear. In particular, overproduction of *trxB1* (encoding TrxR) improves oxidative stress tolerance in *Lb. plantarum* (Serrano et al., 2007). The generation of *Lb. casei* gene disruption mutants for different *trx* genes revealed that the thioredoxin-thioredoxin reductase system is essential for aerobic growth and is the major system used to maintain the intracellular Trx/TrxR (Serata et al., 2012).

This research focuses on *Oenococcus oeni*, the main LAB responsible for malolactic fermentation (MLF). This fermentation is crucial for many vinification processes (Wibowo et al., 1985; Davis et al., 1988; Kunkee, 1991; Lonvaud-Funel, 1999; Bartowsky, 2005). Little is known regarding the Trx-TrxR system and its function in this species, which can grow in wine - a hostile environment containing ethanol and sulphite and with a low pH (Guzzo et al., 2000). In the first sequenced *O. oeni* genome, that of strain PSU-1, three genes were annotated as thiol-disulphide isomerase (*trxA1*, *trxA2*, *trxA3*) and two genes as thioredoxin reductase (*trxB1*, *trxB2*). Nevertheless, the *trxB2* gene was recently designated as a ferredoxin NADPH reductase (OEOE_RS00770).

Studies of oxidative stress in *O. oeni* have described the over-expression of one of the *trxA* genes (OEOE_RS08215) in response to hydrogen peroxide and heat shock (Jobin et al., 1999; Guzzo et al., 2000), as reported for *B. subtilis* (Mostertz et al., 2008). Another *trxA* gene (OEOE_RS01675) has been described as a genetic marker for stress resistance (Renouf et al., 2008).

The purpose of this research was to study the complete thioredoxin system in *O. oeni*. To this end, the presence of the *trx* genes was assessed experimentally in a collection of *O. oeni* strains and *in silico* using published genomes. A phylogenetic study of protein sequences was carried out in order to understand the relationship of *O. oeni* with other LAB species. Finally, to evaluate the role of each *trx* gene in the stress response, the

transcriptional responses of the *trx* genes were studied under optimal growth conditions in rich medium and during MLF in a wine-like medium.

## Materials and Methods

Oenococcus oeni *strains and culture conditions*

Most strains used in this study were isolated from 21 red wines of southern Catalonia of the 2008 vintage, from two different appellations of the origin DOQ Priorat and DO Tarragona. Moreover, five strains were isolated from Italian wines. Other strains used were obtained from commercial companies, and one was the type strain. All strains are described in Table 1.

Stock cultures were thawed from -80ºC and grown in modified MRS broth medium (MRSmf), which is MRS (De Man et al., 1960; Difco Laboratories, Detroit, MI, USA) supplemented with 4 g $l^{-1}$ L-malic acid and 5 g $l^{-1}$ fructose at pH 5.0, at 28ºC in a $CO_2$ incubator. Cells were collected at the end of the exponential phase and inoculated (0.1%) into MRSmf for DNA extraction or to act as the inoculum of an MLF.

*Malolactic fermentation (MLF) in wine-like medium*

Subcultures of the *O. oeni* strains were directly inoculated into flasks containing 100 ml of wine-like medium (WLM) containing 12% (v/v) ethanol at pH 3.4. Inoculation was performed as previously described (Bordas et al., 2013), but we reduced the protein content by 50% and did not add L-cysteine to increase the harshness of the WLM. All assays were performed in duplicate, and inoculum growth was monitored by measuring the absorbance ($OD_{600nm}$) and counting colonies grown on plates made with MRSmf agar (20 g $l^{-1}$ agar). One hour after inoculation in WLM, the cells were collected (1 h point), and the L-malic acid content was measured, then MLF was monitored at periodic intervals, including the midpoint (i.e., 50% consumption of L-malic acid) and end of MLF. Other cell samples were collected at four experimental points (0 h, 1 h, mid-MLF, and end-MLF). L-malic acid consumption was measured using the multianalyser Miura One (I.S.E. S.r.l, Guidonia, Italy) and an enzymatic kit (TDI SL, Barcelona, Spain).

**Table 1** The presence (+) or the absence (-) of thioredoxin genes in *O. oeni* strains as determined by PCR.

| Strains | Genes | | | | | Source[a] | Isolation from wine, place(reference)[b] |
|---|---|---|---|---|---|---|---|
| | *trxA1* | *trxA2* | *trxA3* | *trxB* | *fdr* | | |
| PSU-1 (=ATCC BAA-331) | + | + | + | + | + | ATCC | Red wine, Pennsylvania State University, US |
| CECT 217$^T$ (=ATCC 23279) | - | + | + | + | + | CECT | Red wine, Bordeaux, F |
| ATCC BAA-1163 | - | + | + | + | + | UBo & ISVV | Red wine, Bordeaux, F |
| 1T1 | - | + | + | + | + | URV | Grenache wine, Tarragona, S (Bordas et al., 2013) |
| 2T1 | - | + | + | + | + | URV | " |
| 2T2 | - | + | + | + | + | URV | " |
| 3T1 | - | + | + | + | + | URV | " |
| 3T7 | - | + | + | + | + | URV | " |
| 5T8 | - | + | + | + | + | URV | " |
| 1P1 | + | + | + | + | + | URV | Grenache wine, Priorat, S (Bordas et al., 2013) |
| 1P2 | + | + | + | + | + | URV | " |
| 1P3 | + | + | + | + | + | URV | " |
| 2P2 | + | + | + | + | + | URV | Cabernet Sauvignon wine, Priorat, S (Bordas et al., 2013) |
| 2P10 | - | + | + | + | + | URV | " |
| 3P1 | - | + | + | + | + | URV | " |
| 3P2 | - | + | + | + | + | URV | " |
| 8P4 | - | + | + | + | + | URV | " |
| 8P7 | - | + | + | + | + | URV | " |
| 10P2 | - | + | + | + | + | URV | Grenache wine, Priorat, S (Bordas et al., 2013) |
| 10P4 | - | + | + | + | + | URV | " |
| 13P1 | - | + | + | + | + | URV | Carignan wine, Priorat, S (Bordas et al., 2013) |
| 13P5 | - | + | + | + | + | URV | " |
| 18P7 | - | + | + | + | + | URV | Grenache wine, Priorat, S (Bordas et al., 2013) |
| 19P2 | - | + | + | + | + | URV | Carignan wine, Priorat, S (Bordas et al., 2013) |
| Fn42 | + | + | + | + | + | URV | Monastrell wine, Priorat, S (Masqué and Bordons, 1996) |
| Vi7 | - | + | + | + | + | UVe | Amarone wine Valpolicella, I, (Malacrinò et al., 2003) |
| Al2 | - | + | + | + | + | UVe | " |
| U1 | - | + | + | + | + | UVe | Amarone wine, Valpolicella, I (Zapparoli et al., 2004) |
| Rz81 | - | + | + | + | + | UVe | Bardolino wine, I (Malacrinò et al., 2003) |
| KM334 | - | + | + | + | + | UVe | Chardonnay wine, Franciacorta, I (Torriani et al., 2011) |
| Enoferm Alfa® (Alfa) | - | + | + | + | + | LA (commercial) | Not specified |
| Lalvin VP41® (VP41) | - | + | + | + | + | LA (commercial) | Not specified |
| Viniferm OE 104 (Agv) | - | + | + | + | + | AG (commercial) | Not specified |
| Viniflora® CH11 (CH11) | + | + | + | + | + | CH (commercial) | Not specified |

[a] ATCC: American Type Culture Collection; CECT: Colección Española de Cultivos Tipo, València, Spain; UBo: Université de Bourgogne, Dijon, France; ISVV: Institut des Sciences de la Vigne et du Vin, Université de Bordeaux, France; URV: Universitat Rovira i Virgili, Tarragona, Spain; UVe: Università di Verona, Italy; LA: Lallemand Inc., Montreal, Canada; AG: Agrovin, Alcázar de San Juan, Spain; CH: Chr. Hansen A/S, Hørsholm, Denmark.

[b] US: United States of America; F: France; S: Spain; I: Italy.

*Gene sequences*

O. oeni nucleotide sequences were obtained from the National Center for Biotechnology Information (NCBI). The abbreviations used in this research and the codes of genes from the *O. oeni* strain PSU-1 (accession number in NCBI NC_008528) are shown in Table 2.

*DNA and RNA extraction*

Harvested cells were incubated with lysozyme (50 mg $ml^{-1}$) for 30 min at 37ºC, after which genomic DNA was extracted using a High Pure PCR template kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Total RNA extractions were performed on cells harvested by centrifugation, frozen in liquid nitrogen and stored at -80°C. The extractions were performed using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany) and the RNA was treated with Turbo DNA-free (Life Technologies, USA). Total nucleic acid concentrations were calculated using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany).

*Thioredoxin gene detection by specific PCRs*

To assess the presence of thioredoxin genes in the genomes of *O. oeni* strains, each pair of primers was tested by PCR (Table 2). PCR amplifications were conducted with a BIOTAQ PCR Kit (Bioline, UK) in a final volume of 20 µl, containing 2 µl 10x buffer, 0.2 mM dNTPs, 3.5 mM MgCl2, 0.2 µM of each primer, 0.025 U $\mu l^{-1}$ DNA polymerase, and approximately 10 ng of DNA. The amplifications were performed in a Thermal Cycler 2720 (Applied Biosystems, USA) with the following cycling conditions: 5 min at 94°C; 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 30 s; and 72°C for 7 min. The amplification products were resolved by electrophoresis on 1.4% (w/v) agarose gels run at 100 V for 2 h 45 min and stained with ethidium bromide. DNA molecular weight markers II and VI (proportion 1:2) from Roche Diagnostics (Basel, Switzerland) were used as references.

**Table 2** Gene descriptions and the corresponding primer sequences used in this research. Primers used for gene detection are in bold.

| Target gene and annotation | Primer | Sequence (5'- 3') | Amplicon length (bp) | Reference |
|---|---|---|---|---|
| OEOE_RS01675 | **M12-F** | GTTTCTGAAGACCCGCTTA | 217 | Renouf et al. (2008) |
| Thiol-disulfide isomerase | **M12-R** | TGATGCCCCCTTCGTAAT | | |
| TrxA1 | trxA0350-F | TTGACCATGACCAAGTACG | 126 | This work |
| | trxA0350-R | CGGCTTCAAAATCAGGCTTA | | |
| OEOE_RS07835 | **SEQ1625_F** | TAAAAATTTATAGAGTTGTTTGATACC | 346 | This work |
| Thiol-disulfide isomerase | **SEQ1625_R** | GTAAGTATGAATTTCTATGAACC | | |
| *trxA2* | trxA1625-F | TGGCAGTCTTTGAAACCTGA | 105 | This work |
| | trxA1625-R | CCAAGGGTCGCAATTTAATG | | |
| OEOE_RS08215 | **SEQ1702_F** | GAATTATATGGCAATCTCAG | 330 | This work |
| Thioredoxin | **SEQ1702_R** | CATAAACTTTATTTTGTGTATTTATC | | |
| *trxA3* | trxA1702-F | GCCACTTGGTGTACCCCTTGT | 120 | This work |
| | trxA1702-R | TCCATTTGCCGTTTCCTGGTTT | | |
| OEOE_RS00770 | **SEQ0163_F** | GTCGATTAATGGATAATGAAATA | 1053 | This work |
| Ferredoxin-NADP reductase | **SEQ0163_R** | CTTGCTAAGAGTTGTATCTGCT | | |
| Fdr | trxB0163-F | AGCGAAGTTGCCGATAAAGA | 115 | This work |
| | trxB0163-R | TATCACGCCGATGAATCAAA | | |
| OEOE_RS02695 | **SEQ0566_F** | GTAGGAGATTTTATGACACAGAA | 961 | This work |
| Thioredoxin reductase | **SEQ0566_R** | GATTATATCAGGCTTCTATCTCC | | |
| *trxB2* | trxB0566-F | ATGCCAGCTCAACTCGTTTT | 139 | This work |
| | trxB0566-R | GTCGCTCCGCTAGCAACTAT | | |
| OEOE_RS04805 | 1000-F | AATTCGCACGGATTGTTTTC | 103 | Stefanelli (2014) |
| DNA polymerase III subunit alpha | 1000-R | GCGAACCAGCATAGGTCAAT | | |
| *dpoIII* | | | | |
| OEOE_RS01985 | ldhD-F | GCCGCAGTAAAGAACTTGATG | 102 | Desroche et al. (2005) |
| D-lactate dehydrogenase | ldhD-R | TGCCGACAACACCAACTGTTT | | |
| *ldhD* | | | | |
| OEOE_RS04780 | dnaG-F | TGTGGACGGAGTGGCAATGT | 127 | Desroche et al. (2005) |
| DNA primase | dnaG-PSU1-R | CAGTATTTTCTGTATATTTACTATCG | | This work |
| *dna G* | | | | |
| OEOE_RS00030 | gyrA-F | CGCCCGACAAACCGCATAAA | 95 | Desroche et al. (2005) |
| DNA gyrase subunit A | gyrA-R | CAAGGACTCATAGATTGCCGAA | | |
| *gyrA* | | | | |
| OEOE_RS00025 | gyrB-F | GAGGATGTCCGAGAAGGAATTA | 107 | Desroche et al. (2005) |
| DNA gyrase subunit B | gyrB-PSU1-R | ACCTGCTGGGCATCTGTATTG | | This work |
| *gyrB* | | | | |

### *Real time quantitative PCR*

Reverse transcription, real-time quantitative PCR (qPCR) and primer design were performed according to Olguín et al. (2009). The absence of chromosomal DNA contamination was confirmed by qPCR. The primers designed for this study are listed in

Table 2. To select the most suitable internal control for Reverse Transcription qPCR (RT-qPCR), five genes (*dnaG*, *dpoIII*, *gyrA*, *gyrB*, and *ldhD*) were tested. Based on primer efficiency and Ct values at the four time points, *dpoIII* was chosen for analysis because it varied least between all strains in all of the samples. The amplification efficiency was calculated using the formula $E = [10^{(1/-s)} - 1] \times 100$, where $s$ is the slope of standard curve with several dilutions of cDNA (Beltramo et al., 2006). The threshold value was automatically determined by the instrument, and the amplification efficiency was calculated from raw data using LinRegPCR software (Ruijter et al., 2009; Tuomi et al., 2010). The relative expression ratio value was calculated for fermentation time points compared to the first sampling time point (0 h) according to the Pfaffl equation (Pfaffl, 2001). These results demonstrate the level of cDNA samples in duplicated assays beginning from independent cultures. Each qPCR was performed in triplicate.

To analyse the expression of each gene during growth in MRSmf, the strain used for normalization was the strain with the median expression value for each gene.

*Bioinformatics tools*

BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi) programs - in particular, BLASTN and BLASTX - were used to evaluate sequence conservation and the presence or absence of thioredoxin and thioredoxin reductase genes in different species.

*Phylogenetic tree analysis*

All protein sequences used for phylogenetic analysis were obtained from the NCBI database. Each dataset was aligned using Muscle (Edgar, 2004) and was manually adjusted with Jalview 2.6.1 (Waterhouse et al., 2009). Phylogenetic analyses were performed using the MEGA v6.0 software package (Tamura et al., 2011), and the neighbour-joining method was used for tree reconstruction (Saitou and Nei, 1987). The statistical reliability of phylogenetic tree topology was evaluated by bootstrapping with 1000 replicates (Felsenstein, 1985). Bootstrap values under 70 were not shown on the trees. To infer reference phylogenetic trees, 16S rRNA gene sequences were downloaded from each strain genome page in the NCBI database.

## Results

*In silico analysis of the* O. oeni *PSU-1 thioredoxin system*

On *O. oeni* PSU-1 strain genome sequence, three different genes encoding thioredoxins (named in this study as *trxA1*, *trxA2* and *trxA3,* corresponding to OEOE_RS01675, OEOE_RS07835, and OEOE_RS08215 genes, respectively), one thioredoxin reductase (OEOE_RS00770, named *trxB* in this study*),* and a gene formerly annotated as thioredoxin reductase (*trxB2*) - updated in February 2015 in NCBI as ferredoxin NADPH reductase (OEOE_RS02695, named in this study *fdr*) – are annotated. These genes are dispersed throughout the PSU-1 genome, and only the third quadrant does not contain any of these genes (data not shown). Gene OEOE_RS02695 was included in this study because it was initially annotated as thioredoxin reductase in PSU-1. Although there is no evidence of a relation between these two enzymatic activities (i.e., Trx reductase and Ferredoxin NADPH reductase) in *O. oeni*, we consider that the results obtained for this gene, also involved in redox metabolism, can be of interest and are therefore reported.

The protein sequences encoded by the three *trxA* genes in *O. oeni* PSU-1, as well as those encoded by the *trxB* and *fdr* genes, were compared. The three TrxA proteins share an amino acid identity of 29-38% (coverage of 56-84%), whereas TrxB and Fdr show an identity of 25% (coverage of 81%). Similar low values were reported for *Lb. plantarum* and *Lb. casei* (Serrano et al., 2007; Serata et al., 2012). It is worth noting that each of the thioredoxin proteins in PSU-1 has a different amino acid sequence at the catalytic site. While the two characteristic cysteine residues are conserved, each site varies by two amino acids: CPPC in TrxA1, CGDC in TrxA2 and CTPC in TrxA3.

Bioinformatics tools were used to investigate the evolutionary relationship of the *trx* genes in *O. oeni* PSU-1 with those of related LAB. Only strains matching at least 75% with PSU-1 sequence and showing more than 70% similarity are represented in Table 3. All genes except for *trxA1* were present in the other two *Oenococcus* species (*O. kitaharae* and *O. alcoholitolerans*), and similarity was approximately 70% (coverage 90-100%). The gene *trxA1* was found with high similarity in only seven species of *Lactobacillus* (over 80% for most species). The similarities found for the other *trxA* genes were in most of the cases lower (between 70% and 77%) and were present in the genera *Oenococcus* and *Leuconostoc* for *trxA2* and *Lactobacillus* for *trxA3*. As

expected, the highest similarities were found in species that are more closely phylogenetically related to *O. oeni*.

Because the *O. oeni trxA1* gene was not found in other genera, we examined the phylogenetic inheritance of all genes included in this study. The protein sequences encoded by the five different thioredoxins were used to generate phylogenetic trees (Figures 1 and 2) because protein sequences are more conserved between genera than DNA sequences. As can be seen in Figure 1 and 2, each protein was located in a different subtree with a bootstrap value of 100 for all the proteins with the exception of TrxA3 subtree, that showed a value of 75. Figure 1 shows three different subtrees generated based on the different thioredoxin protein sequences. *O. oeni* TrxA1 is located between different species of *Lactobacillus,* while TrxA2 and TrxA3 have their own subtree within the genus *Oenococcus*. The 16S rRNA tree composed for DNA sequences from different LAB species was used as the reference tree (Figure S1). The sequences obtained for thioredoxin reductases compose another tree, shown in Figure 2, with two subtrees representing the vertical inheritance of these proteins for each genus, similar to that of the 16S tree.

