MOLECULAR STUDY OF THE MECHANISMS OF OENOCOCCUS OENI INVOLVED IN ITS ADAPTATION TO WINE CONDITIONS AND IN THE DEVELOPMENT OF MALOLACTIC FERMENTATION

Nair Temis Olguin

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Molecular study of the mechanisms of *Oenococcus oeni* involved in its adaptation to wine conditions and in the development of malolactic fermentation

Ph Doctoral Thesis

Directed by Dr. Cristina Reguant and Dr. Albert Bordons

Departament de Bioquímica i Biotecnologia

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CERTIFIQUEM

Que aquest treball, titulat "Molecular study of the mechanisms of *Oenococcus oeni* involved in its adaptation to wine conditions and in the development of malolactic fermentation", que presenta **Nair Temis Olguín** per a l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que acompleix els requeriments per poder optar a Menció Europea.

.

Tarragona, 22 de març de 2010

Albert Bordons de Porrata-Doria

Cristina Reguant Miranda

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Volar 12 mil kilómetros lejos de casa para hacer una tesis doctoral no es tarea sencilla. Pero ahora mirando hacia atrás, me doy cuenta de todo lo que he aprendido, tanto a nivel profesional como personal y sin duda, ha valido la pena. He aprendido un montón y muchas de las veces, el aprendizaje ha sido duro, sacando uñas y dientes y otras veces, se sintió como estar de vacaciones. Por eso, gracias…

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Luly y Tati, Graciela, aunque en la distancia, apoyándome siempre. A Horacio (Cafi) a quien le debo muchos de mis pasos y a los hermanos Cozza (Horacio y Eduardo), por todo su apoyo y motivación.

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A mis padres y a Nikos

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I. INTRODUCTION

1. Lactic acid bacteria and wine

Lactic acid bacteria (LAB) comprise a group of Gram-positive, low-GC, acid-tolerant, generally non-mobile, non-sporulating, non-respiring microorganisms. Most LAB only obtain energy from the metabolism of sugar and related compounds so, they are usually found in sugar-rich habitats. Normally, their biosynthetic capacity is limited and their nutritional requirements include aminoacids, vitamins, purins and pyrimidines (Madigan *et al*., 1999).

Through history, they have been used to ferment foods for at least 5000 years (Barnum, 1998). They are best known for their role in the preparation of fermented dairy products, pickling of vegetables, baking, brewing and winemaking.

In wine, LAB are mainly responsible for malolactic fermentation (MLF) which takes place after the alcoholic fermentation carried out by yeasts. In this environment specific strains predominate which are adapted to the wine physical and chemical conditions (Guzzo and Desroche, 2009). Wine LAB, such as *Oenococcus oeni*, have been extensively studied for their predominant role during MLF and numerous reviews have been published (van Vuuren and Dicks, 1993; Lonvaud-Funel, 1999; Versari et al., 1999; Bartowsky and Henschke, 2004; Spano and Massa, 2006). This introduction focuses on the adaptation mechanisms of *O. oeni* to its natural environment (wine) and the influence of its development on the quality of wine. Towards the end, the promising innovative high-throughput technologies will be mentioned and considered for further research.

2. General adaptation mechanisms of *O. oeni* to wine conditions

Nowadays, it is known that MLF is a complex microbiological process which includes a wide range of metabolic traits. Several species of LAB enter into grape juice and wine from the grape berry surface, the stems, leaves, soil and winery equipment. Musts, soon after crushing, include species of the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Oenococcus* (Wibowo *et al.,* 1985; Reguant and Bordons, 2003). At the end of alcoholic fermentation, not only total bacterial counts diminish but the original diversity of the species does also. In most

cases *O. oeni* predominates at the end and after alcoholic fermentation, thus being the main responsible for MLF (Lonvaud-Funel, 1999; Saguir *et al.,* 2009).

Despite being the species more adapted to wine and that the inoculation of selected strains of *O. oeni* is usual, the induction of this fermentation remains problematic (Reguant *et al.,* 2005). The loss of viability when cells are directly inoculated into wine is related to the harsh environmental conditions such as low pH, the presence of short-chain fatty acids, polyphenols, high alcohol content and high SO_2 concentration (Davis *et al*., 1985; Lonvaud-Funel, 1999; Carreté *et al.*, 2006; Spano and Massa, 2006; Rodríguez *et al.,* 2009). In order to understand the intrinsic mechanisms involved in short-term stress response and cellular adaptation of *O. oeni* to wine conditions, a great number of investigations have been made. One of the first mechanisms described is the proton motive force (PMF) generation and the maintenance of internal pH by proton consumption during MLF. Other mechanisms include the potential proton extrusion through the ATPase systems and the stress protein synthesis (Guzzo *et al.,* 2000).

2.1. Bioenergetics and intracellular pH maintenance

Several authors have found that L-malic and citric acids, not energy sources themselves, provided increased growth rates and ATP synthesis when cultured in presence of carbohydrates (Pilone and Kunkee, 1972; Loubiere *et al.,* 1992; Ramos *et al.,* 1994; Salou *et al.,* 1994; Ramos and Santos, 1996). This kind of metabolism not only provides energy to the cell but also participates in the regulation of the internal pH (Salema *et al.,* 1996a).

2.1.1. Fermentation of L-malic acid

MLF is in fact a decarboxylation reaction carried out by the malolactic enzyme (MLE) which decarboxylate L-malate to L-lactate. This enzyme catalyzes the reaction in one step releasing CO_2 in presence of two co-factors: Mn^{+2} and NAD^+. The mechanism by which L-malic acid is taken into the cell has been widely studied (Cox and Henick-Kling 1989; Loubiere *et al.,* 1992; Salema *et al.,* 1994; 1996ab). In *O. oeni*, this transport occurs by an anion uniport

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mechanism where $Hmalate^-$ is rapidly decarboxylated and both products lactate and CO_2 leave the cell in electroneutral process (Salema *et al.,* 1996a; Konings, 2002). However, it is still unclear whether lactate extrusion is carrier mediated or if it leaves the cell by simple electroneutral diffusion (Fig. 1A) (Loubiere *et al.,* 1992; Salema *et al.,* 1994; 1996a).

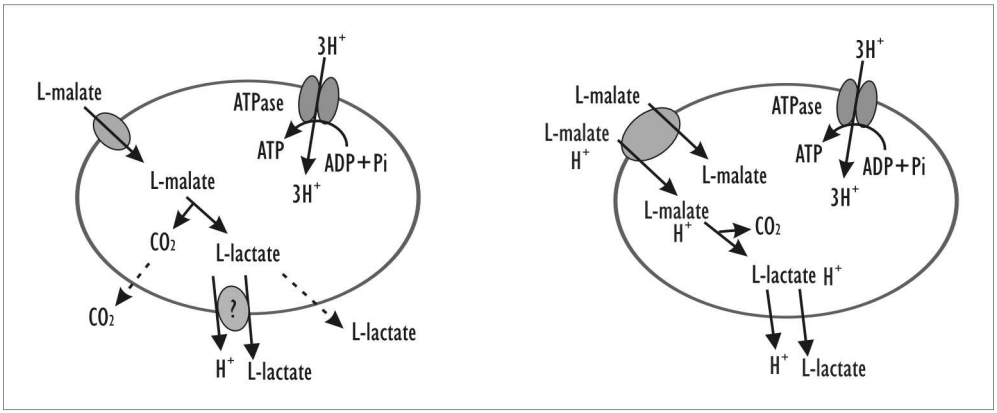

Figure 1. Transport mechanism of L-malate decarboxylation followed by L-lactate extrusion: A) in *O. oeni* (Loubiere *et al.,* 1992; Salema *et al.,* 1994); B) Hypotetical scheme in *Lb. plantarum* (Olsen *et al.,* 1991).

MLF by *O. oeni* then results in the generation of both components of the proton motive force (PMF): the membrane potential ($\Delta\psi$), inside negative, by the inflow of negative charge with $Hmalate^-$ and a pH gradient (ΔpH), inside alkaline, by the internal consumption of protons in the decarboxylation reaction (Ramos *et al.,* 1994). The negative membrane potential built up during MLF has shown to be sufficient to drive the generation of ATP via F_0F_1-ATPase (Cox and Henick-Kling, 1989). These mechanisms are somewhat similar to those proposed for *Lb. plantarum* (Olsen *et al.,* 1991) and *Lactococcus lactis* (Poolman *et al.,* 1991). At low $Hmalate^-$ concentrations (<5 mM), $Hmalate^-$ is transported by proton symport and electroneutral lactate efflux cannot create a $\Delta\psi$. If the $Hmalate^-$ concentration is high (>5 mM), $Hmalate^-$ can enter the cell by a diffusion mechanism and electroneutral lactate efflux creates an electrochemical ΔpH (Fig. 1B). Moreover, an observed growth-stimulating effect of L-malate was attributed to the chemiosmotic transport mechanisms rather than the proton consumption by the malolactic enzyme (Loubiere *et al.,* 1992). It is believed that at the low pH of wine, glycolysis is effectively switched off and, therefore, MLF is probably the only way that *O. oeni* can generate ATP (Bartowsky, 2005).

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The genetic locus involved in malolactic conversion (*mle*) has been identified in *O. oeni* as well as in other LAB (Labarre *et al.,*1996a). Genes for the malolactic enzyme (*mleA*), malate permease (*mleP*) and regulatory protein (*mleR*) are present in a cluster with the first two in a single operon and the *mleR* (LysR-type regulatory protein) transcribed in the opposite direction as shown in Figure 2 (Labarre *et al.,* 1996ab). Recently, it was found a significant increase in the abundance of mRNA of *mleP* derived from *O. oeni* cells incubated in presence of L-malic acid at pH 4.5 and 3.2 (Augagneur *et al.,* 2007). However, the role of the MleR-like protein in *O. oeni* remains unclear since no induction nor repression of the malolactic activity could yet be detected (Labarre *et al.,* 1996b). A gene encoding a LysR-type positive regulator protein required for the expression and induction of MLF was described for *L. lactis* (Renault *et al.,* 1989). Opposite to *O. oeni*, the *mleR* gene in *L. lactis* is present in tandem arrangement (Fig. 2).

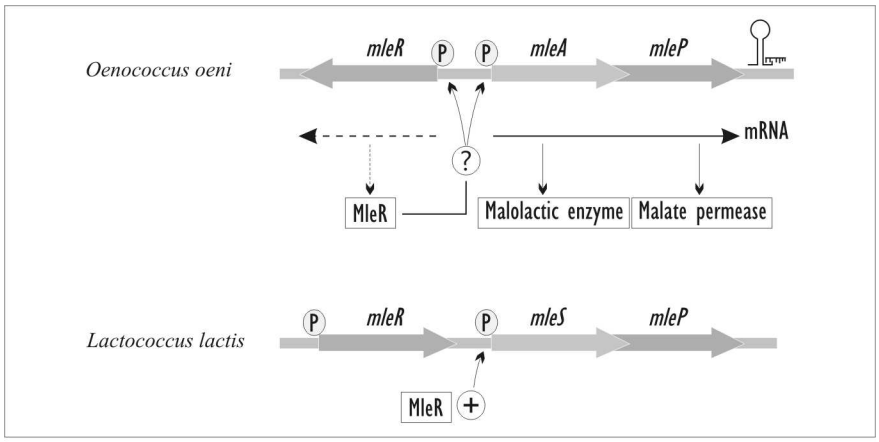

Figure 2. Genetic organization of the *mle* locus of *O. oeni* (Labarre *et al.,* 1996b) and *L. lactis* (Renault *et al.,* 1989). In *L. lactis* the gene coding for malolactic enzyme is named *mleS*.

2.1.2. Fermentation of citric acid

Citrate fermentation-coupled PMF generation in LAB deserves some special attention due to the involvement of more complex metabolic processes (Konings, 2002). Citrate fermentation in *O. oeni* is in addition to MLF important for wine production since citrate is a precursor of aroma compounds as it will be further discussed (Section 3.1). In the pH range of the natural habitat of *O. oeni*, i.e., pH 3.5–5.5, the predominant citrate species is $H_2citrate^-$.

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Thus, citrate enters the cell as H_2citrate$^-$ and the products will leave the cell in their uncharged protonated states (Fig. 3). Internally H_2citrate$^-$ is metabolized to the neutral end products acetic acid, acetoin and CO_2 and in this process one proton is consumed. Citrate metabolism in *O. oeni* therefore, also leads to the generation of both a $\Delta\psi$ and a ΔpH (Konings, 2002) and Ramos *et al.* (1994) also suggested that the citrate-induced PMF is used to generate ATP via F_0F_1-ATPase.

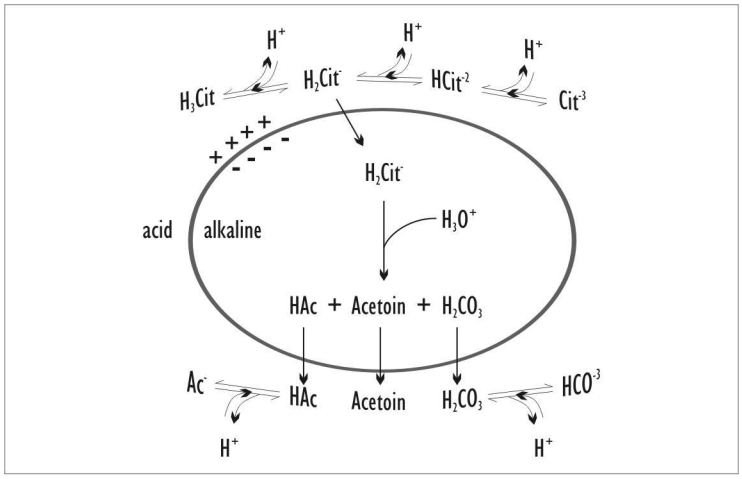

Figure 3. Proposed model for PMF generation by citrate metabolism in *O. oeni*. H_3cit, citric acid; HAc, acetic acid (Ramos *et al.,* 1994).

Citrate transport is somewhat different depending on the LAB species (Konings, 2002; Drider *et al.,* 2004; García Quintans *et al.,* 2008). The citrate metabolic pathway described in *Lactococcus* and *Leuconostoc* is a precursor/product exchange system. The product (lactate) results from the conversion of citrate and also from glucose metabolism, which explains why citrate uptake by *Lactococcus* and *Leuconostoc* is efficient only during lactate production from co-metabolism with a sugar. Related with this, phenolic compounds can reduce the rate of sugar consumption by *O. oeni* and enhance citric acid consumption, increasing the yield of acetic acid (Rozès *et al.,* 2003).

The organization of *cit* genes (Fig. 4) as well as their regulation is also species dependent (Drider *et al.,* 2004; García Quintans *et al.,* 2008). For instance, Martin *et al.* (2005) reported the induction of the *citMCDFGRP* genes of *Weissella paramesenteroides* upon addition of citrate to the growth medium. On the contrary, transcription of *citQRP* and citrate transporter were not induced by the presence of citrate in the growth medium (García Quintans *et al.,* 1998). Martin *et al.* (2005) have shown that CitI (CitR) is involved in the expression of all genes required for

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citrate metabolism in *W. paramesenteroides*, *Leuc. mesenteroides, O. oeni* and *Lb. plantarum*. In these bacteria, two consensus CitI binding operators (O_1 and O_2) could be found in the intergenic region between *citI* and *citM*, suggesting a similar mechanism of transcriptional activation by CitI in conjunction with citrate. On the other hand, sequence analysis of *L. lactis cit* operon shows the absence of O_2 operator which could explain the different regulation observed (Martin *et al.,* 2005).

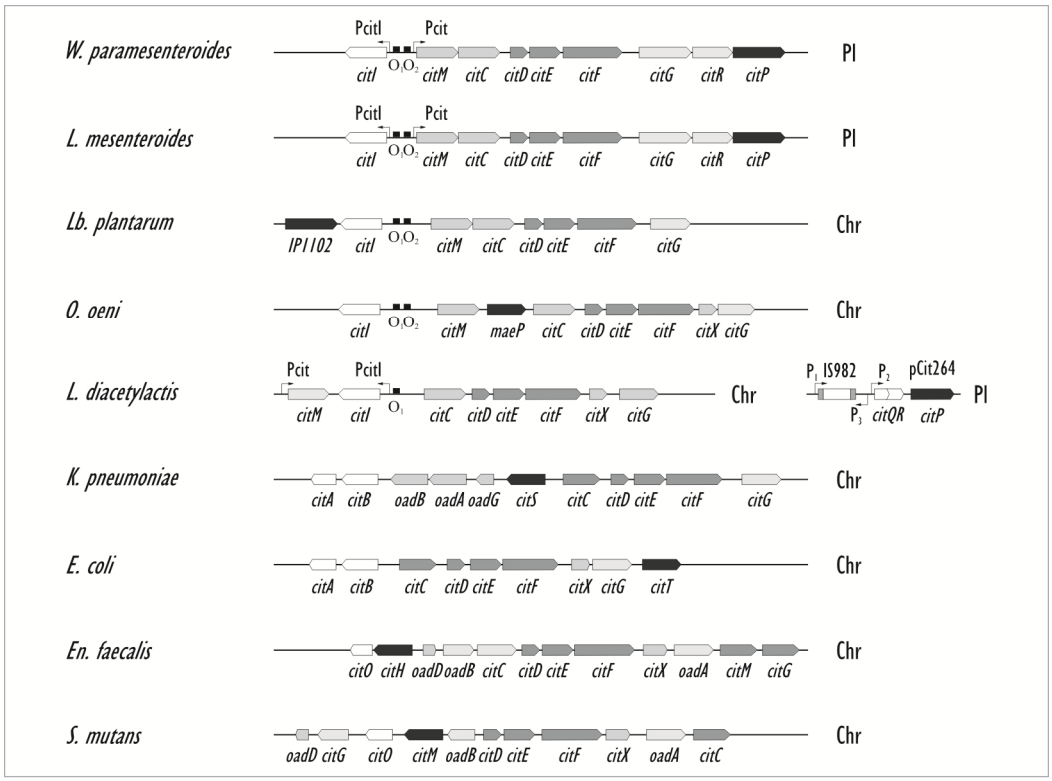

Figure 4. Physical organization of genes encoding proteins involved in citrate transport and conversion into pyruvate. The *cit* genes of the indicated bacteria are depicted. Genes with various functions are indicated by boxes: black, citrate transport; grey, citrate metabolism and white, regulation of gene expression. Plasmidic (Pl) or chromosomal (Chr) location of the *cit* clusters is indicated. O_1 and O_2, operators of the CitI regulator (García Quintans *et al.,* 2008).

In *O. oeni* PSU-1 the genes related to citrate utilization are organized in the citrate lyase gene cluster (*citR*, *citM*, *maeP*, *citC*, *citD*, *citE*, *citF*, *citX*, *citG*) (Fig. 4). A recent work describes the effect of pH on the expression of *maeP* and another putative citrate transporter, *yaeP* (Augagneur *et al.,* 2007). Neither citrate nor pH induced the transcription of *maeP*. On the other hand, the relative *yaeP* transcript level increased in the presence of citrate coupled with higher pH. These authors suggest that *YaeP* could be involved in citrate uptake at pH 4.5 or

above. In either case, there is yet no clear similarity between the transcriptional behaviors of *O. oeni* to that observed in other LAB.

2.1.3. The ATPase system

Regarding to the intracellular pH maintenance and the acquisition of an acid tolerance, the F_0F_1-ATPase plays an important role in LAB. As pointed out above, both malate and citrate fermentations are coupled to the protons consumption thus constituting to the acquisition of the acid tolerance. The role of ATPase in resistance to acid conditions was clearly demonstrated by studying ATPase deficient mutants of *O. oeni* (Tourdot-Maréchal *et al.,* 1999). ATPase mutants were shown to be impaired in malolactic activity suggesting a linkage between MLF and ATPase systems. The absence of malolactic activity in ATPase deficient mutants was further studied at the molecular level (Galland *et al.,* 2003). Both *mle* operon and *mleR* transcripts were not detected in ATPase deficient mutant strains of *O. oeni*.

However, it seems that the F_0F_1-ATPase is not directly affected by malic acid metabolism. Fortier *et al.* (2003) found that the drop in the medium's pH associated with the absence of malic acid was correlated with a higher F_0F_1-ATPase activity and the increase in the amount of its mRNA transcript (*atp*). These results also suggest a reversible activity of this enzyme capable of the extruding protons function as reported for other LAB (Kobayashi, 1985; Kullen and Klaenhammer, 1999; Cotter and Hill, 2003). Further studies are required to clarify the proposed reversible activity of the membrane bound F_0F_1-ATPase. Anyway, ATPase seems to be a good indicator of the physiological state of the cells, since it is affected by different stress compounds (Carreté *et al.,* 2002). Moreover, genes and proteins involved in pH homeostasis and cell protection or repair play a role in acid adaptation, but this role can also extend to more general acid tolerance mechanisms.

Some other systems related to transporters have been associated to the maintenance of the pH homeostasis in *O. oeni* such as the amino acid decarboxylation and the arginine deiminase (ADI) pathway (Liu and Pilone, 1998; Araque *et al.,* 2009; Vincenzini *et al.,* 2009). In these processes the cell take advantage through the electrogenic transport of substrate and product as observed in several bacteria (Poolman, 1993). Interestingly, additional mechanisms described for other LAB (glutamate decarboxylation), have not been observed in *O. oeni* and in

some cases, these mechanisms (ADI) are related to the growth conditions (Liu and Pilone, 1998; Mira de Orduña *et al.,* 2001; Romero *et al.,* 2009). An example of the last case in the fermentation of citrate which can be used only in co-fermentation with hexoses, wereas L-malate can be degraded without need for a co-substrate (Unden and Zaunmüller, 2009).

2.1.4. Hexoses metabolism

As mentioned above, LAB cannot grow with L-malic or citric acid as unique carbon source; therefore these microorganisms need an additional energy source, such as residual fermentable sugars, i.e.: glucose and fructose (Pilone and Kunkee, 1972). For instance, it was reported that one strain of *O. oeni* did not grow in absence of glucose whereas it was stimulated by the presence of malic acid and citric acids in synthetic medium with different glucose concentrations (Saguir and Manca de Nadra, 1996). Pimentel *et al.* (1994) reported that three strains of *O. oeni*, isolated from Portuguese wines, metabolized malate before glucose except at high pH (4.0 and 4.5). It has been also reported that the rate of malic acid consumption is slower when the amino acids leucine, histidine or valine are absent and that every amino acid has its own effect on glucose metabolism (Fourcaisse *et al.,* 1992). For instance, absence of essential amino acid such as arginine, glutamic acid, isoleucine, tryptophan or methionine, inhibited D-glucose utilization. On the other hand, the deficiency of histidine, leucine and valine showed a limited growth and also a partial metabolism of D-glucose (Fourcaisse *et al.,* 1992).

Mixtures of glucose-citrate or glucose-fructose have shown to enhance the specific growth rate and the biomass production of *O. oeni* (Salou *et al.,* 1994). Latter work reported that the presence of citrate provides additional pathways for $NAD(P)^+$ regeneration and allows the diversion of sugar carbon reactions in which ATP is synthesized (Ramos *et al.,* 1996). The increased growth rates and maximal biomass yields of *O. oeni* growing on citrate-glucose mixtures results from increased ATP synthesis both by substrate-level phosphorilation and by chemiosmotic mechanism. On the other hand, glucose catabolism can also be responsible for an inhibitory effect on the malolactic enzyme (Miranda *et al.,* 1997). This inhibition can be explained by the NADH accumulation during glucose metabolism since when additional electron acceptors are provided, the $NAD(P)H/NAD(P)^+$ ratio decreases, with a concomitant decrease in malolactic inhibition. This explanation was also confirmed by Maicas *et al.* (2002)

when testing the aerobic growth of *O. oeni*. M42 strain was found to grow poorly under aerobic conditions with glucose since O_2 inactivated the enzymes of the ethanol-forming pathway, one of the two pathways which reoxidizes $NAD(P)^+$ cofactors in the heterolactic catabolism of glucose. These authors concluded that the regeneration of cofactors is the limiting factor for the aerobic consumption of glucose.

2.2. Membrane, the main cellular barrier

When changes in the environmental conditions affect the physicochemical parameters of the culture medium such as pH, osmotic pressure and ethanol, the first protecting barrier is the cell envelope. In bacteria, the cell membrane performs such vital functions as the maintenance of the PMF and the uptake of nutrients. It is an interface between the external environment and the cellular cytoplasm, the composition of which it helps to regulate. In turn the composition of the membrane will alter in response to changes in the external medium in order to maintain its functions (Russell *et al.,* 1995).

Several works have shown the effect of pH and ethanol over membrane composition and fluidity in *O. oeni*. The presence of ethanol induces an increase in membrane fluidity and permeability leading to the loss of intracellular material such as co-factors and ions (Da Silveira *et al.,* 2002, 2003; Chu-Ky *et al.,* 2005). Exposure to ethanol can also lead to a dissipation of the membrane electrochemical gradient (Da Silveira *et al.,* 2002, 2004). An influx of protons can then occur which will affect cell processes dependent of the pH gradient. Moreover, the malolactic activity was proven to be modified by the presence of ethanol and low pH. At pH 3, the optimal pH for whole-cell malolactic activity, the ethanol effect was negligible until a concentration of 12%. For higher concentrations an increasing inhibition was observed whereas at pH 5 the malolactic activity was slightly increased by ethanol concentrations up to 20% (Capucho and San Romão, 1994).

Cell viability is also affected by ethanol and pH as a consequence of membrane instability (Tourdot-Maréchal *et al.,* 2000; Chu-Ky *et al.,* 2005). Ethanol (10-14%) or combined ethanol-acid (pH 3.5) shocks resulted in high loss of *O. oeni* cell viability. Until now, *O. oeni* population survival to high ethanol concentrations was achieved by previous adaptation to sub-lethal stress conditions such as 8% ethanol (Drici-Cachon *et al.,* 1996; Tourdot-Maréchal *et al.,*

2000). The adaptation to different organic solvents has been attributed to a change in the quantity and quality of the lipid composition in the cell membrane (Weber and de Bont, 1996). Variable observations were reported for *O. oeni* membrane adaptation to the presence of wine or ethanol (Garbay *et al.,* 1995; Garbay and Lonvaud-Funel, 1996; Teixeira *et al.,* 2002; Grandvalet *et al.,* 2008). For instance, the presence of 8% ethanol or low pH (3.5) have shown to induce an increase in the content of saturated fatty acids including cyclopropane fatty acid (CFA) and the rate of proteins/phospholipids. These changes are thought to lead to an increase in membrane rigidity (Grandvalet *et al.,* 2008). Moreover, the level of *cfa* transcripts increased when cells were grown in the presence of ethanol or at low pH, suggesting transcriptional regulation of the *cfa* gene under different stress conditions. Nevertheless, the mechanisms involved in membrane fluidity adjustments in *O. oeni* as well as the nature of lipid-protein interactions remain incomplete.

2.3. Synthesis of molecular chaperones

The stress response involves physiological changes in order to resist and adapt to the environmental conditions. Generally, this response is followed by protein synthesis allowing the maintenance of cell activity and integrity of the different cell constituents (Guzzo and Desroche, 2009). To survive environmental changes, cells synthesize proteins such as chaperones and proteases, above all, to prevent accumulation of abnormal proteins (Gottesman *et al.,* 1997). The cellular chaperone machinery counteracts the aggregation of nonnative proteins, both during de novo folding and under conditions of stress, such as high temperature, when some native proteins unfold (for the outline see Fig. 5) (Hartl and Hayer-Hartl, 2002).

Many chaperones, though constitutively expressed, are synthesized at greatly increased levels under stress conditions and are classified as stress proteins or heat-shock proteins (HSPs) (Hartl and Hayer-Hartl, 2002). HSPs involve both chaperones and proteases which act together to maintain quality control of cellular proteins (Abee and Wouters, 1999). Certain of these proteins, such as molecular chaperones GroES, DnaK and proteins of the Clp family (caseinolytic protein), constitute a common response to different stress (Abee and Wouters, 1999; Sanders *et al.,* 1999; Guzzo and Desroche, 2009).

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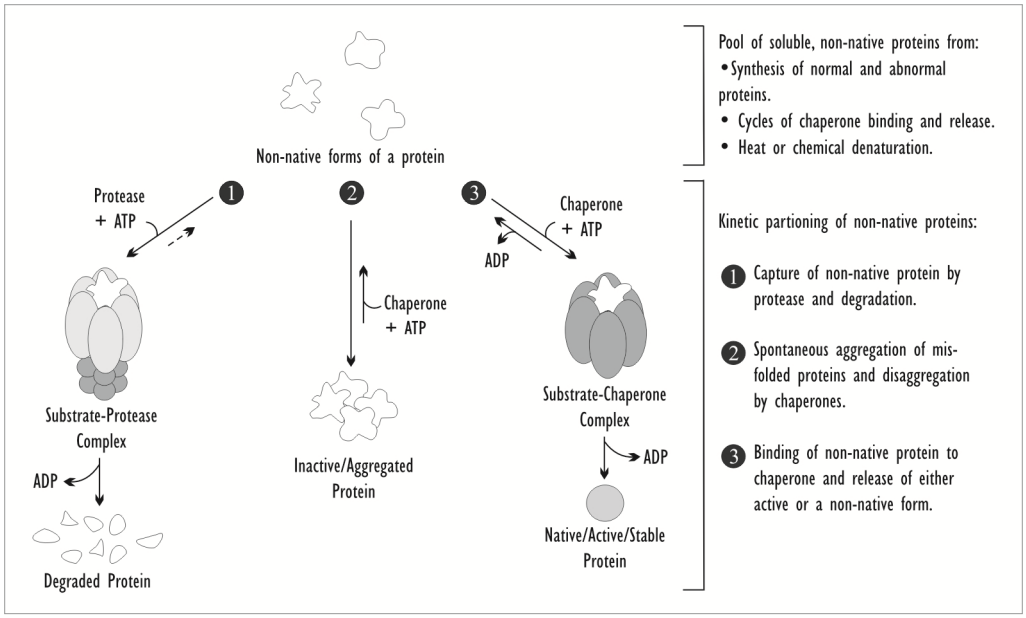

Figure 5. A kinetic model for protein triage. Polypeptides in unfolded and other non-native forms (shown in white) may interact with a number of other cellular proteins. The relative rates at which these occur will determine the ultimate fate of the protein, (1) The may be recognized by proteases (left), almost always leading to degradation; (2) the may aggregate with themselves or other proteins (center); reversal of aggregation may in some cases be promoted by chaperones, thus returning these polypeptides to the pool of soluble proteins; or (3) they may bind chaperones (right). Release from the chaperone may either return the protein to the soluble pool of non-native forms or may result in an active/refolded protein that is no longer a substrate for either protease or chaperone binding. In the absence of chapernes, a competition between the rates of aggregation and degradation will determine the fate of the polypeptide. Unstable proteins obey similar rules; relative rates of degradation and aggregation will determine the sensitivity of degradation to chaperones (Gottesman *et al.,* 1997).

Besides intervention of the HSP proteins, other proteins more specific to different stress take place in the adaptation and resistance of bacteria to stress. For instance, acid shock proteins (ASP); cold shock proteins (CSP); and general stress proteins (GSP). The variety and amount of proteins stress-inducible are variable according to the stress and the LAB studied (Guzzo and Desroche, 2009). A well studied small HPS (smHSP) in *O. oeni* is Lo18. This protein has shown to be greatly induced after heat (42ºC), acid (pH 3) and ethanolic (12%) shocks (Guzzo *et al.,* 1997). This protein is coded by the *hsp18* gene and its expression seems to be controlled at the transcriptional level (Jobin *et al.,* 1997; Coucheney *et al.,* 2005). Lo18 was found to be associated to the cell membrane by week binding thus, suggesting a participation in the membrane fluidity regulation. Moreover, its transcription could be regulated by the level of membrane fluidity (Jobin *et al.,* 1997; Delmas *et al.,* 2001).

Although the induction of HSPs is a universal response, organisms have diverse regulatory mechanisms for controlling HSP synthesis. In *Bacillus subtilis* at least three different

classes of heat-inducible genes can be defined by their common regulatory characteristics: Class I genes (*dnaK* and *groES* operons), which expression involves a σ^A-dependent promoter, an inverted repeat (called the CIRCE element for controlling inverted repeat of chaperone expression) and probably a repressor interacting with the CIRCE element. Class II genes (the majority of general stress genes) are induced at σ^B-dependent promoters by different growth-inhibiting conditions (Hecker *et al.,* 1996). Class III genes (*clpC*, *clpP*, *clpX*, *ftsH*, *trxA*) are controlled by the gene repressor CtsR, which recognizes a tandemly repeated heptad operator sequence (Derré *et al.,* 1999). Class IV genes comprise heat shock genes of unknown regulation. Although the induction of *hsp* genes is a universal response, organisms have diverse regulatory mechanisms for controlling HSP synthesis. For instance, it was reported that *O. oeni* posses a potential CtsR operator upstream from the *clp* genes and the *groESL* and *dnaK* operons, and it was suggested that CtsR controls the expression of most of the *O. oeni* molecular chaperone genes (Grandvalet *et al.,* 2005). In contrast to the diversity of stress response mechanisms described in many gram-positive bacteria, no other known regulator of stress response could be identified in the *O. oeni* genome (Grandvalet *et al.,* 2005; Mills *et al.,* 2005). However, it was also suggested that different mechanisms could be implied in the regulation of stress genes (Jobin *et al.,* 1999; Desroche *et al.,* 2005). A recent study based on monitoring *O. oeni* stress responsive genes after direct inoculation into wine revealed an increase of mRNA rate for *hsp18*, *groEL*, *clpP*, *clpX* and others (Beltramo *et al.,* 2006). In this case, it is worthy of notice that *clpX* gene seems to be regulated by other system not yet known (Grandvalet *et al.,* 2005).

3. Influence of MLF on the organoleptic characteristics of wine

Grape has an undeniable importance in wine aroma and flavor. However, wine has more flavor than the grape juice is fermented from due to the metabolism of grape compounds by yeast and bacteria (Bartowsky and Pretorius, 2009). Research in progress is showing that LAB can modify some of the components and sensory properties of wine, providing a new opportunity to alter the chemistry and possibly the aroma and flavor perception of wine (Fig. 6) (Bartowsky *et al.,* 2002, Matthews *et al.,* 2004; Swiegers *et al.,* 2005).

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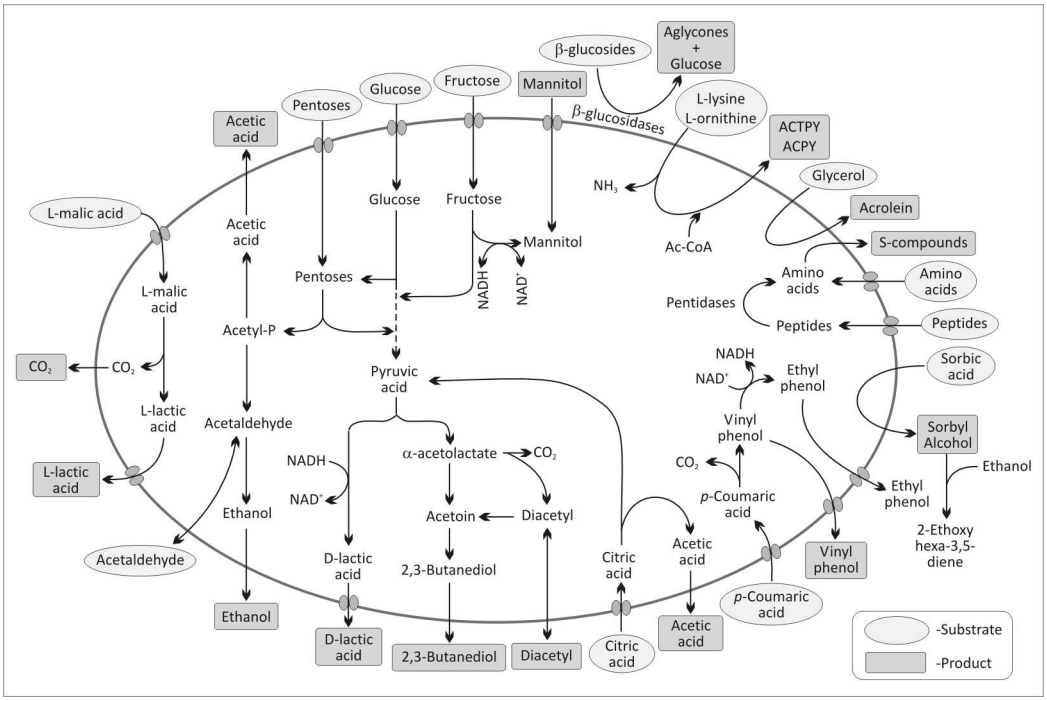

Figure 6. A schematic representation of the biosynthesis and modulation of flavour-active compounds by malolactic bacteria (Swiegers *et al.,* 2005).

During MLF, the main and better studied changes that modify the organoleptic characteristics of wine are those related to malic and citric acid fermentation and the release of aromatic molecules. Fermentation of non-volatile acids as well as changes in the aromatic profile deserve special attention as will be briefly described in the following paragraphs. Nevertheless, LAB are capable of carrying out numerous reactions as part of their metabolism, although to a lesser extent, most likely contribute to the well recognized complex change in the properties of wine (De Revel *et al.,* 1999). The diversity of such reactions is going to be mentioned in Section 3.3.

3.1. Modification of wine acidity: non-volatile acids

The most important change that results from the conversion of the dicarboxylic, L-malic acid to L-lactic acid as described above. This transformation has a dual effect. On the one hand, it deacidifies the wine, in other words, it raises the pH by 0.1 to 0.3 units, an effect that is greater at higher initial quantities of malic acid. It also gives the wine a 'softer mouth feel', replacing the acidic and astringent flavor of the malic acid by the smoother flavor of the lactic acid (Davis

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et al., 1985; Constantini *et al.,* 2009). Thus, this bioconversion is mainly desired in wines made of grapes from cool climates where the concentration of malic acid is often around 4 - 6.5 g l^{-1}, whereas in warm regions, grape juice often contains less than 2 g l^{-1} (Ribéreau-Gayon *et al.,* 2006).

Tartaric acid is relatively stable to bacterial activity and can only be metabolized aerobically by some *Lactobacillus* species with the production of acetic acid, lactic acid and succinic acid. When tartaric acid degradation occurs, it tends to appear as part of a general spoilage scenario (Swiegers *et al.,* 2005; Bartowsky and Pretorius, 2009).

Citrate utilization by LAB is an important metabolic process because of the production of flavoring compounds, such as diacetyl, acetoin, 2,3-butanediol (C_4 compounds), and acetate (Davis *et al.,* 1985; Ribéreau-Gayon *et al.,* 2006). Citric acid is commonly present in wine in the range of 0.1 – 0.7 g l^{-1} and most strains of *O. oeni* are able to metabolize this acid. It has been discussed however, if L-malic acid and citric acid are metabolized simultaneously or sequentially (Pimentel *et al.,* 1994; Saguir and Manca de Nadra, 1996; Nielsen and Richelieu, 1999; Ribéreau-Gayon *et al.,* 2006). Moreover, several parameters (pH, temperature, hexoses) also seemed to affect citrate consumption by *O. oeni* (Pimentel *et al.,* 1994; Salou *et al.,* 1994).

Citric acid is first degraded to acetic acid and pyruvic acid (Fig. 7). Most of the pyruvic acid is then metabolised to D-lactic acid with a smaller proportion going to diacetyl, acetoin and 2,3-butanediol via α-acetolactic acid (Ramos and Santos, 1996). Lower pH favors the production of acetoin in *O. oeni*, whereas D-lactate and acetate production is significantly increased at higher pH values (Ramos *et al.,* 1995). In lower concentrations, diacetyl, acetoin and 2,3-butanediol are felt to add complexity to the wine flavor. In fact, acetoin and 2,3-butanediol do not contribute to the flavor of the wine because of their high aroma thresholds (approximately 150 and 600 mg l^{-1}, respectively). On the contrary, an excess of diacetyl (5 mg l^{-1}) can be overpowering, giving the wine an undesirable butterlike flavor (Davis *et al.,* 1985; Martineau *et al.,* 1995; Nielsen and Richelieu, 1999; Bartowsky and Henschke, 2004). Parameters such as oxygen concentration, wine redox potential and initial citric acid concentration have shown to affect diacetyl levels in wine (Nielsen and Richelieu, 1999). It has been also reported that there is a metabolic shift of the end products from glucose metabolism when citrate is provided along with glucose; ethanol is replaced by acetate and 2,3-butanediol is produced (Ramos and Santos, 1996). Reciprocally, the production of lactate and 2,3-butanediol from citrate is increased in the presence of glucose.

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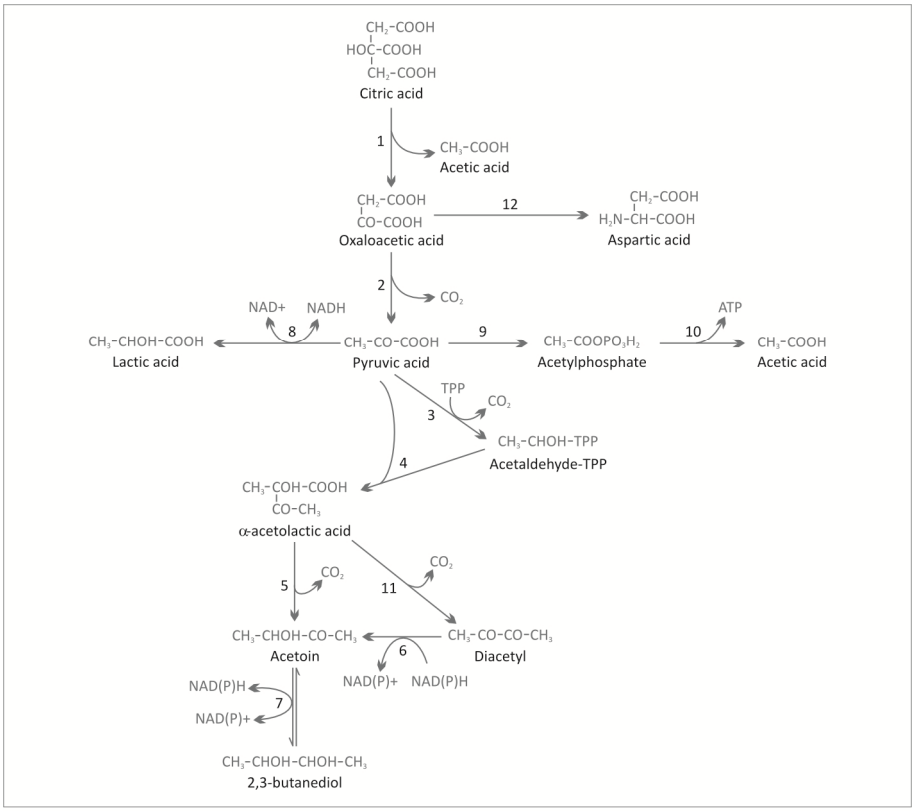

Figure 7. Metabolic pathway of citrate breakdown by *O. oeni*. 1, citrate lyase; 2, oxalacetate decarboxylase; 3, pyruvate decarboxylase; 4, α-oxalacetate synthase; 5, α-oxalacetate decarboxylase; 6, diacetyl reductase; 7, acetoin reductase; 8, lactate dehydrogenase; 9, pyruvate dehydrogenase complex; 10, acetate kinase; 11, nonenzymatic decarboxylative oxidation of α-acetolactate; 12, aspartate aminotransferase. TPP, thiamine PP_i (Ramos *et al.,* 1995).

Sorbic acid has been proposed as possible antiseptic in wine (Ribéreau-Gayon *et al.,* 2006a). All species of LAB including *O. oeni* seem capable to metabolize this acid developing an unpleasant smell of geranium leaves with a reported sensory threshold of 100 ng l^{-1} (Swiegers *et al.,* 2005).

3.2. Aroma modification resulted from β-glucosidase activity

The resulting modifications of MLF are high complex and often involve the reduction of vegetable and herbaceous aromas and the appearance of other fruity, floral, nutty or milky aromas. Improvement of wine volatile composition after MLF has been demonstrated revealing

the importance of β-glucosidase activity (Maicas *et al.,* 1999; Izquierdo-Cañas *et al.,* 2008; Hernandez-Orte *et al.,* 2009).

Specific strains of LAB might present a source for β-glucosidase activity capable to operate under conditions of wine, hence influencing its flavor complexity (Palmeri and Spagna, 2007). Previous work examining performances of cultures derived from 11 commercial preparations of *O. oeni*, responses of β-glucosidase activity to enological pH values, glucose/fructose ratio, and ethanol concentration were determined with synthetic substrates (Grimaldi *et al.,* 2000). On the other hand, Boido *et al.,* (2002) suggested that the limited bacterial glycosidase activity during MLF may not be sufficient to significantly increase the algycon concentration of wine. Nevertheless, it should be noticed that not all strains can contribute to the production of beneficial volatile compounds. This is the reason to suggest the induction of MLF in red wines with selected LAB strains that can offer a positive contribution to the final aroma in wines (Maicas *et al.,* 1999).

Other work has evaluated the β-glucosidase activities of 10 wild strains of *O. oeni* (Barbagallo *et al.,* 2004). Two of these strains retained β-glucosidase activity under oenological conditions. Furthermore, these strains showed a good malolactic performance when inoculated to wine (Barbagallo *et al.,* 2004). Further research showed that different strains of *O. oeni* contributed to the release of volatile aglycons from their glycosylated precursors (Ugliano *et al.,* 2003; D'Incecco *et al.,* 2004). Large differences in the extent of hydrolysis and in the specificity of this activity toward specific aroma precursors were observed and appeared to be related to the chemical structure of the aglycon as well as to individual characteristics of each starter culture (Ugliano *et al.,* 2003). Grimaldi *et al.* (2005) reported that *O. oeni* strains do not necessarily hydrolyse all substrates; instead they may display substrate specificity. This is also in agreement with subsequent findings where free and glycosidically bound volatile compounds of red wine were measured after malolactic fermentation (MLF) with four different commercial starter cultures of *O. oeni* (Ugliano and Moio, 2006). The extent of hydrolysis of glycosides during MLF was dependent on both bacterial strain and chemical structure of the substrate.

3.3. Secondary changes as part of LAB metabolic diversity

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Heterofermentative and facultatively heterofermentative LAB metabolize hexoses and pentoses by the phosphoketolase pathway (Zaünmuller *et al.,* 2006), producing mainly lactate, ethanol and CO_2; and alternatively mannitol, erythritol, acetate and glycerol (Fig. 8). Although these metabolic pathways are well characterized, there is still little information about how the metabolic shift can influence the organoleptic characteristics of wine or when does it happen.

To date, the main concern of winemakers is the production of acetic acid. A small increase in volatile acidity is also attributed to MLF. Two pathways can be involved. Acetic acid can be produced from residual sugar through heterolactic metabolism (phosphoketolase pathway) and the first step in citric acid metabolism produces acetic acid (Ramos and Santos, 1996; Ribéreau-Gayon *et al.,* 2006a).

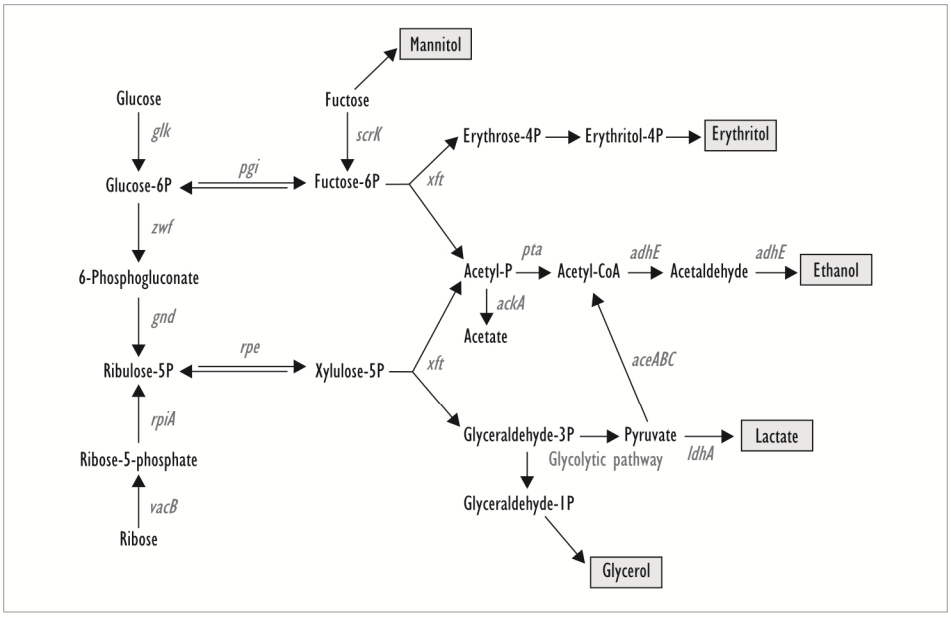

Figure 8. Metabolism of glucose by the phosphoketolase pathway by *O. oeni* PSU-1. Abbreviations: *aceABC* pyruvate dehydrogenase; *adhE* acetaldehyde/ethanol dehydrogenase; *glk* glucokinase; *gnd* 6-phosphogluconate-dehydrogenase; *ldhA* lactate dehydrogenase; *pta* phosphotransacetylase; *pgi* phosphoglucose isomerase; *rpe* ribulose-5-phosphate-isomerase; *scrK* fructokinase; *vacB* ribokinase; *xft* phosphoketolase; *zwf* glucose-6-phosphate-dehydrogenase (adapted from Marcobal and Mills, 2009).

In general, most agree that little acetic acid is formed during MLF even in the presence of significant sugar concentration. However, continued bacterial growth after completion of MLF may lead to considerable acetic acid formation if sufficient fermentable sugars are present (Bartowsky and Henschke, 2004).

Phenolic compounds or polyphenols are natural constituents of grapes and wines. These compounds are very important since they are responsible for many of the organoleptic properties of wines, especially, color and astringency (García-Ruiz *et al.,* 2008). Some data have been reported about *O. oeni* activity on phenolic compounds such as hydroxycinnamic acids (ferulic and coumaric acids). This activity results in the formation of volatile phenols as 4-ethylguaiacol and 4-ethylphenol which are considered phenolic off-flavors when their concentration reaches 4 mg l^{-1} (Cavin *et al.,* 1993). Other interactions involving LAB and phenols have been described for *O. oeni* (Reguant *et al.,* 2000) and also for *L. plantarum* (Rodríguez *et al.,* 2009).

It has been suggested that esterase activity from *O. oeni* and *L. plantarum* may contribute to wine color modification (Hernández *et al.,* 2006; Alexandre *et al.,* 2008). Esterase activity of *O. oeni* and other LAB has also been reported to hydrolyze ester substrates which may also affect wine flavor (Matthews *et al.,* 2007). Furthermore, acetaldehyde metabolism by *Lactobacillus* and *Oenococcus* might play a role in both flavor and color development (Osborne *et al.,* 2000).

Continued investigation into the causes and interactions that give rise to desirable and undesirable flavors in wine must be regarded as more than a challenging preoccupation for wine scientists (Bartowsky and Pretorius, 2009).

4. Moving into the "omics" era

Genome sequencing and functional genomics studies of a variety of LAB are now rapidly providing insights into their diversity and evolution and revealing the molecular basis for important traits such as flavor formation, sugar metabolism, stress response, adaptation and interactions (Siezen *et al.,* 2004). In the current "omics" era, innovative high-throughput technologies allow measuring temporal and conditional changes at various cellular levels (De Keersmaecker *et al.,* 2006). These new technologies are based on comparative and functional versions of genomics, proteomics, transcriptomics and metabolomics coupled with a non-omics glue, bioinformatics (Ward and White, 2002). The ultimate goal in data integration will be to combine the transcriptional, protein interaction and metabolic network to construct comprehensive network models (for the outline see Fig. 9).

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Nowadays, there are 23 complete or high-quality drafts of genome sequences of food or beverage-related LAB species. Of these, 9 sequences are from LAB species that are generally found in wine or musts (Marcobal and Mills, 2009). In particular, access to *O. oeni* genome sequence enables prediction of key pathways involved in central carbon and nitrogen metabolism and facilitates omics research strategies as new tools aiming to predict optimum strategies for efficiently carrying out the MLF with a desirable flavor outcome.

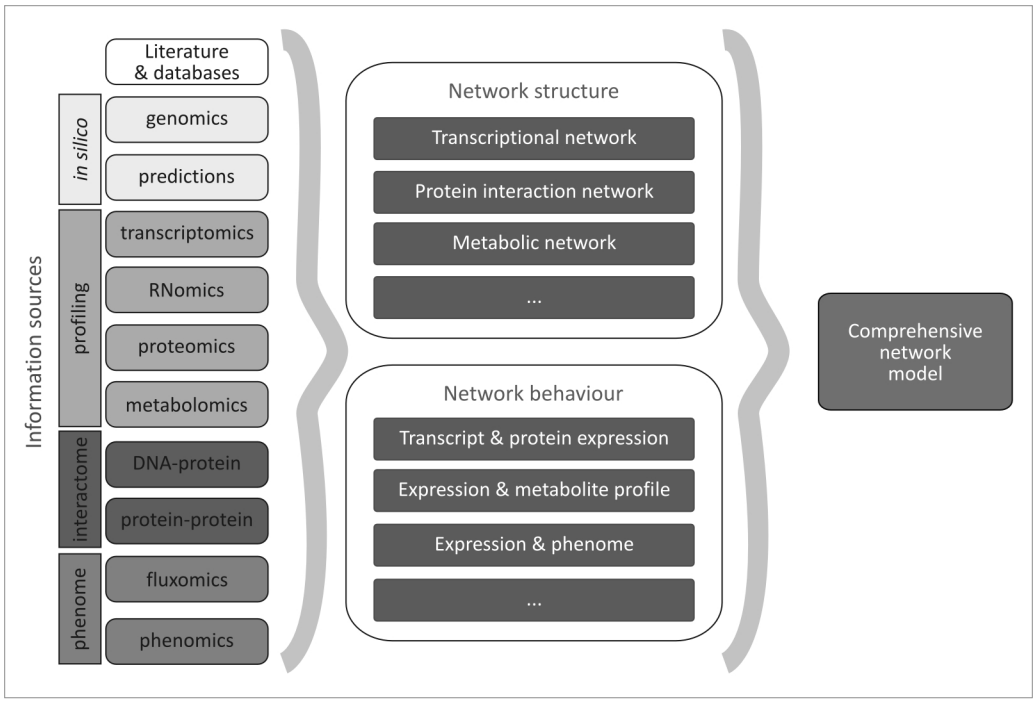

Figure 9. Integration of omics data. Different information sources, i.e. omics data, literature and computational predictions can be integrated to infer the structure of the transcriptional network, the protein interaction network or the metabolic network (network structure). Besides unravelling structures, omics data allows analyzing the responses (mRNA, protein and metabolite profiles) triggered by each of these networks and studying their mutual relation (network behaviour) (De Keersmaecker *et al.,* 2006).

So far, the most representative omics studies performed directly in food matrices have mainly used proteomics and, to a lesser extent, transcriptomics. The major reason for this is the very recent emergence of the application of those techniques to complex systems such as food (Zagorec *et al.,* 2006). Functional analysis of gene expression using comparative transcriptomics and proteomics is providing insight into stress responses and regulation mechanisms in LAB. Preliminary microarray analysis of the *L. plantarum* response to several stress conditions

revealed unanticipated stress response profiles that correlate specifically with lactate- and pH-induced stresses (Siezen *et al.,* 2004).