**Table 3** Thioredoxin gene similarity between different bacterial species and *O. oeni* strain PSU-1. This analysis was performed in January 2015.

| Genes | Species | Strains Accession number | Similarity |
|---|---|---|---|
| *trxA1* | *Lactobacillus acidipiscis* | BACS01000178.1 | 99.0 |
| | *Lb. composti* | BAMK01000023.1 | 98.0 |
| | *Lb. plantarum* | CP004082.1, CP001617.1, CP006033.1, CP005942.1, AL935263.2, CP002222.1, AVFJ01000024.1, ASJE01000208.1, JMEL01000053.1, JOJT01000020.1, JIBX01000028.1, AWTS01000050.1, AGRI01000001.1, ACGZ02000031.1, AYTU01000067.1, BALV01000005.1, JHWA01000027.1, AXDQ01000135.1, AVFI01000068.1, AUTE01000057.1, APFP01000028.1, AWOY01000058.1, AVAI01000136.1, AVAQ01000049.1 | 97.3 |
| | *Lb. fabifermentans* | AYGX01000503.1, AWWK01000075.1 | 84.0 |
| | *Lb. rhamnosus* | CP003094.1, ACIZ01000036.1 | 82.0 |
| | *Lb. pentosus* | FR874854.1, FR871824.1, AKAO01000083.1 | 81.3 |
| | *Lb. parabrevis* | ARTH01000079.1 | 71.0 |
| *trxA2* | *Oenococcus kitaharae* | AFVZ01000001.1, ATZG01000003.1 | 77.0 |
| | *O. alcoholitolerans* | AXCV01000115.1 | 71.0 |
| | *Leuconostoc gelidum* | CP003839.1, AEMI01000016.1 | 73.0 |
| | *Le. pseudomesenteroides* | JDVA01000012.1, JAUI01000015.1, CAKV01000002.1, AEOQ01000025.1 | 72.8 |
| | *Le. citreum* | DQ489736.1, CAGG01000001.1, CAGF01000016.1, CAGE01000001.1 | 72.3 |
| | *Le. gasicomitatum* | FN822744.1 | 72.0 |
| | *Le. inhae* | AEMJ01000491.1 | 72.0 |
| | *Le. mesenteroides* | CP000574.1, JRGZ01000004.1, CP003101.1, CP000414.1, JAUJ01000011.1, ATAZ01000107.1, ACKV01000007.1 | 70.6 |
| | *Le. argentinum* | AEGQ01000033.1 | 70.0 |
| | *Le. lactis* | JMEA01000004.1 | 70.0 |
| | *Le. carnosum* | BACM01001214.1 | 70.0 |
| *trxA3* | *O. kitaharae* | AFVZ01000001.1, ATZG01000003.1 | 75.0 |
| | *O. alcoholitolerans* | AXCV01000115.1 | 71.0 |
| | *Lb. acidophilus* | CP002559.1 | 70.0 |
| | *Lb. amylovorus* | CP002338.1, CP002609.1, CBBM010000054.1 | 70.0 |
| | *Lb. kitasatonis* | BALU01000013.1 | 70.0 |
| *trxB* | *O. kitaharae* | AFVZ01000001.1, ATZG01000003.1 | 75.0 |
| | *O. alcoholitolerans* | AXCV01000115.1 | 72.0 |
| *fdr* | *O. kitaharae* | AFVZ01000001.1, ATZG01000003.1 | 67.0 |
| | *O. alcoholitolerans* | AXCV01000115.1 | 66.0 |

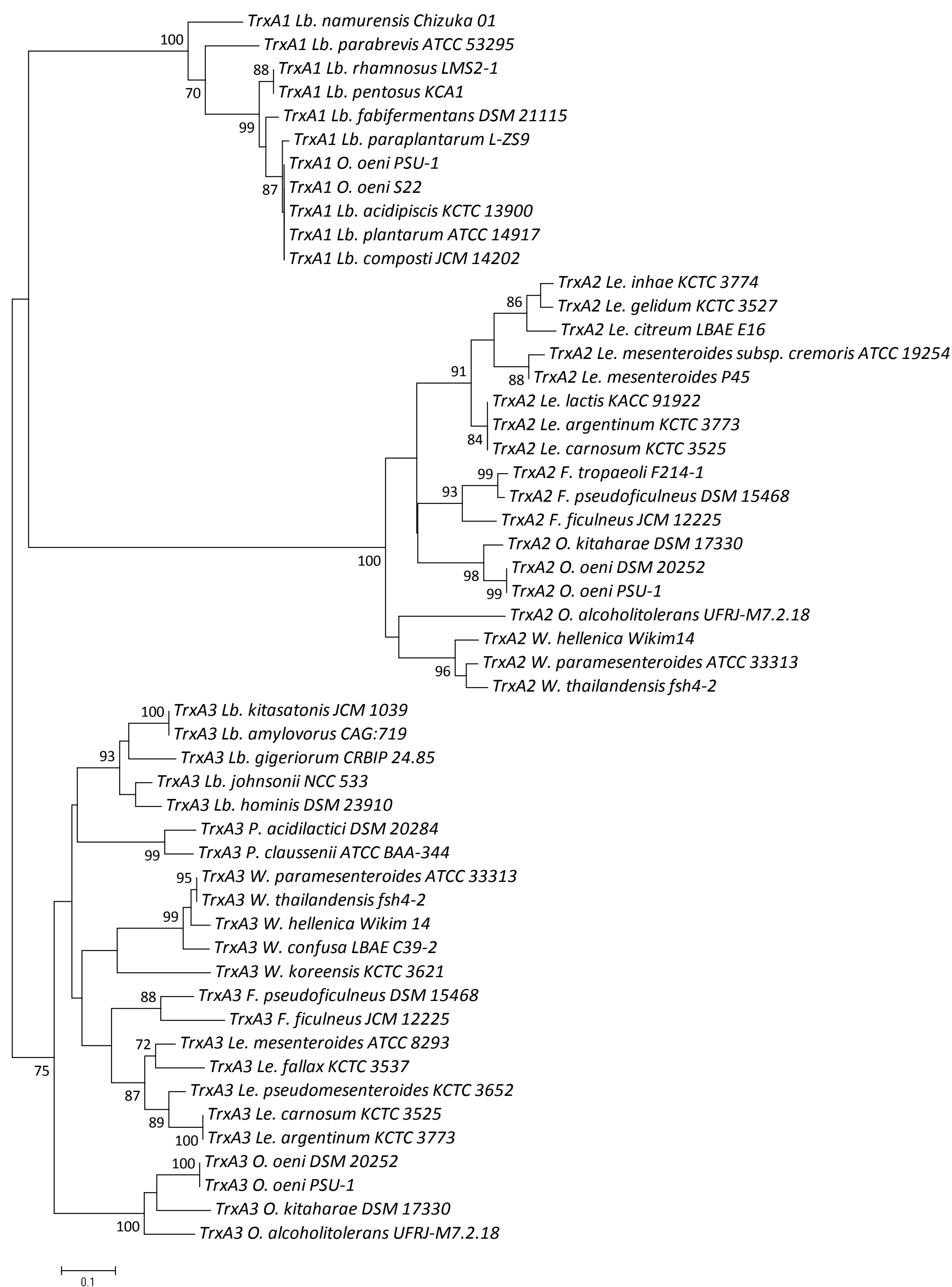


**Figure 1** Phylogenetic tree based on 52 thioredoxin protein sequences obtained via Blastp similarity to *O. oeni* PSU-1 thioredoxin protein. The tree was inferred using the Poisson model and neighbour joining. Bootstrap values (1000 replicates) are shown as a percentage at the nodes (values below 70% are not shown). The scale bar represents the number of substitutions per site. The tree was constructed using MEGA v5.0 software. Abbreviations: *Lb*., *Lactobacillus*; *O*., *Oenococcus*; *Le*., *Leuconostoc*; *F*., *Fructobacillus*; *W*., *Weissella*; *P*., *Pediococcus; L*., *Lactococcus*.

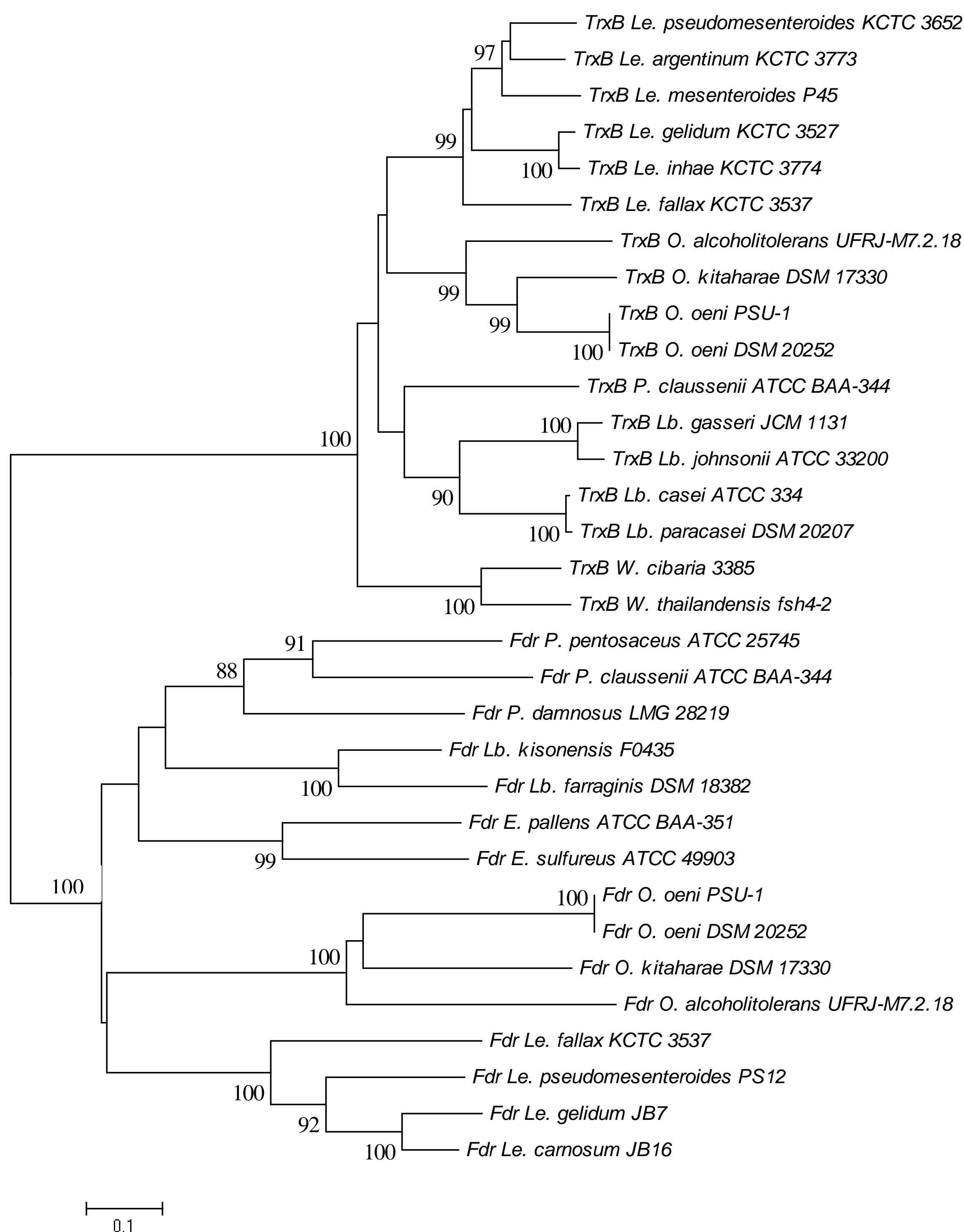


**Figure 2** Phylogenetic tree based on 32 reductase protein sequences obtained via Blastp similarity to *O. oeni* PSU-1 TrxB and Fdr proteins. The tree was inferred using the Poisson model together with neighbour-joining. Bootstrap values (1000 replicates) are shown as a percentage at the nodes (values below 70% are not shown). The scale bar represents the number of substitutions per site. The tree was constructed using MEGA v5.0 software. Abbreviations: see Figure 1.

*Detection of* trx *and* fdr *genes in* O. oeni *strains*

PCR detection of *trx* and *fdr* genes was carried out for a collection of different *O. oeni* strains (Table 1). The results revealed the presence of four genes (*trxA2*, *trxA3*, *trxB* and *fdr*) in all strains, whereas *trxA1* was absent from most strains. This gene was detected in only 7 out of 34 strains. Five of those strains had been isolated from the same area (Priorat): three (1P1, 1P2 and 1P3) from Grenache wines of the same cellar, one (2P2) from a Cabernet Sauvignon wine, and one (Fn42) from a Monastrell wine. This gene was also detected in PSU-1, as expected, and in the commercial strain CH11.

*In silico* analysis based on different published genomes of *O. oeni* strains in NCBI (data used July 2015) using bioinformatics tools (Table S1) indicated the presence of four out of five genes analysed in all strains, with high percentages of similarity (99-100%). *trxA1* was the least common of these genes and was found in only 7 out of 60 strains. Surprisingly, although the BLASTN result for ATCC BAA-1163 did not detect *trxA2,* this gene was amplified by PCR. A further sequence analysis revealed that this strain lacks approximately 1600 bp between two contigs compared to PSU-1, explaining this discordance (Stefanelli, 2014). Most of the strains sequenced contained the two thioredoxin reductase genes described in PSU-1. Only the strains IOEB_B16, IOEB_9803 and S12 lack the *trxB1* gene.

*Expression of the* trx *and* fdr *genes by* O. oeni *strains in rich medium*

The expression levels of the *trxA, trxB* and *fdr* genes at exponential growth in MRSmf were determined for the *O. oeni* strains listed in Table 1. The relative expression of these genes was detected by RT-qPCR (Figure 3). Although *trxA1* was detected in some strains (Table 1), the expression of this gene was only observed in the strains PSU-1 and Fn42, even when using a higher concentration of cDNA for RT-qPCR (data not shown).

Figure 3 shows the log2 value of the relative expression between each strain in relation to the median value for each gene. Values equal to or higher than 1 indicate over-expression, whereas values equal to or lower than -1 indicate inhibition. Most of the genes detected exhibited the same expression across strains. In certain strains, *trxA2* was over-expressed up to 10-fold compared to the strain with the median value. In contrast, *trxA3* was over-expressed or inhibited in a similar number of strains. The genes *trxB*

and *fdr* had similar expression values, although *trxB* was over-expressed more frequently than *fdr*.

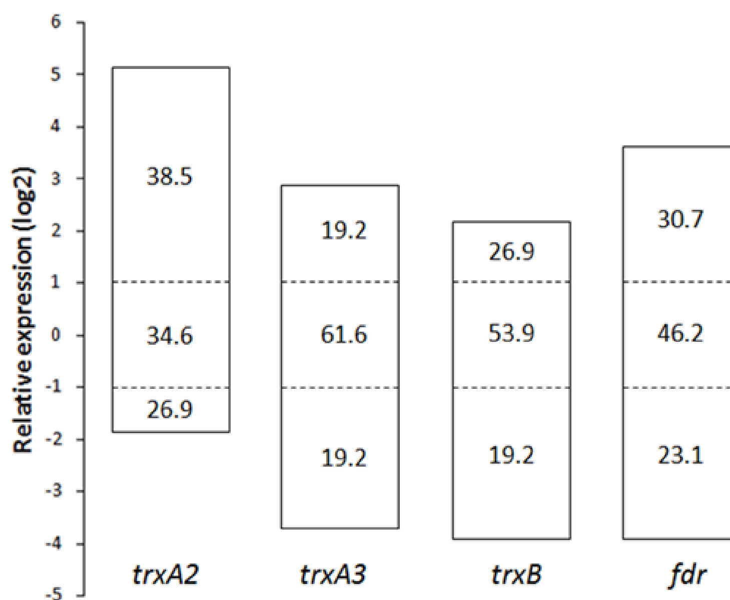


**Figure 3** Relative expression (RE) levels of two *O. oeni* thioredoxin genes, *trxB* and *fdr,* at a late exponential phase. The plot shows the percentages of strains whose genes are over-expressed above the median strain (upper box > 1) or inhibited (lower box < -1) below the median strain. Data shown are% of strains that have higher or lower RE values relative to the median strain. The central box corresponds to the% of strains that presented the same expression values as the median strain.

*Expression of* trx *and* fdr *genes in* O. oeni *strains under wine-like conditions*

To evaluate the expression of *trx* genes under wine-like conditions, malolactic fermentations were generated using different *O. oeni* strains in WLM (12% ethanol and pH 3.4). The reference strain PSU-1, the type strain CECT 217$^T$, two strains (3P2 and Fn42) from the *Universitat Rovira i Virgili* collection, and five strains from the *Università di Verona* collection (Vi7, Al2, U1, Rz81 and KM334) were used (Table 1). All strains except Rz81 completed MLF in between 2-4 days.

An evaluation of relative expression levels during MLF was conducted over a time course of four experimental points: 0 h, 1 h, mid-MLF, and end-MLF. The condition 0 h (before inoculation in WLM) was used as a calibrator. The genes assayed were the four ubiquitous genes *trxA2*, *trxA3*, *trxB* and *fdr* for the strains Vi7, Al2, U1, Rz81, KM334, 3P2 and 217$^T$, whereas for PSU-1 and Fn42, the expression of *trxA1* was also analysed. Figure 4 shows the relative expression level among five of the nine strains analysed.

These five strains were chosen to represent each transcriptional profile of the analysed genes, which was similar for some of the studied strains. Over-expressed genes presented values greater than two, whereas inhibited genes presented values below 0.5. The expression profile of PSU-1 (Figure 4A), which is similar to that observed for Fn42 (Figure S2), showed the increasing expression of all genes as MLF proceeds. Over-expression at the end of MLF was significant for all genes with the exception of *trxA2* in PSU-1. Most of the Italian strains (Al2, Vi7, U1, KM334) showed a similar expression profile (represented by Al2 in Figure 4B; the rest of the strains are shown in Figure S2). This profile revealed the noticeable over-expression of *trxA3* at the end of MLF in comparison to the other genes.

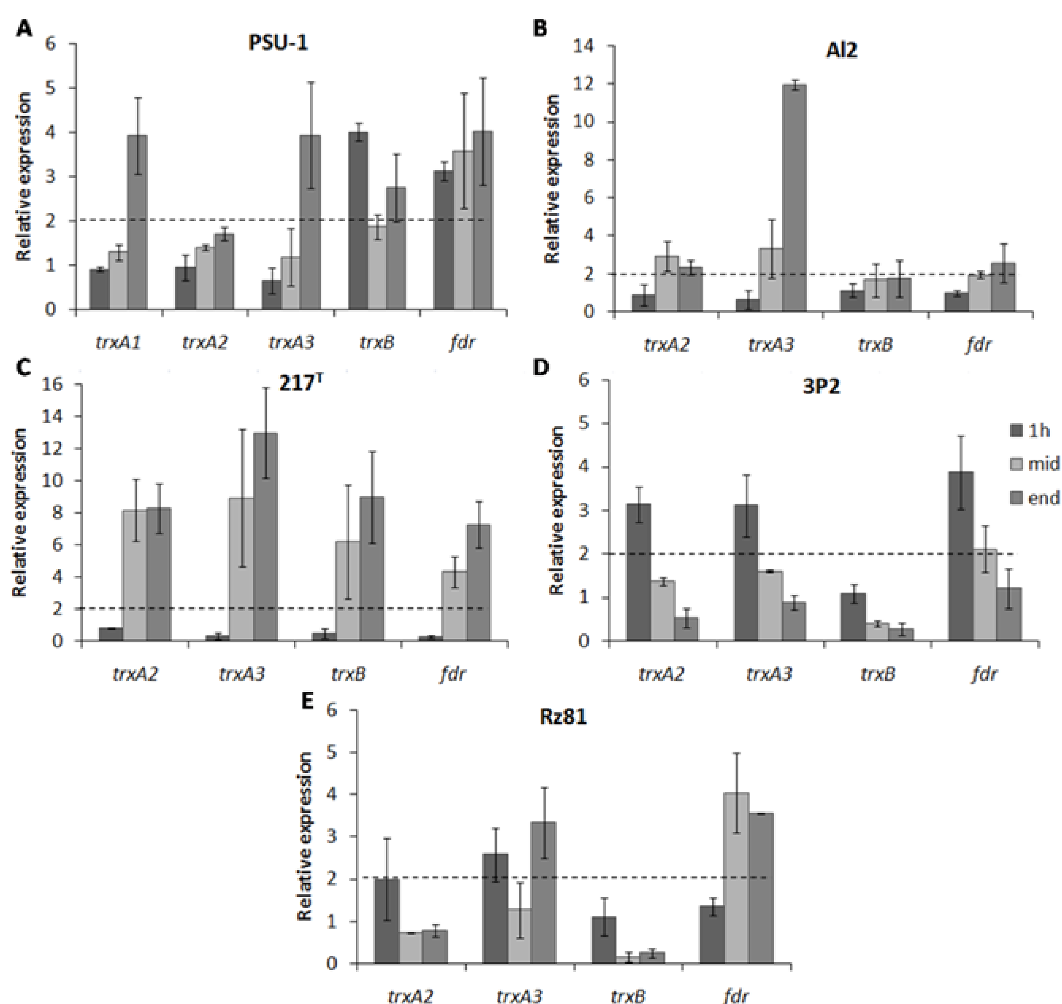


**Figure 4** Relative expression (RE) levels of *O. oeni* thioredoxin genes during MLF in WLM (pH 3.4 and 12% ethanol) over time (1 h, middle MLF and end of MLF) in strains representative of different transcriptional profiles. The calibrator condition used was at the time of inoculation. Data shown are means with SEM values (n = 2).