Furthermore, some reports have recently included the application genomics and proteomics in the study of *O. oeni* (Mills *et al.,* 2005; Zé-Zé *et al.,* 2008; Cecconi *et al.,* 2009; Borneman *et al.,* 2010). For instance, Beltramo *et al.* (2006) carried out a reverse transcription quantitative PCR (RT-qPCR) to quantify the transcript level of 13 genes that could play a role in adaptation of *O. oeni* in wine. Their results provided information on the temporal expression of *O. oeni* genes during growth in a wine-like medium enabling a better understanding of the overall response of this species to stress during MLF. Further investigation including array technology would allow scientists to work with a higher number of genes.

Using a proteomic approach, Da Silveira *et al.* (2004) have provided evidence for and active ethanol adaptation response of *O. oeni* at the cytoplasmic and membrane protein levels. Moreover, Cecconi *et al.* (2009) also contributed to a better knowledge of the physiological adaptation of *O. oeni* to wine conditions. These authors found a different protein behavior from non-acclimated and ethanol-acclimated cells after their inoculation into wine. These variations were mainly attributed to differential modulation of specific proteins involved in stress response and in sugar and amino acid metabolism (Cecconi *et al.,* 2009).

Although there is still much room for improvement, these studies represent the first step towards data integration related to *O. oeni* and its inclusion into the omics era.

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II. HYPOTHESIS AND OBJECTIVES

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HYPOTHESIS AND OBJECTIVES

O. oeni is the predominant lactic acid bacteria in the malolactic fermentation (MLF) of wines. This bioconversion is encouraged primarily to lower wine acidity, increase microbiological stability and enhance subtle changes in flavour and aroma complexity. Although MLF can start randomly, the beginning is often delayed leading to possible alterations in wine quality. These delays are due to the difficulties in the bacterial population development as a consequence of the harsh environmental conditions of wine. In other words, despite of being the species more adapted to wine, *O. oeni* has to face multiple stressors such as low pH, high ethanol concentration and the presence of sulphur dioxide that can cause an inhibition of cell growth. To avoid the problems arising from the slowdown of MLF, the inoculation of selected *O. oeni* strains is usual. However, the induction of this fermentation remains problematic since, in some cases, the inoculated strains fail to develop.

Regarding to the adaptation mechanisms of *O. oeni* to wine conditions, there is still little information at the molecular level. Some studies have been focussed in the response of *O. oeni* to specific stressors such as temperature and pH. However, there are no studies addressing the global relationship between different mechanisms involved in such response. Recently the complete sequence of *O. oeni* strain PSU-1 has been published, opening the door to genetic and transcriptional studies that will contribute to a better understanding of *O. oeni* behaviour in wine. Thus, it is necessary the application of the new available technologies for studying the adaptation mechanisms of *O. oeni* to wine conditions, which will also allow a more precise characterization between different strains.

In view of the above, the main objective of this thesis is to address a global study, both transcriptional and functional, of genes and proteins involved in *O. oeni* adaptation to wine conditions and the development of MLF. The hypothesis is that the results of a global analysis will allow a better characterization between different strains based on their capacity of adaptation to wine conditions. This hypothesis will be tested through the following objectives:

- ✓ To study the transcriptional response of the citrate pathway genes of *O. oeni* under the effects of ethanol and pH. Because citrate metabolism has been associated to a possible acid

stress response by maintaining the intracellular homeostasis and it is also a precursor of flavoring compounds, we wanted to analyze the influence of ethanol and pH on this pathway.

✓ To evaluate the relationship between the transcriptional and functional behavior of several stress responsive genes under wine related conditions in different *O. oeni* strains. Considering that the metabolic activity may vary among strains under the same growing conditions, we wanted to investigate a possible link between that activity and the expression of related genes.

✓ To further analyze *O. oeni* β-glucosidase activity and to analyze the transcription of one of the sequences coding for this enzyme during MLF in red wine. As β-glucosidases have been target of much attention because of their relationship with the increase in flavor compounds during MLF, we aimed to contribute to this knowledge by including a transcriptional study using different *O. oeni* strains as well as different growing conditions.

✓ To evaluate the protein expression pattern of *O. oeni* after ethanol shock using two-dimensional gel electrophoresis. After the transcriptional analysis, we considered interesting to analyze the influence of ethanol over the protein profile of *O. oeni* using the new available technology as an approach to further studies.

All the research work carried out in this PhD thesis has been supported by grant AGL2006-03700ALI from the Spanish Ministry of Education and Science, for the project entitled "Multigenic study of the mechanisms of *Oenococcus oeni* involved in its adaptation to wine conditions and in the development of malolactic fermentation", with Cristina Reguant as main investigator. For doing the thesis, Nair Olguín has enjoyed a predoctoral fellowship (2006-2010) from the Catalan government (Generalitat de Catalunya). An international stage has been done in l'Institut National de la Recherche Agronomique (INRA), Jouy-en-Josas, Paris, France. It was supported by a mobility grant (TME2008-01339) from the Spanish Ministry of Education.

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III. RESULTS

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i. Influence of ethanol and pH on the gene expression of the citrate pathway in *Oenococcus oeni*

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ABSTRACT

The consumption of citrate by the malolactic bacterium *Oenococcus oeni* changes the aromatic profile of wines due to the production of volatile compounds such as diacetyl and acetic acid. In this study, the expression of genes related to citrate utilization in the *O. oeni* strain PSU-1 was investigated to further understand the role of this metabolic pathway in the adaptation to wine environment and its impact on organoleptic qualities. Different conditions of ethanol content (0% and 10%) and pH (3.5 and 4.0) were assayed to evaluate the transcriptional response to both these stress factors. In the presence of ethanol, metabolic and transcriptional behavior was different than the observed when ethanol was absent. The expression of citrate pathway genes was mainly affected by ethanol, while pH showed a lower effect. Among the studied genes, *citE*, *ackA* and *alsD* were the genes revealing a distinctive transcriptional response. The differences observed in gene expression were in correlation with the different content of end products such as acetic acid and diacetyl. The increment of gene expression observed in the presence of ethanol at low pH suggests the participation of citrate metabolism in the response to stress conditions.

INTRODUCTION

Oenococcus oeni is the main species responsible for wine malolactic fermentation (MLF) in which the dicarboxylic L-malic acid is decarboxylated into the monocarboxylic L-lactic acid. The change in flavor and aroma complexity is an indirect effect of the loss of acidity during MLF (Davis *et al*., 1985; Lonvaud-Funel, 1999).

O. oeni can metabolize other organic acids present in wine such as citric acid (Lonvaud-Funel *et al*., 1984; Hugenholtz, 1993). The metabolism of malic and citric acids consumes protons, and generates both a membrane potential and a pH gradient, (Cox and Henick-Kling, 1989; Loubiere *et al*., 1992; Ramos *et al*., 1994; Salema *et al*., 1996), thus allowing ATP synthesis. These mechanisms have been associated to a possible acidic stress response (Martín *et al*., 2004; Tourdot-Maréchal *et al*., 1999). Moreover, citrate utilization leads to the production of flavoring compounds, such as diacetyl, acetoin, butanediol and acetate (Fig. 1). Diacetyl is responsible for one of the most evident flavor changes during MLF and confers a ‘‘buttery’’ character to wine (Bartowsky and Henschke, 2004). Nevertheless, it seems that the presence of diacetyl in wine is the result of a nonenzymatic decarboxylative oxidation of a-acetolactic acid (ALA) since diacetyl synthetase has never been isolated from lactic acid bacteria (Nielsen and Richelieu, 1999). The formation of acetate is another consequence of citrate utilization by *O. oeni* (Fig. 1). As it is well known, the increase of the volatile acidity detrimentally affects wine aroma.

Although enzymatic activities of the citrate pathway have been studied in *O. oeni* (Ramos *et al*., 1995), little is known at the molecular level. Thanks to the genome sequencing of *O. oeni* PSU-1 (Genbank NC_008528) the genes related to citrate utilization have been identified in this strain (Mills *et al*., 2005). These genes are organized in the citrate lyase gene cluster (*citR*, *maeP*, *citC*, *citD*, *citE*, *citF*, *citX*, *citG*). Martín *et al*. (2005) have showed that CitI (CitR) is involved in the expression of all genes required for citrate metabolism in *Weissella paramesenteroides* and that the organization of cit clusters and putative CitI operators are similar in *O. oeni*, suggesting a similar mechanism of transcriptional activation by CitI for both microorganisms. The genes involved in the butanediol pathway and pyruvate fermentation have been also located in the *O. oeni* PSU-1 genome (Wagner *et al*., 2005). A recent work (Augagneur *et al*., 2007) describes the effect of pH on the expression of two putative citrate transporters, *maeP* and *yaeP*. To the best of our knowledge, there are no other studies of the transcriptional response of genes related to citrate metabolism in *O. oeni*. In this respect, it

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would be useful to recognize which genes are differentially expressed under different winemaking conditions and how these differences affect the organoleptic characteristics of wine. As suggested by Mills *et al*. (2005), one potential application of the transcriptional profile analysis may be the selection of *O. oeni* starter cultures in terms of MLF performance and improvement of wine bouquet.

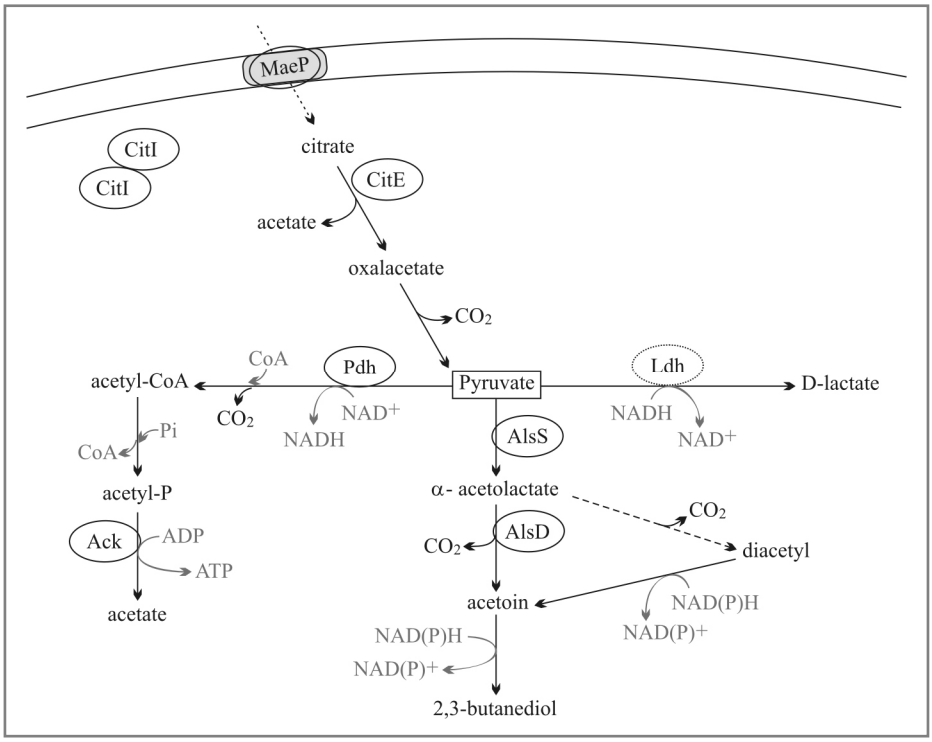

Figure 1. Main pathways for citrate/pyruvate metabolism by *O. oeni*. Genes analyzed in this study: citI – transcriptional activator; *maeP* – putative citrate permease; *citE* – citrate lyase; *pdh* – pyruvate dehydrogenase; *ackA* – acetate kinase; *ldh* – lactate dehydrogenase; *alsS* – α-acetolactate synthase; *alsD* – α-acetolactate decarboxylase. Dashed arrow toward diacetyl denotes a nonenzymatic reaction.

The aim of this work was to study the transcriptional response of the citrate pathway genes of *O. oeni* under the effect of different wine stress factors (presence of ethanol and low pH). Seven genes (Table 1), coding for enzymatic activities relevant in the organoleptic modification of wine, were selected. The expression of these genes was quantified in the *O. oeni* strain PSU-1 by means of reverse transcription real-time quantitative polymerase chain reaction (RT- Real-Time qPCR). The production of metabolites from citrate utilization was also monitored to evaluate the correlation with the expression of their related genes.

MATERIALS AND METHODS

Growth conditions

O. oeni PSU-1 was grown at 28 ºC in a medium based on FT80 (Cavin *et al*., 1989) modified by the addition of 0.5 g l^{-1} of citrate and meat extract (5 g l^{-1}) instead of casamino acids. This medium, as FT80, contained 5 g l^{-1} of L-malic acid, 5 g l^{-1} of glucose and 3.5 g l^{-1} of fructose, and it was referred to as cFT80 medium. The PSU-1 strain was used because its genome sequence is currently available (Genbank NC_008528).

Cultures were initiated by direct inoculation of *O. oeni* PSU-1 grown at pH 4.0 in cFT80 medium. Cells were collected at the end of the exponential phase ($OD_{600nm} \approx 1$) and inoculated to a final concentration of 10^7 CFU ml^{-1} in four flasks with 500 ml of cFT80 medium in the following conditions: a) pH 4.0 without ethanol (referred as pH4); b) pH 4.0 with 10% (vol/vol) ethanol (pH4OH); c) pH 3.5 without ethanol (pH 3.5); and d) pH 3.5 with 10% (vol/vol) ethanol (pH3.5OH). They were incubated at 28 ºC. All assays were performed in duplicate and the growth was monitored by measuring absorbance at 600 nm and counting plates in MRS medium (De Man *et al*., 1960), supplemented with L-malic acid (4 g l^{-1}) and fructose (5 g l^{-1}) at pH 5.0. Growth measures of absorbance were calibrated against cell dry weight measurements. For the biomass determination, cells were harvested and dried at 100 ºC to a constant weight. A calibration curve was constructed to relate O.D. at 600 nm and dry weight. A change of 1 unit of O.D. was equivalent to 0.5 g of dry matter per liter.

RNA extraction

O. oeni cells were harvested by centrifugation, frozen in liquid nitrogen and kept at -80 ºC until RNA extraction. Total RNA extractions were performed according to Chomczynski and Sacchi (1987). After isolation, the RNA was purified using Roche RNeasy kit according to the manufacturer's instruction (Mannheim). Purified RNAs were suspended in 50 ml of 0.1% DPC (dimethylpyrocarbonate)-treated water. RNA concentrations were calculated by measuring absorbance at 260 nm using Thermospectronic Genesys 10 UV Spectrophotometer (Thermo Fisher Scientific).

Gene sequences

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Nucleotide sequences of *O. oeni* strain PSU-1 were obtained from the National Center for Biotechnology Information (NCBI). The sequence references of the genes from *O. oeni* PSU-1 (NC_008528) are the following: *citI* OEOE_0417 (location complements 402339–403298); *citE* OEOE_0422 (location 407032–407946); *maeP* OEOE_0419 (location 404701–405684); *pdh* OEOE_0331 (location 322550–323971); *ackA* OEOE_1249 (location complement 1169761–1170954); *alsS* OEOE_1703 (location 1622268–1623950); *alsD* OEOE_1704 (location 1623953–1624669); and *ldh* (AJ831540).

Reverse transcription and real-time qPCR

cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems) as recommended. Absence of chromosomal DNA contamination was confirmed by real-time PCR. Primers were designed (Table 1) to be about 18–22 bases long, to contain over 50% G/C and to have a melting temperature (Tm) above 60 ºC. The length of the PCR products ranged from 90 to 123 bp. Clone Manager Professional Suite software and ABI Primer Express program (Applied Biosystems) were used to select primer sequences, and analyze secondary structures and dimer formation. *O. oeni ldhD* gene, coding for lactate dehydrogenase, was used as internal control, using the primers described by Desroche *et al*. (2005).

Table 1. Gene descriptions and the corresponding primer sequences, designed in this work (see text).

Target gene	Description	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon length (bp)
maeP	Putative citrate transporter	GTGGGGCTTTATTGTTGCT	AAAGGCGTAACCAAACCG	123
citE	Citrate lyase β subunit	CCGCACGATGATGTTTGTTCC	GCTCAAAGAAACGGCATCTTCC	108
citI	Citrate lyase regulator	GAGTAATCGACGCCTTTTCAGC	CAGCCTTTTCAACCATCCTTCC	117
pdh	Pyruvate dehydrogenase (E3)	CGGCCTATGCTTTTCACG	AATCGCCTTCCAGATCGG	95
ackA	Acetate kinase	GCTGATGCGCTTGTTTTCACG	AATGCCCAAGAAAGCCAGACG	90
alsS	Acetolactate synthase	CAAAACGCAGCTTTCATGG	TGTGCCGTCAAAATTCCG	116
alsD	α acetolactate decarboxylase	GCCGCAATTAGAGTACACG	CGCGACCTTTGAAAATGGC	93

Real-time PCR was performed in 25 ml final volume containing 5 ml of cDNA dilution, 1.5 ml of each primer at 10 nmol ml^{-1}, 4.5 ml of RNAse free-water and 12.5 ml of SYBR Green

Master Mix (Applied Biosystems). Amplifications were carried out using an ABI Prism 5700 Sequence Detection System (Applied Biosystems) with an initial step at 95 ºC for 10 min followed by 40 cycles of 95 ºC for 15 s, 60 ºC for 1 min and 72 ºC for 30 s. An additional step starting between 90 and 60 ºC was performed to establish a melting curve, and was used to verify the specificity of the real-time PCR reaction for each primer pair.

The efficiencies of amplifications were 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). In this study, the threshold value was automatically determined by the instrument. Results were analyzed using the comparative critical threshold ($\Delta\Delta C_T$) method in which the amount of target RNA was adjusted to a reference (internal target RNA) as previously described (Livak and Schmittgen, 2001). Measures were taken for each condition from cDNA that had been synthesized from RNA extracted from two independent cultures. They were taken in triplicate for each gene.

Chemical analysis

MLF was monitored by measuring L-malic acid, citric acid, acetic acid, D-glucose, D-fructose, D-lactic acid and L-lactic acid using Boehringer enzymatic kits (Mannheim). Medium samples without cells were stored at -20 ºC for further analysis. pH measurements were taken using a GLP31 pH-meter (Crison Instruments).

The concentration of diacetyl was detected by high-pressure liquid chromatography (HPLC Agilent 1100 Series). The methodology used was adapted from de Revel *et al*. (2000) by using the shorter column Zorbax Eclipse XDB-C8 (150 mm x 4.6 mm x 5 mm) purchased from Agilent Technologies and changing the separation and elution programmes of the mobile phase: water/acetic acetic acid 0.5%-methanol 0/20, 2/50, 6/58, 10/64, 15/75, 16/100, 18/100, 22/0, 23/20, 28/20 (time in min/% methanol).

RESULTS

Growth and metabolic evolution during and after MLF

For all the assays, four samples were taken: at the time of inoculation (T0), one day (T1); two days (T2) and seven days (T3) after inoculation. In all conditions, the population of *O. oeni*

PSU-1 inoculated was around 1 x 10^7 CFU ml^{-1} (O.D. at 600 nm 0.1), which is similar to the values used for starter cultures in winemaking. There was no loss of viability after inoculation. However, the bacterial population barely increased in the presence of ethanol but noticeably incremented when ethanol was absent (Fig. 2H).

The consumption of L-malic acid was very fast in the absence of ethanol and it took one more day in cultures with ethanol (Fig. 2A). In accordance with utilization of L-malic acid, L-lactic acid was produced (Fig. 2B) in an approximatively equimolar ratio (Table 2), although the molar yield of L-lactic acid produced from L-malic was slightly higher at pH 3.5 that at pH 4.

Under the studied conditions, citrate was completely consumed in all cases (Fig. 2G). When ethanol was present, citrate metabolization was delayed in comparison to MLF, while it was simultaneous to MLF in absence of ethanol. Nevertheless, the molar ratio of citrate consumption in relation to the biomass present was clearly higher in presence of ethanol (Table 2).

At the end of growth (T3), glucose was totally consumed in the absence of ethanol, although it took one day longer at pH 3.5 (Fig. 2C). Fructose had a similar pattern of consumption but it was not fully exhausted (Fig. 2D). In cultures with ethanol, both glucose and fructose were barely consumed. As expected, D-lactic acid was produced in all conditions in quantities that were inversely proportional to sugar consumption (Fig. 2E).

The production of acetic acid was higher in the absence of ethanol (Fig. 2F) but the molar ratio of acetate produced in relation to the consumption of all potential substrates, both sugars and citrate (Table 2, fifth row), was definitively higher in the assays where ethanol was present (about 0.8) than in those without ethanol (about 0.5). Since *O. oeni* is an heterofermentative species, these quantities of acetic acid could be produced from glucose or fructose, besides from citric acid. Regarding acetate produced from sugars by this species, it has been suggested (Salou *et al*., 1994) a stoichiometric conversion of 1 mol of glucose (or fructose) to 0.6 mol of acetate. According to this conversion, the acetic acid produced from citrate was calculated subtracting the amount of acetic acid hypothetically produced from sugars to the total amount detected. However, this calculation yields negative values for the assays without ethanol. That would mean that no acetic acid would have been produced from the citrate consumed. Obviously, this is not biochemically possible, since the metabolization of citrate generates a minimum of 1 equimolar amount of acetate as result of citrate lyase activity.

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Moreover, additional acetic acid can be produced by the pyruvate dehydrogenase pathway (Fig. 1).

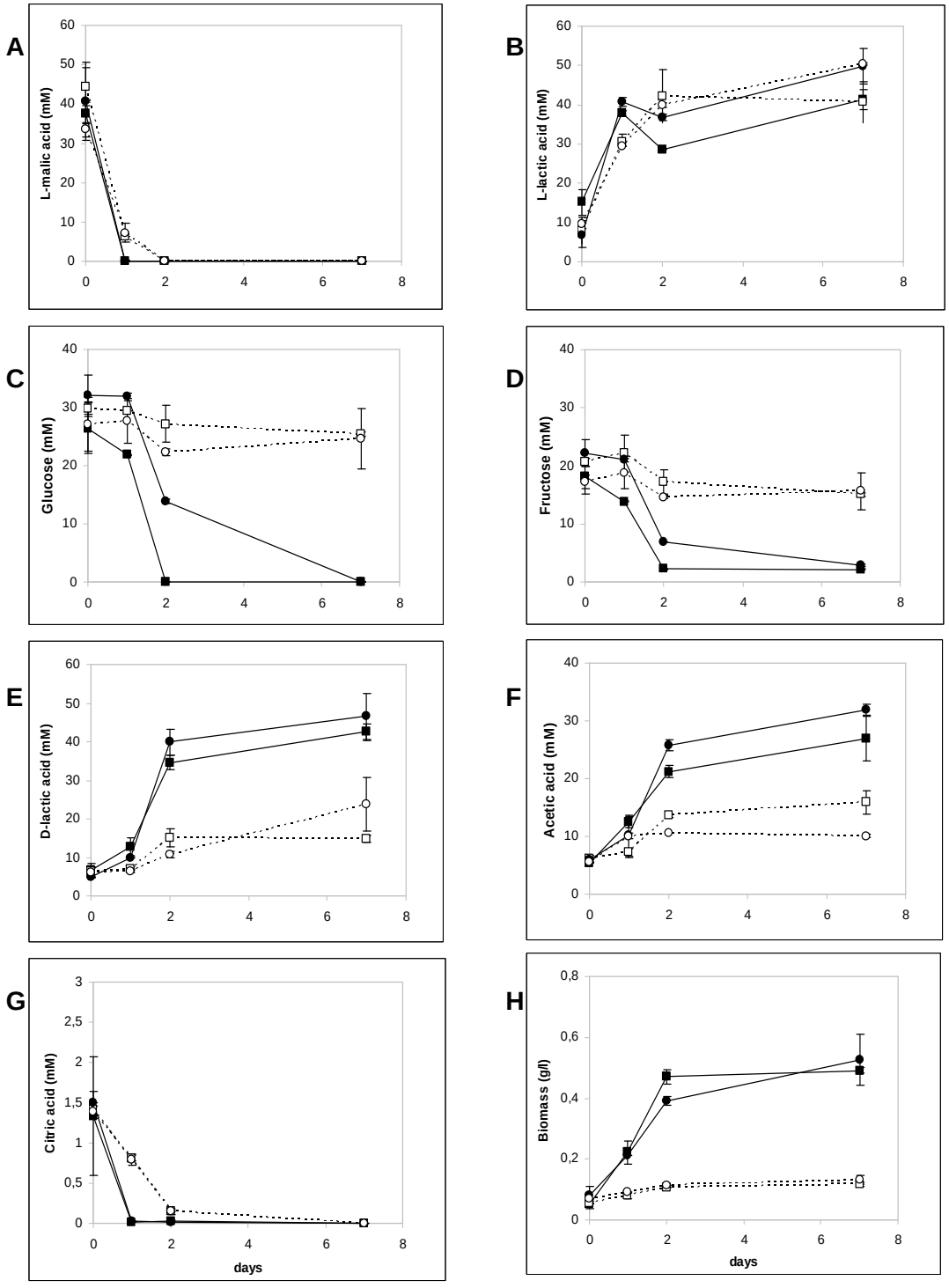

Figure 2. Growth and metabolic monitoring of *O. oeni* PSU-1 during and after MLF in cFT80 medium, at pH 4.0 (squares) and pH 3.5 (circles), in the absence (solid lines and filled symbols) and presence of 10% ethanol (dashed lines and empty symbols). A: L-malic acid. B: L-lactic acid. C: D-glucose. D: D-fructose. E: D-lactic acid. F: Acetic acid. G: Citrate. H: Biomass, dry weight. Data shown are mean values with standard deviations (n = 2).

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Therefore, for the assays without ethanol, a minimum of 1 equimolar conversion of citrate to acetate was fixed to calculate the maximum possible conversion of sugars to acetic acid. Under these conditions, the molar ratio acetic acid produced/sugars consumed yielded 0.47, while the molar ratio acetic acid produced/citrate consumed was about 1.1 both at pH 4 and pH 3.5 (Table 2).

For the assays with ethanol, considering the molar conversion of 0.6 from sugars to acetic acid suggested by Salou *et al*. (1994), molar ratios of 2.66 and 1.46 were obtained at pH4OH and pH3.5OH respectively, which are clearly higher than those without ethanol.

Diacetyl was detected at the end of the assay at very low concentrations (2 x 10^{-4} mM) only when ethanol was present. This value is below its taste threshold (Martineau and Henick-Kling, 1995).

Table 2. Molar ratios of substrates consumption and metabolites production at the end of growth cultures (7 days) of *O. oeni* PSU-1, calculated from data of Figure 2.

Condition	pH 4.0	pH 4.0 10% Ethanol	pH 3.5	pH 3.5 10% Ethanol
L-lactic acid produced (mmol) per L-malic acid consumed (mmol)	0.69	0.74	1.06	1.22
Citric acid consumed (mmol) per biomass (g, dry weight)	2.70	12.14	2.84	10.57
Acetic acid (mmol) produced per substrates consumed (glucose + fructose + citric) (mmol)	0.49	0.86	0.49	0.81
Acetic acid (mmol) produced from glucose and fructose (mmol) *	0.48	0.84	0.47	0.75
Acetic acid (mmol) produced from citric acid **	0	2.6	0	1.5

* Hypothesizing that acetic acid produced from citrate was 1 equimolar in all conditions (see text)

** Hypothesizing that acetic acid produced from glucose or fructose was 0.6 equimolar in all conditions (see text).

Changes in expression of citrate metabolism genes

In this work, primers to quantify the expression of citrate pathway genes were designed (Table 1). Efficiencies for all the primer pairs were close to 100%, making them appropriate for the analysis by the comparative critical threshold method ΔΔCT (Livak and Schmittgen, 2001).

To study the time course of gene expression, the relative expression (RE) levels were calculated at T1, T2 and T3 for all conditions. T0 at pH 4 was defined as the calibration condition. As previously determined by Desroche *et al*. (2005), a relative expression level higher than two means that the gene is over-expressed. It should be noted that MLF concluded at T1 in the absence of ethanol but took one more day (T2) in the presence of ethanol (Fig. 2A). In this way, T1 reflects gene expression during MLF in assays with ethanol but represents the end of MLF in assays without ethanol.