The strain 217$^T$ (Figure 4C) exhibited the increasing over-expression of all genes from the middle to the end of MLF. In contrast, 3P2 (Figure 4D) exhibited the decreasing relative expression of all genes after one hour of inoculation. Finally, Rz81 (Figure 4A) presented the inhibition of *trxB* in mid-MLF and end-MLF, while *fdr* was over-expressed under both conditions. Regarding *trxA*, the *trxA3* gene showed over-expression at one hour after inoculation and at the end of MLF, while *trxA2* did not exhibit relevant changes.

## Discussion

Previous studies have described the relationship between two *O. oeni* thioredoxins - *trxA1* (Renouf et al., 2008) and *trxA3* (Guzzo et al., 2000) - and stress response. However, this is the first study examining the complete Trx system in *O. oeni*.

The *trx* gene content can vary depending on species. Among LAB, the presence of four thioredoxins (*trxA*) and two thioredoxin reductases (*trxB*) was detected in *Lb. plantarum* (Molenaar et al., 2005), although one of these *trxB* genes has recently been annotated as ferredoxin NADPH reductase (lp_2585). In the *Lb. casei* strain Shirota, four *trxA* genes and one *trxB* gene have been described (Serata et al., 2012), and in *L. lactis,* two *trxA* genes and one *trxB* gene are present (Jordan et al., 1996).

The genome of the reference strain PSU-1 - the only completely assembled genome available for *O. oeni* - contains three thioredoxins (*trxA*) and one thioredoxin reductase (*trxB*). Moreover, a gene initially annotated as a thioredoxin reductase has been reannotated as a ferredoxin NAPDH reductase. *In silico* analysis revealed that the *trxA* gene sequences of *O. oeni* are more similar to homologous genes in other genera than to the *trxB* and *fdr* genes. This indicates a high specificity of thioredoxin reductase and ferredoxin NADPH reductase in the *Oenococcus* genus. Ferredoxin NADPH reductase (FNR) has been linked to the reduction of oxidized TrxA in photosynthetic organisms (Buchanan et al., 2002; Balmer et al., 2003). However, the sequence found in *O. oeni* PSU-1 does not contain the catalytic motif required for Trx reduction, which includes two cysteine residues (Balsera et al., 2013). In other LAB species, such as *Lb. plantarum* (Serrano et al., 2007) and *Lb. casei* (Serata et al., 2012), the presence of a ferredoxin NADPH reductase gene lacking the di-cysteine motif and homologous to

*yumC* in *Bacillus subtilis* (Seo et al., 2004) has been described. This protein is proposed to belong to a new group of FNRs that should be added to the established plant-type, bacteria-type, and mitochondria-type FNR groups (Seo et al., 2004). Although little is known regarding the role of YumC-like proteins in Gram-positive bacteria, a functional study in *L. lactis* has associated this activity with nucleotide reduction, which would enhance the beneficial effect of deoxynucleosides on aerobic growth (Chen et al., 2015).

A distribution analysis of a collection of 33 *O. oeni* strains from different sources and 60 genomes available at NCBI revealed that *trxA1* is poorly distributed among this species because it is included in the genomic island described by Bon et al. (2009), whereas the other studied genes are ubiquitous. This distribution was also observed directly in the phylogenetic tree analysis of this protein. TrxA1 shares great similarity with different thioredoxin sequences from *Lactobacillus* species and is located in a region containing several annotated transposases (OEOE_RS01600, OEOE_RS01605, OEOE_RS01705) that are also present in the genome of *Lb. casei* KL1-Liu (JXNV01) with high similarity (approximately 99%). This finding suggests the horizontal gene transfer (HGT) of this DNA region from *Lactobacillus* to some *O. oeni* strains. Because few strains have incorporated this region in the genome, it is difficult to determine whether the functions encoded by this region increase fitness. No evidence regarding malolactic performance has been found in the strains studied in this work. Renouf et al. (2008) described *trxA1* (OEOE_RS01675) as a genetic marker for stress resistance; however, it has also been reported that the presence of certain stress responsive genes, including *trxA1*, does not always correlate with the ability of strains to perform MLF (Renouf et al., 2008; Favier et al., 2012). Multiple genetic and phenotypic factors therefore affect the behaviour of *O. oeni* strains under the harsh wine conditions.

The thioredoxins TrxA2 and TrxA3 have been inherited vertically, as they follow the same structure as 16S rRNA gene sequence tree. The amino acid composition of the TrxA2 catalytic site corresponds to that described by Björnberg et al. (2014) for a new group of thioredoxins, TrxD in *L. lactis* that differs from the classical Trx of *E. coli*.

The analysis of transcription during optimal growth revealed a wide diversity among strains in terms of relative gene expression of *trxA2* and *fdr*. For these two genes, more than 30% of the strains exhibited significantly higher relative expression values compared to the median. In contrast, the expression of *trxA3* and *trxB* genes was similar

to the median for more than 50% of the analysed strains. According to these results, there is variable strain-dependent expression of the *trx* and *fdr* genes during optimal growth that are more substantial for *trxA2* and *fdr*.

The expression of Trx system genes during MLF in wine-like medium (WLM) was investigated in PSU-1, $217^T$ and other strains isolated from Italian and Spanish wines that exhibited different characteristics, such as oenological properties, growth in MRSmf, and MLF kinetics. In spite of the different origins of the strains used, some exhibited similar *trx* and *fdr* expression profiles. A similar profile was observed for several Italian isolates: Vi7, Al2, U1 isolated from Amarone wine, and KM334 isolated from Chardonnay wine. The two analysed strains containing the *trxA1* gene - PSU-1 and Fn42 - also presented some similarities, with *fdr* high expression at the end of MLF. However, some strains, such as 3P2, $217^T$ and Rz81, had unique expression patterns. Therefore, the transcriptional behaviour of the Trx system depends on the strain. While over-expression was observed primarily at the end of MLF in most cases, there were exceptions, such as the 3P2 strain. However, a common trend was the strong over-expression of the *trxA3* gene at the end of the MLF in almost all strains, with the exception of 3P2, which exhibits over-expression one hour after inoculation. This gene corresponds to *trxA* studied in the strain ATCC BAA-1163, which was found to be constitutively expressed during all growth phases and to be over-expressed in the presence of hydrogen peroxide and after heat shock (Jobin et al., 1999; Guzzo et al., 2000). Thus, the *trxA3* gene may play a crucial role during MLF in relation to stress adaptation. *trxA3* also varies least in its relative expression among the strains during optimal growth, indicating that it is expressed consistently in most strains and suggests that it plays a fundamental role.

In contrast, the expression of the thioredoxin reductase gene (*trxB*) is evident only in two of the five transcriptional profiles assessed, indicating that the activation of this gene under wine-like conditions is strain-dependent. However, the over-expression of *fdr* is marked in four of the five expression patterns represented. The role of the protein encoded by this gene in *O. oeni* is unknown, although these results suggest its relevance in the response to wine-like stress conditions. In *Lb. plantarum*, the expression of *fdr* (*trxB2*) and the thioredoxin *trxA2* has been associated with the responses to heat shock and reductive stress (Serrano et al., 2007).

This study provides the first data on the Trx system in *O. oeni* with respect to the genes present in the species, other species and genera, and their expression modulation. Further research is needed to clarify the biological role of each Trx and Fdr. As suggested Serata et al. (2012) for *Lb. casei*, it is possible that each thioredoxin plays a different role in cellular processes, as suggested by their low amino acid identities and the different behaviours of *trx* mutants. In this study, we demonstrated the involvement of the *O. oeni* Trx system in the response to some wine stress conditions and confirmed the essential role of *trxA3* in different strains. Future studies will focus on the molecular regulation of this system and its relevance for determining strain-dependent stress resistance.

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## Supplementary data

**Table S1** Similarity (expressed as per unit bases) of the thioredoxin gene sequences between the genomes of *O. oeni* strains available in the NCBI database compared to the *O. oeni* strain PSU-1 genome. This analysis was performed in July 2015.

| Strains | Genes | | | | | Source |
|---|---|---|---|---|---|---|
| | *trxA1* | *trxA2* | *trxA3* | *trxB* | *fdr* | |
| ATCC BAA-1163 | - | - | 0.99 | 0.99 | 0.99 | University of Burgundy, France |
| AWRIB202 | - | 1 | 1 | 1 | 0.99 | AWRI |
| AWRIB304 | - | 1 | 1 | 1 | 0.99 | " |
| AWRIB318 | - | 1 | 1 | 1 | 0.99 | " |
| AWRIB418 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| AWRIB419 | - | 1 | 1 | 1 | 0.99 | " |
| AWRIB422 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| AWRIB429 | - | 1 | 1 | 1 | 0.99 | " |
| AWRIB548 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| AWRIB553 | - | 1 | 1 | 1 | 0.99 | " |
| AWRIB568 | - | 1 | 1 | 1 | 0.99 | " |
| AWRIB576 | - | 1 | 1 | 1 | 0.99 | " |
| AWRIB129 | - | 1 | 1 | 1 | 1 | " |
| IOEB_B10 | - | 1 | 1 | 1 | 0.99 | ISVV, University of Bordeaux, France |
| IOEB_B16 | - | 0.99 | 0.99 | 0.99 | - | " |
| IOEB_CiNe | - | 1 | 1 | 1 | 0.99 | " |
| IOEB_C23 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| IOEB_C28 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| IOEB_C52 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| IOEB_L18 3 | - | 1 | 1 | 1 | 0.99 | " |
| IOEB_L26 1 | - | 1 | 1 | 0.99 | 0.99 | " |
| IOEB_L40 4 | 0.99 | 1 | 1 | 1 | 0.99 | " |
| IOEB_L65 2 | 0.98 | 1 | 1 | 1 | 0.99 | " |
| IOEB_S277 | - | 1 | 1 | 1 | 0.99 | " |
| IOEB_S436a | - | 1 | 1 | 1 | 0.99 | " |
| IOEB_S450 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| IOEB_VF | - | 1 | 1 | 1 | 0.99 | " |
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| IOEB_0501 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| IOEB_0502 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| IOEB_0607 | - | 1 | 1 | 0.99 | 0.99 | " |
| IOEB_0608 | 0.98 | 1 | 1 | 1 | 0.99 | " |
| IOEB_1491 | 0.99 | 1 | 1 | 0.99 | 0.99 | " |
| IOEB_8417 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| IOEB_9304 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| IOEB_9517 | - | 1 | 1 | 1 | 0.99 | " |
| IOEB_9803 | - | 0.99 | 0.99 | 0.99 | - | " |
| IOEB_9805 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| OM22 | - | 1 | 1 | 1 | 0.99 | University of Foggia, Italy |
| OM27 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| OT3 | - | 1 | 0.99 | 1 | 0.99 | " |
| OT4 | - | 1 | 0.99 | 1 | 0.99 | " |
| OT5 | - | 1 | 0.99 | 1 | 0.99 | " |
| OT25 | - | 1 | 1 | 1 | 0.99 | " |
| S11 | - | 1 | 1 | 1 | 0.99 | ISVV, University of Bordeaux, France |
| S12 | - | 0.99 | 0.99 | 0.99 | - | " |
| S13 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| S14 | - | 1 | 1 | 0.99 | 1 | " |
| S15 | - | 1 | 1 | 1 | 0.99 | " |
| S19 | 0.99 | 1 | 1 | 0.99 | 0.99 | " |
| S22 | 0.99 | 1 | 1 | 0.99 | 0.99 | " |
| S23 | 0.98 | 1 | 1 | 1 | 0.99 | " |
| S25 | - | 1 | 1 | 0.99 | 0.99 | " |
| S28 | - | 1 | 1 | 1 | 0.99 | " |
| S161 | - | 1 | 1 | 1 | 0.99 | " |
| X2L | - | 1 | 1 | 0.99 | 0.99 | CERELA-CONICET, Tucumán, Argentina, (Mendoza et al., 2015) |
| 399 | - | 1 | 1 | 0.99 | 1 | University of Chile |
| 139 | - | 0.99 | 0.99 | 0.99 | 0.99 | " |
| 49 | - | 1 | 0.99 | 0.99 | 0.99 | " |
| 565 | - | 1 | 1 | 0.99 | 1 | " |

AWRI: The Australian Wine Research Institute; ISVV: Institut des Sciences de la Vigne et du Vin

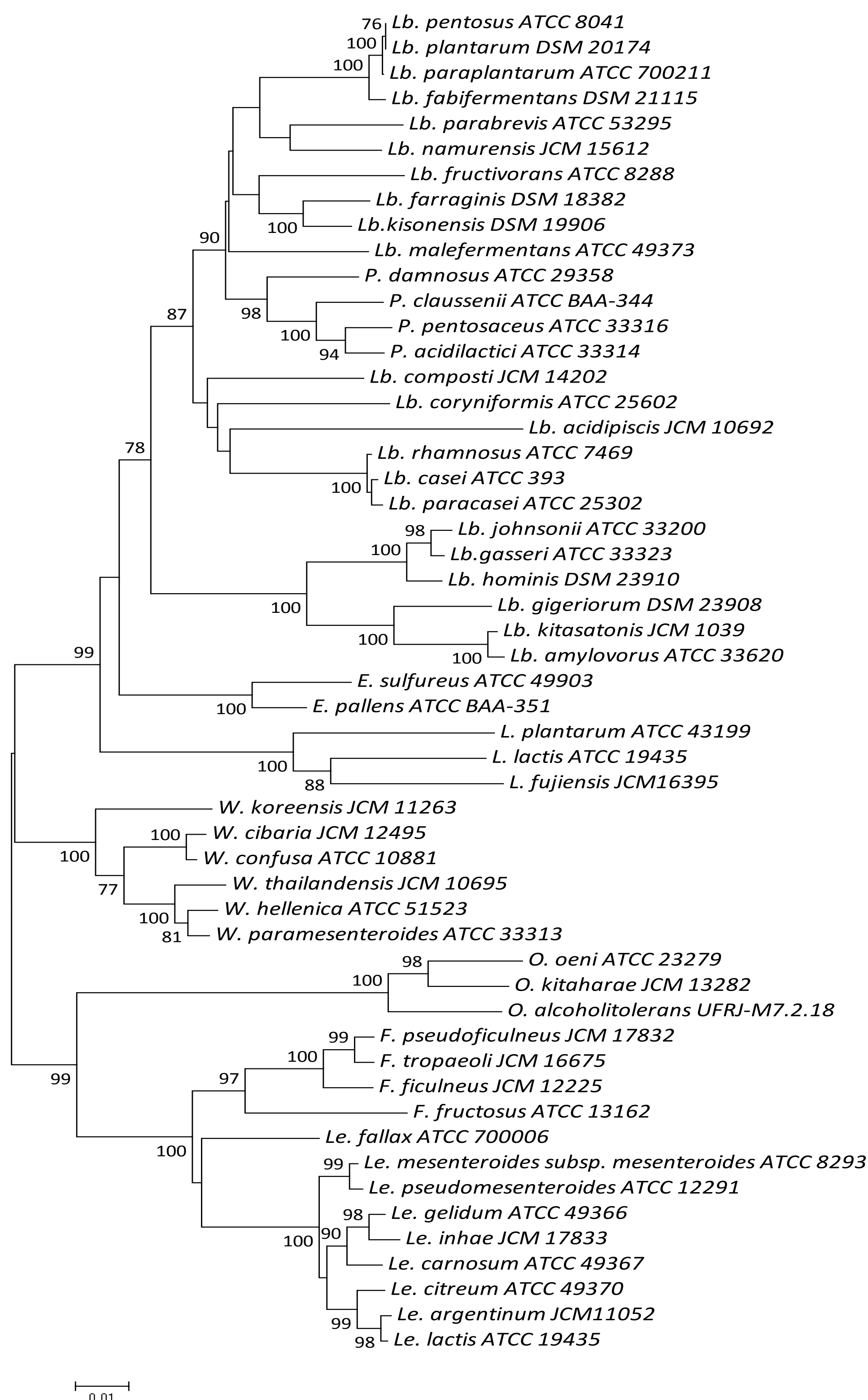


**Figure S1** Phylogenetic tree based on 53 16S rDNA gene sequences of type strains. The tree was inferred using the Poisson model together neighbour joining. Bootstrap values (1000 replicates) are shown as a percentage at the nodes (values below 70% are not shown). The scale bar represents the number of substitutions per site. The tree was constructed with MEGA v5.0 software. Abbreviations: see Figure 1.

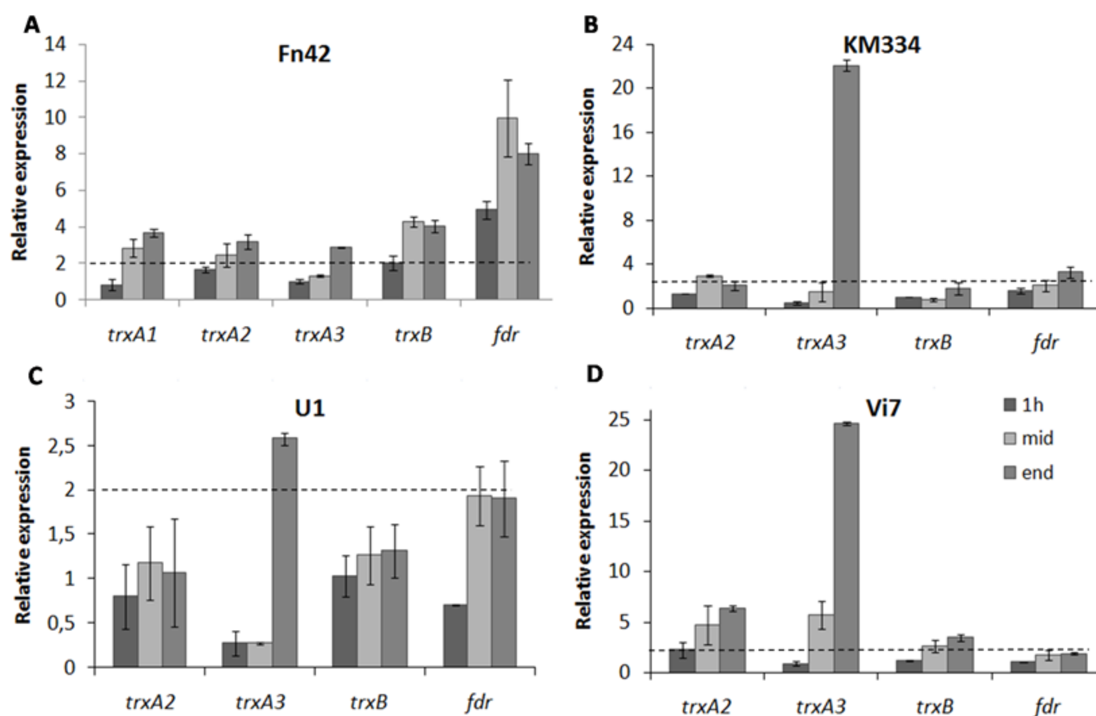


**Figure S2** Relative expression (RE) levels of *O. oeni* thioredoxin genes during MLF in WLM (pH 3.4 and 12% of ethanol) over time (1 h, middle MLF and end of MLF) compared to other strains. The calibrator condition used was at the time of inoculation. Data shown are mean values with SEM values (n = 2).

# 4. GENERAL DISCUSSION AND PERSPECTIVES

Lactic acid bacteria (LAB) play a key role as natural or selected starters in food fermentation processes, contributing to the sensory properties and the microbiological stability of the final product. *O. oeni* is the main LAB responsible for the malolactic fermentation (MLF) in wine. This decarboxylation of L-malic acid into L-lactic acid and the overall action of *O. oeni* into wine diminish the acidity of the final product, stabilize the microbial community and change the aroma profile. Since 1990, the URV LAB research team is working in different aspects of the MLF. Factors like high ethanol content, low pH and phenolic compounds presence have been linked to a delay in the start of MLF (Reguant et al., 2005, 2000; Rozès et al., 2003). The development of a reproducible method for molecular typing strains of *O. oeni* (Reguant and Bordons, 2003; Zapparoli et al., 2000) provided the study of population dynamics of these strains during wine MLF. Also, a better knowledge of the molecular processes involved in *O. oeni* tolerance to wine was required. Therefore the URV LAB research team has studied on *O. oeni* the role of specific metabolisms like citrate (Olguín et al., 2009) and arginine degradation (Araque et al., 2016, 2013, 2011, 2009), the ATPase activity (Carreté et al., 2002), the relative expression of certain genes under wine stress conditions (Bordas et al., 2015; Olguín et al., 2010) and the characterization of the ethanol effect using microarrays and proteomic techniques (Olguín et al., 2015). In parallel, the isolation and selection of autochthonous strains of *O. oeni* isolated from nearby vineyards have enlarged the collection of highly ethanol-tolerant strains as promising starters (Bordas et al., 2013).

Besides the different works carried out, one of the main objectives in the group was to unveil the adaptation of *O. oeni* into wine after the alcoholic fermentation in order to prevent a possible stuck of MLF process. Nowadays, using some of the isolates from the Priorat region (South of Catalonia, Spain) and taking advantage of the high-through techniques available, the study of the acclimation to wine seems more feasible.

In the light of these previous results, the objective of this thesis was to evaluate the *O. oeni* acclimation to wine conditions in order to have a better understanding of which mechanisms are activated. Also, the attention was focused on two cellular redox systems, glutathione and thioredoxin, not studied in this bacterium yet.

In a first instance, in this thesis, the possible stress related gene targets of *O. oeni* genomes were studied. Taking advantage from the current amount of data sequences of *O. oeni* strains, a comparative genetic analysis using the genetic annotation was performed (**Chapter I**). The study of the genomic composition of good oenological starters is important in order to unveil possible markers and targets for the selection of *O. oeni* strains to overcome the stressed wine conditions. According to previous data, the presence of 16 significant genetic markers was successfully used for determining the resistance of indigenous *O. oeni* strains (Renouf et al., 2008). Thus, it seems that the presence and the expression of genetic markers can be used as indicators of strain resistance and adaptation to wine related conditions. **Chapter I** reported the distribution of selected sequences among all the 57 genomes of *O. oeni* published in database in May 2015 and the identification of seven genomic regions starting from a subset of 106 genes related to stress. Some of these candidate genomic regions harboured genes affected by wine-like conditions reported in the literature and **Chapter II**, which could be added to the complex mechanisms so far described in *O. oeni*. Thus, R2 of **Chapter I**, enclosed several genes down-regulated under wine-like conditions (**Chapter II**), most of them being transcriptional factors or involved in sugar metabolism. Genes contained in R6 were widespread among most *O. oeni* genomes available, being most of them affected under wine adaptation (**Chapter II**). Some of the highlighted genes were related to redox reactions like the 2,5-diketo-D-gluconic acid reductase (OEOE_RS00320) harboured in R1 and the ferritin (OEOE_08440) enclosed in R7, both of them affected under wine stress conditions (Stefanelli 2014; Olguín et al. 2015; **Chapter II**). It is worth noting that some of these regions, like R1, harbours hypothetical proteins affected under wine-like conditions whose function is still unknown.

Harboured in the seven genomic regions identified for their putative flexibility among the genomes available for *O. oeni*, specific genes were pointed out conforming part of the core-genome of *O. oeni*, and others scarcely spread. As other authors had already

reported some of these regions using other methods, the approach used in **Chapter I** seems a good strategy to find putative flexible genomic regions. The genomic island (GI) already described (Bon et al., 2009) is only shared by few *O. oeni* sequenced strains, and it could be detected in some isolates from Priorat vineyards in a different composition as in the known PSU-1 genome. It is not clear if this GI can contribute to better oenological characteristics, as there was no difference in the MLF performance. However, this region (R3 in **Chapter I**) harbours some stress related genes and a thioredoxin annotated in PSU-1 genome, which was used as a oenological genetic marker (Renouf et al., 2008) and has been over expressed under wine-like conditions (**Chapter V**). Moreover, in **Chapter V**, the phylogenetic analysis confirmed that this GI would come from *Lactobacillus* species.

Looking for genetic mobile elements as transposases in PSU-1 genome, Stefanelli (2014) analysed the genomic region named R5 in **Chapter I**, but no foreigner source could be established. The *in silico* analysis of **Chapter I** revealed the different genetic compositions of this short GI between *O. oeni* and *Lactobacillus spp.* Due to the possibility of a large number of LAB genomes available for analysis, it has been confirmed that this new horizontal gene transfer in *O. oeni* is older than the GI of R3 as it is more widespread. However, further work is needed to understand how the gene threonine dehydrogenase (*trhD*) sandwiched between the two pairs of transposases could benefit *O. oeni* as it is the fourth gene codifying for this type of enzyme in *O. oeni* PSU-1 genome. However, it is worth noting that this *trhD* (OEOE_RS03560) was down-regulated under stress wine conditions (Stefanelli, 2014) and the *trhD* (OEOE_RS01895) was repressed as well under WLM adaptation (**Chapter II**).

Apart from the stress related genes harboured in *O. oeni* it was important to determine the activated pathways under wine conditions, especially when these metabolic traits are also related to cell adaptation and stress response. To achieve this goal, in **Chapter II** the first eight hours of stress response of *O. oeni* PSU-1 into wine-like medium before the start of MLF were analysed using “omic” techniques, such as DNA microarrays and two complementary proteomic techniques (2D-DIGE and iTRAQ labeling). Currently, two transcriptomic analysis using *O. oeni* under wine conditions have been published (Costantini et al., 2015; Olguín et al., 2015), being this last one from the URV LAB research team. On the other hand, the proteomic works published are still scarce for *O.*

*oeni* species. Starter cultures cells have no time for a gradual adaptation and they have to survive a direct shock. These first hours after shock may switch on different pathways to overcome its new environment. A global approach of the mechanisms implicated in this crucial period was accomplished in **Chapter II**, mimicking wine conditions and using "omic" techniques. It is worth noting that the differences found between transcriptomic and proteomic data suggested the relevance of post-transcriptional mechanisms and the complexity of the stress response in *O. oeni* adaptation, as have been suggested by other authors (Guzzo et al., 2000).

In this **Chapter II**, genes involved in translation function showed an over-expression during the adaptation period to wine. Some of the corresponding proteins of these genes as well were more abundant after the inoculation. On the other hand, a strong transcriptional inhibition of carbohydrate metabolism related genes was observed. However, the malate transport and citrate consumption over expression indicated the use of L-malate and citrate associated to stress response and as an alternative energy source to sugar metabolism. Regarding the amino acid metabolism, certain proteins involved in glutamine and glutamate metabolism showed an increased abundance revealing the key role of nitrogen uptake under stressful conditions. Also, several peptidases were up regulated both at gene and protein level.

Like other proteomic works with *O. oeni* under wine conditions (Costantini et al., 2015; Olguín et al., 2015), genes and proteins related to cell envelope showed significant changes. Those changes are in agreement with the fact that one of the main functions of the envelope and membrane is to be a barrier and protect the inside of the cell from the environment.

Even though the great amount of information reported in **Chapter II**, more than 600 genes and 100 proteins different regulated after inoculation into wine, further research should deepen into the highlighted metabolisms. Also, it is worth noting that there are 100 genes differently expressed annotated as hypothetical proteins which function is still unknown. It is suggested that in few years more genes and proteins will be connected and their involvement in wine adaptation will be unveiled in a more particular way. Likewise, *O. oeni* database functional annotation still needs integration of the experimental data and converging all the microbiological pathways in one unique platform in order to have a more unified approach and results.

In wine, ethanol is one of the most stressful factors which must be counteracted by *O. oeni* (Maitre et al., 2014; van Bokhorst-van de Veen et al., 2011). *O. oeni* deals with oxidative stress by other processes than the extend catalase system. Thioredoxin (Trx) is ubiquitous in bacteria, whereas the glutathione (GSH) antioxidant system is lacking in some bacteria. Indeed, the glutathione system in *O. oeni* is only linked to the use of this antioxidant because of the lack of the biosynthesis route (Pophaly et al., 2012). Therefore, both redox systems appear a key point in the study of this bacterium to stress response.

**Chapter II** supported the relevance of the thioredoxin and glutathione systems in the adaptation of *O. oeni* to wine related stress. While the unique annotated gene for a thioredoxin reductase (*trxB*) for PSU-1 was activated under wine stress conditions, two thioredoxins proteins were up-regulated 6 h after inoculation. So it may indicate a possible reduction process of cytoplasmic proteins carried out by thioredoxins in order to counteract stress. It is worth noting that one of these Trx was activated as well by heat and hydrogen peroxide shocks (Jobin et al., 1999) and that all this Trx system was activated under MLF process (**Chapter V**). Regarding the other important redox system, in **Chapter II** the glutathione reductase (GshR) was significantly more abundant in *O. oeni* PSU-1 after inoculation into WLM. However, transcriptomic data revealed that some of GSH genes were inhibited; indicating that translational regulation of these functions would be prevalent under the studied conditions.

In other LAB the protective role of GSH has been elucidated, but in *O. oeni* there was only a preliminary study using GSH in MLF (Rauhut et al., 2004). Thus, **Chapter III** and **Chapter IV** describe the effect of GSH addition during growth in wine stress conditions and unveil the complete set of genes of GSH system in PSU-1 strain. **Chapter III** determined that the GSH uptake from the medium by a collection of 30 strains was strain-dependent. This observation is not surprising since the putative transporter for GSH is still not well defined. Also, the action of GSH outside the cell is unclear and it could be associated to a protective effect or stress reducer of the media. Also, no correlation between GSH uptake versus MLF could be established.

From the screening of the 30 strains, three strains were selected as a reference for different GSH uptake: 3P2, PSU-1 and $217^T$. The fatty acid (FA) composition of this three strains growing with GSH indicated a change in FA composition, being the most

relevant an increase of cyclopropane FA. Moreover, together with the anisotropy results obtained, it may indicate that the addition of GSH could resemble the stationary cellular membrane. Cells grown with GSH showed an improved survival against ethanol shock (14% v/v). Moreover, after the shock the cells grown with GSH taken at exponential phase had a similar membrane recovery from stationary cells. The changes in cell membrane in stationary phase are well known (Teixeira et al., 2002) and they are linked to a more protective cellular state that may be used to cope with different stresses. Therefore, each of the selected strains was grown under wine-acclimation conditions of low pH (3.4 and 4) and 6% ethanol content in order to determine the possible beneficial effect of GSH addition. **Chapter III** reported that the greater the GSH uptake the greater the growth of the strain even though the presence of some stress in the media. Therefore, this antioxidant addition could be useful for the adaptation of MLF starter cultures to the stressful wine conditions.

The study of the *gshR* expression in *O. oeni* was obtained (Bordas et al., 2015) because its relation to adaptation to wine (Silveira et al. 2004; Cecconi et al. 2009; Costantini et al. 2015). This gene related to GSH system has been the only studied and identified in other works. For this reason, **Chapter IV** focused on the identification of the seven genes involved in the GSH redox system found in all *O. oeni* strains (*gshR*, glutathione peroxidase - *gpo*, three glutaredoxin-like genes and two subunits of an hypothetical transporter) and their relative expression (RE) under wine acclimation conditions and GSH addition. The three selected strains from **Chapter III** were used. The *gshR* was over-expressed due to the ethanol effect in 3P2 and $217^{T}$ regardless of the addition of GSH. The activation of *gshR* in these strains is in accordance with previously described results in wine-like medium with 12% ethanol (Bordas et al., 2015) and in proteomic studies in which GshR has been involved in the adaptation to ethanol (8% v/v) (Silveira et al. 2004) and in ethanol-adapted cells (5% v/v) (Cecconi et al., 2009). For PSU-1, the GSH addition caused a down-regulation of the *gpo* gene at pH5, pH4 and pH4Et6. Regarding one of the subunit transporter, *cydC*, a transcriptional induction was measured in strain 3P2 at the condition pH4Et6, the harshest one.

As the annotation of the three genes of glutaredoxin-like in PSU-1 was not so clear it demanded a more profound inquiry. **Chapter IV** described the three glutaredoxin-like proteins and their differences in their active site, commonly described for other

organisms such as CXXC for dithiol Grx and CXXS for monothiol Grx (Lillig et al., 2008). OEOE_RS04215 has a glutaredoxin motif, CPQC, which is similar to the one present in other species and identical to an annotated glutaredoxin of *Bacillus* sp. Moreover, it is the only glutaredoxin gene tested that presented a transcriptional activation due to the GSH addition in the strain with a higher capacity of GSH uptake, 3P2. So, despite the current annotation, we suggest that OEOE_RS04215 (*rdx3* in **Chapter IV**) could codify for an "authentic" glutaredoxin. On the other hand, because of the genomic proximity of OEOE_RS00645 (*rdx2* in **Chapter IV**) to several genes annotated as subunits of RNR (OEOE_RS00640, OEOE_RS00650 and OEOE_RS00655)as in *Lactococcus lactis* (Jordan et al., 1996), and being the only transcriptional change to ethanol stress, this gene was considered to be codifying for a NrdH-redoxin in PSU-1. Finally, OEOE_RS00435 (*rdx1* in **Chapter IV**), although being annotated as Grx, it has no active site prediction in NCBI. Even though, the phylogenetic analysis indicated that Rdx1 is closer to the other two dithiol Grx found in *O. oeni* PSU-1 than to monothiol Grx described in other microorganisms, there was not sufficient information to conclude which would be the active site in Rdx1. Therefore, the specific function of this protein is to be elucidated. For further studies, in order to know the concrete activity of each glutaredoxin-like protein, these enzymes may be included when comparing enzyme activity and gene expression of GSH system. Moreover, because of the presence of three copies of the same type of enzyme in the small genome of *O. oeni* (1.8 Mb) seems very interesting to study.

For the final chapter of the thesis, the complete thioredoxin system in *O. oeni* was studied (**Chapter V**). In the first sequenced strain PSU-1, three thioredoxins (*trxA*) and one thioredoxin reductase (*trxB*) are annotated. Also, it is worth noting that in this work due to the old annotation of a second *trxB*, a ferredoxin reductase (*fdr*) was also included in the project. The *in silico* analyses conducted using the genetic sequences of other LAB species, revealed a more similar DNA sequence between the different *trxA* than the *trxB*. The expression profile of *trx* genes in different *O. oeni* strains isolated from Catalan and Italian wines concluded this system as over-expressed during MLF. The *trxA1*, as it is harboured in a GI (**Chapter I**), was only present in few genomes but still over-expressed in the strains that enclosed it. The first known thioredoxin, *trxA3* in **Chapter V** (OEOE_RS08215), presented the highest over expression in most of strains

while the relative expression of *trxB* and *trxA2* was widely strain dependent. According to previous data, *trxA3* was highly over-expressed under hydrogen peroxide and heat shock (Jobin et al., 1999). From this analysis also the gene *fdr* (OEOE_RS00770) was activated under wine-like conditions and activated at gene level during inoculation to wine (**Chapter II**). However, no relationship between MLF activity and gene expression could be established. The next step, already underway, is evaluating the activity of these enzymes in different strains under different conditions.

In summary, this work has been focused on *O. oeni* molecular response to wine related conditions. Three different approaches have been used: comparative genomic, transcriptomics and proteomics. From them two main systems have been selected and studied due to their relevance in redox processes and the few present knowledge available in this species. In conclusion, a complete description of both systems from the genome composition to their expression into wine stress conditions has been reported.

Finally, the results obtained and the techniques used have opened new doors and will be useful for future studies of *O. oeni* adaptation to wine. These data should be considered for further analysis using other *O. oeni* strains and different conditions. Also, the increase in popularity of autochthon isolates starters can lead to focus the attention on the impact of the genomic features of the nearby *O. oeni* strains as some of them harboured specific genomic regions that may increase the natural genetic diversity of species for technological purposes.

The identification of genes and proteins affected under wine-like media can be useful to go deep into some concrete pathways and their regulation, like sugar or nitrogen metabolism. Results obtained should be also complemented by other studies using enzymatic assays to ensure the activity of certain key proteins like TrxR and GshR. In fact, this research is being carried out hoping to correlate these new results to those obtained so far. The beneficial effect of GSH could be finally correlated with the activities of the enzymes involved, thus, finishing the characterisation of specific strains since their GSH capitation to its use. GSH transport and how GSH positive effect is conducted are still two points to cover. Regarding the thioredoxin system it could be interesting, thanks to the new technologies of asRNA in *O. oeni* (Darsonval et al., 2016, 2015), to construct mutants for each of the genes to unveil their role and essentiality in this species.

# 5. CONCLUSIONS

The main conclusions obtained from this PhD Thesis are:

1. Comparative genomic approach using the genes annotated as stress related from *O. oeni* PSU-1 and all the *O. oeni* sequenced genomes, has allowed the identification of genomic regions in which interesting target genes involved in stress wine response can be detected.
2. Genomic R2 and R6 have been for the first time identified and they harbour genes affected during wine adaptation or wine stress conditions.
3. The region R5 from strain PSU-1 is a new genomic island provided by horizontal gene transfer from a *Lactobacillus* species quite spread among the *O. oeni* genomes available, and harbours a threonine dehydrogenase gene.
4. The known genomic island correlated with R3 has been found in indigenous isolates of *O. oeni* from wines of South Catalonia.
5. Inoculation of *O. oeni* PSU-1 into wine-like medium induces mainly genes related to translation process and promotes the down-regulation of carbohydrate metabolism.
6. The nitrogen metabolism of *O. oeni* PSU-1 is affected during the first hours before MLF, being up-regulated several peptidases both at gene and protein level and certain proteins involved in glutamine and glutamate metabolism.
7. The identification of genes and proteins from both redox systems glutathione (GSH) and thioredoxin (Trx) confirms their involvement under wine stress conditions.
8. GSH uptake in *O. oeni* is strain dependent and the greater its uptake, the greater the beneficial effect even in the presence of stress conditions.
9. GSH addition provokes changes in fatty acid membrane composition of *O. oeni*, being the increase of cyclopropane fatty acids the most relevant change. The membrane acquires a similar profile as in stationary phase, which results in a more resistant membrane.

10. *O. oeni* grown with GSH showed an improved survival against ethanol shock (14% v/v) and an increased biomass during the adaptation to wine stress conditions. Therefore, GSH addition could be useful for the adaptation of MLF starter cultures.
11. The complete GSH redox system in *O. oeni* PSU-1 genome has seven genes: *gshR*, *gpo*, two subunits of a putative transporter and three glutaredoxin-like proteins.
12. The *gshR* overexpression in *O. oeni* is due to the ethanol effect regardless the addition of GSH.
13. OEOE_RS04215 is the only annotated glutaredoxin gene of *O. oeni* activated due to the GSH addition.
14. Thioredoxin system is activated under wine stress conditions during MLF, being *trxA3* the most affected in most of the *O. oeni* strains tested. The other *trxA2* and *trxB* are activated in a strain dependent manner.
15. The *trxA1* present in a genomic island is over-expressed in the *O. oeni* strains which harbour it at the end of MLF.
16. The ferredoxin reductase of *O. oeni* PSU-1 is activated under wine stress conditions in most of the strains tested, in a strain-dependent manner.

# CONCLUSIONI

Le principali conclusioni ottenute da questa Tesi di Dottorato sono:

1. La genomica comparativa, effettuata sui geni annotati come correlati allo stress di *O. oeni* PSU-1 e di tutti i genomi sequenziati di *O. oeni*, permette l'identificazione di regioni genomiche in cui possono essere rilevati interessanti geni bersaglio coinvolti nella risposta allo stress da vino.
2. Sono state identificate, per la prima volta, le regioni genomiche R2 e R6 che portano geni influenzati durante l'adattamento al vino o per le condizioni stressanti del vino.
3. La regione R5 dal ceppo PSU-1 è una nuova isola genomica, fornita dal trasferimento genico orizzontale da una specie di *Lactobacillus*, molto diffusa tra i genomi disponibili di *O. oeni*, e ospita un gene che codifica per una treonina deidrogenasi.
4. L'isola genomica correlata con R3 è stata trovata in ceppi autoctoni isolati da vini del sud della Catalogna.
5. L'inoculo di *O. oeni* PSU-1 nel *wine-like medium* induce principalmente geni legati alla processo di traduzione e promuove la regolazione negativa del metabolismo dei carboidrati.
6. Il metabolismo dell'azoto di *O. oeni* PSU-1 è influenzato durante le prime ore che precedono la FML, in quanto sono sovra-regolate, sia a livello di geni e di proteine, varie peptidasi e alcune proteine coinvolte nel metabolismo del glutammato e glutammina.
7. L'identificazione di geni e proteine da entrambi i sistemi redox del glutatione (GSH) e tioredossina (Trx) conferma il loro coinvolgimento in condizioni di stress da vino.
8. L'incorporazione del GSH dipende dal ceppo, e maggiore è la sua incorporazione, maggiore è l'effetto benefico, anche in presenza di condizioni di stress.