Expression of genes involved in citrate utilization

In the absence of ethanol, the expression of genes of the citrate lyase cluster did not show a relevant transcriptional response, except for *maeP* at T3 (Fig. 3C). In this case, the RE level at pH4 showed a 13-fold change and a 3-fold change at pH 3.5.

In the presence of ethanol, *cit* and *maeP* genes generally increased their expression. It was observed a clear transcriptional response of *citE* to ethanol content at T1, showing a 16-fold and 11- fold change at pH4OH and pH3.5OH, respectively. The expression of this gene decreased progressively along time (Fig. 3). The *maeP* and *citI* genes showed a moderate increase in expression at T1 (Fig. 3A). The RE levels of *citI* decreased to control values at the end of MLF (T2) and remained steady (Fig. 3B and C). By contrast, *maeP* highly incremented its expression at the end of MLF both at pH4OH and pH3.5OH (Fig. 3B). After MLF, *maeP* diminished its transcriptional response to ethanol (Fig. 3C).

Expression of genes involved in acetate production from pyruvate

The expression of *pdh* was not significantly affected either by pH or ethanol during MLF. Nevertheless, some days after the end of the fermentation (Fig. 3C), *pdh* showed a noticeable increase of RE in the absence of ethanol.

On the other hand, a general increase of the expression of *ackA* was observed at T1, especially in when ethanol was present (Fig. 3A). Further in time, the expression of this gene remained similar to the control in response to ethanol. Nonetheless, *ackA* was over-expressed at T3 both at pH4 and pH3.5 without ethanol.

Expression of genes involved in diacetyl production

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The acetolactate synthase gene *alsS* showed few changes in expression. In the presence of ethanol, a 3-fold change was observed at pH4OH during MLF (Fig. 3A). After MLF, *alsS* expression increased in 4-fold only at pH4.5 (Fig. 3C).

Oppositely, the transcriptional response of *alsD* was remarkable. In the presence of ethanol, *alsD* was over-expressed during MLF, showing a 14-fold change at pH4.5OH (Fig. 3A). After MLF, *alsD* response decreased. It is worth to note the increase observed at T3, where a 13-fold and 21-fold change was detected at pH4 and pH3.5, respectively (Fig. 3C).

DISCUSSION

In *O. oeni*, the catabolism of citric acid in wine has been described to be delayed in comparison with malic acid decarboxylation (Nielsen and Richelieu, 1999; Bartowsky and Henschke, 2000). In this study, *O. oeni* PSU-1 showed a concomitant degradation of citrate and malic acid in the absence of ethanol. Notwithstanding, when ethanol was present, more than half of citrate content was utilized once L-malic acid was almost depleted. Nevertheless, the determination of molar yield of this slower consumption of citrate has shown that biomass present was more effective when ethanol was present, at both pHs, 4 and 3.5, than in absence of ethanol (Table 2, second row). This implies an activation of metabolic activity of citrate consumption in this stress condition.

In cultures without ethanol, glucose and fructose were consumed toward the end of MLF. This consumption was delayed at

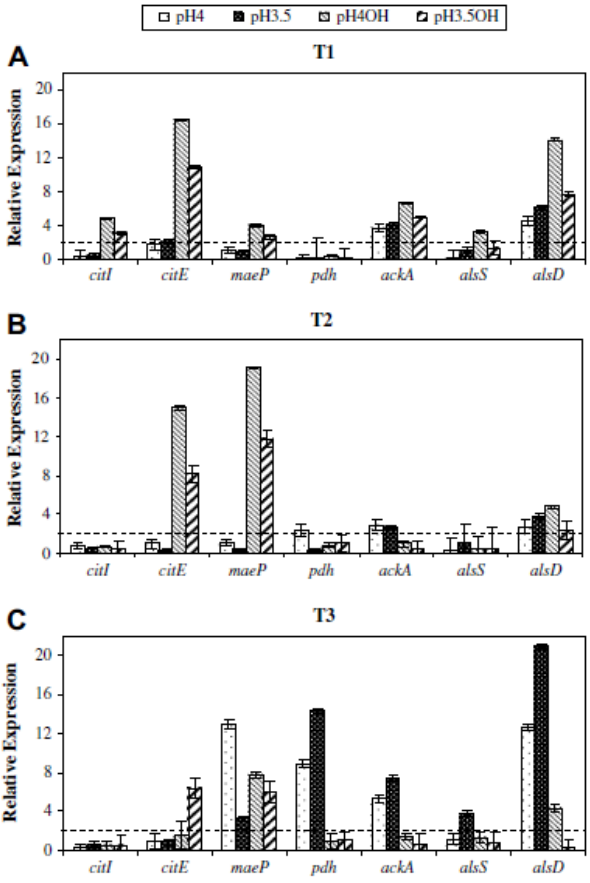

Figure 3. Relative expression (RE) levels of seven *O. oeni* genes related to citrate utilization in four culture conditions (pH 4.0 and pH 3.5, 0% and 10% of ethanol) along time: A) T1; B) T2 and C) T3. The calibrator condition used was pH 4 at the time of inoculation (T0). Data shown are mean values with standard deviations (n = 2).

pH3.5OH. These results are in agreement with previous studies that showed the preference of *O. oeni* for L-malate instead sugars as energy source for maintenance at low pH (Pimentel *et al*., 1994). In the absence of ethanol, higher levels of acetic acid were produced, very probably as a consequence of sugar fermentation. By contrast, there was almost no growth in the presence of ethanol, thus sugars were barely consumed and low concentrations of acetic acid were produced. Nevertheless, the yields of acetic acid produced per biomass (Table 2, fourth row) were similar or higher in the presence of ethanol, due to the low quantity of cells. In fact, molar ratios showed that yields of acetate produced from all substrates available for cells (both sugars and citrate) were higher in the assays where ethanol was present. In this sense, there was a significant increased molar ratio of acetate production from citrate when cells were exposed to ethanol. Thus, these results suggest that under the stress generated by the presence of ethanol, *O. oeni* may show an increased metabolic flux from citrate to acetate.

Changes in gene expression were also different depending on the presence or absence of ethanol. Ethanol produced a stronger transcriptional response than pH decrease did. It has been reported that ethanol increases membrane fluidization, which produces intracellular acidification (da Silveira *et al*., 2002; Chu-Ky *et al*., 2005). In this study, the external decrease of pH was moderate (pH 4–3.5). However, the ethanol shift from 0% to 10% may have had a greater effect on internal acidification. The incremented gene expression of the citrate pathway genes observed when ethanol is present, suggests the participation of citrate metabolism in the adaptation to acidic conditions. This fact is associated to the generation of proton motive force driven by ATPase (Mills *et al*., 2005) which helps to maintain internal pH homeostasis (Fortier *et al*., 2003). On the other hand, an inhibitory effect of pH on gene transcription could be observed in the presence of ethanol, since genes responding to this factor showed higher expression levels at pH4OH than at pH3.5OH. Another phenomenon observed for several genes, such as *maeP*, *pdh*, *ackA*, *alsS* and *alsD*, was the increase of their RE levels at the end of the assay in the absence of ethanol. This increment was probably not associated to specific experimental conditions but to the entry into the stationary phase of growth, as described for other *O. oeni* genes (Desroche *et al*., 2005; Beltramo *et al*., 2006).

Regarding citrate uptake and utilization, their related genes were over-expressed in the presence of ethanol. However, less changes were observed during MLF when ethanol was absent. The gene coding for the *cit* operon activator, *citI*, was over-expressed only during MLF in the presence of ethanol, in correlation with *citE* higher levels of transcription. In spite of the

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moderate changes observed in the absence of ethanol, the maximum RE levels of *citE* (around 2-fold change) were detected at the end of citrate consumption (T1). Altogether, our results suggest that *citE* expression is increased while citrate is being consumed, both with or without ethanol. Even though citrate was consumed in all conditions, the putative citrate transporter *maeP* increased its expression only in the presence of ethanol. Augagneur *et al*. (2007) described that, oppositely to other lactic acid bacteria, neither citrate nor pH induced the transcription of *maeP* in *O. oeni*. The role of this transporter should be further elucidated in order to understand its mechanism under wine conditions.

When both citrate and sugars are utilized, the intermediate pyruvate can be metabolized through the pyruvate dehydrogenase pathway producing additional acetic acid (Wagner *et al*., 2005). Of the studied genes involved in this pathway, *ackA* showed a transcriptional response in accordance with acetic acid production. At T1, this gene was generally over-expressed and the concentrations of acetate were similar in all conditions. Once finished MLF, acetate was still being produced in the absence of ethanol and the RE levels of *ackA* remained over 2-fold in these conditions. On the contrary, acetate was not produced in the presence of ethanol and the expression of *ackA* decreased to levels similar to the control or lower. These results suggest that the RE levels of *ackA* might be an appropriate indicator of acetate production. The other gene related to pyruvate utilization, *pdh*, did not show changes of RE levels associable to acetic acid production. In *O. oeni*, it has been described the low enzymatic activity of pyruvate dehydrogenase in cells grown on glucose. Under the same conditions, a high activity of acetate kinase was observed (Wagner *et al*., 2005). These results correlate with our transcriptional data, since growth in our cultures would have been sustained mainly by glucose and fructose present in the media.

In relation to the diacetyl/acetoin pathway, *alsS* and *alsD* genes of *O. oeni* strain Lo84.13 have been reported to be in a single, constitutively expressed operon (Garmyn *et al*., 1996). According to our results, the expression of the *alsS* gene varied occasionally but it could not be appreciated a clear trend in response to any of the studied conditions. By contrast, the expression of *alsD* gene was increased during MLF. Ramos *et al*. (1995) showed the increase of aacetolactate decarboxylase enzymatic activity at low pH. On the other hand, α-acetolactate synthase activity did not vary at pH below 6 and remained at low levels. These data would be in agreement with transcriptional results since *alsD* gene expression was induced by effect of pH. Moreover, it was revealed the transcriptional response of *alsD* to ethanol. In spite of the

organization of *alsS* and *alsD* in a single operon, Garmyn *et al*. (1996) detected the presence of two transcripts, a monocistronic unit including *alsS–alsD* and a single transcript of *alsD*. The downstream initiation at an internal promoter may explain the presence of the individual transcript of *alsD*. Accordingly, the transcription of this gene could be regulated independently to *alsS*, responding to stress conditions such as low pH or presence of ethanol.

In this study, diacetyl was detected at very low concentrations and remained in the media only in the presence of ethanol. The production of low amounts of diacetyl may be linked to the high expression levels of *alsD* during MLF under our experimental conditions. Diacetyl is formed chemically from the oxidative decarboxylation of alpha-acetolactic acid (ALA), an unstable intermediate compound produced from citrate metabolism (Nielsen and Richelieu, 1999; Ramos *et al*., 1995). On the other hand, diacetyl concentration also depends on ALA transformation by *AlsD* to acetoin or its reduction by diacetyl reductase also to acetoin. Moreover, the capacity to produce diacetyl is strain dependent (Bartowsky and Henschke, 2004). Therefore, the expression pattern of *alsD* gene may be different depending on the strain.

In summary, this study shows the induction of the transcriptional response of genes involved in the citrate pathway due to the presence of ethanol at low pH, particularly during MLF. These results suggest the involvement of citrate metabolism in cell adaptation to intracellular acidification. Genes showing a distinctive transcriptional response could be used in the future as indicators of citrate utilization of different *O. oeni* strains under wine conditions. Among cit genes, *citE* was the gene revealing a major change indicating citrate consumption during MLF. Related to acetic acid production, the increase of *ackA* expression was in accordance with acetic acid generation. On the other hand, the increment of *alsD* transcriptional levels may be linked to the low production of diacetyl. These differences in the expression patterns were in agreement with the differences in the content of compounds affecting organoleptic characteristics, such as acetate and diacetyl, and with the calculated molar ratio balances between substrates and products, which has shown also the deviation of metabolic activities in stress conditions such as the presence of ethanol. Altogether, the molecular approach of this study provides new data for a better understanding of the contribution of *O. oeni* to wine aromatic profile.

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ii. Multigenic expression analysis as an approach to understand *Oenococcus oeni* behaviour under wine conditions.

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ABSTRACT

The correct performance of wine malolactic fermentation (MLF) depends on the metabolic characteristics of the *Oenococcus oeni* strain/s responsible for this process. This study characterized four *O. oeni* strains showing a different behaviour in terms of malolactic performance, citric acid utilization related to acetic acid production, and stress adaptation. Metabolic evolution and their associated enzymatic activities were studied. Moreover, the transcriptional response of the genes related to MLF, citrate metabolism and stress response was compared among strains. A higher initial expression of both the malolactic enzyme and the encoding gene *mleA* could be related to a faster MLF. Citrate lyase (*citE*) initial transcriptional levels resulted to be an indicative of early citrate consumption. Moreover, strains showing a better performance under wine-like conditions, presented much higher relative expression of several stress responsive genes, highlighting the role of *hsp18*, *clpP*, *ctsR* and *rmlB*. Finally, the inter-strain comparison of transcriptional levels of selected genes, at different times of MLF, resulted to be a useful tool for strain characterization in accordance with their metabolic behaviour.

INTRODUCTION

Oenococcus oeni is the most adapted wine-associated lactic acid bacteria and is almost exclusively used for induction of malolactic fermentation (MLF) in red, white and sparkling base wines (Wibowo *et al*., 1985). MLF is the bacterial-driven decarboxylation of L-malic acid to L-lactic acid and carbon dioxide, and brings about deacidification, flavor modifications and microbial stability of wine (Bartowsky, 2005). In wine, *O. oeni* has to face multiple stresses such as ethanol, sulfur dioxide concentration, nutrient depletion, temperature and low pH that influence population development (Vaillant *et al.,* 1995; Versari *et al.,* 1999).

In order to survive in such environmental conditions, *O. oeni* uses several mechanisms that have been associated to a possible stress response and adaptation. The metabolism of malate and citrate involves the proton consumption and the generation of both membrane potential and pH gradient (Cox and Henick-Kling, 1989; Loubiere *et al.,* 1992; Salema *et al.,* 1996; Augagneur *et al.,* 2007), thus allowing ATP synthesis and the deacidification of intracellular and extracellular media. Moreover, citrate utilization leads to the production of flavoring compounds, such as diacetyl, acetoin, butanediol and acetate, thus contributing to the organoleptic characteristics of wine (Ramos *et al.,* 1996). Until recently, little was known at the molecular level about malate and citrate metabolism. Nowadays, and thanks to the genome sequence of *O. oeni* PSU-1 (Genbank NC_008528) it was possible to investigate the genes involved in this pathway and their level of transcription under different growth conditions (Beltramo *et al.,* 2006; Augagneur *et al.,* 2007; Olguín *et al.,* 2009).

Another mechanism involved in *O. oeni* adaptation to low pH and the presence of ethanol is the adjustment of membrane fluidity by modifying its composition (Teixeira *et al.,* 2002; Da Silveira *et al.,* 2003; Gandvalet *et al.,* 2008). For instance, *O. oeni* cells respond to culture in the presence of ethanol by increasing their cyclopropane fatty acid (CFA) content which is thought to counteract the effect of ethanol on membrane fluidity (Teixeira *et al.,* 2002). In addition, the increase in CFA content observed in acid- and ethanol-grown cells was also related to a higher level of *cfa* transcripts suggesting a transcriptional regulation of this gene under different stress conditions (Grandvalet *et al.,* 2008).

The synthesis of heat-shock proteins, chaperones and proteases by *O. oeni* under stress conditions has also been studied (Jobin *et al.,* 1997; Guzzo *et al.,* 1997, 2000; Beltramo *et al.,* 2004). Recent studies have confirmed at the molecular level the transcriptional regulation of the small heat-shock protein Lo18 (Coucheney *et al.,* 2005). Additionally, *O. oeni* cells exhibiting

an increased synthesis of Lo18 showed a greater ability to survive in wine and to perform MLF. Furthermore, a multigenic analysis aiming to quantify the transcriptional level of 13 genes that could be involved in *O. oeni* adaptation to wine conditions has been recently carried out (Beltramo *et al.,* 2006). Using one strain of *O. oeni* these authors assessed the overall response to stress during MLF. However, the use of other *O. oeni* strains as well as other genes would be necessary for a better understanding between the level of gene induction and cell adaptation mechanisms. According to this, evaluation of the metabolic pathways at the transcriptional level could also contribute to understand both, cell adaptation and secondary metabolite production.

The aim of this work was to evaluate the relationship between the transcriptional and functional behaviour of several stress responsive and metabolic related genes under wine–like conditions in different *O. oeni* strains. The expression of twelve genes (Table 1) was quantified in four strains of *O. oeni* by means of reverse transcription real-time quantitative polymerase chain reaction (RT-Real-Time qPCR). The production of metabolites from citrate utilization as well as the activity of related enzymes was monitored to evaluate the correlation with the expression of the corresponding genes. MLF was also evaluated by both enzyme activity and gene expression.

MATERIALS AND METHODS

Oenococcus oeni strains

The strains used in this study were selected from a previous screening according to their metabolic behaviour. Four strains were chosen due to their differences in growth rate, malate and citrate consumption time and acetate production: PSU-1 (ATCC BAA-1163), CECT 217^T (type strain), Microenos (Laffort, S.A.) and Z42 (own collection).

Growth conditions

O. oeni strains were grown in MRS broth medium supplemented with D,L-malic acid (8 g l^{-1}) and fructose (5 g l^{-1}) at pH 5.0. Cells were collected at the end of the exponential phase (OD_{600nm} = 1) and inoculated after centrifugation to a final concentration of 10^7 CFU ml^{-1} in 500 ml flasks containing cFT80 medium (Olguín *et al.,* 2009) at pH 4.0 modified by the addition of 1 g l^{-1} of citric acid. Following the same procedure, when cultures reached again the end of the

exponential phase, they were inoculated in 2 l flasks containing cFT80 medium at pH 3.5 with 12% (vol/vol) ethanol and reduced amount of sugar (glucose 1 g l^{-1} and fructose 1 g l^{-1}). The incubation conditions were always at 28ºC in a CO_2 incubator. All assays were performed in duplicate and the growth was monitored by measuring absorbance and counting plates in MRS medium (De Man *et al.,* 1960) supplemented with L-malic acid (4 g l^{-1}) and fructose (5 g l^{-1}) at pH 5.0.

RNA extraction

Cells were harvested by centrifugation, frozen in liquid nitrogen and kept at -80ºC until RNA extraction. Total RNA extractions were performed as described Chomczynski and Sacchi (1987) and then purified using Roche RNeasy kit according to the manufacturer's instructions (Mannheim). RNA concentrations were calculated by measuring absorbance at 260 nm using Thermospectronic Genesys 10 UV Spectrophotometer (Thermo Fisher Scientific).

Nucleotide sequences

Nucleotide sequences of *O. oeni* PSU-1 were obtained from the National Center for Biotechnology Information (NCBI). The sequence references of the genes from *O. oeni* PSU-1 (NC_008528) are the following: *mleA* (X82326); *citE* OEOE_0422 (YP_810046); *ackA* OEOE_1249 (YP_810790); *alsD* OEOE_1704 (YP_811215); *ddl* OEOE_0672 (YP_810247); *rmlB* OEOE_1447 (YP_810973); *cfa* (AJ831546); *ctsR* (AJ831549); *hsp18* (X99468); *clpX* (Y15953); *clpP* (AJ606044); *trxA* (XT93091) and *ldhD* (AJ831540).

Reverse transcription and real-time qPCR

cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems) as recommended. Three pair of primers were designed (Table 1) to be about 18-22 bases long, to contain over 50% G/C and to have a melting temperature (Tm) above 60ºC. The length of the PCR products ranged from 90 to 156 bp. Ten pair of primers were taken from previous works (Desroche et al. 2005; Beltramo et al. 2006; Olguín et al. 2008, 2009). *O. oeni ldhD* gene, coding for lactic acid deshydrogenase, was used as internal control, as described Desroche *et al.* (2005).

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Real-time PCR was performed in 25 µl final volume containing 5 µl of diluted cDNA, 1.5 µl of each primer at 5 µM, 4.5 µl of RNAsa free-water and 12.5 µl of SYBR Green Master Mix (Applied Biosystems). Amplifications were carried out using a Real Time PCR System 7300 (Applied Biosystems) with an initial step at 95ºC for 10 min followed by 40 cycles of 95ºC for 15 s, 60ºC for 1 min and 72ºC for 30 s. An additional step starting between 90 and 60ºC was performed to establish a melting curve, and was used to verify the specificity of the real-time PCR reaction for each primer pair.

Table 1. Primers used in this work.

Target gene	Description	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplicon length (bp)	Reference
mleA	Malolactic enzyme	CCGACAATTGCTGATACAATTGAA	GGCATCAGAAACGACCAGCAG	156	Beltramo *et al.* 2006
citE	Citrate lyase β subunit	CCGCACGATGATGTTTGTTCC	GCTCAAAGAAACGGCATCTTCC	108	Olguín *et al.* 2009
ackA	Acetate kinase	GCTGATGCGCTTGTTTTCACG	AATGCCCAAGAAAGCCAGACG	90	Olguín *et al.* 2009
alsD	α acetolactate decarboxylase	GCCGCAATTAGAGTACACG	CGCGACCTTTGAAAATGGC	93	Olguín *et al.* 2009
ddl	D:alanine-D:alanine ligase	CCTAAGCGAGCCTAATACAC	CCTGTTGGCTTCGATTCATTCC	92	This work
rmlB	dtdP-glucose-4,6-dehydratase	TATCCGCAATGCGCAATTGG	GAACGGTCAACATGCGATTCAG	93	This work
cfa	Cyclopropane fatty acid synthase	TGGTATTACATTGAGCGAGGAG	CGTCTTTGAGATCACGATAATCC	113	Beltramo *et al.* 2006
ctsR	Master regulator of stress response	GGGCCATGGCAGAAGCTAATATTTCAG	CGATCGGAGTTTCCAAAGAGAC	119	Desroche *et al.* 2005
hsp18	Stress protein Lo18	CGGTATCAGGAGTTTTGAGTTC	CGTAGTAACTGCGGGAGTAATTC	102	Beltramo *et al.* 2006
clpX	Clp ATPase protein	GAAGCGTGTTAACGAGTCC	CAGCGACTGAGCCAAATAAG	112	This work
clpP	ClpP protease	CGGTACCAAAGGCAAGCGTTTTAT	CTCTTCCGAGTCTTCAAAAGTTGAT	131	Beltramo *et al.* 2006
trxA	Thioredoxin	GCCACTTGGTGTACCCCTTGT	TCCATTTGCCGTTTCCTGGTTT	120	Beltramo *et al.* 2006
ldhD	D-lactate deshydrogenase	GCCGCAGTAAAGAACTTGATG	TGCCGACAACACCAACTGTTT	102	Desroche *et al.* 2005

In this study, the threshold value was automatically determined by the instrument. Results were analyzed using the comparative critical threshold ($\Delta\Delta C_T$) method in which the amount of target RNA was adjusted to a reference (internal target RNA) as previously described (Livak and Schmittgen, 2001). Measures were taken for each condition from cDNA that had been synthesized from RNA extracted from two independent cultures. They were taken in triplicate for each gene.

Inter-strain comparison of relative gene expression data

In order to compare the transcriptional response of the studied genes among the assayed strains, and evaluate the relationship with the different malolactic and metabolic behaviour, the relative expression level (RE) was represented in some cases as an inter-strain comparison. This

means that RE was calculated taking as reference (calibrating condition) the strain showing the lowest relative expression for the studied gene at the analyzed time, that corresponds to the strain showing the highest ΔC_T value ($\Delta C_T = C_T$ studied gene – C_T ldhD). Therefore, the strain chosen as reference has a RE value of one, while RE for the rest of the strains represents the time fold of over-expression for that gene, in the same conditions, compared to the strain taken as reference. It is worth to notice that ldhD (internal control) variations were below one unit showing an average C_T close to 21 in all sampled times and strains.

Chemical analysis

MLF was monitored by measuring L-malic acid, citric acid, acetic acid, D- glucose and D-fructose using Boehringer enzymatic kits (Mannheim). The concentration of diacetyl was detected by high-pressure liquid chromatography (HPLC Agilent 1100 Series). The methodology used was adapted from de Revel *et al.* (2000) by using the shorter column Zorbax Eclipse XDB-C8 (150mm x 4.6mm x 5µm) purchased from Agilent Technologies and changing the separation and elution programmes of the mobile phase: water/acetic acetic acid 0.5%-methanol 0/20, 2/50, 6/58, 10/64, 15/75, 16/100, 18/100, 22/0, 23/20, 28/20 (time in min/% methanol).

Enzymatic analysis

O. oeni cellular extracts were obtained by agitation on a vortex of a mixture of the bacterial suspension in 10 mM of Tris-HCl buffer (pH 8.0) with glass beads. After centrifugation at 15 000 rpm for 10 min, the supernatant fluid was transferred into a sterile tube and kept at -20ºC for further analysis. The malolactic (Labarre *et al.,* 1996), citrate lyase (Cogan, 1981), acetate kinase (Cogan, 1987) and α-acetolactate decarboxylase (Ramos *et al.,* 1995) activities were performed as previously described. Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as standard protein. Enzymatic activity units (U) were defined as µmol min^{-1}.

RESULTS

Strains selection

The selection of strains for this study was based on a preliminary screening of twelve *O. oeni* strains in order to choose those showing a different behaviour in terms of malolactic performance and citrate utilization. *O. oeni* PSU-1 was selected as a reference strain, since it has been subject of previous studies (Olguín *et al.,* 2009). Moreover, PSU-1 genome sequence has been used for primer design in this and previous work (Table 1). On the other hand, results of strains screening (data not shown) were also taken into account. In terms of malolactic performance, PSU-1 developed a slower MLF than other strains. Likewise, the type strain of *O. oeni* species, CECT 217T, was also selected because it showed a delayed MLF. Furthermore, 217T produced the highest amount of acetate among all tested strains. Strains Z42 and MO were selected because of its fast MLF performance and population viability under wine-like stress conditions. Therefore, four strains were finally selected as models of study, showing slow (PSU-1 and 217T) and fast (Z42 and MO) MLF, and one of them showing a higher acetic acid production (217T) compared to the others.

Metabolic evolution during MLF and growth

Regarding malolactic performance, strains Z42 and MO finished MLF in about two days, while fermentations with 217T and PSU-1 were slower and lasted five days (Fig. 1A). All strains grew during MLF (Fig. 1B) although PSU-1 showed a lower growth. Maximum biomass values observed coincided in all strains with the end of MLF.

Regarding citrate utilization, this was higher in strain 217T (Fig. 1C), being close to 4 mM, representing the consumption of 87% of the initial citrate in the medium. MO showed also an active citrate metabolism during MLF, consuming the 58 % of citrate. However, Z42 and PSU-1 showed lower citrate utilization, consuming only 40 and 35% of initial citrate, respectively. The molar relation of citrate consumption and acetate production was close to one in MO, 217T and PSU-1, and a little bit higher (1.3) in Z42 (Fig. 1C and D). The consumption of sugars was poor in general (Fig. 1E and F). Nevertheless, it is worth to notice the higher consumption of fructose by 217T between the middle and end of MLF. Diacetyl concentrations detected in all fermentations were very low, always showing values below the taste threshold (data not shown).

Expression of genes involved in malate and citrate metabolism

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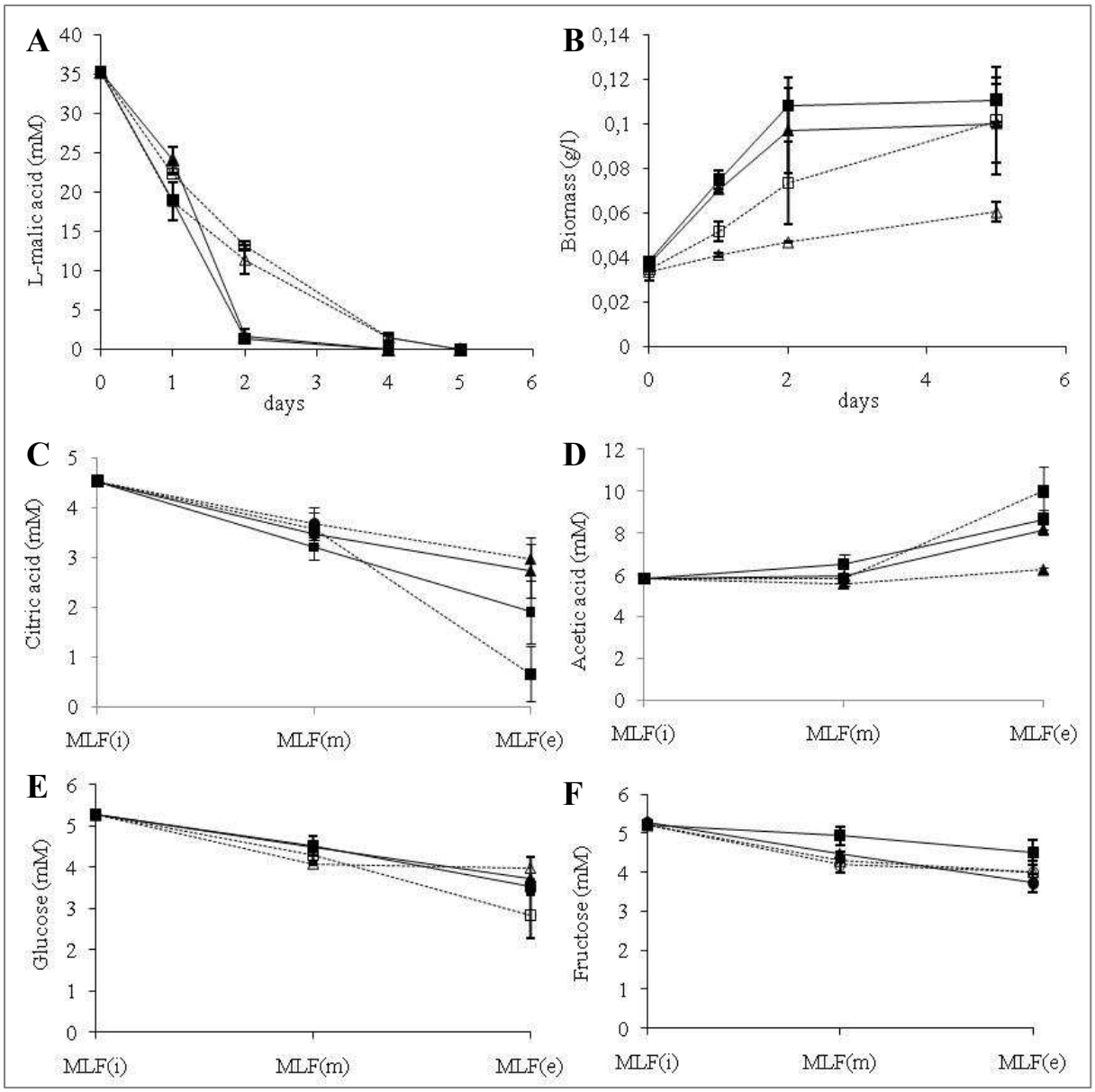

Figure 1. Evolution of biomass and metabolites along MLF in the four studied strains. Z42 (black triangles, solid line), MO (black squares, solid line), PSU-1 (empty triangles, dashed line), 217T (empty squares, dashed line). A: Biomass, dry weight. B: L-malic acid. C: citric acid. D: acetic acid. E: D-glucose. F: D-fructose. E: D-lactic acid. F: Acetic. Data shown are mean values with standard deviations (n=2).

The inter-strain comparison of relative expression (RE) of *mleA* (Fig. 2) showed a clear difference among strains that performed a fast MLF (Z42 and MO) and slow MLF (PSU-1 and 217T). Z42 and MO showed an over-expression of 35-fold and 45-fold, respectively, in comparison to 217T and PSU-1, both showing a RE close to one. Along MLF strains PSU-1 and 217T increased their *mleA* expression compared to the inoculum (Fig. 3). Despite this increase,

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since transcriptional levels were initially much lower for 217T and PSU-1, these strains showed a similar *mleA* expression to that in Z42 and MO at the end of MLF.

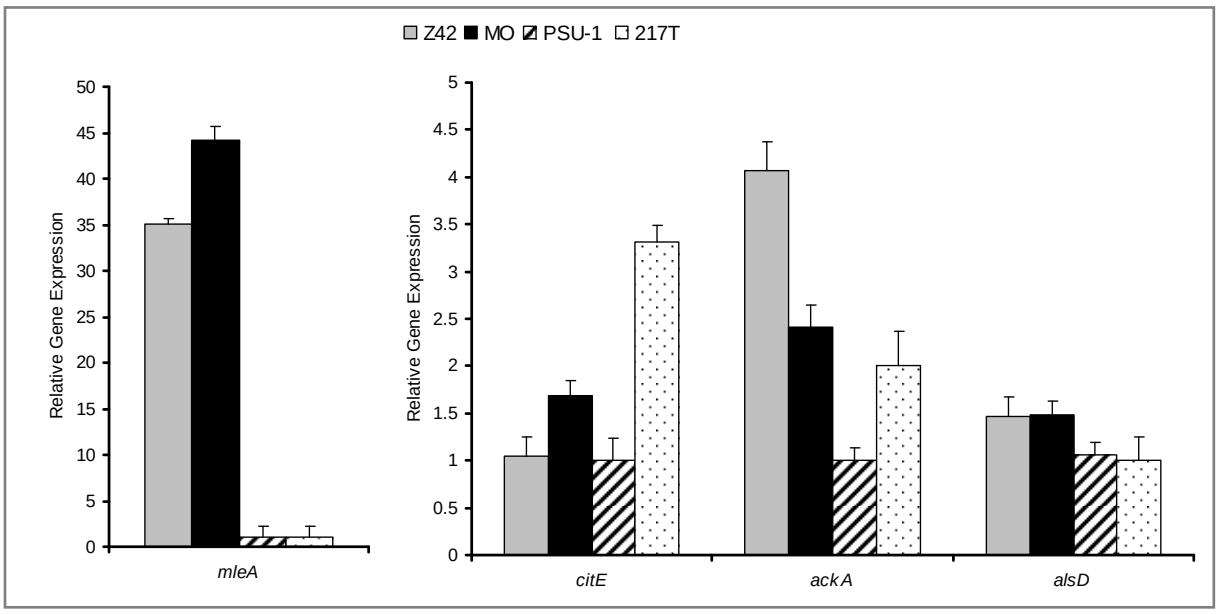

Figure 2. A Inter-strain comparison at different times of MLF of the relative expression of genes *mleA, citE*, *ackA* and *alsD*, taking as reference condition the strain showing the lowest ΔC_T value for each gene.

As can be seen in Figure 2, citrate lyase gene (*citE*) showed an initial over-expression of 3-fold in 217T compared to the rest of strains. However, in the middle of MLF, very low transcriptional levels of *citE* were detected in 217T, while Z42 showed a relevant over-expression compared to the time of inoculation (Fig. 3). At the end of MLF all the studied strains behaved similarly, increasing *citE* expression. At this time of fermentation were detected the maximum values of *citE* for all strains (lowest ΔC_T values). Regarding acetate kinase gene (*ackA*) and acetolactate decarboxylase (*alsD*) a similar trend was observed. In the inocula, few differences were observed among strains for these genes, with the exception of the over-expression of *ackA* in Z42 compared to the other strains (Fig. 2). Likewise, along MLF they did not vary significantly (Fig. 3).

Enzymatic activities related with MLF and citrate metabolism

Regarding enzymatic activity measurements (Table 2), strains 217T and PSU-1 showed malolactic enzyme specific activities of around 30% and 20% lower than strains Z42 and MO.

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In the middle of MLF the malolactic activity detected decreased in general and was comparable for all strains.

Citrate lyase activity was similar for all strains at the time of inoculation. However, in the middle of MLF the activities increased in general and were around 30% higher in strains MO and 217T than in Z42 and PSU-1. No relevant changes were detected for acetate kinase enzymatic activity between inoculation and mid MLF. It is worth of notice the lower values obtained with 217T for this enzyme. Regarding acetolactate decarboxylase, Z42 and MO showed around 30% higher enzymatic activities than PSU-1 and 217T at the time of inoculation. Z42 and MO did not vary this enzymatic activity along MLF, whereas PSU-1 increased its activity to similar levels than the former strains. In the case of 217T, the activity remained lower

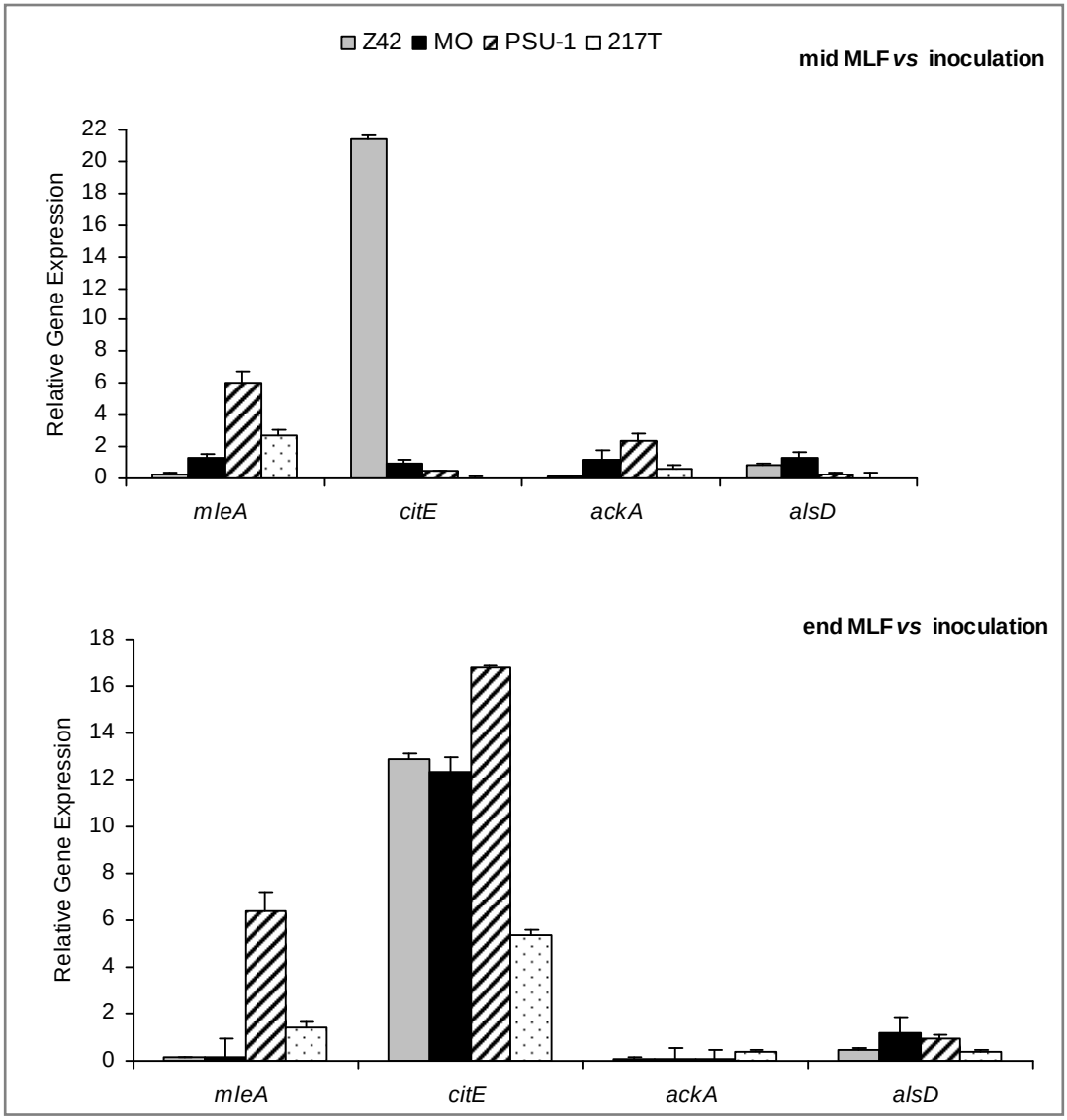

Figure 3. Evolution of relative expression of *mle, citE*, *ackA* and *alsD* genes in the middle and end of malolactic fermentation, taking as reference condition the time of inoculation.

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and even decreased a little in the middle of MLF.

Table 2. Mean values (n=2) of specific enzymatic activities (U mg^{-1} total protein) at time of inoculation (inoc) and in the middle of MLF (mid).

	Z42		MO		PSU-1		217T	
U/mg total protein	**inoc**	**mid**	**inoc**	**mid**	**inoc**	**mid**	**inoc**	**mid**
Malate decarboxylase	1.78	0.53	1.85	0.62	1.46	0.50	1.23	0.45
Citrate lyase	0.11	0.32	0.09	0.48	0.04	0.29	0.04	0.42
Acetate kinase	0.26	0.25	0.26	0.21	0.17	0.23	0.15	0.15
Acetolactate decarboxylase	0.82	0.75	0.89	0.98	0.59	0.73	0.55	0.41

Transcriptional response of stress related genes

The transcriptional behaviour of several genes related to stress response was compared among the four studied strains (Fig. 4 and 5). These genes can be grouped according to the function that they encode for: stress proteins (*hsp18*, *clpP*, *clpX* and *ctsR*), redox balance maintenance (*trxA*) and membrane and cell wall biosynthesis (*ddl*, *cfa* and *rmlB*). They were chosen among a collection of stress related genes according to results obtained in a previous work (Olguín *et al.,* 2008), in which relevant transcriptional changes of these genes were observed in response to ethanol in strain PSU-1.

Some common trends were observed depending on the fermentation stage, mainly once MLF was started. At mid MLF it was observed the increase in expression of some genes in strain Z42 such as *hsp18*, *clpP* and *clpX* (Fig. 4) in comparison to the time of inoculation. The relevant increase in *clpX* results in similar transcriptional levels of this gene compared to the other strains (Fig. 5), since clpX RE in Z42 was initially very low. In fact, the most relevant changes for stress related genes can be appreciated in the inter-strain comparison of transcriptional levels in the middle of MLF (Fig. 5), when strains Z42 and MO showed considerably higher transcriptional values than the other two strains for all the genes except for *clpX* and *ddl*, for which similar values were detected for all strains. PSU-1 showed also a relative over-expression of some genes (*hsp18*, *trxA* and *rmlB*) in comparison to 217T, strain which showed a general low transcription of stress responsive genes. At the end of MLF (Fig. 4), a general over-expression was observed for PSU-1 genes, compared to time zero, with the

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exception of *rmlB*, *hsp18* and *trxA*. On the other hand, *hsp18* and *trxA* and showed the highest value in strain MO whereas *rmlB* remained similar for all strains.

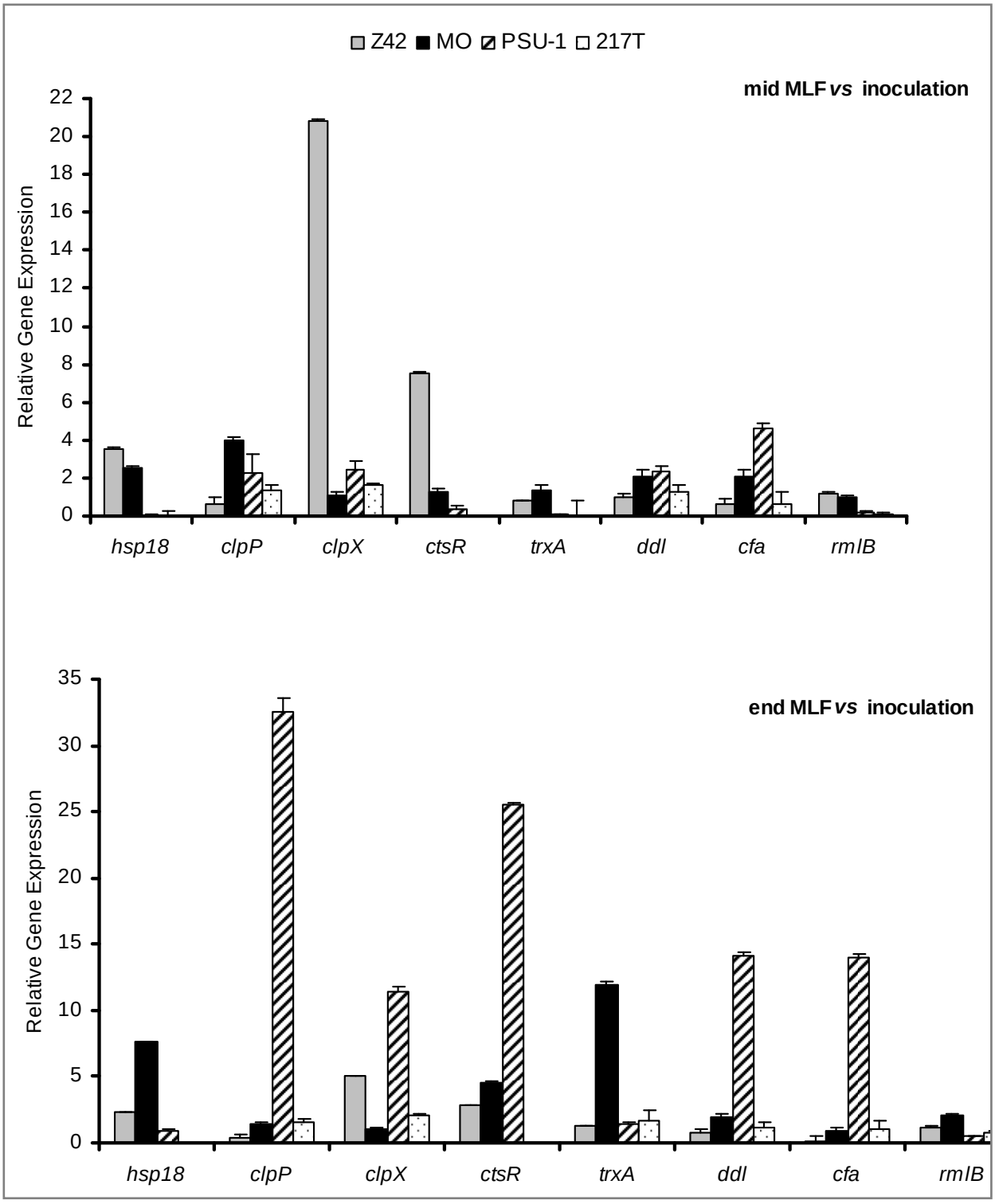

Figure 4. Evolution of relative expression of several genes related to stress response in the middle and end of malolactic fermentation, taking as reference condition the time of inoculation.

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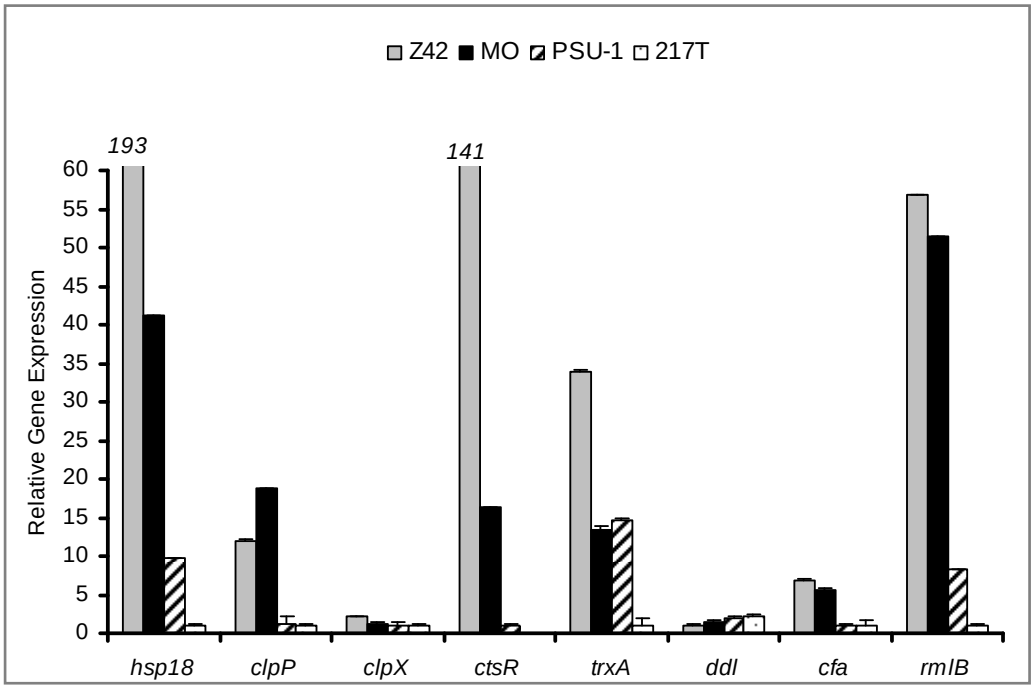

Figure 5. Inter-strain comparison at different times of MLF of the relative expression of genes related to stress response, taking as reference condition the strain showing the lowest ΔC_T value for each gene. Numbers in italics on unfinished columns indicate the RE value out of the scale used for the graph.

DISCUSSION

This work has evaluated the different behaviour of four *O. oeni* strains by means of metabolic performance, enzymatic activities evolution and gene expression comparison. Two strains, Z42 and MO, were chosen in accordance to their faster MLF performance, in contrast with PSU-1 and 217T, which showed a delayed MLF. Moreover, strain 217T showed a higher capacity to produce acetic acid. The fermentation assays carried out with these strains in modified cFT80 medium reflected the different metabolic traits of the four strains. MLF with 217T and PSU-1 lasted twice than those with Z42 and MO, and the maximum production of acetate was observed for 217T. Although PSU-1 and 217T showed a similar malolactic performance, 217T grew better than PSU-1 along fermentation, reaching biomass values close to those observed for the faster MLF strains. This growth could be sustained by the higher consumption of citrate and glucose observed during the second half of MLF for this strain, which would also account for the increase in volatile acidity. The co-metabolism of glucose with citrate has already been described in *O. oeni* (Ramos and Santos 1996), showing that the combination of the two compounds results in a higher yield of biomass than the consumption of these compounds separately (Salou *et al.,* 1994). A major consequence described of the reorientation of metabolic pathways resulting from the mixotrophic conditions is the increase in

the acetate yield associated with a higher level of ATP production (Ramos *et al.,* 1994, Salou *et al.,* 1994). This additional ATP production would have provided to 217T strain an extra source of energy for growth, although this had no consequences on FML duration. The rest of the strains showed a lower consumption of glucose than 217T. Likewise, fructose was barely consumed by any of the studied strains, including 217T.

The inter-strain comparison of malate decarboxylase gene (*mleA*) at the time of inoculation showed relevant differences among strains. Transcriptional levels *mleA* were much higher in fast MLF strains, Z42 and MO, than in slow MLF strains, PSU-1 and 217T. These relative gene expression (RE) values could be correlated with the higher specific enzymatic activities, which were also higher in Z42 and MO at this initial step. However differences observed among strains in enzymatic activity were not so accused as in gene expression. Along MLF transcriptional levels of *mleA* were equalized among strains. This is in accordance with the similitude observed in malolactic activities detected at mid MLF among strains, which suffered a general decrease. According to these results, the initial malolactic enzyme activity, related to its transcriptional level, would be the determining factor in MLF velocity. Coucheney *et al*. (2005) reported the malolactic enzymatic activity of freeze-dried *O. oeni* strains as a discriminating parameter to evaluate their potential as malolactic starter cultures. These authors could relate a higher malolactic activity at low pH (3.0) with a better performance of MLF in wine. The differences of specific enzymatic activities reported by Coucheney *et al.* (2005) among strains showing a distinct malolactic performance are very close to the presented in our work. Similar results have been observed for *O. oeni* in other environments such as cider, where a higher specific enzymatic activity of the inoculum could be related to a faster MLF performance (Herrero *et al.,* 2003).

Regarding citrate metabolism, an initial over-expression (3-fold) of citrate lyase gene (*citE*) was observed in the inoculum of strain 217T, compared to the other strains. MO also showed slightly higher transcriptional levels of *citE* than Z42 and PSU-1. However, at this initial step, enzymatic activities did not reflect relevant differences among strains and were generally very low. Citrate lyase specific activities increased during MLF and higher values were observed for MO and 217T. This increment resulted in the higher rate of citric acid consumption by these two strains in the second half of MLF. In other lactic acid bacteria species it has been described the activation of citrate lyase with the addition of citrate (Mellerick and Cogan 1981, Hugenholtz 1993, Bekal *et al.,* 1998). Therefore, the availability of acid citric in

the new media after inoculation might have activated citrate lyase activity during MLF. The evolution of *citE* along MLF showed an early activation of the transcription of this gene in Z42 at mid MLF, which was observed later for all strains at the end of MLF. At this point, 217T was the strain showing a lower *citE* RE. This could be due to the fact that citric acid had been almost depleted by this strain. The stronger activation of *citE* transcription towards the end of MLF in the strains that barely consumed citric acid during MLF, could be an indicative of the delayed use of this substrate as mechanism for cell survival once L-malate has been exhausted. Altogether, seems that the initial over-expression of *citE* could be an indicative for a higher citrate consumption during MLF. This would be in accordance with previously reported results (Olguín *et al.,* 2009). These results confirm that the citric acid metabolism characteristics is strain dependent as described for other LAB, such as *Lactococcus* (Hugenholtz, 1993).

Acetate kinase gene (*ackA*) expression could be related in a previous work with the higher production of acetic acid (Olguín *et al.,* 2009). In this work, *ackA* RE did not vary significantly along MLF. Likewise, the inter-strain comparison of its initial transcriptional response showed some differences among strains but they could not be related to the different production of acetic acid observed for the studied strains. This was in correlation with the enzymatic activities which barely varied during the fermentations. Studies with the Gram positive model bacterium *Bacillus subtilis* showed that the expression of *ackA* is stimulated by the presence of glucose (Grundy *et al.,* 1993). In the present study, initial concentrations of sugars, including glucose, were much lower than in the previously reported work (Olguín *et al.,* 2009). Observed sugar consumption and acetate production in these assays were also much below than the detected in the previous study. Therefore, with low glucose concentration, similar to sugar content in wine, *ackA* was not so active. In this case, the higher production of acetic acid by strain 217T should be associated mainly to citrate lyase utilization of citric acid.

The enzymatic activity of α-acetolactate decarboxylase is related to lower concentrations of diacetyl, since it transforms α-acetolactate (diacetyl precursor) into acetoin. No relevant differences were observed for α-acetolactate decarboxylase gene expression (*alsD*) in the initial inter-strain comparison neither along MLF. This would be in agreement with Garmyn *et al.* (1996), who described the organization of *alsD* and *alsS* genes in a single operon constitutively expressed. However, previous results obtained with PSU-1 (Olguín *et al.,* 2009) revealed a transcriptional increase of *alsD* in response to lower concentrations of ethanol than the used in this work (10% vol/vol). This would suggest that *alsD* transcription could be

differently regulated depending on medium conditions, such as ethanol content, pH or sugar content. Garmyn *et al.* (1996) detected the presence of two transcripts, a monocistronic unit including *alsS–alsD* and a single transcript of *alsD*. The downstream initiation at an internal promoter may explain the presence of the distinct regulation of *alsD* depending on the conditions. It is worth to notice that initial *alsD* transcriptional levels were much higher than the rest of citrate metabolism studied genes in all strains (data not shown). Therefore, even if the expression of this gene did not vary along MLF, its high constant transcription would account for the low diacetyl concentrations detected. This would be in accordance with the measured α-acetolactate decarboxylase enzymatic activities, which barely changed and were already high at the time of inoculation compared to the other citrate metabolism activities.