9. L'aggiunta di GSH provoca cambiamenti nella composizione degli acidi grassi di membrana, e il cambiamento più rilevante è quello relativo all'aumento degli acidi grassi del ciclopropano. La membrana acquisisce un profilo simile a quello della fase stazionaria, che si traduce in una membrana più resistente.
10. *O. oeni* coltivate con GSH hanno mostrato un miglioramento della sopravvivenza contro l'etanolo (14% v/v) e una maggiore biomassa durante l'adattamento a condizioni di stress da vino. Pertanto, l'aggiunta di GSH potrebbe essere utile per l'adattamento di starter nella FML.
11. Il sistema redox GSH completo nel genoma di *O. oeni* PSU-1 è composto da sette geni: *gshR*, *gpo*, due sub unità di un trasportatore putativo e tre proteine glutaredossina-simile.
12. La sovra-espressione di *gshR* è dovuta all'effetto etanolo, indipendentemente dall'aggiunta di GSH.
13. OEOE_RS04215 è l'unico gene glutaredossina annotato di *O. oeni* attivato a seguito dell'aggiunta di GSH.
14. Il sistema tioredossina è attivato in condizioni di stress da vino durante la FML, essendo *trxA3* il gene più influenzato nella maggior parte dei ceppi testati. Gli altri geni *trxA2* e *trxB* vengono attivati in modo ceppo-dipendente.
15. Il gene *trxA1*, presente in un'isola genomica, è sovra-espresso alla fine della FML nei ceppi che lo posseggono.
16. Il gene della ferredossina reduttasi di *O. oeni* PSU-1 è attivato in condizioni di stress da vino nella maggior parte dei ceppi testati in modo ceppo-dipendente.

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UNIVERSITAT ROVIRA I VIRGILI
OENOCOCCUS OENI RESPONSE TO WINE STRESS: ROLE OF GLUTATHIONE AND THIOREDOXIN SYSTEMS
Maria del Mar Margalef Català



# ANNEX 1

## *Curriculum Vitae*

Mar was born on 31 st August 1988 in Tarragona, Spain. In 2006 she took up studies in Biotechnology at the Universitat Rovira and Virgili (URV) in Tarragona. She obtained her degree in 2010. During her degree project research at the Department of Biochemistry and Biotechnology of the same University she worked in the field of dehydration tolerance of *Saccharomyces cerevisiae* under the supervision of Prof. Ricardo Cordero Otero. In the same group she carried out the master's Oenology thesis working on characterisation of the Quantitative Trait Locus (QTLs) of *Saccharomyces cerevisiae* in response to dehydration tolerance. During this period she did part of her thesis in the Institute of Genetics, University of Nottingham, Queen's Medical Centre (Nottingham, UK) under the supervision of Prof. Gianni Liti.

After master's graduation in 2011, Mar started working as a PhD student in the lactic acid bacteria team from the Oenological Biotecnological group of URV. Her thesis was supervised by Prof. Cristina Reguant and Prof. Albert Bordons from the same university, and Prof. Sandra Torriani from University of Verona. Thesis research was mainly focused on the main mechanisms involved in *Oenococcus oeni* adaptation to wine stress and the study of the redox systems glutathione and thioredoxin, not yet studied in this bacterium. At the second year she went to the Institue Jules Guyot (Dijon, France) to learn, during a short stay, the membrane anisotropy technique under the supervision of Prof. Jean Guzzo. During the longer stay in the group of Prof. Sandra Torriani and under the supervision of Prof. Giovanna Felis, she worked in the field of genomic comparison analysis focused on *O. oeni*.

## Publications

**Margalef-Català, M.**, Araque, I., Weidmann, S., Guzzo, J., Bordons, A., Reguant, C., 2016. Protective role of glutathione addition against wine-related stress in *Oenococcus oeni*. Accepted for publication in Food Research International.

**Margalef-Català, M.**, Araque, I., Albert Bordons, A., Reguant, C., Bautista-Gallego, J., 2016. Transcriptomic and proteomic analysis of *Oenococcus oeni* adaptation to wine stress conditions. Frontiers in Microbiology 7:1554. doi: 10.3389/fmicb.2016.01554

**Margalef-Català, M.**, Stefanelli, E., Araque, I., Karoline Wagner, K., Felis, G., Bordons, A., Torriani, S., Reguant, C., 2016. Variability in gene content and expression of the thioredoxin system in *Oenococcus oeni*. Food Microbiology pp. 23-32. doi: 10.1016/j.fm.2016.08.005

López-Martínez, G., **Margalef-Català, M**., Salinas, F., Liti, G., Cordero-Otero, R., 2015. *ATG18* and *FAB1* Are Involved in Dehydration Stress Tolerance in *Saccharomyces cerevisiae*. PLoS ONE: Mar 24;10(3): e0119606. doi: 10.1371/journal.pone.0119606.

López-Martínez, G., Rodríguez, B., **Margalef-Català, M**., and Cordero-Otero, R., 2012. The STF2p Hydrophilin from *Saccharomyces cerevisiae* Is Required for Dehydration Stress Tolerance. PLoS ONE: 7(3):e33324. doi: 10.1371/journal.pone.0033324

## List of oral conferences

Variability in gene content and expression of the thioredoxin system in *Oenococcus oeni*. (Red de Bacterias Lácticas, 2016 Madrid, Spain) **Conference in Spanish**.

Proteomic study of the adaptation of *Oenococcus oeni* to wine conditions. (Symposium International Oenology, 2015 Bordeous, France) **Conference in English**.

Better adaptation of *Oenococcus oeni* due to glutathione's addition. (Sociedad Española de Microbiología, 2014 Zaragoza, Spain) **Conference in Spanish**.

Effect of the addition of glutathione on growth and *Oenococcus oeni*'s adaptation to stress. (Red de Bacterias Lácticas, 2014 Calahorra, Spain) **Conference in Spanish**.

Implication of glutathione and thioredoxin system into the adaptation of *Oenococcus oeni* to wine. (Red de Bacterias Lácticas, 2012 Tarragona, Spain) **Conference in Spanish**.

**List of conference posters**

**Margalef M.**, Araque I., Bordons A., Reguant C. Characterization of the GSH uptake and its effect on the growth of *Oenococcus oeni* strains. National Congress of Oenological research (Gienol). Tarragona, Spain June 2015.

**Margalef M**. GSH and Trx system are involved in the adaptation of *O. oeni* into wine. Ph.D. Day in Verona, Italy. 19th February 2015.

**Margalef M.**, Araque I., Bordons A., Reguant C. GSH and Trx systems are involved in the adaptation of *O. oeni* to wine conditions. 2nd Workshop of research in Oenology and Viticulture in Catalonia (CEICS). Tarragona, Spain 10th December 2014.

**Margalef M**. GSH and Trx system are involved in the adaptation of *O.oeni* into wine. Ph.D. Day in Verona, Italy. 26th February 2014.

Margalef M., Weidmann S., Bordons A., Reguant C., Guzzo J. Effect of GSH addition on the membrane fluidity of *Oenococcus oeni* and under ethanol stress. Spanish Society of Microbiology (SEM). Hospitalet de Llobregat, Spain 10-13 July 2013.

**Margalef M.**, Araque I., Bordons A, Reguant C. Effect of GSH addition on the acclimation of *Oenococcus oeni* strains. Congress of Oenological research (Gienol). Madrid, Spain 18-21 June 2013.

Bordas M, **Margalef M**, Araque I., Bordons A., Reguant C. Molecular mechanisms of adaptation of the acid lactic bacteria *Oenococcus oeni* to harsh wine conditions. 1st Workshop of research in Oenology and Viticulture in Catalonia (CEICS). Tarragona, Spain 4th June 2013.

**Margalef M.**, Araque I., Bordons A., Reguant C. Study of thioredoxin genes of *Oenococcus oeni* and their relation to the resistance to harsh wine conditions. IV Congress of Industrial Microbiology and Microbial Biotechnology (SEM). Salamanca, Spain 14-16 November 2012.

Bordas M., Araque I., **Margalef M.**, Rozès N., Reguant C., Bordons A. Potential stress markers in *Oenococcus oeni*: a comparative study of gene expression in wine-like media and in Penedès wine. 6th International Meeting on Biotechnology (BioSpain). Bilbao, Spain 19-21 September 2012.

**Margalef M.**, Bordas M., Araque I., Rozès N., Bordons A., Cordero R., Reguant C. Transcriptomic changes in *Oenococcus oeni* during its adaptation to wine harsh conditions. 6th International Meeting on Biotechnology (BioSpain). Bilbao, Spain 19-21 September 2012.

# ANNEX 2

## *ATG18* and *FAB1* Are Involved in Dehydration Stress Tolerance in *Saccharomyces cerevisiae*

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**Abstract**

Recently, different dehydration-based technologies have been evaluated for the purpose of cell and tissue preservation. Although some early results have been promising, they have not satisfied the requirements for large-scale applications. The long experience of using quantitative trait loci (QTLs) with the yeast *Saccharomyces cerevisiae* has proven to be a good model organism for studying the link between complex phenotypes and DNA variations. Here, we use QTL analysis as a tool for identifying the specific yeast traits involved in dehydration stress tolerance. Three hybrids obtained from stable haploids and sequenced in the *Saccharomyces* Genome Resequencing Project showed intermediate dehydration tolerance in most cases. The dehydration resistance trait of 96 segregants from each hybrid was quantified. A smooth, continuous distribution of the anhydrobiosis tolerance trait was found, suggesting that this trait is determined by multiple QTLs. Therefore, we carried out a QTL analysis to identify the determinants of this dehydration tolerance trait at the genomic level. Among the genes identified after reciprocal hemizygosity assays, *RSM22*, *ATG18* and *DBR1* had not been referenced in previous studies. We report new phenotypes for these genes using a previously validated test. Finally, our data illustrates the power of this approach in the investigation of the complex cell dehydration phenotype.

**Keywords:** anhydrobiosis, dehydration stress, *FAB1*, *ATG18*, yeast

## Introduction

Almost all yeast-based food industries are steadily expanding their use of active dry yeast (ADY) because of its greater genetic stability at room temperature and lower transport and storage costs. Unfortunately, most laboratory-developed industrial yeast strains, as well as strains isolated from industrial environments, have the biotechnological handicap of losing viability during the drying process (Dupont *et al.*, 2014). Therefore, such strains are excluded from the commercial catalogues of yeast manufacturers, awaiting a breakthrough that would allow their desiccation to be optimized. In a previous study, we performed a genetic screen of the *Saccharomyces cerevisiae* deletion library for mutants sensitive to dehydration stress (Rodríguez-Porrata *et al.*, 2012b). Among the genes characterized as essential for overcoming dehydration stress, only five (*SIP18*, *STF2*, *GRE1*, *YJL144w*, and *NOP6*) were found to have protective effects against dehydration stress when over-expressed (Rodríguez-Porrata *et al.*, 2012b; López-Martínez *et al.*, 2012). Recent studies investigating whether the response to desiccation involves regulation at the transcriptional and/or translational level detected changes in genes involved in lipid binding and synthesis, protein synthesis and mobility, and metabolism (Novo *et al.*, 2007; Singh *et al.*, 2005; Miermont *et al.*, 2007; Rossignol *et al.*, 2006; Nakamura *et al.*, 2008). However, correlations were rare between these transcriptomic studies and genetic screens using the *S. cerevisiae* deletion library of mutants sensitive to dehydration stress (Rodríguez-Porrata *et al.*, 2012b; Ratnakumar *et al.*, 2011; Cubillos *et al.*, 2011). In contrast, haploid strains overexpressing yeast genes encoding hydrophilic proteins (Stf2p, Sip18p, Gre1p, Yjl144wp, and Nop6p), which are essential for overcoming dehydration stress, are tolerant of dry conditions (Rodríguez-Porrata *et al.*, 2012b; López-Martínez *et al.*, 2012).

On the other hand, Rodríguez-Porrata *et al.* (2012a) showed that the knockout mutants for four nuclear apoptotic-related genes with mitochondrial functions (Δ*aif1*, Δ*nuc1*, Δ*cpr3*, and Δ*qcr7*) were hyper-tolerant of dehydration stress. Most *S. cerevisiae* genes involved in qualitative traits related to their basic biology have been identified using recombinant DNA techniques. However, many phenotypes important to industrially appear to be quantitative traits that are determined by quantitative trait loci (QTLs), such as growth temperature, ethanol tolerance, acetic

acid production, sporulation rate, sake aromatic compounds production, and nitrogen utilization (Cubillos *et al.*, 2011; Yang *et al.*, 2013; Hu *et al.*, 2007; Marullo *et al.*, 2007; Deutschbauer *et al.*, 2005; Katou *et al.*, 2008; Ambroset *et al.*, 2011). Considering the large amount of genetic variability in industrial yeast, a characteristic as crucial as dehydration tolerance is likely controlled by multiple QTLs that cannot be identified by conventional molecular genetic approaches.

In this paper, we performed QTL analysis on 96 segregants derived from a cross between two haploid strains derivatives of two strains of wine yeast using statistical linkage analysis between dehydration tolerance characteristics and DNA marker genotype data. We functionally characterized two QTLs encompassing six genes involved in dehydration stress tolerance that contribute to the natural phenotypic variation in the paternal strains (Cubillos *et al.*, 2011).

## Materials and Methods

### *Strains and plasmids*

Table 1 summarizes the yeast strains and plasmids used in this study. The *RIM15*, *BST1*, *BUD27*, *BLM10*, *YFH7*, *FAB1*, *ATG18*, *CBT1*, *MRP49*, *RSM22*, and *DBR1* genes were deleted using a short-flanking homology PCR technique in which *URA3* was the selectable marker (Figure S1B) in the *Mat α* and *Mat a* versions of the WA ($Hyg^R$), WA ($Nat^R$), WE ($Hyg^R$), and WE ($Nat^R$) strains (Schiest and Gietz, 1989). Degenerative primers (shown in Table S1) were used to amplify the *URA3* deletion module from the pNSU114 plasmid (Louis and Borts, 1995). Transformants were obtained using the lithium acetate transformation protocol and selected by plating on synthetic glucose media lacking uracil (Schiest and Gietz, 1989). $URA^+$ transformants were selected and restreaked to obtain single colonies, for which integrations were confirmed by PCR using the primer pair URA3Fw and GENERv, a reverse primer that anneals at the downstream region of the deleted gene (Table S1). The URA3 module was deleted from the WE, *Δatg18* strain by transforming single mutant strains with the PCR DNA fragment obtained using the ATGufw-ATGurv primer pair from the *atg18*::*URA3* locus. The transformants, which were able to grow in the presence of 5FOA and unable to grow on $SC^{-ura}$ medium, were further evaluated by PCR. The validated WE, *Δatg18u*

strain was further transformed, as mentioned previously, to obtain the WE, *Δatg18u*, *Δfab1* strain. Haploid strains with opposite mating types were crossed on yeast peptone dextrose agar (YPDA) medium supplemented with 100 μg·ml$^{-1}$ hygromycin B and 200 μg·ml$^{-1}$ nourseothricin sulfate. Diagnostics for isolates from individual colonies were made with the *MAT* locus by PCR using WA ($Nat^{R}$) and WE ($Hyg^{R}$) as tester strains (Huxley *et al.*, 1990). Recombinant DNA techniques were carried out according to standard protocols (Sambrook *et al.*, 2001). The amplification reactions contained a 1x PCR buffer, 1.25 mM dNTPs, 1.0 mM $MgCl_2$, 0.3 μM of each primer, 2 ng·μl$^{-1}$ template DNA, and 3.5 U DNA Polymerase in a total volume of 100 μl. All reactions were performed using a PCR thermal cycler for 25 cycles, as follows: denaturation, 2 min at 94°C; primer annealing, 30 s at 55°C; and primer extension, 1.5 min at 68°C.

**Table 1** Strains and plasmid used in the study.

| Strain | Relevant characteristics | References |
|---|---|---|
| BY4742 | *MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0* | Bryant and Stevens, 1998 |
| DBVPG6044 (WA $Hyg^{R}$) | *MATa, ho::HygMX, ura3::KanMX* | Kim *et al.*, 2000 |
| DBVPG6044 (WA $Nat^{R}$) | *MATα, ho::NatMX, ura3::KanMX* | Kim *et al.*, 2000 |
| DBVPG6765 (WE $Hyg^{R}$) | *MATa, ho::HygMX, ura3::KanMX* | Kim *et al.*, 2000 |
| DBVPG6765 (WE $Nat^{R}$) | *MATα, ho::NatMX, ura3::KanMX* | Kim *et al.*, 2000 |
| Y12 (SA $Hyg^{R}$) | *MATa, ho::HygMX, ura3::KanMX* | Kim *et al.*, 2000 |
| YPS128 (NA $Hyg^{R}$) | *MATa, ho::HygMX, ura3::KanMX* | Kim *et al.*, 2000 |
| WE/NA | WE $Nat^{R}$/NA $Hyg^{R}$ | Cubillos *et al.*, 2011 |
| WE/WA | WE $Nat^{R}$/ WA $Hyg^{R}$ | Cubillos *et al.*, 2011 |
| WA/WE | WA $Nat^{R}$/ WE $Hyg^{R}$ | This work |
| WE/SA | WE $Nat^{R}$/ SA $Hyg^{R}$ | Cubillos *et al.*, 2011 |
| 96 spores WE/NA | F1 from WE $Nat^{R}$/NA $Hyg^{R}$ | Cubillos *et al.*, 2011 |
| 96 spores WE/WA | F1 from WE $Nat^{R}$/ WA $Hyg^{R}$ | Cubillos *et al.*, 2011 |
| 96 spores WE/SA | F1 from WE $Nat^{R}$/ SA $Hyg^{R}$ | Cubillos *et al.*, 2011 |
| WA, *Δrim15* | *MATα, ho::NatMX, rim15::URA3* | This work |
| WA, *Δbst1* | *MATα, ho::NatMX, bst1::URA3* | This work |
| WA, *Δbud27* | *MATα, ho::NatMX, bud27::URA3* | This work |
| WA, *Δblm10* | *MATα, ho::NatMX, blm10::URA3* | This work |
| WA, *Δyfh7* | *MATα, ho::NatMX, yfh7::URA3* | This work |
| WA, *Δfab1* | *MATα, ho::NatMX, fab1::URA3* | This work |
| WA, *Δatg18* | *MATα, ho::NatMX, atg18::URA3* | This work |
| WA, *Δcbt1* | *MATα, ho::NatMX, cbt1::URA3* | This work |
| WA, *Δmrp49* | *MATα, ho::NatMX, mrp49::URA3* | This work |
| WA, *Δrsm22* | *MATα, ho::NatMX, rsm22::URA3* | This work |
| WA, *Δdbr1* | *MATα, ho::NatMX, dbr1::URA3* | This work |
| WE, *Δrim15* | *MATα, ho::NatMX, rim15::URA3* | This work |
| WE, *Δbst1* | *MATα, ho::NatMX, bst1::URA3* | This work |
| WE, *Δbud27* | *MATα, ho::NatMX, bud27::URA3* | This work |
| WE, *Δblm10* | *MATα, ho::NatMX, blm10::URA3* | This work |
| WE, *Δyfh7* | *MATα, ho::NatMX, yfh7::URA3* | This work |
| WE, *Δfab1* | *MATα, ho::NatMX, fab1::URA3* | This work |
| WE, *Δatg18* | *MATα, ho::NatMX, atg18::URA3* | This work |
| WE, *Δrpl2a* | *MATα, ho::NatMX, rpl2a::URA3* | This work |
| WE, *Δcbt1* | *MATα, ho::NatMX, cbt11::URA3* | This work |
| WE, *Δmrp49* | *MATα, ho::NatMX, mrp49::URA3* | This work |
| WE, *Δrsm22* | *MATα, ho::NatMX, rsm22::URA3* | This work |
| WE, *Δdbr1* | *MATα, ho::NatMX, dbr1::URA3* | This work |
| WE, *Δatg18u* | *MATa, ho::HygMX, atg18::ura3* | This work |
| WE, *Δatg18u*, *Δfab1* | *MATa, ho::HygMX, atg18::ura3, fab1::URA3* | This work |

| | | |
|---|---|---|
| WA/*Δrim15*^WE | WA *Hyg^R*/WE, *Δrim15* | This work |
| WA/*Δbst1*^WE | WA *Hyg^R*/WE, *Δbst1* | This work |
| WA/*Δbud27*^WE | WA *Hyg^R*/WE, *Δbud27* | This work |
| WA/*Δblm10*^WE | WA *Hyg^R*/WE, *Δblm10* | This work |
| WA/*Δyfh7*^WE | WA *Hyg^R*/WE, *Δyfh7* | This work |
| WA/*Δfab1*^WE | WA *Hyg^R*/WE, *Δfab1* | This work |
| WA/*Δatg18*^WE | WA *Hyg^R*/WE, *Δatg18* | This work |
| WA/*Δrpl2a*^WE | WA *Hyg^R*/WE, *Δrpl2a* | This work |
| WA/*Δcbt1*^WE | WA *Hyg^R*/WE, *Δcbt1* | This work |
| WA/*Δmrp49*^WE | WA *Hyg^R*/WE, *Δmrp49* | This work |
| WA/*Δrsm22*^WE | WA *Hyg^R*/WE, *Δrsm22* | This work |
| WA/*Δdbr1*^WE | WA *Hyg^R*/WE, *Δdbr1* | This work |
| WE/*Δrim15*^WA | WE *Hyg^R*/WA, *Δrim15* | This work |
| WE/*Δbst1*^WA | WE *Hyg^R*/WA, *Δbst1* | This work |
| WE/*Δblm10*^WA | WE *Hyg^R*/WA, *Δblm10* | This work |
| WE/*Δyfh7*^WA | WE *Hyg^R*/WA, *Δyfh7* | This work |
| WE/*Δfab1*^WA | WE *Hyg^R*/WA, *Δfab1* | This work |
| WE/*Δatg18*^WA | WE *Hyg^R*/WA, *Δatg18* | This work |
| WE/*Δcbt1*^WA | WE *Hyg^R*/WA, *Δcbt1* | This work |
| WE/*Δmrp49*^WA | WE *Hyg^R*/WA, *Δmrp49* | This work |
| WE/*Δrsm22*^WA | WE *Hyg^R*/WA, *Δrsm22* | This work |
| WE/*Δdbr1*^WA | WE *Hyg^R*/WA, *Δdbr1* | This work |
| WA/*Δatg18u*^we, Δ*fab1*^we | WA *Nat^R*/WE, *Δatg18u, Δfab1* | This work |
| **Plasmid** | | |
| pNSU114 | | Weedon *et al.*, 2008 |

*Growth conditions and desiccation-rehydration process*

Yeast strains were grown in shake flasks at 150 rpm in SC medium containing 0.17% yeast nitrogen base, 2% glucose, 0.5% $(NH_4)_2SO_4$, and 25 $mg \cdot l^{-1}$ uracil. The desiccation-rehydration process and yeast viability assays were performed as previously described (Rodríguez-Porrata *et al.*, 2011).