Regarding the stress related genes, it could be established a clear relationship between a considerable higher transcription of *hsp18*, *clpP*, *ctsR*, *trxA*, *cfa* and *rmlB* in the middle of MLF, observed in strains Z42 and MO, compared to the other strains, with their better MLF performance and growth under wine-like conditions. The heat shock protein Hsp18 has been defined as one of the stress proteins of *O. oeni* playing a relevant role in the response to different environmental conditions (Guzzo *et al.,* 1997, 1998, 2000; Beltramo *et al.,* 2005). Furthermore, the evaluation of Hsp18 abundance and its gene expression has been successfully used as a parameter to evaluate the state of adaptation of different strains (Coucheney *et al.,* 2005). On the other hand, ClpP is a stress responsive protease that can act independently or associated with ClpX ATPase to degrade larger specific substrates (Makovets *et al.,* 1998). The different behaviour observed between *clpP* and *clpX* gene transcriptional evolution in this work can be explained by the fact that *clpP* is regulated by *ctsR*, whereas *clpX* could depend on other mechanisms, not yet characterized (Beltramo *et al.,* 2004). CtsR has been reported as the master regulator of *O. oeni* stress response since is the responsible for the control of the transcription of most Clp proteins, except ClpX, and Hsp18 (Grandvalet *et al.,* 2005). At the middle of MLF, strains Z42 and MO showed a remarkably higher expression of *ctsR*, and genes under its control *hsp18* and *clpP*, than the other two strains. These results suggest that the inter-strain comparison of the expression of these three genes under wine-like conditions can be an indicative of a higher capacity of strain adaptation and, in consequence a faster MLF. Likewise, some genes related to cell wall and membrane biosynthesis, mainly *rmlB* (dTDT-glucose-4,6-dehydratase), but also *cfa* (cyclopropane fatty acid synthase), showed a relevant over-expression in fast MLF strains Z42 and MO. These results would be in agreement with the described by Silveira *et al.*

(2004) that described *rmlB* as one of the proteins over-expressed in the presence of ethanol in a global proteomic study. In the same way, Beltramo *et al.* (2006) found a relevant over-expression of *cfa* under wine conditions and thioredoxin gene (*trxA*) also showed higher RE in the inter-strain comparison at mid-MLF in the strains Z42 and MO, as well as PSU-1. This gene is related to the maintenance of redox intracellular balance and would be responding to the oxidative stress caused by ethanol.

The evolution of stress related genes along MLF showed a late transcriptional response of PSU-1. The over-expression of these genes would indicate a late recovery of this strain, which was the one showing the slowest growth. In contrast, 217T showed all along MLF low transcriptional levels of stress related genes, although it grew better than PSU-1. This strain could have sustained its survival and growth mainly thanks to sugar and citrate consumption, with the production of additional ATP and the protection against intracellular acidification associated to citrate utilization (Martín *et al.,* 2004).

In summary, this work has evaluated the different behaviour of four *O. oeni* strains by means of measuring the evolution of metabolites that may have an impact on organoleptic qualities, the related enzymatic activities and their gene expression and the transcriptional response of several stress responsive genes. The initial actitivities of malate decarboxylase and citrate lyase would determine a faster MLF and earlier citrate consumption, respectively. The inter-strain comparison of the transcriptional levels of the corresponding genes resulted to be a useful tool, indicative of different metabolic traits. Similar conclusions are extracted from the inter-strain comparison of some stress related genes in the middle of MLF, mainly *hsp18*, *clpP*, *ctsR* and *rmlB*. These data can be helpful for the characterisation of *O. oeni* strains for their use as malolactic starters. More research is needed in order to study in depth the regulation of mechanisms such as citrate utilization and acetate production, which can drastically change wine quality, and mechanisms leading to a better stress adaptation which can ensure a correct MLF performance.

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iii. β-glucosidase activity and *bgl* gene expression of *Oenococcus oeni* strains in model media and Cabernet Sauvignon red wine.

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ABSTRACT

Strains of *Oenococcus oeni* performing malolactic fermentation might prove an interesting source of β-glucosidase enzymes, which can release aromatic volatile compounds from their flavorless glycoconjugate precursors present in wine. Many studies have been conducted in wine-like culture conditions but little is known about the activity of these strains in real wine conditions. In this work, real Cabernet Sauvignon red wine was used to test β-glucosidase activity and *bgl* gene expression for four *O. oeni* strains. Assays in wine were compared with wine-like medium and mixtures of wine and MRS. β-glucosidase activity was detected in both whole-cell and supernatant fractions for all four strains tested although results varied depending on the conditions. The highest activity was found in wine, with values of 4 U mg^{-1}, and the activity was inhibited in the presence of fructose. To measure *bgl* expression, the RNA of *O. oeni* was extracted directly from red wine for the first time. Using real-time qPCR, the relative expression level of the *bgl* gene was evaluated. The expression of this gene varied depending on the strains and it was over-expressed in samples of wine diluted with MRS. These results could be of importance to the wine industry and in studies of strain adaptation to wine related to the selection of starters.

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INTRODUCTION

Many white wines and almost all red wines undergo secondary, or malolactic, fermentation (MLF), which is in fact a decarboxylation process in which dicarboxylic L-malic acid is decarboxylated into monocarboxylic L-lactic acid. This bioconversion is carried out by lactic acid bacteria, mainly *Oenococcus oeni*. Microbiological stability and flavor modification of wine are also outcomes of MLF (Bartowsky *et al.,* 2002).

The most distinctive flavor compound associated with MLF is diacetyl, which leads to the typical buttery flavor of some wines (Bartowsky and Henschke, 2004). Nevertheless, many volatile compounds in wine can be released from their flavorless glycoconjugate precursors by enzymic hydrolysis. For instance, grapes contain compounds called "aroma precursors", the most active of which are glycosides, mainly linalool, nerol and geraniol (Palmeri and Spagna, 2007). These glycoconjugates can be either monoglucosides or disaccharide glycosides in which glucose is further replaced with α-L-arabinofuranosyl, α-L-rhamnopyranosyl, β-D-xylopyranosyl or β-apiofuranosyl, which is then bound to the aglycone (D'Incecco *et al.,* 2004). The existence of such bound forms is interesting because these glycosides can produce flavoring aglycones when hydrolytic reactions take place (Palmeri and Spagna, 2007).

These bound volatile aglycones are released either by acid hydrolysis, which usually occurs slowly during wine storage, or by the action of glycosidase enzymes. Typically, the target glycosides are β-D-glucose and diglycoside conjugates, with the latter comprising glucose and a second sugar unit, α-L-arabinofuranose, α-L-rhamnopyranose or β-D-apiofuranose. Volatile aglycones present as monoglucosides are released via β-D-glucosidase (glucopyranosidase), whereas diglycoside-bound aglycones are sequentially released by α-L-arabinofuranosidase, α-L-rhamnopyranosidase or β-D-apiofuranosidase followed by β-D-glucosidase (D'Incecco *et al.,* 2004; Grimaldi *et al.,* 2005b).

Specific strains of LAB well-adapted to perform MLF might represent a source of β-glucosidase enzymes capable of operating under the physicochemical conditions of wine, thereby influencing its flavor complexity (Barbagallo *et al.,* 2004). Recently, several studies have been published on commercial strains of *O. oeni*, providing evidence of the potential value of the glycosidic activities of these bacteria for enhancing the flavor of wines (Grimaldi *et al.,* 2000; 2005ab; Boido *et al.,* 2002; D'Incecco *et al.,* 2004). Most of these studies focused on the enzyme activity of *O. oeni* during MLF in wine-like culture conditions, and demonstrated that β-glucosidase activity is mainly affected by pH, temperature, sugar and ethanol content.

Moreover, the extent of β-glucosidase activity also seems to be strain dependent (Barbagallo *et al.,* 2004; Grimaldi *et al.,* 2005a; Saguir *et al.,* 2009). However, little is known about these activities in real wine conditions, especially in red wine.

Previous studies have also tried to characterize gene coding for a putative β-glucosidase enzyme from *Lactobacillus plantarum* and from a commercial strain of *O. oeni*. Spano *et al.* (2005) found that the β-glucosidase gene from *L. plantarum* was apparently regulated by abiotic stresses such as those affecting the enzyme activity described by other researchers. To the best of our knowledge, there are no other studies of the transcriptional response of the *O. oeni* β-glucosidase gene. Therefore, it would be interesting to determine whether there is a relationship between glucosidase activity and gene expression and whether it is strain dependent. The results could potentially be used in the selection of *O. oeni* strains for the production of starter cultures based on their MLF performance and improvement of wine bouquet.

The aim of this work was to study the β-glucosidase activity of four *O. oeni* strains during MLF in different culture conditions including Cabernet Sauvignon red wine. We also quantified the expression of one of the *O. oeni* genes codifying for a β-glucosidase enzyme by means of reverse transcription real-time quantitative polymerase chain reaction (RT – Real-Time qPCR). The relationship between enzyme activity, gene expression and culture media conditions was also evaluated.

MATERIALS AND METHODS

Oenococcus oeni strains

Four *O. oeni* strains were used in this study: CECT 217^T (ATCC 23279^T) obtained from the Colección Española de Cultivos Tipo (València, Spain) (referred to as 217 in the text); strain Microenos® B1 obtained from Laffort S.A. (referred to as MO); strain Z42 from our collection, previously isolated from a Catalan wine; and strain PSU-1 (ATCC BAA-331), originally isolated from red wine at Pennsylvania State University.

Experimental conditions

Subcultures of *O. oeni* strains were grown in MRS broth medium (De Man *et al.,* 1960) supplemented with L-malic acid (5 g l^{-1}) and fructose (4 g l^{-1}) at pH 5.0. The incubation

conditions were at a constant 28ºC in a CO_2 incubator. Cells were collected at the end of the exponential phase ($OD_{600nm} \approx 1$) and inoculated (10%) into the different experimental media.

All assays were performed in duplicate and the evolution of MLF was monitored by L-malate consumption using Boehringer enzymatic kits (Mannheim). Viable populations were counted in MRS medium plates with 2% agar, supplemented with L-malic acid (4 g l^{-1}) and fructose (5 g l^{-1}) at pH 5.0. Viability was expressed as dry weight using a calibration curve relating the two parameters.

MRS culture medium

Each subculture of the *O. oeni* strains was directly inoculated in two flasks with 200 ml of fresh modified MRS broth medium as described above.

cFT80 wine-like medium

For cFT80 medium plus 12% ethanol the inoculation procedure was followed as described previously (Olguín *et al.,* 2009). Subcultured cells were inoculated into cFT80 medium at pH 4.0. When cultures reached the end of the exponential phase, they were inoculated in cFT80 medium at pH 3.5 plus 12% (vol/vol) ethanol and the amount of sugar was reduced (glucose 1 g l^{-1} and fructose 1 g l^{-1}).

Microvinification trials

Subcultures of the *O. oeni* strains were directly inoculated in two flasks containing 200 ml of Cabernet Sauvignon wine. The wine data were as follows: pH 3.46, 12.15 % (vol/vol) ethanol, 1.89 g l^{-1} of L-malic acid, 0.04 g l^{-1} of total sugars, free SO_2 5 mg l^{-1} and total acidity 7.4 g l^{-1} of tartaric acid. The wine was provided by the Institut Català de la Vinya i el Vi (INCAVI, Reus, Catalonia, Spain). Before inoculation wines were treated with 0.08% (vol/vol) of dimethyldicarbonate (DMDC) to prevent yeast and bacterial contamination.

Wine dilutions

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To test the extent of the influence of wine composition on β-glucosidase activity and *bgl* gene expression, we performed an MRS plus wine assay using the strain PSU-1. The following dilutions were used: 100% MRS; 75% MRS plus 25% wine (75-M); 50% MRS plus 50% wine (50-MW); 25% MRS plus 75% wine (75-W); and 100% wine (100-W).

Determination of β- glucosidase activity

Samples (1 ml) were taken from each experimental condition. These were separated into biomass (whole cells) and supernatant fractions by centrifugation (8,000 x *g* for 20 min) and each assayed for β-glucosidase activity separately.

For cultures grown in MRS and cFT80 media, enzyme activity was directly determined using a modified previously reported method (D'Incecco *et al.,* 2004). The supernatant fraction was used directly for analysis. For whole cell activity, the pellet was resuspended in 1 ml of sodium acetate buffer 0.1 M pH 5.1. Then, 100 µl of sample was added to 300 µl of substrate [3 mM of *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) diluted in 0.1 M sodium acetate buffer, pH 5.1] and incubated for 30 min at 37ºC. Next, 400 µl 1 M Na_2CO_3 was added to stop the reaction and allow the *p*-nitrophenolate anion to turn yellow. The reaction tubes were centrifuged and the assay was read against the blank at 400 nm up to a maximum absorbance of 1.0 unit using a Thermo Spectronic Genesys 10 UV Spectrophotometer (Thermo Fisher Scientific). From this measurement, the concentration of liberated *p*-nitrophenol (*p*-NP) was determined from a calibration curve taken from standard *p*-NP solutions. Unless otherwise indicated, one unit (U) of β-glucosidase activity corresponds to 1 µmol of *p*-nitrophenol released per minute per 1 mg dry weight cells (U mg^{-1}).

The same protocol was used for wine samples. However, several pretreatments were also performed in order to test matrix interference. Supernatant samples from sterile MRS and wine were previously assayed for β-glucosidase activity as follows: i) direct measurement of enzymatic activity; ii) absorbance measurement after treatment with 800 mg l^{-1} of polyvinylpyrrolidone (PVP) (Landete *et al.,* 2004); and iii) absorbance measurement after wine filtration through a 1 cm layer of polyvinylpolypyrrolidone (PVPP) (Zamora, 2003). In each case, 8 units of β-glucosidase pure enzyme (SIGMA G-0395) were added to the samples before treatment in order to record any possible loss of enzyme. Whole cell samples from MRS and wine were directly assayed for β-glucosidase activity or after filtration through Whatman® nº3

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filter paper. Viability was also tested in order to compare results between filtered and non-filtered samples. All pretreatment assays were performed in triplicate and then subjected to statistical analysis using SPSS Inc. software version 17.0 (Chicago, IL, USA).

RNA extraction

O. oeni cells were harvested by centrifugation, frozen in liquid nitrogen and kept at -80ºC until RNA extraction. Total RNA extractions were performed using the Roche RNeasy kit according to the manufacturer's instructions (Mannheim). RNA concentrations were calculated by measuring absorbance at 260 nm using a Thermo Spectronic Genesys 10 UV Spectrophotometer (Thermo Fisher Scientific). The quality of the RNA samples was checked on a 1% agarose gel electrophoresis with 1 x TAE buffer stained with ethidium bromide and visualized in a UV transilluminator prior to reverse transcription and real-time PCR.

Nucleotide sequences

Nucleotide sequences of *O. oeni* PSU-1 were obtained from the National Center for Biotechnology Information (NCBI). The sequence references of the genes from *O. oeni* PSU-1 (NC_008528) are the following: *bgl* OEOE_0224 (YP_809876) coding for 6-phospho-beta-glucosidase (EC: 3.2.1.86) and *ldhD* (AJ831540) coding for lactate dehydrogenase.

Reverse transcription and real-time qPCR

cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems) as recommended. Primers were designed to be about 18-22 bases long, to contain over 50% G/C and to have a melting temperature (Tm) above 60ºC. Clone Manager Professional Suite software was used to select primer sequences and analyze secondary structures and dimer formation. The primers *bgl*-F (5'- GACGAGGATCTTTCCCAATG-3') and *bgl*-R (5'-GGTTCGATACCGTACTTGTG-3') produced an amplicon of 102 bp. The primers *ldhD*-F and *ldhD*-R were those described by Desroche *et al.* (2005).

Real-time PCR (qPCR) was performed in 25 µl final volume containing 5 µl of diluted cDNA, 1.5 µl of each primer at 5 µM, 4.5 µl of RNAse free-water and 12.5 µl of SYBR® Green Master Mix (Applied Biosystems). Amplifications were carried out using the 7300 Real-Time

PCR System (Applied Biosystems) with an initial step at 95ºC for 10 min followed by 40 cycles of 95ºC for 15 s, 60ºC for 1 min and 72ºC for 30 s. An additional step starting between 90 and 60ºC was performed to establish a melting curve, and was used to verify the specificity of the real-time PCR reaction for each primer pair.

During qPCR analysis, the threshold value was automatically determined by the instrument. Results were analyzed using the comparative critical threshold ($\Delta\Delta C_T$) method in which the amount of target RNA was adjusted to a reference (internal target RNA) as previously described (Livak and Schmittgen, 2001). Measures were taken for each condition from cDNA that had been synthesized from RNA extracted from two independent cultures of each tested condition. They were taken in triplicate for each gene.

RESULTS

Influence of the medium on L-malic acid consumption

For MRS and cFT80 assays, three samples were taken: at the time of inoculation (T0), at twenty-four hours (T24) and at forty-eight hours (T48) after inoculation. For wine and MRS-wine combinations an additional sample was taken at six hours (T6) after inoculation.

The ability to perform MLF was evaluated for *O. oeni* strains cultured in MRS, cFT80, wine and wine dilutions (Table 1). The percentage of MLF completed in MRS and cFT80 media, 48 hours after inoculation, was higher in strains Z42 and MO, showing similar values, close to 95% or higher. Strains PSU-1 and 217 showed differences in malolactic performance between cFT80 and MRS media, values were higher in the later case. In wine, all the strains had a similar evolution and fermented between 31 and 41 % of initial L-malate in 48 hours. In the fermentation of wine dilutions with PSU-1 strain, L-malate consumption decreased in correlation with the increase of wine content in the medium. However, the most noticeable decrease was observed between 75-W and 100-W, where L-malate consumed in not diluted wine was the half than in diluted wine.

On the other hand, the rate of L-malate consumption in relation to biomass was similar for all four strains growing in MRS and wine (Table 1). Thus, L-malic acid in wine was depleted more slowly than in MRS due to the lower amount of viable cells, since in MRS cells grew more than one-log unit whereas in wine population viability decreased (data not shown). On the other hand, L-malate consumption was more enhanced in cFT80 than in the other assayed

media. Strain PSU-1 behaved differently in wine dilutions. Interestingly, values for the rate of malate consumption in 75-M, 50-MW and 75-W were higher than those in MRS and wine (100-W).

Table 1. Influence of the growth media on L-malic acid consumption of four *O. oeni* strains after 48 hours incubation. 75-M, 50-MW, 75-W and 100-W correspond to wine-MRS dilutions (see text). Data are the mean values of duplicate assays.

	Strain	**cFT80**	**MRS**	**75-M**	**50-MW**	**75-W**	**100-W**
% MLF completed	PSU-1	73.49	98.39	87.00	84.77	82.03	37.78
	217	62.46	78.88				31.05
	Z42	95.12	95.16				40.79
	MO	96.38	98.39				33.91
mg L-malate consumed per mg^{-1} dry weight	PSU-1	13.56±2.23	2.40±0.23	5.21±0.42	5.90±0.52	5.14±0.51	2.14±0.49
	217	13.55±2.63	1.12±0.17				1.76±0.45
	Z42	13.43±2.97	2.02±0.43				2.31±0.55
	MO	13.57±3.34	2.01±0.24				1.92±0.47

β-glucosidase activity

MRS and cFT80 culture media

The results of an initial survey performed on cultures of *O. oeni* strains in MRS medium showed that the four strains have β-glucosidase activity in both cell and supernatant fractions. As shown in table 2, all strains were able to de-glycosylate the *p*-NPG substrate. The four strains presented decreased β-glucosidase activity in MRS medium at T24 and T48. Strains PSU-1, Z42 and MO presented an increase in β-glucosidase activity in the cell fraction at T24 and T48, reaching values similar to those of the supernatant fraction. Strain 217 presented a similar pattern but had the lowest enzyme activity.

We found a clear difference in initial β-glucosidase activity among strains in cFT80 medium (Table 2). Strains Z42 and MO presented a higher level of activity than PSU-1 and 217. These activities were also lower than those from MRS and wine, which is probably due to the prior pre-adaptation step in cFT80 without ethanol. In addition, results show a variable distribution of β-glucosidase activity between cell and supernatant fractions during T0 and T24. However, the same pattern can be distinguished for all four strains: β-glucosidase activity was

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found to diminish towards T48 in both supernatant and whole-cell fractions, and this diminution was higher for the cell fraction, not being detected any activity at T48.

Table 2. Total β-glucosidase activity of four *O. oeni* strains growing cultures in cFT80 wine-like media and Cabernet Sauvignon wine and its distribution in whole cell and supernatant. Data are the mean values of duplicate assays.

	Strain	β-glucosidase activity (U mg^{-1})[1]		
		T=0h	T=24h	T=48h
cFT80	PSU-1	0.48 (17:83)	nd	0.10 (100:0)
	217	0.41 (20:80)	nd	0.03 (100:0)
	Z42	0.73 (36:64)	0.18 (98:2)	0.14 (100:0)
	MO	0.86 (35:65)	0.26 (56:44)	0.16 (100:0)
Wine	PSU-1	1.92 (100:0)	4.28 (87:13)	4.05 (84:16)
	217	2.0 (100:0)	3.94 (85:15)	3.88 (83:17)
	Z42	1.92 (100:0)	4.11 (86:14)	4.24 (82:18)
	MO	2.18 (84:16)	4.05 (85:15)	4.5 (83:17)

[1]Enzyme activity is expressed as μmol of p-nitrophenol liberated per minute by 1 mg dry weight cells (U mg^{-1}). [§]Numbers in parenthesis are the relative distribution (%) between the two culture fractions assayed (supernatant:whole cell). nd: not detected.

Cabernet Sauvignon wine

Due to potential wine matrix interference during the enzymatic assay, a pretreatment step using PVPP or PVP filtration was performed in both the whole-cell and supernatant fractions (see Materials and Methods). The results obtained from both the treated and non-treated samples were subjected to statistical analysis. We found that there was no significant difference within the different treatments for either supernatant or cell fraction within a confidence interval of 99% ($P<0.01$). Moreover, the potential loss of viable cells was determined before and after cell fraction filtration through Whatman® paper. We found no significant difference between non-filtrated (8.19 ± 0.28 Log CFU ml^{-1}) and filtrated samples (8.05 ± 0.05 Log CFU ml^{-1}). As we observed no wine matrix interference, we chose to follow the β-glucosidase activity testing protocol for wine samples as we had done with previous samples.

In wine it was observed a different pattern to that present in cFT80 culture media. Both the cell-associated and supernatant fractions presented a two-fold increase in β-glucosidase activity at T6 (data not shown) that remained almost unaffected at T24 and T48 (Table 2).

Combinations of MRS and wine

Total β-glucosidase activity (cell fraction and supernatant) was evaluated for strain PSU-1 in wine dilutions (Fig. 1). The values of each dilution (75-M, 50-MW, 75-W) and wine (100-W) are expressed in relation to the activity present in MRS medium at the same time. β-glucosidase activity displayed the same evolution under all conditions: it increased at T6, decreased to its initial values at T24 and increased again at T48. Notably, the values of enzyme activity in 100-W were almost 100% higher than those observed in the other conditions at T6 and T24 and even higher at T48.

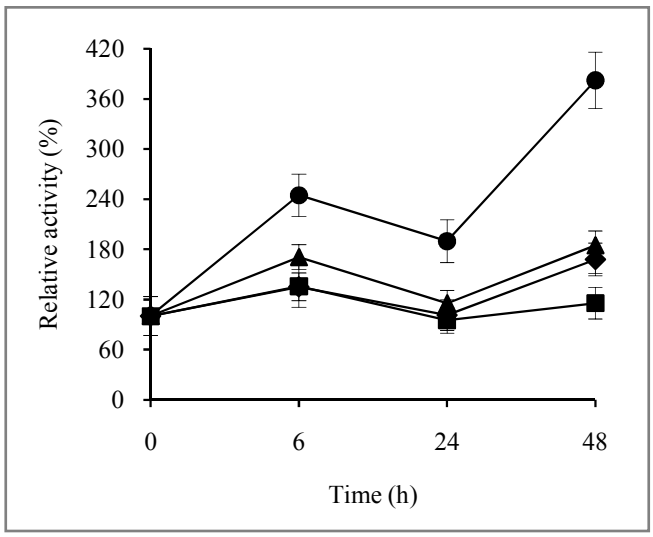

Figure 1. Influence of wine concentration on total β-glucosidase activity of *O. oeni* PSU-1: (■ 75-M); (◆ 50-MW); (▲ 75-W); (● 100-W). Values are the mean of two determinations and are expressed relative to the activity of the given time in MRS medium. Data are the mean values of duplicate assays.

RNA extraction from wine

Prior to the gene expression analysis, the quality of the RNA obtained from the wine samples of each of the strains was evaluated on agarose gel electrophoresis (Fig. 2a). According to these results, the extraction technique proved to be suitable for the extraction of RNA from all samples. The amplified fragments were also evaluated as products of the *bgl* gene (Fig. 2b).

β-glucosidase gene expression

The relative expression level (RE) of the *bgl* gene was evaluated in all strains cultured in MRS, cFT80 and wine (Fig. 3). As described by Desroche *et al.* (2005), a relative expression

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level higher than two means that the gene is over-expressed. The calibrator condition used was time zero (T0) at the moment of inoculation. In MRS medium, an over-expression of the *bgl* gene was observed in Z42 and MO strains (7.36 and 6.32-fold respectively) at T24 in MRS medium (Fig. 3A). The studied *bgl* gene was down-regulated in cFT80 medium at T24 for all the strains, except for MO, which did not vary (Fig. 3B). However, strain 217 presented an over-expression (6.63-fold) after 48 hours. In wine, 217 was also the only strain showing over-expression (5.4-fold) but in this case after 24 hours. The rest of the strains did not modify their *bgl* expression along time in wine (Fig. 3C).

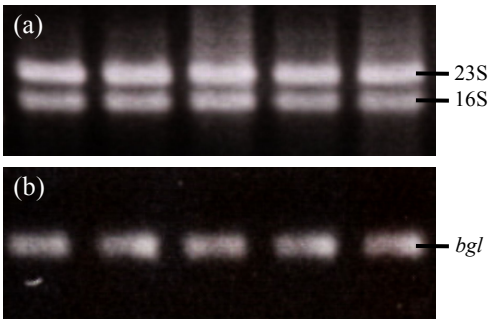

Figure 2. RNA extraction directly from Cabernet Sauvignon red wine. (a) RNA extracted from different samples of *O. oeni* PSU-1 cultured in wine; (b) 102 bp fragment of the *bgl* gene after PCR amplification using the RNA extracted in (a).

The relative expression level (RE) of the *bgl* gene was also measured in wine dilutions and 100-W (Fig. 4). In this case, only strain PSU-1 was tested and samples corresponding to T6 are also represented. The calibrator condition used for this comparison was MRS corresponding to each of the sampling times. The *bgl* gene presented a diverse pattern of induction depending on the culture conditions. An over-expression of the *bgl* gene was observed for samples taken from 75-M at T24 (7.53-fold); 50-MW at T48 (2.57-fold); 75-W at T6 (3.45-fold) and T48 (2.30-fold). Samples taken from 100-W presented an RE level, which is consistent with the results described above (Fig. 3C). In this respect, it is worth noting that the *bgl* gene was not over-expressed in MRS (Fig. 3A).

DISCUSSION

Several studies have been conducted for the purpose of finding a suitable method for selecting resistant strains able to perform MLF which include a quality criterion related to improving the aromatic profile of wines (Maicas *et al.,* 1999; Ruiz *et al.,* 2010). Previous studies of *O. oeni* have focused on investigating the activities of β-glucosidases and related enzymes (Boido *et al.,* 2002; D'Incecco *et al.,* 2004; Grimaldi *et al.,* 2005ab). As far as we are concerned, this is the first study including the expression analysis of one of the *O. oeni* β-glucosidase genes. Furthermore, we examined *bgl* gene expression and enzyme activity in several culture conditions in order to provide a better understanding of enzyme behavior during MLF.

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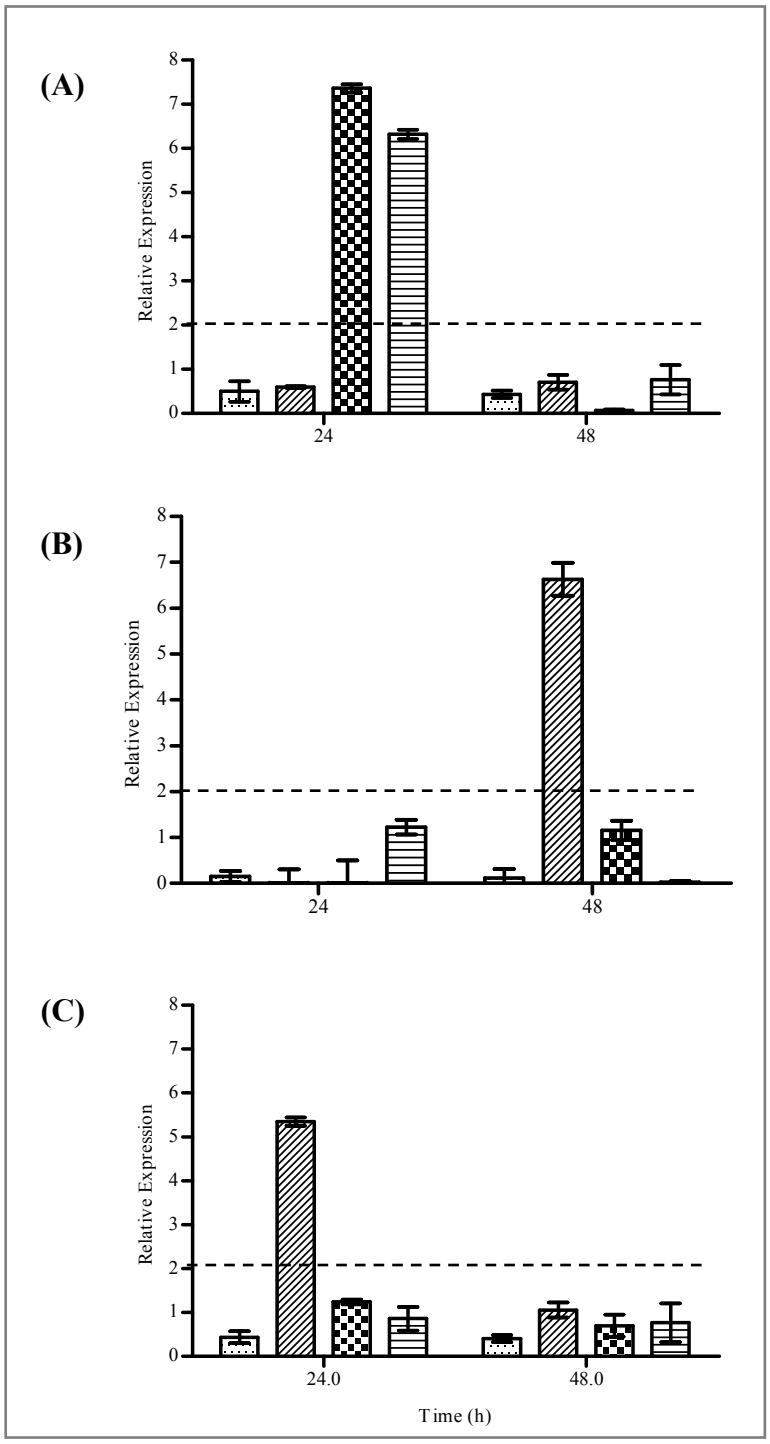

Figure 3. Relative expression (RE) of the *bgl* gene of *O. oeni* PSU-1 ( ), 217 ( ), Z42 ( ) and MO ( ) cultured in MRS (A), cFT80 (B) and Cabernet Sauvignon wine (C). The calibrator condition used was time zero (T=0h) at the moment of inoculation. Data are the mean values of duplicate assays.

Under the studied media, β-glucosidase activity was detected in all tested strains. However, the level of enzymatic activity was highly variable depending on the culture

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conditions. Under optimal growth conditions (MRS), higher values of enzymatic activity were observed than in wine-like cFT80 medium. Nevertheless, activities were lower in MRS than in red wine. These results suggest that β-glucosidase activity would not be related to cell growth or survival since populations grew, as expected, in MRS while in the other media barely grew or even decreased in viability. This would be in accordance with the described by Grimaldi *et al.* (2000) that reported similar β-glucosidase activity values for different growth phases in several *O. oeni* strains. Likewise, no evidence of a linkage between β-glucosidase activity and malolactic activity was found.

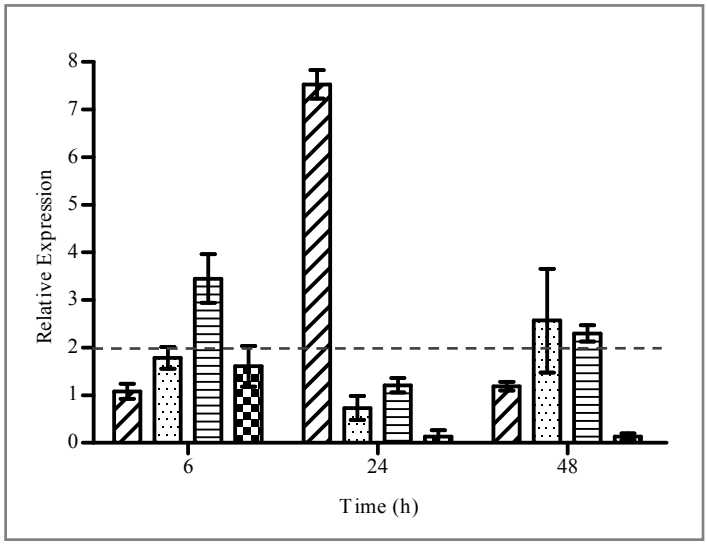

Figure 4. Relative expression (RE) levels of the *bgl* gene of *O. oeni* PSU-1 grown in different concentrations of Cabernet Sauvignon red wine: (▨) 75-M; (⬚) 50-MW; (▤) 75-W; (▩) 100-W. The calibrator condition used was MRS corresponding to each of the given times. Data are the mean values of duplicate assays.

The most relevant differences in β-glucosidase activity were observed the between the two media with similar ethanol content and pH, cFT80 and red wine. An enhancement or inhibition of β-glucosidase activity after the addition of ethanol, depending on the content, has previously been reported (Grimaldi *et al.,* 2000; Barbagallo *et al.,* 2004). Our results demonstrate that this variation does not only depend on the ethanol concentration but also on the medium's matrix. When measured in cFT80 12% ethanol, we found an inhibition in β-glucosidase activity. Meanwhile, this activity was enhanced in wine with a similar pH (3.5) and ethanol concentration to that of cFT80. Regarding β-glucosidase activity inhibition by pH, it has been suggested that some strains are more affected by the pH conditions of the medium than

others (Ugliano *et al.,* 2003). In the case of *O. oeni*, a loss of activity ranging from 57 to 88% of maximum value has also been observed within the pH range of 3.5 to 4.0 (Grimaldi *et al.,* 2000). However, the presence of ethanol at pH 3.5 would enhance β-glucosidase activity (Barbagallo *et al.,* 2004). This synergistic effect could account for the high β-glucosidase activity observed in red wine but not for the results in cFT80 medium. Therefore, other factors, related to wine composition, would be the activating agents. Among them, it should be considered the presence of β-glucosidase substrates in red wine but not in cFT80.

In order to understand the role of wine, besides ethanol and pH, in β-glucosidase activation, serial wine dilutions with MRS were assayed. The relative enzymatic expression of β-glucosidase showed a similar pattern in all wine dilutions, showing an initial increase at 6 hours, decrease after 24 hours of inoculation and again a final increase after 48 hours. The higher the proportion in wine the higher the β-glucosidase activity detected, being maximum in 100% wine. However, the increase observed did not maintain a linear correlation with wine content in the dilutions. Therefore, not only the presence of a determinate compound in wine could account for the β-glucosidase activation. In this sense, despite difficulties in comparing culture media, there is one parameter that may be associated with the highest increase of glucosidase activity in not diluted wine. It has been reported that glucose and especially fructose cause the inhibition of β-glucosidase activity in some *O. oeni* strains (Grimaldi *et al.,* 2000; 2005a). Inhibition could be observed at a concentration as low as 0.01% (Grimaldi *et al.,* 2005a). The presence of MRS, thus sugars, in wine dilutions could have masked partially the activation by wine compounds. This could also explain in part the lower activities detected in cFT80, which also contains glucose and fructose.

Regarding the strain behavior, the level of population development and enzymatic activity was similar within the four tested strains in all assayed conditions. According to a previous study, different *O. oeni* strains can display differing degrees of β-glucosidase activity, although some strains are found to have a similar pattern (Saguir *et al.,* 2009). Our results suggest that in the tested conditions, the four *O. oeni* strains can be considered in the same group based on their level of activity. However, it would be more appropriate to test this hypothesis by studying the activity of these strains over a longer period of time and with other wine varieties because strains can respond in different ways to the wine matrix (Gockowiak and Henschke, 2003).

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We observed noticeable differences between the biomass and the supernatant fractions in the distribution of β-glucosidase activity. Several authors have discussed glucosidase localization because activity occurs in whole-cell, intercellular and parietal fractions (Barbagallo *et al.,* 2004; Michlmayr *et al.,* 2010). Our results are consistent with previous work in which β-glucosidase activity was detected in both whole-cell and supernatant fractions and in which activity varied depending on the isolate and the phase of growth at which the sample was collected (Grimaldi *et al.,* 2000).

Our results showed no correlation between of the level of *bgl* expression and the observed enzyme activity. The pattern of expression was also different among the four strains tested, regardless of the culture condition. Surprisingly, the *bgl* gene was not affected in strain PSU-1 growing in MRS, cFT80 or wine. It was however over-expressed in samples from wine dilutions. Moreover, an over-expression of this gene was previously observed when this strain was grown in cFT80 10% ethanol (Olguín *et al.,* 2008). So, it is possible that the effect on *bgl* gene expression may be augmented by moderate ethanol content (3-10%). Analyzing a β-glucosidase gene from *L. plantarum*, Spano *et al.* (2005) found that 12% ethanol repressed the expression of this gene. Thus, it is possible that *bgl* gene expression is induced in certain growing conditions and it may also be strain dependent. Moreover, it is known that the β-glucosidase gene is widespread among wine lactic acid bacteria and that *O. oeni* possesses several different sequences, which code for potential β-glucosidases (YP_811088, YP_809979, YP_809980, YP_810767). Our study is the first to provide information about the RE level of one of these sequences. So it would be interesting to include all the sequences coding for potential β-glucosidases in future studies, and to isolate and characterize each one of these enzymes in order to improve our understanding of them. However, preliminary work in this direction shows that the gene studied in this work is the most expressed, among other *bgl* genes, in different *O. oeni* strains (data not shown). Altogether, it seems that in the presence of 12% ethanol other regulation mechanisms, at the post-transcriptional and/or post-traductional level, would be involved in β-glucosidase activity.

In summary, this study contributes to the knowledge of *O. oeni* β-glucosidase activities and *bgl* gene expression under different culture conditions. According to our results, some *O. oeni* strains are able to show a high β-glucosidase activity even at low survival rates in wine, which is an important consideration for the wine industry. To the best of our knowledge, this is the first time that the RNA of *O. oeni* has been extracted directly from red wine for rt-qPCR

analysis, offering interesting perspectives for further research. However, more work is still needed in order to better understand the relationship between β-glucosidase activity and gene expression.

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iv. Influence of ethanol shock on *Oenococcus oeni* cytoplasmic proteome.

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ABSTRACT

Bacterial development and malolactic fermentation are not always successful due to the harsh environmental conditions of wine. The survival of *Oenococcus oeni* commercial starter cultures requires insight into the mechanisms involved in the tolerance and adaptation to ethanol conditions. In this study, a proteomic approach was undertaken to evaluate the evolution of the cytoplasmic protein pattern of *O. oeni* PSU-1 strain after 12% ethanol shock using two-dimensional gel electrophoresis (2-DE). A total of 45 spots in the pH range 4-7 showed a significant volume variation after five hours of ethanol shock. Proteins related to cell wall components such as RmlB, MurC, MurD and GlmS, which are involved in murein biosynthesis, showed decreased expression after ethanol shock. This suggests that ethanol stress causes important changes in cell wall composition. Also, more than half of the identified proteins, all of which were down-expressed in the presence of ethanol, were related to protein biosynthesis and protein stability. Our results suggest that protein biosynthesis and protein stability, together with cell envelope biosynthesis are the main cellular mechanisms affected by ethanol toxicity.

INTRODUCTION

It is generally known that *Oenococcus oeni* is the most important of the lactic acid bacteria involved in wine malolactic fermentation (MLF). However, bacterial development and MLF are not always successful due to the harsh environmental conditions of wine (Davis *et al.,* 1985; Malherbe *et al.,* 2007). Several studies on the *O. oeni* response under such stress conditions as pH, temperature, sulfite concentration and ethanol content were reported (Versari *et al.,* 1999). However, ethanol seems to be one of the most limiting parameters for *O. oeni* survival in wine. Therefore, if control over MLF in the wine industry is to be improved, it is essential to understand the mechanisms involved in ethanol stress and tolerance in *O. oeni*.

Ethanol toxicity is generally attributed to the interaction of ethanol with membranes at the aqueous interface, resulting in perturbed membrane structure and function (Weber and Bont, 1996; Beney and Gervais, 2001). Studies with *O. oeni* have shown that exposing cells to ethanol increases the permeability of the cytoplasmic membrane, and enhances passive proton influx and the concomitant loss of intracellular material (Da Silveira *et al.,* 2002). The permeability of the membrane for protons dissipates the proton motive force and affects ATP synthesis, which is no longer available for growth (Capucho and San Romão, 1994; Salema *et al.,* 1996; Weber and Bont, 1996). This could explain the high mortality of *O. oeni* that was observed when cells were directly inoculated into a wine-like medium supplemented with 12-16% ethanol (Chu-Ky *et al.,* 2005; Silveira *et al.,* 2002).

Nonetheless, ethanol has no significant effect on malolactic activity in concentrations up to 12% but it does strongly inhibit cell growth (Capucho and San Romão, 1994). These authors suggest that the mechanisms regulating cell growth are more sensitive to ethanol than the malolactic enzyme itself. On the other hand, we have recently found that some citrate pathway genes of *O. oeni* are over expressed in the presence of ethanol, suggesting that the citrate metabolism takes part in the response to stress conditions such as the presence of ethanol (Olguín *et al*., 2009)..

Two-dimensional gel electrophoresis (2-DE) has provided invaluable information about the adaptative response of microorganisms to changes in external conditions (Champomier-Vergès *et al.,* 2002). For instance, Da Silveira and co-workers (2004) found that ethanol triggers alterations in the protein patterns of *O. oeni* cells that are directly stressed with 12% ethanol for 1 hour and cells pre-adapted in 8% ethanol. It was recently shown that acclimated cell cultures

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in a wine-like medium supplemented with 10% ethanol survived better in wine probably due to the differential expression of proteins (Cecconi *et al.,* 2009).

The aim of the present study was to evaluate the evolution of the cytoplasmic protein pattern of *O. oeni* PSU-1 after 12% ethanol shock using 2-DE. To separate the proteins we chose a p*I* range between 4 and 7 on the basis of the known distribution of proteins for other lactic acid bacteria (Champomier-Vergès *et al.,* 2002). In order to study the sole effect of ethanol on *O. oeni* cells, the assays were performed in MRS medium at pH 5.0 (De Man *et al.,* 1960).

MATERIALS AND METHODS

Growth conditions

O. oeni PSU-1 was cultured at 30ºC in 2 l flasks containing MRS broth medium supplemented with L-malic acid (4 g l^{-1}) and fructose (5 g l^{-1}) at pH 5.0. When cultures reached the late exponential phase ($OD_{600nm} \approx 1$) they were divided into two sterile flasks. Immediately, 12% (v/v) of ethanol was added to one flask and 12% (v/v) of water was added to the other (control). The latter was used as a control assay in order to evaluate the possible effect of culture dilution on the proteome. At this moment, the quantities of L-malic acid and fructose remaining in the medium were 0.03 g l^{-1} and 0.28 g l^{-1}, respectively. The pH of the medium was 4.35. Both flasks were incubated at 28ºC. All assays were performed in triplicate using independent cultures and the growth was monitored by counting plates in MRS medium (De Man *et al.,* 1960), supplemented as described above. Samples were taken at time zero just before water/ethanol was added and at one, three and five hours after addition.

L-malic acid and fructose contents were measured using Boehringer enzymatic kits (Mannheim) on culture supernatants stored at -20ºC until use. pH measurements were taken using a GLP31 pH-meter (Crison Instruments, Barcelona, Spain).

Protein extract preparation

Cells cultured in the presence or absence of ethanol were harvested by centrifugation, washed with 10 mM of Tris-HCl buffer (pH 8.0) and frozen at -80ºC until analysis. Pellets were then resuspended to a final OD_{600nm} $ml^{-1} \approx 60$ in 0.1 M of Tris-HCl buffer (pH 7.5) and cellular

extracts were obtained using a cell disrupter (BASIC Z; Constant Systems Ltd., Daventry, United Kingdom) at a pressure of 2.5 kbar. The suspension was first centrifuged at 4,500 x *g* for 15 min at 4ºC to remove unbroken cells and cellular debris. The supernatant was ultra-centrifuged at 50,000 x *g* for 30 min at 4ºC to remove cell envelope components. Protein concentration was estimated using the Bradford method according to the manufacturer's instructions (Coomassie Protein Assay Reagent; Pierce Biotechnology, Rockford, IL).

Sample preparation and protein electrophoresis

Protein samples and 2-DE were performed as described by Sánchez *et al.* (2005), with some modifications. Cytosolic extract was treated with 1 µl of Benzonase (Merck, KGaA) and 1 µl of 1M $MgCl_2$ to remove nucleic acids. The mixture was vortexed and centrifuged at 3,500 x *g* for 2 min at 4ºC. After the addition of four volumes of deionized water and vigorous vortexing samples were centrifuged at 3,500 x *g* for 10 min at 4ºC. The upper phase was removed and proteins were precipitated by adding 3 volumes of methanol and centrifuging at 3,500 x *g* for 4 min. The pellets were then resuspended in solubilization buffer.

The second dimension electrophoresis was run at 11 mA gel^{-1} for 15 h at 4ºC.

Image analysis

Detection and volume quantification of spots was made with Prodigy SameSpots software (Nonlinear Dynamics), including images of at least three gels of each time and condition. A sample taken at time zero (just before water/ethanol addition) was chosen as the reference. Protein expression was deemed to have changed if the mean normalized spot volume varied at least twofold and was confirmed by analysis of variance at a significant level of $P < 0.05$. Reproducibility was assessed by performing three independent experiments and sets of five gels were analyzed.

Identification of proteins by peptide mass fingerprinting

Individual spots were excised from gels and submitted to tryptic digestion, and mass spectrometry analyses were performed as previously described (Guillot *et al.,* 2003). The mass of the peptides was determined by MALDI-TOF MS on a Voyager DE STR instrument

(Applied Biosystems) at the PAPSSO platform of the INRA Center in Jouy-en-Josas. Identification of protein was performed against the *O. oeni* NC_008528 database.

RESULTS

Population development

Growth of *O. oeni* PSU-1 in MRS medium and the effect of ethanol over population development were monitored by counting plates (data not shown). In all conditions, cell populations of more than 10^7 CFU ml^{-1} were detected.

Cytoplasmic protein patterns

The protein profile of *O. oeni* PSU-1 at T0 was characterized to generate a standard grid, which was used for subsequent comparative studies of samples obtained after water/ethanol addition. A high-resolution map was obtained that revealed approximately 215 spots (Fig. 1).

The comparison of cytoplasmic profiles from 12% ethanol- or 12% water-treated cultures for each sampling time revealed quantitative and qualitative modifications of the spots patterns that could be recognized by simple visual comparison of the two conditions. Further analysis of the gels confirmed that ethanol- and water-treated populations had a different response. Two spots were down-expressed in the control condition (water addition). One of these spots could not be identified whereas the other one corresponds to the spot no.7 which was also down-expressed in ethanol-treated samples (Fig. 1). After ethanol treatment, one spot (spot 36) was up-regulated and 44 were down-regulated.

Among the 45 spots which expression varied when compared to the proteome reference gel (T0), 35, corresponding to 31 proteins, could be identified by mass spectrometry (MALDI TOF) and comparison to the genomic database of *O. oeni* NC_008528 (Fig. 1, Table 1). It is noteworthy that spot 36 was the only one detected that increased in response to ethanol shock (Fig. 2). This spot has a molecular mass of approximately 22 kDa and a p*I* of 4.5. Da Silveira *et al*. (2004) also found some similar spots harboring the same pattern, suggesting that these proteins are typical ethanol stress-induced.

As shown in table 1, the proteins identified are involved mainly in nucleotide transport and metabolism (22.86%); translation, ribosomal structure and biogenesis (17.14%); cell

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envelope biogenesis (14.26%); and posttranslational modification, protein turnover, and chaperone functions (11.43%). Seven spots are classified in five other functional categories and for the last five spots (14.29%) no function could be predicted.

Finally 3 proteins (PyrG, PyrE and Zwf) were each matching with 2 distinct spots (spots 1 and 2, 7 and 8, 26 and 27, respectively).

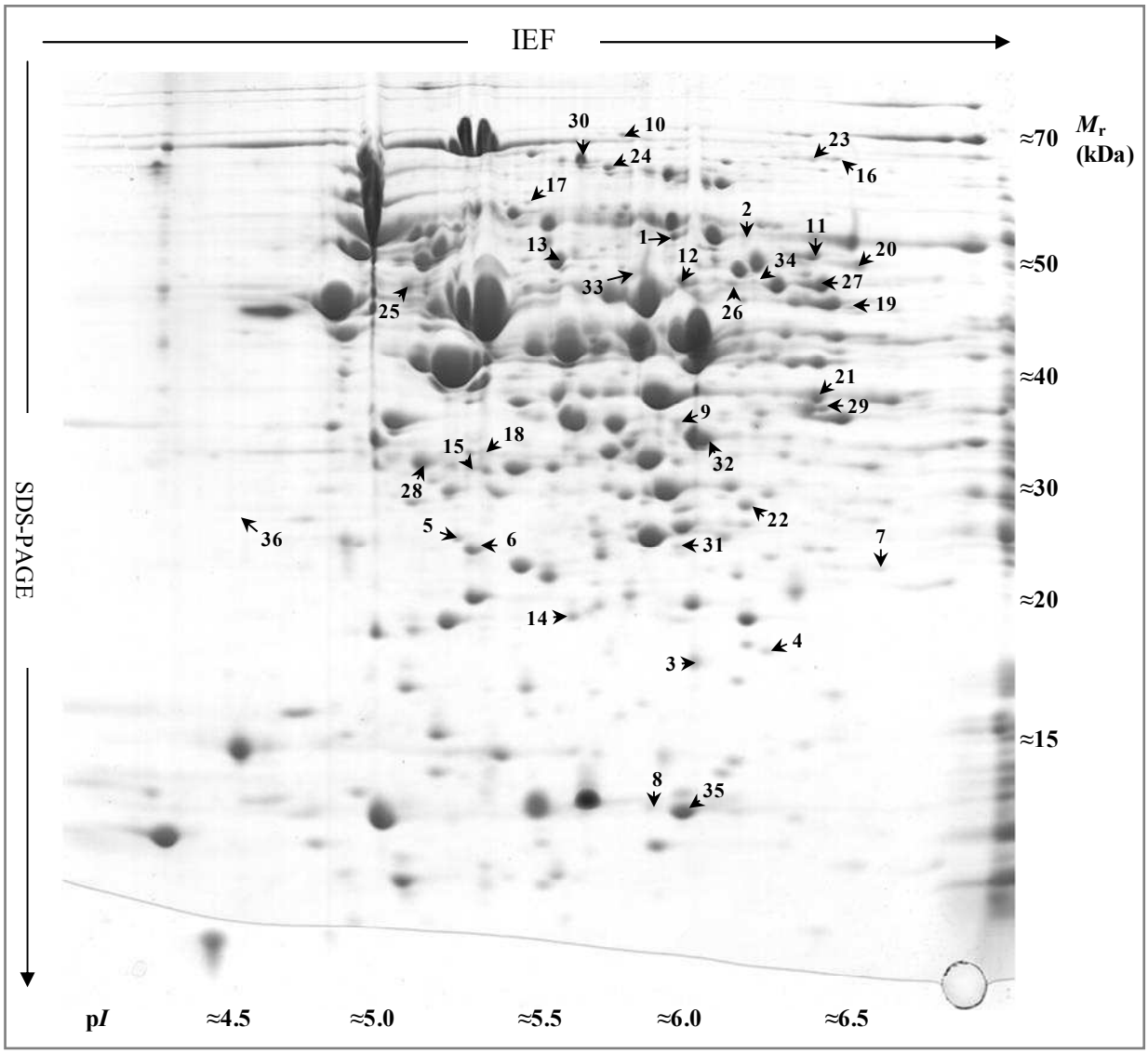

Figure 1. Reference map of cytoplasmic proteins extracted from *O. oeni* PSU-1 cells at the late-exponential phase of growth in MRS (t=0h) before water or ethanol addition. The 36 differentially expressed spots are indicated by spot number as reported Table 1. Molecular masses in kilodaltons (right side) and p*I* ranges (bottom) are indicated.

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Table 1. Identification of the differentially expressed proteins of *O. oeni* PSU-1 growing in the presence of ethanol.