*Linkage analysis*

Linkage analysis was performed using the rQTL software, and the LOD score was calculated using a normal model (Cubillos *et al.*, 2011; Broman *et al.*, 2003; Salinas *et al.*, 2012). Briefly, the significance of a QTL was determined from permutations. For each trait and cross, we permuted the phenotype values within tetrads 1,000 times and recorded the maximum LOD score each time. A QTL was considered significant if its LOD score was greater than the 0.05 tail of the 1,000 permuted LOD scores.

*RNA isolation and cDNA synthesis*

The total RNA was obtained from: WE, WA, WE, *Δatg18*, WE, *Δfab1*, WA, *Δatg18*, WA, *Δfab1*, and WA/*Δatg18u*^*WE*, *Δfab1*^*WE* yeast cells using a GeneJET RNA Kit (Thermo Scientific, Lithuania) according to the manufacturer's protocol. The RNA was resuspended in 100 µL RNase-free water. The DNase I RNAase free kit (Fermentas, Thermo Scientific) was used to remove the 16 genomic DNA from the RNA

preparations. The RNA was quantified with a spectrophotometer (Nanodrop 1,000 Spectrophotometer, Thermo Scientific) at an absorbance of 260 nm and tested for purity (by the A260/280 ratio) and integrity by denaturing gel electrophoresis. The first strand of cDNA was reverse transcribed from 1 μg total RNA from each sample using a First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific) according to the manufacturer's protocol. An identical reaction without the reverse transcription was performed to verify the absence of genomic DNA. The cDNA was subsequently amplified by PCR using yeast strain specific couple of primers forward-reverse for: *ATG18*, *FAB1*, *ALG9* and *TAF10* genes (Table S1).

*Real-time RT-PCR*

Quantitative PCR for *ATG18* and *FAB1*, was carried out using a Real Time qPCR kit according to the manufacturer's protocol and was analysed on a Real-Time PCR Detection System. The thermal cycling was composed of an initial step at 50°C for 2 min followed by a polymerase activation step at 95°C for 10 min and a cycling step with the following conditions: 40 cycles of denaturation at 95°C for 15s, annealing at 63°C for 1 min, and extension at 72°C for 1 min. Oligonucleotides of varying lengths produce dissociation peaks at different melting temperatures. Therefore, at the end of the PCR cycles, the PCR products were analysed using a heat dissociation protocol to confirm that a single PCR product was detected by the SYBR Green dye. The fluorescence data was acquired at the 72°C step. The threshold cycle ($C_t$) was calculated using a software to indicate significant fluorescence signals above the noise during the early cycles of amplification. The software calculated copy numbers for the target samples from the Ct using interpolation from the standard curve. The relative levels of expression of the target genes were measured using *ALG9* and *TAF10* mRNA as an internal control and calculated according to Livak and Schmittgen (2001).

*Microscopy*

Cultures of strains were grown to the stationary phase in selective medium. The cells were washed with 1× PBS buffer (pH 7.4) and fixed in 70% ethanol for 10 min at room temperature. Fluorescence was viewed using a fluorescence microscope. A digital camera and a software were used for image acquisition.

*Statistical analysis*

To determine the statistical significance of data the results were analysed by one-way ANOVA, the Shapiro-Wilk test and the Scheffé test were carried out using a statistical software package. Statistical significance was set at $p<0.001$.

## Results

*Variation in dehydration stress tolerance in recombinant yeast populations*

Using a colony-counting assay, desiccation tolerance was assessed for a set of three recombinant populations of 96 segregants generated from a cross of divergent *S. cerevisiae* isolates (WE [Wine European] x WA [West African], WE x NA [North American], and WE x SA [Sake]) previously described (Figure S1A) (Cubillos *et al.*, 2011). The mean CFU (colony-forming units) per ml value for survival after rehydration was calculated, taking into account the viability before drying (Figure 1A-C). The *W* value obtained from the Shapiro-Wilk test carried out with the three sets of segregants were lower than 0.5, therefore, for an α level of 0.05, the phenotypic distributions of segregants did not show a normal distribution, suggesting a polygenic contribution to cellular desiccation tolerance (Figure 1A-C). The highest number of transgressive segregants (24%) was observed in the cross between the low dehydration stress-resistant strains WE (20.3%) and WA (49.4%) (Figure 1A). However, when the highly sensitive WE strain was crossed with the resistant SA and NA strains (75.9% and 70.5%, respectively), approximately 5.5% of segregants exceeded the phenotypic range of their parents by at least 2 SD, criteria previously used to name these segregants as transgressive, (Figure 1B-C) (Marullo *et al.*, 2006).

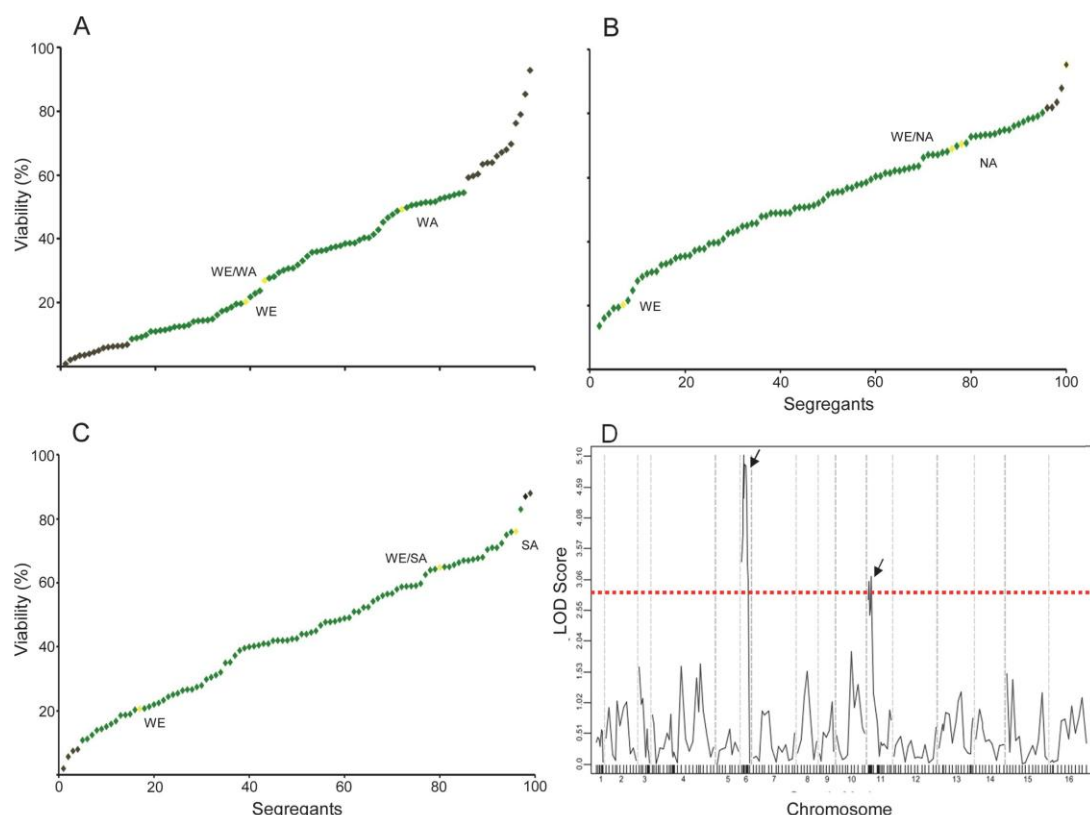


**Figure 1** Viability rate variation after dehydration stress. Viability rate values are shown on the *y*-axis for the 96 ranked segregants of the WE x WA cross (A), WE x NA cross (B), and WE x SA cross (C). Dots indicate segregants with transgressive phenotypes (exceeding two parental standard deviations, black), parental and hybrid strains (yellow), and segregants within the phenotypic range of the parental strains (green). D) Linkage analysis for dehydration stress tolerance from WE/WA segregants. The chromosomes are displayed on the *x*-axis, and LOD viability values, according to each molecular marker across the 16 yeast chromosomes, are displayed on the *y*-axis. The significant LOD score threshold is indicated by a red line and was determined by a permutation test. The significant QTLs are indicated by arrows.

By running a linkage analysis using ~200 previously reported genotype markers, we evaluated whether the different genotypes correlated with the viability trend observed in the WE/WA strain segregants (Cubillos *et al.*, 2011). Only the genetic markers *Y034W*, *BST1*, *FRS2*, *RPN11*, *ROG3*, *TRP3*, and *FAS1* showed significant differences ($p<0.005$). The same analysis performed for the segregants from the WE/NA and WE/SA strains did not show any correlation between genomic region and cell viability.

*Identification of QTLs involved in dehydration tolerance*

To identify the QTL intervals responsible for natural phenotypic variations in dehydration stress, linkage analysis was performed based on the cellular viability

after stress induction and the genotypes of the 96 F1 segregants (Cubillos *et al.*, 2011, Louis and Borts, 2009). In total, two significant regions were mapped using the marker regression model and permutation method in the WE x WA cross, allowing the identification of 15 candidate genes (Figure 1D; Table 2). A region in chromosome XI (from 37 to 137 kb) with a peak LOD score of 3.10 was identified and after further inspections, we identified seven candidate genes (*CBT1*, *YKT6*, *FAS1*, *MRP49*, *RSM22*, *DBR1* and *AVT3*) within this QTL. In the second QTL (Chr VI, LOD 5.1), eight candidate genes (*RIM15*, *BST1*, *BUD27*, *BLM10*, *YFH7*, *FAB1*, *ATG18* and *ROG3*) were identified between 65 KB and 196 KB. After a sequence alignment, only 11 of the genes encompassed by either QTL interval (*RIM15, BST1 BUD27, BLM10, YFH7, FAB1, ATG18, CBT1, MRP49, RSM22* and *DBR1*) contained single nucleotide polymorphisms (SNPs) (Table 2). Furthermore, the SNPs did not create premature stop codons in the coding sequence of the WE and WA strains. Among these genes, only *BUD27*, *FAB1*, and *CBT1* were found to be necessary for the yeast to overcome desiccation stress (Rodríguez-Porrata *et al.*, 2012b; Ratnakumar *et al.*, 2011; Salinas *et al.*, 2012).

**Table 2** The position in the genome, significance value, genes in the respective regions and the differences in the amino acid sequence for each gene in WE strain versus WA are described. – allele without mismatch

| Chromosome | QTL's | Position (cM) | LOD | Gene / Position | Position of amino acid change *WA* allele→*WE* allele |
|---|---|---|---|---|---|
| VI | Y034w | 65 | 3.85 | *RIM15* / 69.11 | 161 E → K; 240 S→G; 249 E→D; 251 T→S; 366 T→S; 399 V→A; 771 R→P; 1020 T→I; 1022 C→Y |
| | BST1 | 84 | 5.11 | *BST1* / 84.14 | 202 A→T; 221 N→D; 253 A→P; 432 N→D; 438 K→M; 506 Q→L; 610K→R; 636S→W; 849 D→V |
| | | | | *BUD27* /90.9 | 32 Δ→E; 33D→Y; 75 S→F; 177 E→G; 182 D→E |
| | HTX10 | 111 | 4.95 | *BLM10* / 123.47 | 99 Q→R; 220 T→A; 258 G→A; 729 S→N; 759 I→V; 791 N→D; 902 C→Y; 1102 R→K; 1315 G→S; 1444 D→N; 1586 P→A; 1592 R→C; 1698 T→A; 1782 G→D; 1861 D→Y; 1900 I→V; 1971 M→I |
| | ARS605 | 136 | 4.93 | - | |
| | RPN11 | 153 | 4.50 | *YFH7* / 159.29 | 109 V→I; 138 A→T; 149 V→A |
| | YFR016c | 180 | 3.32 | *FAB1* / 184.50 | 120 S→N; 126 N→S; 333 A→S; 583 Δ→N; 1273 N→D; 1300 Y→H; 1524 G→E; 1604 R→M; 1780 P→S; 1878 I→M; 1882 S→A; 1884 Q→Δ |
| | | | | *ATG18* / 194.81 | 195 N→S |
| | ROG3 | 196 | 2.40 | - | |
| XI | TRP3 | 37 | 2.72 | *CBT1* / 47.15 | 29 S→G; 109 T→A |
| | ARS1103 | 58 | 3.03 | - | |
| | YKT6 | 75 | 2.46 | - | |
| | FAS1 | 103 | 2.58 | - | |
| | TP05 | 121 | 3.10 | *MRP49* / 133.72 | 131 G→R |
| | PIR1 | 142 | 2.34 | *RSM22* / 159.45 | 228 E→K; 474 D→S; 619 S→N |
| | | | | *DBR1* / 167.61 | 223 Q→R; 286 K→E; 325 N→D |
| | AVT3 | 173 | 1.16 | - | |

*Dissection of the QTLs associated with stress tolerance*

To identify causative genes within the mapped QTL intervals on chromosomes VI and XI, we generated a set of haploid strains with deletions in the candidate genes (Table 1). Then, their desiccation tolerance capacity was assessed (Figure 2). After rehydration, four strains (WA, Δ*bud27*; WA, Δ*fab1*; WA, Δ*atg18*; and WA, Δ*cbt1*) exhibited a similar reduction in cell viability values, which were ~20% lower than in the WA strain (49%). Surprisingly, the same set of gene deletions in the WE genetic background showed the opposite effect, with viability values ~30% higher than the WT. In addition, both versions of the Δ*dbr1* strain showed significantly higher viability values after dehydration stress compared with the WT WA and WE strains (20% and 80%, respectively). Furthermore, the WE, Δ*rsm22* strain displayed 30% higher viability than its reference strain, whereas the WA, Δ*rsm22* strain had similar viability to the WA strain. The viabilities of the Δ*rim15*, Δ*bst1*, Δ*blm10*, Δ*yfh7*, and Δ*mrp49* strains were not significantly different from the WT strains, WA and WE, suggesting that these genes are not involved in desiccation-rehydration stress resistance. Therefore, two-thirds of the WE mutants enhanced dehydration stress tolerance, suggesting that the $BUD27^{WE}$, $FAB1^{WE}$, $ATG18^{WE}$, $CBT1^{WE}$, and $RSM22^{WE}$ alleles have a detrimental effect on the ability of the WE strain to overcome this type of stress.

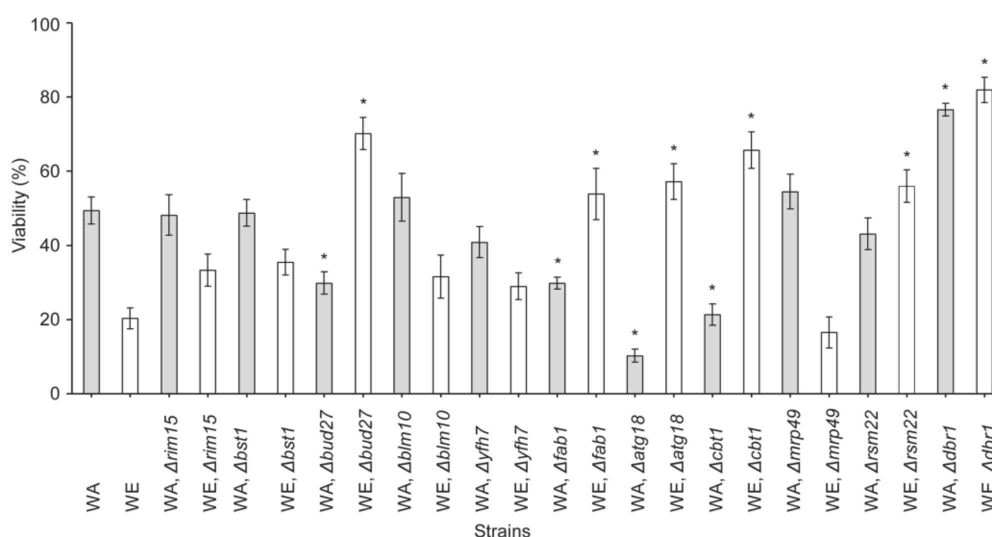


**Figure 2** Effect of knockout haploid strains on yeast viability after dehydration-rehydration stress (DRS). The scale of viability (%) indicates the percentage of experimental values for the different strains. The values shown are means of $n$ = 3 independent samples ± SD. *Significant differences ($p$<0.01) between knockout and its own parental strains.

To confirm the impact of these alleles on dehydration stress, we used a reciprocal hemizygosity analysis (Figure S1B) (Marullo *et al.*, 2006). A set of isogenic hybrid strains was developed by crossing the haploid knockout strains with the complementary WA (*Nat*$^R$) or WE (*Hyg*$^R$) strain [e.g., WA (*Nat*$^R$) x WE Δ*rim15* (*Hyg*$^R$) or WA Δ*rim15* (*Hyg*$^R$) x WE (*Nat*$^R$), Table 1]. The desiccation tolerance of the hemizygous strains was measured (Figure 3). The WA/Δ*bud27*$^{WE}$ strain showed ~40% higher viability than the WA/WE strain, which correlated with the increased viability of the WE, Δ*bud27* strain after stress induction, suggesting an adverse effect of the *BUD27*$^{WE}$ allele on stress resistance. Additionally, the WE/Δ*bud27*$^{WA}$ strain could not be obtained, suggesting a certain level of incompatibility between the *BUD27*$^{WE}$ allele and the WA genetic background. After dehydration stress induction, the hybrid strains carrying *FAB1*$^{WA}$, *ATG18*$^{WA}$, *CBT1*$^{WE}$, and *RSM22*$^{WA}$ showed viability values nearly 30% higher than the hybrids carrying *FAB1*$^{WE}$, *ATG18*$^{WE}$, *CBT1*$^{WA}$, and *RSM22*$^{WE}$ and the reference strains. The detrimental effects of the *FAB1*$^{WE}$, *ATG18*$^{WE}$, *CBT1*$^{WA}$, and *RSM22*$^{WE}$ alleles on overcoming dehydration stress were concomitant with the enhanced viability values obtained for the WE, Δ*fab1*, WE, Δ*atg18*, WA, Δ*cbt1*, and WE, Δ*rsm22* strains (Figure 2). Furthermore, hybrids carrying either the *DBR1*$^{WE}$ or *DBR1*$^{WA}$ allele exhibited 30% higher viability than the heterozygous strains (Figure 3). From the cell viability results for the WA, Δ*dbr1*, WE, Δ*dbr1* and heterozygous strains, a correlation can be assumed between the increasing number of *DBR1* allele copies per cell and the decreasing desiccation survival rate. The desiccation tolerances of a collection of 4,850 viable mutant haploid strains (BY4742) were previously assessed (Rodríguez-Porrata *et al.*, 2012b; Shima *et al.*, 2008). For the genes above, only the Δ*rsm22* and Δ*dbr1* strains (BY4742 background) exhibited significantly higher viability values after stress induction (73% and 77%, respectively) compared with the BY4742 strain. The viability of the Δ*rim15*, Δ*bst1*, Δ*bud27*, Δ*yfh7*, Δ*fab1*, Δ*atg18*, and Δ*cbt1* strains did not significantly differ from the reference strain (34%) (Rodríguez-Porrata *et al.*, 2012a). However, the BY4742, Δ*mrp49* strain showed 20% viability, which contrasts with the unchanging viability values for the WA, Δ*mrp49* and WE, Δ*mrp49* strains. These results confirm that *RSM22*$^{WE}$, which has 98% sequence identity to the *RSM22*$^{BY4742}$, *DBR1*$^{WA}$, *DBR1*$^{WE}$, and *DBR1*$^{BY4742}$ gene products, has a detrimental effect on dehydration stress tolerance.

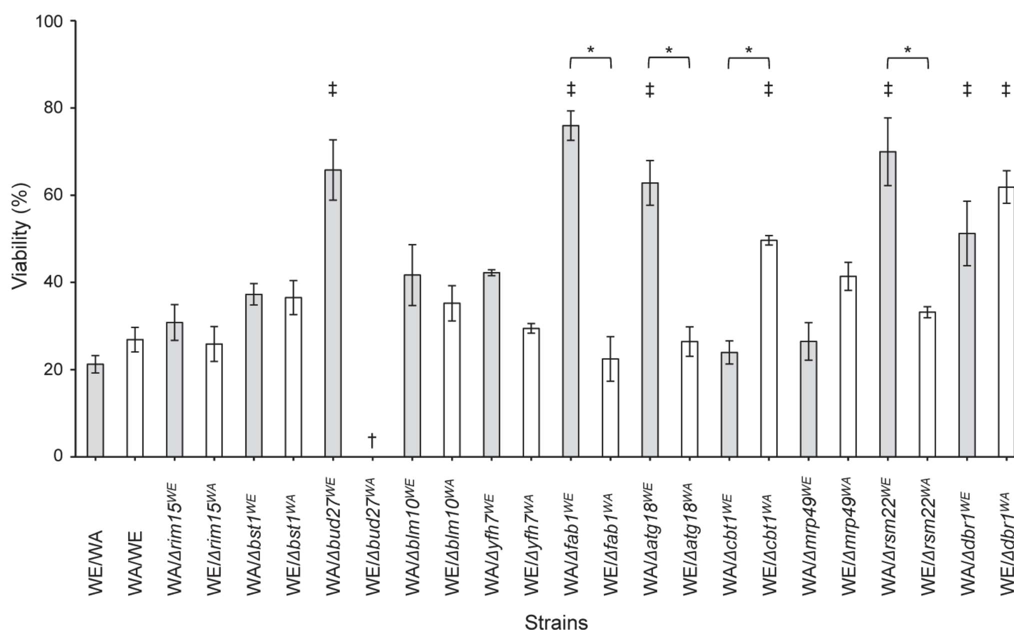


**Figure 3** Hybrid viability after DRS. The scale of viability (%) indicates the percentage of experimental values for the different strains. The values shown are the means of at least $n = 3$ independent samples ± SD. † Non-viable strain. *Significant differences at $p<0.01$ between hemizygous strains. ‡ Significant differences at $p<0.01$ between the hemizygous and reference strains.

### *The ATG18$^{WE}$ allele compromises vacuole function*

Atg18p is a key component in retrograde membrane trafficking from the vacuole to the Golgi apparatus via the endosome and is also an apparent effector and modulator of phosphatidylinositol (3,5)-bisphosphate [PtdIns(3,5)*P*2] (Efe *et al.*, 2007). It should be noted that the vacuole is responsible for amino acid storage and therefore represents the cellular reserve of nitrogen and phosphate. When yeast cells are exposed to starvation conditions, such as upon entrance into the stationary phase or during sporulation, vacuolar hydrolases are up-regulated to obtain recycled nutrients through the turnover of macromolecules (Klionsky *et al.*, 1990). It follows then that malfunctions in the nutrient storage or recycling machinery are likely to compromise cell viability. Homozygous diploid Δ*atg18* is defective in autophagy prior to vacuole fusion of autophagosomes, causing the development of cell sensitivity to nitrogen starvation and non-sporulating cells (Barth *et al.*, 2001). The hybrid carrying *ATG18$^{WA}$* showed 35% higher asci formation than the WE (*Nat$^{R}$*)/WA (*Hyg$^{R}$*) and WA (*Nat$^{R}$*)/WE (*Hyg$^{R}$*) strains, at 7% and 3%, respectively. However, the hybrid carrying *ATG18$^{WE}$* showed the lowest asci formation, at 0.5% of the total cells (Figure 4A). The

wild-type and hemizygous strains were first grown to the mid-log phase and then shifted to nitrogen starvation conditions, and their viability was determined over time (Figure 4B). The hybrid strains survived nine days of nitrogen starvation with no significant decrease in viability. In contrast, the number of viable cells for the hybrid carrying *ATG18* $^{WE}$ and the hybrid carrying *ATG18* $^{WA}$ decreased by up to 60% and 20%, respectively, over the same time period. Additionally, Δ*atg18* cells exhibited phenotypic defects, including non-acidic and conspicuous vacuoles and the loss of osmotic stress tolerance (Yamamoto *et al.*, 1995). To determine putative changes in vacuole morphologies, samples of aerated wild-type, WA/Δ*atg18*$^{WE}$, and WE/Δ*atg18*$^{WA}$ cells in the stationary phase were analysed by fluorescence microscopy using FM4-64 and the blue fluorescent dye Arg-CMAC, which accumulates in acidic vacuoles (Figure 4D).

Both Δ*atg18* hemizygous strains had larger vacuoles than the WE/WA cells, but the hybrid carrying *ATG18*$^{WE}$ showed abnormal vacuolar acidification compared with the hybrid carrying *ATG18*$^{WA}$ and the WE/ WA strains. To assess the consequences of the *ATG18*$^{WE}$ allele, the osmotic sensitivity was tested when the cells were grown on media containing 1 M NaCl or 1 M sorbitol at 28°C and 37°C (Figure 4C). On the 1 M NaCl plates, the hybrid carrying *ATG18*$^{WA}$ showed better growth performance at 37°C and 28°C relative to the hybrid carrying *ATG18*$^{WE}$. No significant growth differences were exhibited between hybrids for the other serial dilutions grown on YPD and 1 M sorbitol at 37°C and 28°C. The data indicates that *ATG18*$^{WE}$ may not provide adequate nutrient storage to tolerate starvation conditions, thereby inducing both low cell viability under nitrogen starvation conditions and impaired asci formation. The *ATG18*$^{WE}$ allele was more sensitive to osmotic stress at high temperatures than the *ATG18*$^{WA}$ allele, which correlated with the differences in dehydration tolerance observed for these alleles. Furthermore, the ionic osmotic sensitivity showed by the hybrids carrying either the *ATG18*$^{WA}$ or the *ATG18*$^{WE}$ allele reverted to a resistant phenotype when the cells were grown at a high temperature.

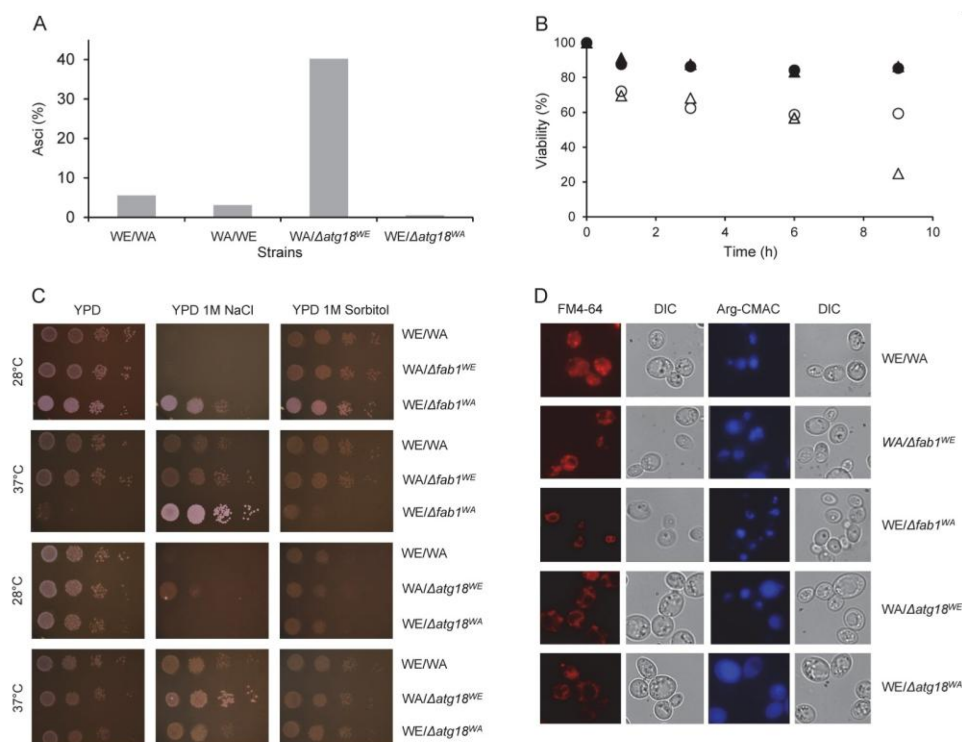


**Figure 4** Phenotypic characterization of *ATG18* and *FAB1* alleles. A) After 48 hours on 1% K-acetate, the count of asci were expressed as a percentage of total cells. B) Effect of nitrogen starvation on cell viability of the Δ*atg18* strains. The hybrid WE (*Nat*$^R$)/WA (*Hyg*$^R$) (●), WA (*Nat*$^R$)/WE (*Hyg*$^R$) (▲), WE/*ATG18* $^{WA}$ (o), and WA/*ATG18* $^{WE}$ (Δ). The scale of viability (%) indicates the percentage of viable cells for the different strains against the time in starvation medium. Values are the mean of triplicate measurements, and the standard deviation was less than 15%. C) *FAB1*$^{WA}$ and *ATG18*$^{WE}$ rescue cells from ionic-hyperosmotic stress at 37°C plated on YPD medium, YPD medium containing 1 M NaCl, and 1 M sorbitol. D) Hemizygous cells show vacuole fragmentation and vacuole acidification deficiency. Each pair of image columns show phase microscopy of the same field, which shows cells stained with FM4-64 to visualize vacuole membrane, pH vacuolar dye cell blue Arg-CMAC, and the differential interference contrast (DIC) images.

### *The FAB1*$^{WE}$ *allele enhances osmotic ionic stress tolerance*

Retrograde membrane traffic from the vacuole to the Golgi apparatus via the endosome depends on PtdIns(3,5)*P*2 (Gary *et al.*, 2002; Dove *et al.*, 2002). The kinase FAB1p generates PtdIns(3,5)*P*2 via phosphatidylinositol (3)-phosphate phosphorylation (Gary *et al.*, 1998; Dove *et al.*, 2004). Abnormal levels of PtdIns(3,5)*P*2 were observed in Δ*atg18* yeast cells, suggesting that Atg18p is an inhibitor of the Fab1p kinase (Merz and Westermann, 2009). Yamamoto *et al.* (1995) suggested that *fab1* mutations in yeast cells cause aberrant chromosome segregation, defects in cell surface integrity, and deficiencies in vacuole morphology and function.

To determine the incidence of *FAB1* alleles in vacuole activity, WA/Δ*fab1*$^{WE}$ and WE/Δ*fab1*$^{WA}$ cells were grown on medium containing 1 M NaCl or 1 M sorbitol at 28°C and 37°C (Figure 4C). The hybrid carrying *FAB1*$^{WE}$ grew on 1 M NaCl at 28°C, whereas the hybrid carrying *FAB1*$^{WA}$ and the WE/WA strain did not. However, all of the strains grew similarly on 1 M sorbitol. At 37°C, the hybrid carrying *FAB1*$^{WE}$ was osmoremediated on 1 M NaCl but was not recovered on 1 M sorbitol. The data indicates that ionic osmotic stress rescues the growth of *FAB1*$^{WE}$ hemizygous cells at this non-permissive temperature. The vacuolar morphology and activity of hybrid-carrying *FAB1*$^{WA}$ or *FAB*$^{WE}$ in the stationary phase were analysed using FM4-64 and Arg-CMAC dyes, respectively (Figure 4D). The vacuolar acidity Arg-CMAC dye profile of the hemizygote cells was similar to that of the reference cells. However, Arg-CMAC and FM4-64 staining revealed vacuolar fragmentation in the hybrid carrying *FAB1*$^{WE}$, which contrasts with the single large vacuole per cell observed in both the hybrid carrying *FAB1*$^{WA}$ and the WE/WA strain. The *FAB1*$^{WE}$ allele is more sensitive than the *FAB1*$^{WA}$ allele to osmotic stress at high temperatures, which correlates with the differences in dehydration tolerance observed for these alleles. Alternatively, an isogenic strain was developed by crossing the haploid double knockout strain WE, Δ*atg18u*, Δ*fab1* with the complementary WA (*Nat*$^{R}$) strain (Table 1). The WA/Δ*atg18u*$^{WE}$, Δ*fab1*$^{WE}$ strain showed ~60% higher viability than the WA/WE strain, which was correlated with the increase in viability of the WE, Δ*atg18u*, Δ*fab1* strain after dehydration stress, which showed 65% viability (data not shown). Surprisingly, the double knockout WA, Δ*atg18u*, Δ*fab1* strain could not be obtained. To exclude putative artificial regulatory effect of the deletions over the genes *ATG18* or *FAB1*, which are in the same chromosome at a distance of 3.5 kb, we quantified their expression in samples from WA; WE; WA, Δ*fab*1; WA, Δ*atg18*; WE, Δ*fab*1; WE, Δ*atg18* and WA/Δ*atg18u*$^{WE}$, Δ*fab*1 strains (Figure S2). Our data showed no statistically significant differences between the controls and the strain samples in the expression of any of the tested genes.

*The CBT1 and RSM22 alleles do not show respiratory deficiencies*

From a gene pool identified after a large-scale functional analysis of respiratory-deficient yeast, the mutant Δ*cbt1 and* Δ*rsm22* strains showed impaired respiratory performance (Merz and Westermann, 2009). The mitochondrial small ribosomal

subunit protein Rsm22p participates in mitochondrial mRNA translation, and Cbt1p is involved in mt mRNA stabilization. Both of these proteins are essential for respiratory growth. To assess the putative effects of these alleles on respiration activity, serial dilutions of the wild-type, WA/Δ*cbt1*$^{WE}$, WE/Δ*cbt1*$^{WA}$, WA/Δ*rsm22*$^{WE}$, and WE/Δ*rsm22*$^{WA}$ strains were plated on YPD and YPG media and incubated at 28°C for 24 h and48 h. No significant differences in growth were observed between the different hybrids on YPG medium with glycerol as the respiratory carbon source (Figure 5A), suggesting that the *CBT1* and *RSM22* alleles do not significantly affect the respiratory activity of hybrid cells. Therefore, both the hybrid carrying *CBT1*$^{WE}$ and the hybrid carrying *RSM22*$^{WA}$ enhance dehydration tolerance with no apparent variation in cellular respiration.

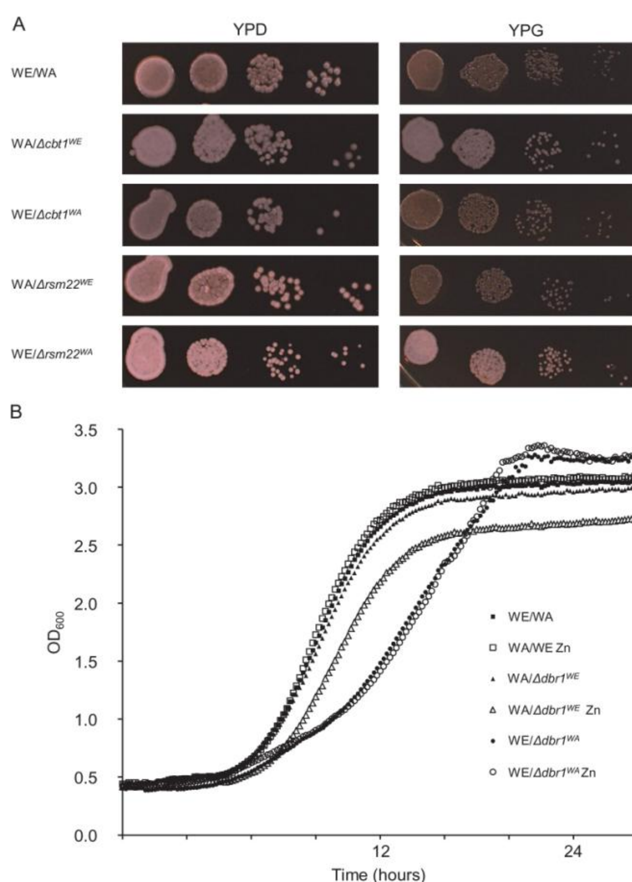


**Figure 5** Phenotypic characterization of *CBT1*, *RSM22*, and *DBR1* alleles. A) *CBT1* and *RSM22* alleles did not show respiratory deficiency. Serial dilutions of heterozygous and hemizygous strain cells were plated on YPD medium and YPG medium containing 2% glycerol, which were grown at 28°C for one and two days, respectively. B) The hybrid carrying *DBR1*$^{WA}$ shows defective competitive fitness. Optical density at 600 nm (OD600) was monitored every 10 min as a growth measure at 28°C of the strains in SD medium and SD medium containing 3.5 mM $ZnCl_2$.

*The DBR1$^{WA}$ allele provides competitive disadvantages to yeast cells*

The RNA lariat debranching enzyme Dbr1p is involved in intron turnover, which is required for efficient Ty1p transposition (Chapman and Boeke, 1991). The phenotypes already described for the Δ*dbr1* strain include decreasing competitive fitness and lower resistance to zinc deficiency (Breslow *et al.,* 2008; North *et al.*,2012). We aimed to ascertain the growth performance of the Δ*dbr1* hemizygous strains in minimal medium and minimal medium supplemented with 1 μM, 3.5 mM, or 7 mM zinc dichloride (Figure 5B shows the growth with 3.5 mM $ZnCl_2$). Hybrids carrying *DBR1$^{WA}$* and *DBR1$^{WE}$* exhibited doubling times (DT) that were 5.8 min and 67.7 min higher, respectively, than the WE/WA strain. Both the hybrid carrying *DBR1$^{WE}$* and the reference strain showed similar DT in media with or without Zn, but the hybrid carrying *DBR1$^{WA}$* exhibited a 24.8 min higher DT in the presence of Zn than when grown in minimal medium alone.

**Discussion**

Most of the genetic determinants of dehydration tolerance in yeast are still unknown. In this paper, two dehydration-tolerant QTLs were identified using a segregating population. By analysing strains with deleted genes in each QTL and by reciprocal hemizygosity assays, six genes have been confirmed to affect the capacity of yeast cells to survive dehydration and rehydration, namely the *BUD27*, *FAB1*, and *ATG18* genes mapped to QTLs on chromosome VI and the *CBT1*, *RSM22*, and *DBR1* genes in QTLs on chromosome XI. Furthermore, their phenotypic effects have been estimated. The genes *ATG18*, *RSM22*, and *DBR1* were not found to be necessary for desiccation tolerance in yeast cells (Rodríguez-Porrata *et al.*, 2012b; Ratmakumar *et al.*, 2011). The fact that the genes mapped in our results do not fully coincide with previous genetic studies carried out with the *S. cerevisiae* deletion libraries of mutants sensitive to dehydration stress may indicate that different cellular mechanisms for overcoming stress imposition were caused by dissimilar selective forces exerted during the evolution of the yeast strains, or because the mutations present in the laboratory strains used for these studies are the effectors of these particular phenotypes (Lettre *et al.*, 2008; Maher, 2008; Weedon *et al.*, 2008; Romano *et al.*,

2010). Therefore, small discrepancies among the genes associated with cell dehydration tolerance from different studies support the idea that different allelic combinations exert different effects. The nitrogen-deficient sporulation medium contains acetate as a carbon source to promote high levels of respiration, which induce sporulation in diploid yeast strains. In *S. cerevisiae*, the Δ*atg18* mutant is defective in sporulation but does not exhibit impaired vacuolar acidification (Barth *et al.*, 2001). The sequences of the $ATG18^{WA}$ and $ATG18^{WE}$ alleles revealed seven non-identical nucleotides. However, only one point mutation at nucleotide 584, from G to A, causes a single amino acid change of a serine to an asparagine residue (S195N; Table 2). Multiple sequence alignment of the WE and WA ATG18 alleles with 25 sequences of the *ATG18* gene from different *S. cerevisiae* strains annotated in the *Saccharomyces* Genome Database (SGD), as well as the Atg18p sequence characterized in this study, showed that the S residue is present in 16 genes, the N in eight genes, and the R in only one. This residue is located in the N-terminal region before the two WD40 domains and within a patch of highly conserved residues in Atg18p from *Pichia pastoris*, *Schizosaccharomyces pombe*, and *Homo sapiens* (Guan *et al.*, 2001). The immediate response of yeast cells to osmotic challenge involves the release of calcium from the vacuole and the formation of fragmented vacuoles (Li and Kane, 2008). Our results suggest that the $FAB1^{WE}$ allele principally affects vacuolar morphology, which might allow the hybrid carrying $FAB1^{WE}$ to adapt quickly to ionic stress. However, 1 M sorbitol osmotic stress at 37°C is lethal to these cells when the WE/ WA strain and the hybrid carrying $FAB1^{WA}$ are adapted. The $FAB^{WA}$ and $FAB1^{WE}$ allele sequences revealed 15 non-identical nucleotides, producing differences in 12 residues (Table 2); however, only the N1273D and Y1300H mutations are located in a region of conserved residues within the Zn-finger domain (Shaw *et al.*, 2003). Furthermore, none of these residues have a high identity ratio among the Fab1p sequences from the 28 *S. cerevisiae* strains (SGD). Fab1p governs vacuole homeostasis by generating PtdIns(3,5)*P2* on the vacuolar membrane. Atg18p colocalizes with Fab1p, and its deletion causes an abnormal elevation in the levels of PtdIns(3,5)*P2*, which suggests that Atg18p is also a negative regulator of the Fab1p kinase pathway (Efe *et al.*, 2007). The hybrid carrying $FAB^{WA}$ and the hybrid carrying $ATG18^{WE}$ exhibit an osmotic pressure-

dependent growth phenotype (Figure 4C), indicating that the genes are essential for growth only at high temperatures in the presence of osmotic ionic stress. At the permissive temperature, the hybrids carrying *FAB1*$^{WA}$ and the hybrid carrying *ATG18*$^{WE}$ exhibited extremely defective growth. These phenotypes are comparable to the ones exhibited by some of the temperature-sensitive isolated vacuolar protein sorting (*vps*) mutants, which require one or more vacuolar functions at the permissive temperature that cannot be provided at 37°C by other vacuolar components in these mutant cells (Bryant and Stevens, 1998).

The *DBR1* gene is conserved in humans (*hDBR1*) and maintains the same function in both human and yeast cells (Kim *et al.*, 2000). Among other phenotypes of the Δ*dbr1* strain, decreases in competitive fitness and Zn deficiency stress resistance have been previously described (Breslow *et al.*, 2008; North *et al.*, 2012). The growth fitness of a strain with the *DBR1*$^{WE}$ allele is affected and this strain is less sensitive to Zn stress than the *DBR1*$^{WA}$ allele, for which the opposite effect on growth is observed. The *DBR1*$^{WA}$ allele had $K^{286}$ and $N^{325}$ residues in the putative HMM domain, replacing $E^{286}$ and $D^{325}$, respectively (Table 2), which are 100% conserved in other Dbr1p deduced from the genomic sequences of 26 different *S. cerevisiae* strains (SGD). The deduced sequence of Cbt1$^{WA}$ showed two residue differences with Cbt1$^{WE}$, S29G, and T109A. In addition, three mutations were observed between the deduced peptide sequences of the *RSM22*$^{WA}$ and *RSM22*$^{WE}$ genes: E228K, D474S, and S619N (Table 2). These mutations do not affect the respiratory capacity of the different strains, thus enabling the separation of dehydration stress tolerance from respiration capacity. However, the above-mentioned variations in the sporulation efficiency of the *ATG18* hemizygous strains are not due to a pleotropic effect of the *RSM22* or *CBT1* alleles that affects cellular respiration.

The genetic approach used in this study, with a population of 96 segregants, allowed the detection of yeast dehydration resistance QTLs. The *RSM22* and *ATG18* genes enclosed within these QTLs that provide dehydration tolerance to the cell were not referenced in previous studies. Additionally, a detrimental effect on dehydration stress tolerance was shown to be provided by *DBR1* gene products. Our results further the understanding that dehydration stress tolerance is not a phenotype that results from the individual addition of independent genes. Furthermore, the

monogenic approach is not suitable for summarizing all of the epistatic effects driven by a group of alleles. Currently, the successful long-term storage of living cells is of critical importance, but the contradictory results with complex eukaryotic cells make the application of a simpler model desirable. There are a number of advantages, including ease of growth and modification and well-characterized cell physiology, genetics and biochemistry, which make yeast cells the model of choice for anhydrobiotic engineering.

### Author contributions

Conceived and designed the experiments: GL and RCO. Performed the experiments: GLM, MMC andFS. Analyzed the data: GLM, GL and RCO. Contributed reagents/materials/analysis tools: GLM, FS, GL, RCO. Wrote the paper: GLM, GL and RCO.

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**Supplementary data**

**Table S1** Primers used in this study.

| Primer | Nucleotide sequence (5'-3') |
|---|---|
| **HXT10fw** | ACGAAATCCCATACGCAAAT[1] |
| **HXT10rv** | AGCCGATGAGATGAGGATTG[1] |
| **ARS605fw1** | CCATGTAGCTTATCGCAGCA[1] |
| **ARS605rv1** | CCAAGCAGTTCTCTAGCTCCA[1] |
| **ARS605fw2** | TGGCACTTCGTATATGCAACA[1] |
| **ARS605rv2** | AGGAACCAAAAATCGCCAAC[1] |
| **YFR016Cfw2** | TGACCTTTCAATTTAGAAGATTTCC[1] |
| **YFR016Crv2** | CTGGTGTGCTTGATTACTCTGG[1] |
| **ARS1103fw** | TGGGCTATTTCATCCCATTG[1] |
| **ARS1103rv** | GAGAAGGAGGCAGCAGGAG[1] |
| **YKT6fw** | CGCCACCCAATAAGAAAAA[1] |
| **YKT6rv** | CTTGTTTGGTGTCGGCATAA[1] |
| **TP05fw** | TCGTCAGCTAAAGCAGGTAACA[1] |
| **TP05rv** | ACAAAGTCTGTGTTTATTGGATCA[1] |
| **PIR1fw** | TGAGAATTGTAGCATTACGATCTTCT[1] |
| **PIR1rv** | GCCTTTTTATGTCCTGCCAAT[1] |
| **PIR3fw1** | AGGTATGTGCGCAGCTCTTT[1] |
| **PIR3rv1** | CCGGTGACCTTACCGATCTA[1] |
| **PIR3fw2** | GAGCAACCATTTCCGAATGT[1] |
| **PIR3rv2** | ACGGCGAAATATGCCAAA[1] |
| **RIM15fw** | ATTATTCTCAGATTTGCTTTCAAGCAAAGTTTTTATTCAGTTATTTTTTTAATTAT CTTTATCTTAAAATTTATAGCTTTTCAATTCAATTCATCAT[1] |
| **RIM15rv** | AGCATTTTCCCTTTTTTTTTTTCCCTTTCCTTTCTCTTGCCTCATTTGATAGAATAG ATAAGCCCAGTAGAGGAAGACAGAAGCTTTTTCTTTCCAATT[1] |
| **RIM15A1fw** | ATTCTGCTTTTAATATTTCCAGATTT[1] |
| **BST1fw** | CAAGCTTTTTTCTTGCCATGATCTAGAACTCTCAGGCAATATATACAGTTAATCT TTTTTTACTGGGTTGTAGTTCAGCTTTTCAATTCAATTCATCAT[1] |
| **BST1rv** | CACACTCGAAATACTCCCTCTACTTTAAAGCATTGGCCTATATCTTAGGCTTACC ATCATACAAAAATCTTCATTTCGTTAAGCTTTTTCTTTCCAATT[1] |
| **BST1A1fw** | TGATCAAAATTTACGGCTTTGA[1] |
| **BUD27fw** | GAATTTTATAGTAAACAGGTATCCTCAGACTGTAATAGCCAAGCTTTTCAATTCA ATTCATCAT[1] |
| **BUD27rv** | GTTAATATAGATTCTGATTTACTTTCTGTCTCCATATGGGTAGCTTTTTCTTTCCA ATT[1] |
| **BUD27A1fw** | AATTTTGTGGTCGGATCGTG[1] |
| **BLM10fw** | TGTATTTGCATACATAAACTTTATCATTGTTCGTTAGCTAGCTTTGCACATTAATT TTTCGATTTGTTACCGCCAAAGCTTTTCAATTCAATTCATCAT[1] |
| **BLM10rv** | AATCAGCAGATAGCTCCAGCTATTTGTTTAGATGTACATATATGTCTAGATATGT GCTTAATATCCTATACTAATATGAATAGCTTTTTCTTTCCAATT[1] |
| **BLM10A1fw** | GGCCGAGGTATCCCTTAGAA[1] |
| **YFH7fw** | AACCTTGTTAGGTTAATTTCACTAGTACTATACATATTTTATCCTGTATCATACCA GAGGATCATTCTAGCCACAAAGCTTTTCAATTCAATTCATCAT[1] |
| **YFH7rv** | TGCCGTTTTTGCTTGCGCGCCTTAATTATCTGTATTCAGTTCGATTTTACAAAAAT ATATACAAGGTTCCGCCTAACCTTCAGCTTTTTCTTTCCAATT[1] |
| **YFH7A1fw** | TGGGCTTATCAGACTTGTCCA[1] |
| **FAB1fw** | ATAAAGGGCCAAACAAAAAACTATTTCGAATAGCAAGGTAGCTTCCATCCTGTA CATGCAAGACCGTCACACAGCAAGCTTTTCAATTCAATTCATCAT[1] |

| | |
|---|---|
| **FAB1rv** | TACTGAAAGTTAAAGAACACTAATGTGCGTGATAGTGTATAAAAAAAAGTTACA GAATATAACTTGTACACGTTTATGTATAGCTTTTTCTTTCCAATT[1] |
| **FAB1A1fw** | TAACTCTCCCTCTCCCCCTCT[1] |
| **ATG18fw** | CACGACCCTCCCTTATTAATCAGTTAGTAATAGTGTTCCAGTTAACTCTGTATCCT TTTCTTCTTCGGCCTGACAAAGCTTTTCAATTCAATTCATCAT[1] |
| **ATG18rv** | AGATTATACGCAGGAGTTTATATAAACTATATTGTGTATGCGTTGTGACGTACGG AAGGCAGCGCGAGACACTTCCGTGATAGCTTTTTCTTTCCAATT[1] |
| **ATG18A1fw** | CATTCGGAAGTGCGACAATA[1] |
| **RPL2Afw** | ACAATCACATGGTTGTTAAATCACGGTGCTGACATACCCATAGCTTTTCAATTCA ATTCATCAT[1] |
| **RPL2Arv** | GAACTGGTTTGTTACGTGGTTCTCAAAAGACCCAAGATTAGAGCTTTTTCTTTCC AATT[1] |
| **RPL2AA1fw** | AACTTGGCAGCACCTTGTCT[1] |
| **CBT1fw** | GATTGATCAGAAGTTTACTGCGCTTTTGGGTAAAGAAGCATTAAACAAAGGAGA GAGAAATATTGCAAGGAAAAAAAGCTTTTCAATTCAATTCATCAT[1] |
| **CBT1rv** | AGCCAGTGCTATAGTCACCAAATAATACGCATTATATATGGATATGTACAGTTCG CAGATCTTTATGGCATATTTATCGTTAGCTTTTTCTTTCCAATT[1] |
| **CBT1A1fw** | GCCATTTGCCTATAGCTTGG[1] |
| **MRP49fw** | AGTTTTGAATTTACATATTTCCATGAAGGGCAATGTTTTTTGATATATACATGAA CAAACTTATCGAGAGAAAGCTAGCTTTTCAATTCAATTCATCAT[1] |
| **MRP49rv** | CAGGATATCTGTAAGAATCGGCCATAAAACTCATTAATAGAAGAACAGTATAAC ATAAGTGAGCCTGCTACAATAAGAAGAAGCTTTTTCTTTCCAATT[1] |
| **MRP49A1fw** | TCTCCTCCTGCATTACCATTG[1] |
| **RSM22fw** | ATATTCACGTATGTAGAATATTAAAGTATTGAATATATTAATATTATTACTTTATT TCCAGTTACTTACAATTTCCAGCTTTTCAATTCAATTCATCAT[1] |
| **RSM22A1fw** | GTTACCTGCGAATCCTGCTC[1] |
| **DBR1fw** | GTATGACTAAAAATTCTCTCAAGAAGGCTTGGCTTTAAGCTCTAATTCCGTCTGC ATTCGTAATAGAAATATCTCTAGCTTTTCAATTCAATTCATCAT[1] |
| **DBR1rv** | AAATGAGCAGGAGAAAGTCATATGGCGAACGTAAATATGTAACTAAAAATTAA GATGGGCAGACATTTATCATTTTGCTTAAGCTTTTTCTTTCCAATT[1] |
| **DBR1A1fw** | GTCCCCCACCATTTATGAAC[1] |
| **ATGufw** | CACGACCCTCCCTTATTAATCAGTTAGTAATAGTGTTCCAGTTAACTCTGTATCCT TTTCTTCTTCGGCCTGACAAATCACGGAAGTGTCTCGCGCTGC |
| **ATGurv** | AGATTATACGCAGGAGTTTATATAAACTATATTGTGTATGCGTTGTGACGTACGG AAGGCAGCGCGAGACACTTCCGTGATTTGTCAGGCCGAAGAAG |
| **S8rv** | CCTCTAGGTTCCTTTGTTACTTCT[2] |
| **MATaspe** | ACTTCCACTTCAAGTAAGAGTTTG[3] |
| **MATαspe** | GCACGGAATATGGGACTACTTCG[3] |
| **MATfla** | AGTCACATCAAGATCGTTTATGG[3] |
| **ATG18fw** | GAAACTTCCCGTTGAAACCA[1] |
| **ATG18rv** | CCGGATACTCGGATGTGTCT[1] |
| **FAB1fw** | TTGATCGCATTTTGCTTGAG[1] |
| **FAB1rv** | TTGGGCATTCAAGTTCATCA[1] |
| **ALG9fw** | GCCGTCTACGAGCAATTTTC[1] |
| **ALG9rv** | TCTGGCAGCAGGAAAGAACT[1] |
| **TAF10fw** | CCAGGATCAGGTCTTCCGTA[1] |
| **TAF10rv** | AGCTCTCGCCTGACTGTTGT[1] |

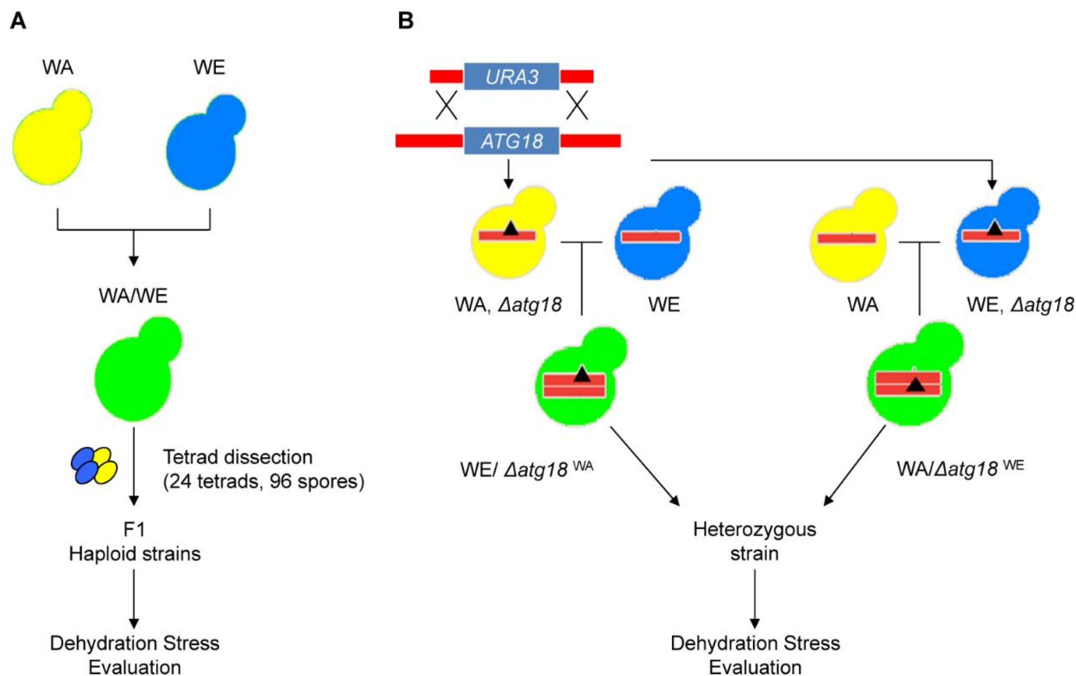


**Figure S1** Diagrams of strain generation. A) Production of F1 population (Liti *et al.*, 2009). B) Haploid strains were disrupted for the identified genes (e.g., *ATG18*) using *URA3* and used to develop heterozygous diploid strains by reciprocal hemizygous crossover.

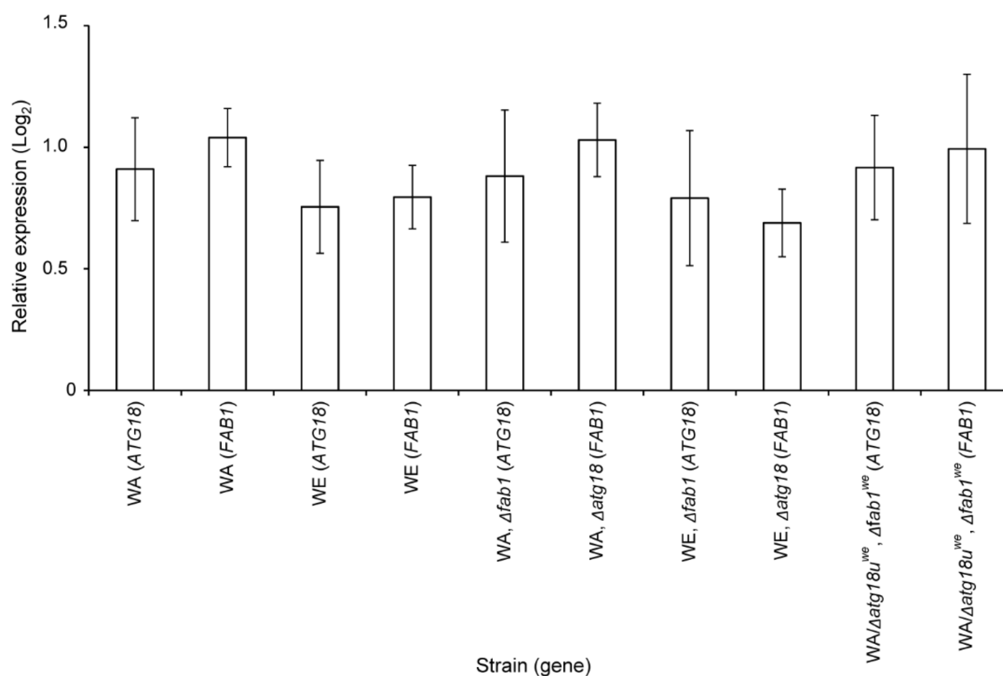


**Figure S2** Quantitative real-time PCR analysis of gene expression before stress. Data represent mean relative expression ± SD (y axis, $Log_2$ values) of each individual gene (show at the bottom) before dehydration of different strains. Genes *ALG9* and *TAF10* were simultaneously used as constitutive reference genes as determined by the geNorm algorithm (Vandesompele *et al.*, 2002). Relative expression was calculated using REST-MCS v2 software (Pfaffl *et al.*, 2002).