Spot no[§].	Gene symbol	Gene name	Functional category Protein name (EC number)	Fold change*	Theoretical M_r (kDa)	Theoretical p*I*
			Nucleotide transport and metabolism			
1-2	OEOE_1786	*pyrG*	CTP synthase (UTP-ammonia lyase) (EC 6.3.4.2)	-5.6	60.11	5.52
3	OEOE_0138	*nrdL*	Ribonucleotide reduction protein	-5.4	18.05	5.52
4	OEOE_1069	*apt*	Adenine/guanine phosphoribosyltransferase or related PRPP-binding protein (EC	-3.6	19.25	5.7
5	OEOE_1124	*hpt*	2.4.2.7)	-2.7	20.93	4.99
6	OEOE_0437		Hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8)	-2.1	24.74	5.07
7-8	OEOE_0263	*pyrE*	Deoxynucleoside kinase (EC 2.7.1.113) Orotate phosphoribosyltransferase (EC 2.4.2.10)	-3	23.13	5.67
			Coenzyme metabolism			
9	OEOE_1036	*pdxS*	Pyridoxine biosynthesis enzyme, SOR/SNZ family	-3.1	31.44	5.58
			Translation, ribosomal structure and biogenesis			
10	OEOE_0982	*proS*	Prolyl-tRNA synthetase (EC 6.1.1.15)	-5.2	64.33	5.41
11	OEOE_0321	*glnS*	Glutamyl- and glutaminyl-tRNA synthetase (EC 6.1.1.17 – 6.1.1.24)	-2.7	57.05	5.94
12	OEOE_0440	*serS*	Seryl-tRNA synthetase (EC 6.1.1.11)	-2.4	49.86	5.68
13	OEOE_1694	*gatA*	Asp-tRNAAsn/Glu-tRNAGln aminotransferase A subunit (EC 6.3.5.6 – 6.3.5.7)	-2.4	52.5	5.31
14	OEOE_0806	*def*	N-formylmethionyl-tRNA deformylase (EC 3.5.1.88)	-2.3	21.08	5.27
15	OEOE_1699	*map*	Methionine aminopeptidase (EC 3.4.11.18)	-2.2	29.95	5.08
			Energy production and conversion			
16	OEOE_1248	*eutG*	Iron-binding alcohol dehydrogenase / aldehyde dehydrogenase family domain (EC1.1.1.1)	-3.8	99.1	6.04
			Cell envelope biogenesis			
17	OEOE_0635	*glmS*	Glucosamine 6-phosphate synthetase, amidotransferase and phosphosugar isomerase domains (EC 2.6.1.16)	-3.7	66.17	5.28
18	OEOE_0565	*galU*	UDP-glucose pyrophosphorylase (EC 2.7.7.9)	-2.9	32.56	5.37
19	OEOE_1269	*murC*	UDP-N-acetylmuramate-alanine ligase (EC 6.3.2.8)	-2.7	48.13	6.03
20	OEOE_1147	*murD*	UDP-N-acetylmuramoylalanine-D-glutamate ligase (EC 6.3.2.9)	-2.5	48.65	6.16
21	OEOE_1447	*rmlB*	dTDP-D-glucose 4,6-dehydratase (EC 4.2.1.46)	-2.1	37.46	5.8
			Posttranslational modification, protein turnover, chaperones			
22	OEOE_1114	*sufC*	Fe-S-cluster assembly ABC-type transport system, ATPase component	-2	28.22	5.71
23	OEOE_0514	*clpC*	ATP-binding subunit of Clp protease and DnaK/ DnaJ chaperones (subunit of DnaK/J)	-3.2	91.48	5.86
24	OEOE_0640	*clpE*	ATP-binding subunit of Clp protease and DnaK/ DnaJ chaperones (subunit clpE)	-2.5	81.29	5.37
25	OEOE_1309	*dnaK*	Molecular chaperone	-2.9	66.2	4.89
			Carbohydrate metabolism			
26-27	OEOE_0135	*zwf*	Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49)	-2.9	55.67	6.44
28	OEOE_1523	*gnd*	6-phosphogluconate dehydrogenase (EC 1.1.1.44)	-2.1	32.86	4.83
			Amino acid transport and metabolism			
29	OEOE_0845	*appF*	ABC-type oligopeptide transport system, ATPase component	-2.7	34.49	5.74
			Signal transduction mechanisms			
30	OEOE_0807	*typA*	Stress response membrane GTPase	-3.1	68.22	5.24
			General function prediction only			
31	OEOE_1270	*arc1*	EMAP domain	-2.3	22.47	6.1
32	OEOE_0070	*ara1*	Aldo/keto reductase related enzyme	-2.1	31.68	5.6
33	OEOE_1072	*obg*	Predicted GTPase	-3	48.2	5.47
34	OEOE_0531		Lactoglutathione lyase or related lyase	-2.5	13.46	4.71
35	OEOE_1705		Methylmalonyl-CoA epimerase (EC 5.1.99.1)	-2.5	16.22	5.62

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Table 1. Continued

	Not identified	
36		+6.0

[§]Several spot nos. for the same protein entry indicate that the protein was identified in several spots. *Maximum fold-change among four samples of each spot.

Protein expression kinetics during O. oeni growth

As mentioned above, image analysis showed only subtle changes of the protein kinetics in the control assay with water added. Therefore, all further analysis focused on gels obtained from ethanol-treated cultures. The fold-change value was derived from the mean normalized volumes of four groups of three gels. Each group corresponded to time 0 hours (reference gel), 1, 3 and 5 hours. When more than two groups were used, only the maximum fold-changes are shown (see comments Table 1 about which fold is indicated).

As mentioned above, 35 spots were down-regulated and one up-regulated after ethanol treatment. On the basis of their predicted function, proteins were classified into ten different functional categories (see Table 1). The kinetic changes were also analyzed and seven different kinetics were observed. Those are represented for some representative proteins in Fig. 2. Within a given functional category, several kinetics could be observed as for instance spots no 2 and no 7, both belonging to pyrimidine biosynthetic pathwa.

DISCUSSION

In this study we followed *O. oeni* cytoplasmic protein changes during the first five hours after 12% ethanol shock. We chose to compare the ethanol treated samples to a reference map obtained from untreated cells, collected at the late exponential growth phase. This map allowed us to distinguish 215 spots. In a similar study, the use of the same pH gradient allowed the resolution of only 100 spots of *O. oeni* (Da Silveira *et al*., 2004). Nevertheless, these authors could identify five differentially expressed proteins among which one (the phosphocarrier protein HPr) was significantly over-expressed after 12% ethanol shock. Another protein, identified as the glutathione reductase (GR), was also up-regulated after a 12% ethanol shock but not in 8% ethanol pre-adapted cells. Using a larger gradient pH 3-10 Cecconi *et al*. (2009) showed approximately 400 spots. These authors also found that GR was up-regulated in 5% ethanol acclimated cells suggesting that maintenance of the redox balance plays an important

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role in alcohol adaptation. None of these two proteins were found to be up-regulated in the present study.

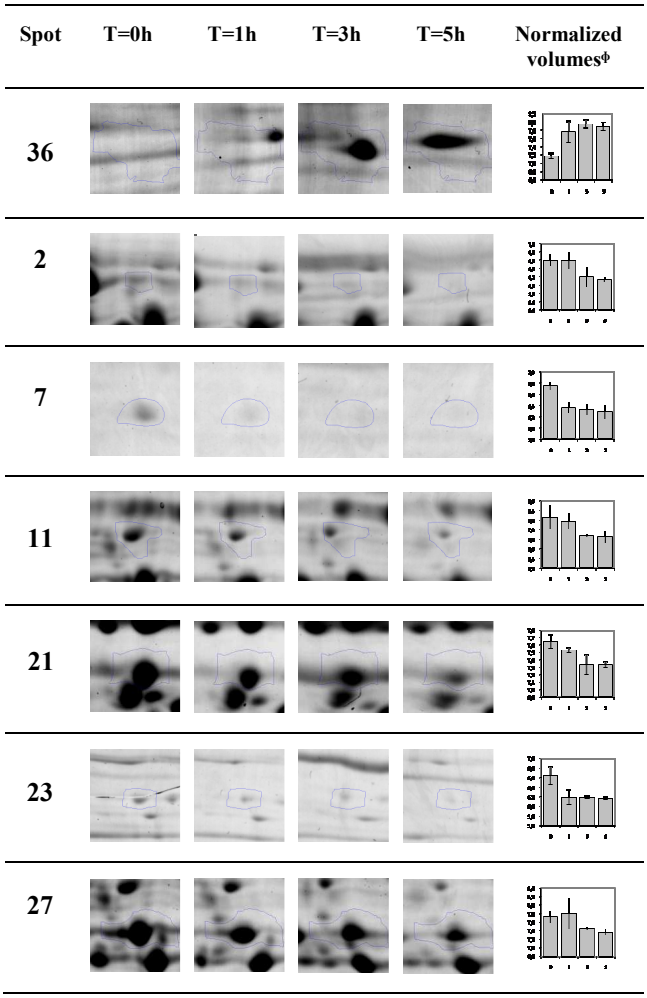

Figure 2. Kinetics of expression of some spots of representative proteins at time 0, 1, 3, and 5 h after ethanol shock. Diagrams show the logarithm of normalized volumes for each represented spot ($P < 0.05$).

In addition, Da Silveira and co-workers (2004) found that one spot, identified as the dTDP-D-glucose 4,6-dehydratase (RmlB) involved in cell wall lipopolysaccharide biosynthesis, was present in the membrane-associated protein extract of cells pre-adapted in 8% ethanol but was not detected in the control condition or after 1 hour of 12% ethanol shock. Our results show that RmlB was present in all the samples of cytoplasmic fractions although it decreased over time in the presence of ethanol. Interestingly, we have observed an initial down-regulation of *rmlB* gene expression in the presence of 12% ethanol (manuscript in preparation) and an

increase in *rmlB* when cells were grown in cFT80 supplemented with 10% ethanol (Olguín *et al.,* 2008). This may suggest that *rmlB* is initially down regulated after ethanol shock, and thus the RmlB protein level decreases, but is subsequently over-expressed when cells are adapted, so it may be an indicator of cell acclimation. We have also observed that, depending on the *O. oeni* strain used, there is a correlation between high levels of *rmlB* transcripts and a better malolactic performance (manuscript in preparation). In addition, we show in the present study that proteins such as MurC, MurD and GlmS, which are involved in murein biosynthesis, are down-regulated after a 12% ethanol shock (Table 1). Altogether, these results suggest that ethanol stress causes important changes in cell wall composition. In this regard, it has been reported that resistance to stress depends on genes involved in peptidoglycan and teichoic acid biosynthesis (Delcour *et al*., 1999). Nonetheless, there is little biochemical or genetic data available on the biosynthesis pathways of the cell wall constituents in lactic acid bacteria. Further study is required in this area.

We found only one spot that was up-regulated after ethanol treatment, and that most were down-regulated, as has been reported (Da Silveira *et al*., 2004). However, the number of up- or down-regulated proteins seems not to be a conclusive proof of cellular viability. For instance, Zapparoli (2004) assessed total protein analysis for differentially aged *O. oeni* cultures and found that old cultures were more resistant to starvation conditions than younger ones. Surprisingly, the older cultures presented three-times more down-regulated (43) than up-regulated proteins (15).

Considerable debate has been generated about whether or not the adaptation of *O. oeni* cells to ethanol conditions is related to *de novo* synthesis of proteins such as the stress protein Lo18 and changes in the membrane lipid composition (Guzzo *et al*., 1997; Tourdot-Maréchal *et al*., 2000; Grandvalet *et al*., 2008). Our results suggest that protein synthesis or stability is diminished when cells are directly submitted to 12% ethanol. As shown in Table 1, more than half of the down-regulated proteins (53.13%) are related to protein biosynthesis and protein stability. These proteins are associated to *i*) nucleotide transport and metabolism (18.75%); *ii*) translation, ribosomal structure and biogenesis (18.75%); *iii*) posttranslational modification, protein turnover and chaperones (12.50%); and *iv*) amino acid transport and metabolism (3.12%). PdxS, a biosynthetic enzyme of pyridoxine (vitamin B_6) known to be involved as pyridoxal-5'-P in numerous reactions of the amino acid metabolism, has also been observed to be down-regulated (John, 1995).

Two proteins that are also down-expressed in the presence of ethanol are related to the carbohydrate metabolism (Zwf and Gnd). They are key enzymes in the oxidative branch of the phosphoketolase pathway, the main one of the obligate heterofermentative LAB such as *O. oeni*, and the second of these enzymes generate reduced nicotinamide nucleotides. They are also related to the aforementioned group of nucleotide-transport and metabolism proteins, which Da Silveira *et al*. (2004) found to decrease in the presence of ethanol.

According to our results, protein stability would be compromised during the early response to ethanol stress. The molecular chaperone DnaK, a stress-induced in other lactic acid bacteria (Kilstrup *et al.,* 1997; Lim *et al*., 2000), is down-regulated after ethanol shock (Table 1). Likewise, other chaperone proteins identified in this study such as ClpC and ClpE, which are associated with acid adaptation (Budin-Verneuil *et al*., 2005), are down-regulated in the presence of ethanol, thus suggesting a reduced stress response and a negative impact on cell survival.

The decrease in protein expression can be explained by the increase in membrane fluidity after ethanol shocks (Da Silveira *et al*., 2002, 2003; Chu-Ky *et al*., 2005). It has been shown that the fluidity of the cytoplasmic membrane in *O. oeni* cells increases when ethanol is added in a concentration-dependent manner. A protective response, then, should occur immediately. This is also in agreement with our findings since 14.26% of the down-regulated proteins are related to cell envelope biogenesis. As has been discussed previously, proteins or other compounds that are already in the cytoplasm are more likely to be called upon to stabilize the cytoplasmic membrane (Da Silveira *et al*., 2003). This hypothesis may also explain the appearance of a colony polymorphism after plating cultures submitted to ethanol, that we observed (data not shown) and which was previously reported by Zapparoli (2004). Da Silveira *et al*. (2002) have also found that ethanol induced population heterogeneity in which some cells developed a mechanism that partially increased the efficiency of their cytoplasmic membrane as a barrier.

Nonetheless, this heterogeneity does not seem to be related to spontaneous mutations (Zapparoli, 2004) which confirms the hypothesis that ethanol adaptation and survival are associated to a rapid adjustment of membrane permeability independent of the novo protein synthesis (Da Silveira *et al*., 2003). Our results suggest that protein biosynthesis and protein stability, together with cell envelope biosynthesis are the main cellular mechanisms affected by ethanol toxicity. Therefore, the formulation of nutrient additives for enhancing MLF should

include not only amino acids and peptides, but also nucleotides, vitamins such as pyridoxine, and other cell wall components.

It is also worth pointing out that, during our experiment, cells were ethanol-shocked when no L-malic acid remained in the medium. So there was no metabolic possibility of compensating for the effect of ethanol. Malate consumption could have yielded ATP (Cox and Henick-Kling, 1989), thus leading to a better maintaining of the proton motive force and leading to a better cell survival. We had previously observed that the molar ratios of consumed L-malic acid and citrate were higher for 10% ethanol growing cultures than for cells growing in the absence of ethanol (Olguín *et al.,* 2009).

Until now, *O. oeni* population survival in the presence of high ethanol concentrations was achieved by adapting them to sub-lethal stress conditions (Drici-Cachon *et al.,* 1996; Tourdot-Maréchal *et al.,* 2000). Adaptation in the presence of 8% ethanol decreases the fluidity of the membrane by changing its composition (Da Silveira *et al.,* 2002, 2003; Grandvalet *et al.,* 2008). However, the extent of this adaptation is strain dependent and varies also depending on the pH of the medium and the growth phase it is performed (Garbay *et al.,* 1995; Drici-Cachon *et al.,* 1996). However, the acquisition of ethanol resistance in *O. oeni* is a complex phenomenon that seems to depend on the nature and duration of the shock and culture conditions (Teixeira *et al.,* 2002). For instance, Cecconi *et al*. (2009) found that ethanol-acclimated *O. oeni* cells induced proteins involved in amino acid and sugar metabolism. On the other hand, in our experimental conditions (without prior ethanol adaptation) we found the opposite behavior for this group of proteins (Table 1).

Finally, we observed different classes of kinetics of the expression level of proteins, suggesting a complex response to ethanol during the first 5 hours of exposure (Fig. 2). Indeed, one protein (spot no 36) increased after one hour and its amount remained stable, whereas other spots decreased after one hour and remained stable (spots no 7 or 23), or decreased only after 3 hours (spots no 2, 11, 27), or showed a gradual decrease depending on time (spots no 11 or 21). However we could not assess a clear-cut link between functional categories and the type of kinetics.

In conclusion, this study shows that exposure of *O. oeni* to 12% ethanol leads to modification of its cytoplasmic proteome. Most proteins showed a significantly decreased fold-change, which indicates the cellular mechanisms that are most affected by ethanol. This information may be useful for the design of starter culture pre-adaptation procedures or

malolactic nutrient formulation. Overall studies that include cytoplasmic- and membrane-associated proteins are necessary if the physiological development of *O. oeni* under different environmental conditions is to be understood.

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IV. DISCUSSION AND PERSPECTIVES

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DISCUSSION AND PERSPECTIVES

In addition to the obvious, such as the grape variety and quality, yeast and lactic acid bacteria play a key role in the organoleptic characteristics of wine. Yeasts have caught the attention of researchers since Pasteur's times and their activity conceived responsible for the main and most complex changes in the characteristics wine. Malolactic fermentation (MLF) has instead been considered for many years as a simple process, mostly characterized by the decarboxylation of L-malic acid. However, recent studies involving new analytical methods have demonstrated the occurrence of much more complex modifications during MLF (Maicas *et al.,* 1999; Ruiz *et al.,* 2010).

In a first instance, we were interested in the citrate metabolism by *O. oeni*. Although enzymatic activities of this pathway had been studied, little was known about the molecular mechanisms involved. Likewise, the potential application of such data could be used for the selection of *O. oeni* strains for their use as starter cultures. Currently, such strains are mainly selected due to their MLF performance in terms of L-malic acid degradation but citrate utilization and secondary metabolites production is still a field to be exploited. Moreover, metabolic activities can also be different depending on wine conditions. Higher pH and ethanol content like those expected in coming years due to the climate change may also affect *O. oeni* metabolism. Thus, a better understanding of the metabolic behavior under different conditions may be useful for strain selection, especially when these metabolic traits are also related to cell adaptation and stress response.

Through our first experiment, we were able to determine the metabolic activity of citrate consumption in absence and presence of ethanol. Despite a lower population development in presence of ethanol, the pathway of citrate consumption seemed to be activated in *O. oeni* PSU-1. This observation is not surprising since citrate, as well as malate metabolism, have been associated to a possible acidic stress response (Tourdot-Maréchal *et al.,* 1999). From an organoleptic point of view, we found that higher levels of acetate were produced in presence of ethanol. Interestingly, the yields of acetate produced from citrate and sugar metabolism were higher in presence of ethanol even when sugar consumption was higher in absence of ethanol. These results suggest that under the stress generated by the presence of ethanol, *O. oeni* may

respond by increasing the metabolic flux from citrate to acetate and consequently, higher production of ATP.

From gene expression analysis under different pHs and ethanol concentrations, we could determine that ethanol produced a strong transcriptional response. This is also in agreement with the above suggestion that citrate utilization seems to be activated under ethanolic conditions. Our hypothesis is that in presence of ethanol, the expected membrane fluidization and consequent intracellular acidification may require the activation of a homeostatic mechanism. In this case, citrate metabolism is enhanced in order to increase proton consumption and ATP generation. The expression of *citE*, *ackA* and *alsD* genes was also correlated to the different content of end products such as acetic acid and diacetyl under the studied conditions. This correlation could be useful as a strain selection marker according to citrate metabolism. However, the evaluation of one strain in one test condition is not sufficient to ensure this behavior. Thus, we decided to evaluate these genes using other strains with different MLF performances as well as more restrictive growth conditions. We have also evaluated the expression of some genes already known as stress responsive.

We evaluated four *O. oeni* strains showing different behavior in terms of malolactic performance using a higher ethanol concentration than in our previous experiment. Again, we observed that the initial over-expression of *citE* gene could be correlated with higher citric acid consumption and acetic acid production. In these conditions strain PSU-1 consumed less citrate than the other strains. Therefore, citrate consumption seems to be conditioned by both strain and growth conditions. On the other hand, the relative expression level of *ackA* gene and AckA activity were independent of acetic acid production. Thus, it seems that the relative expression of *citE* can be correlated with citrate consumption and acetic acid production as observed for strains 217T and MO, independently of AckA activity. Moreover, a correlation between *mleA* gene expression and faster MLF was also observed. These results indicate that both *citE* and *mleA* genes could be used as markers for strain characterization according to their metabolic behavior.

Regarding to stress related genes, a clear relationship could be established between faster MLF strains (Z42 and MO) and slow MLF strains (PSU-1 and 217T). Faster MLF strains grew better than slow MLF strains indicating that cell survival is an important factor for the speed of MLF as previously reported (Coucheney *et al.,* 2005). A higher transcription of *hsp18*, *clpP*, *ctsR*, *trxA*, *cfa* and *rmlB* was observed in Z42 and MO at the middle of MLF. Together with

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mleA, *hsp18* expression has been used as a parameter to evaluate the state of adaptation of different strains (Coucheney *et al.,* 2005). Thus, our results show the same transcriptional behavior and contribute by adding other five potential markers (*clpP*, *ctsR*, *trxA*, *cfa* and *rmlB*).

As pointed out somewhere else (Sanders *et al.,* 1999), stress-induced proteins are molecular markers for the fitness of starter cultures and could be used as positive indicators for a culture that is fully adapted to resist an upcoming stress condition. A recent genomic approach based on 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. Nevertheless, more research is needed to further understand both metabolic and stress related mechanisms. Likewise, it has been possible to obtain DNA of samples taken directly from wine thus, allowing the evaluation of the presence of some of these markers in active strains. However, there is no current information about the evaluation of gene expression in such conditions. Therefore, our next objective was to evaluate both the RNA extraction technique and the level of gene expression in strains inoculated in red wine.

Likewise, we thought that would be useful to evaluate the enzyme activity of the target gene. For this, it was first necessary to find a suitable protocol for the enzymatic analysis and RNA extraction without the interference of phenolic compounds and other wine related macromolecules. We also decided to include another feature of *O. oeni* metabolism related with the organoleptic characteristics of wine such as β-glucosidase activity. The main interest in this third study was to evaluate β-glucosidase gene expression since it was not made before in *O. oeni,* whereas it has been reported that the over-expression of *BGL1* in *Saccharomyces cerevisiae* does indeed influence the aroma profile of wine (Van Rensburg *et al.,* 2005). Thus, *bgl* gene may also be used as a marker for *O. oeni* strain selection if its expression is correlated with the enzyme activity.

Our results showed that both β-glucosidase activity and *bgl* gene expression can be measured from samples taken directly from wine. However, we were not able to establish a clear relationship between enzyme activity and gene expression. It was possible nonetheless to compare the enzyme activity of four strains in different growing conditions. According to previous data, β-glucosidase activity was highly variable showing an enhancement or an inhibition depending on the presence of ethanol and fructose. On the other hand, we observed

the highest β-glucosidase activity in red wine despite a lower activity in other culture media (wine-like medium, MRS-broth and MRS-wine dilutions). Thus, it is possible that other factors as part of the complex wine matrix may be stimulating β-glucosidase activity. In the same way, *bgl* gene expression seems to be strain dependent and its induction also dependent of growing conditions.

It is also worthy of notice that we measured the total enzyme activity as cell associated and supernatant fractions. It may be also interesting to evaluate the extract fraction in further investigations since it was previously suggested that the enzyme in whole cells may be partially immobilized (Barbagallo *et al.,* 2004). Moreover, the observed enzyme activity may be the sum of several β-glucosidases. It is known that there is more than one sequence coding for this enzyme in *O. oeni* genome. Thus, it would be interesting to include all these sequences when comparing enzyme activity and gene expression.

It is also of general concern that starter cultures may be able to modify tannins and anthocyanins of wine. However, previous study has reported that selected strains of *O. oeni* presenting p-NP-β-Glucopyranoside, p-NP-α-Glucopyranoside and p-NP-β- xylopyranoside activities did not present tannase or anthocyanase activities (Ruiz *et al.,* 2010). Moreover, when considering glucosidase activity, a wide range of activity has been demonstrated in different *O. oeni* strains. It should be considered that a high glucosidase activity may increase the concentration of some desirable end products until an undesirable level. So, it is important to continue evaluating the potential activity of *O. oeni* strains as well as it would be of great interest to find a molecular marker related to this behavior.

For the final and perhaps more ambitious work of this thesis, we aimed to address our investigation into the 'omics' era. Not only to have a glance into the global analysis technology but also to contribute with one of the most investigated subjects and yet still challenging: the 'ethanol response'. Mimicking wine conditions or using real wine during *O. oeni* culture, ethanol concentration seems to be always the most limiting factor for cell development. In general, an enhancement of population survival and development was observed after cell acclimation or pre-exposure to stress condition. However, and despite the extensive work done so far, little is known about the molecular mechanisms of *O. oeni* response to high ethanol concentrations. A better comprehension of these mechanisms will allow us to understand why the starter cultures are not always successful, and perhaps even to improve this step in winemaking.

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The first consideration in mind is that in its natural environment, *O. oeni* can naturally adapt to the gradual increase in ethanol concentration. However, when using starter cultures, cells have no time for a gradual adaptation but they have to survive a direct shock. These first few hours after shock could be critical for cell survival. Therefore, we decided to evaluate *O. oeni* response during this period, especially considering ethanol as the main factor for cell growth inhibition. Thus, in order to study the sole effect of ethanol without the interference of other parameters such as low pH and lack of nutrients, the assays were performed in MRS medium at pH 5.0.

The obtained results allow us to have an idea about the main mechanisms affected by ethanol toxicity. But mainly, our intention was to study the global changes occurring in the protein expression by using one of the available new techniques such as 2-dimentional gel electrophoresis. These data should be considered for further analysis using other *O. oeni* strains and different conditions. It should be also complemented by other studies using other global analysis techniques such as transcriptomics. In fact, we have continued our research by including this analysis technique. We are currently analyzing the new data hoping to correlate these new results to those obtained so far. This would allow us to understand if the protein level found in our work is determined at the transcriptional level or if other factors are involved in protein diminution. Likewise, these studies will contribute to data generation which is a prerequisite for successful system biology and networks reconstruction and integration (De Keersmaecker *et al.,* 2006), field that has been barely started in prokaryotes.

On the other hand, this study was thought to understand *O. oeni* response to ethanol shock as it may occur for starter cultures. However, it would be also interesting to evaluate cell response in real conditions. It is known that several strains of *O. oeni* are present in the grape surface and at the beginning of alcoholic fermentation. Then, a gradual selection seems to occur until one or two *O. oeni* strains are the main responsible for MLF. But what is the main factor involved in this selection? Is it the inoculated yeast strain, the interaction among *O. oeni* strains, the presence of genetic polymorphisms? Does this selection depend on the global environment conditions? And how does the cell respond during this selection? It is obvious that much more information is needed.

In summary, this work has been focused in *O. oeni* molecular response to wine related conditions. We have considered both the metabolic behavior and the adaptation or stress-responsive mechanisms. The relative expression level of some of the studied genes could be

considered in further studies as markers for cell survival and the content of the metabolic end products affecting the organoleptic characteristics of wine. We have made a first attempt to correlate the results obtained in wine-like medium to those obtained in red wine. Even though our results are not conclusive, these techniques proved to be useful for future studies. In addition, the proteomic approach undertaken showed that the main affected cytoplasmic proteins after ethanol shock correspond to those related to protein biosynthesis and stability as well as cell envelope biosynthesis. The next step in already underway and is evaluating the results obtained from a transcriptomic approach, and to compare these results with those obtained from the proteomic approach. Likewise, systems microbiology is a challenging research domain that still needs for computational platforms in order to generate comprehensive microbiological insights that could be used for industrial applications.

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V. GENERAL CONCLUSIONS

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GENERAL CONCLUSIONS

The general conclusions obtained from this PhD Thesis are:

✓ **Ethanol influence both gene expression and secondary metabolite production from the citrate pathway**

In the presence of ethanol, metabolic and transcriptional behavior of *O. oeni* strain PSU-1 was different than the observed when ethanol was absent. Ethanol produces a strong transcriptional response of *cit* genes, especially during the first stages of MLF whereas pH has a lower effect. The expression of *citE*, *ackA* and *alsD* genes can be correlated with citrate consumption and the final content of acetic acid and diacetyl. The increment of gene expression observed in the presence of ethanol at low pH suggests the participation of citrate metabolism in response to stress conditions.

✓ **Multigenic expression analysis can be used as a tool for strain characterization in accordance with their metabolic behavior.**

A higher initial expression of both the malolactic enzyme and the encoding gene *mleA* could be related to a faster MLF. Citrate lyase gene (*citE*) initial transcriptional levels resulted to be an indicative of early citrate consumption. Moreover, strains showing a better performance under wine-like conditions, presented much higher transcriptional levels of several stress responsive genes, highlighting the role of *hsp18*, *clpP*, *ctsR* and *rmlB*.

✓ **A higher β-glucosidase activity is detected from samples taken directly from wine in comparison with the detected activity in model conditions. Therefore, wine matrix has an important influence beyond ethanol content and pH.**

It is possible to evaluate both, β-glucosidase activity and *bgl* gene expression from samples taken directly from red wine.

✓ **Protein biosynthesis and protein stability, together with cell envelope biosynthesis are the main cellular mechanisms affected by ethanol toxicity**

Ethanol shock causes a significant variation in the content of cytoplasmic proteins. Protein synthesis is diminished when cells are directly submitted to 12% ethanol. The main affected proteins are associated to: *i*) nucleotide transport and metabolism; *ii*) translation, ribosomal structure and biogenesis; *ii*) posttranslational modification, protein turnover and chaperones; and *iv*) amino acid transport and metabolism.

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VI. ANNEXES

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Poster presentations

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Nair Olguín, Albert Bordons, Cristina Reguant. Estudio transcripcional sobre el metabolismo del citrato en *Oenococcus oeni* durante la fermentación maloláctica. XXI Congreso Nacional de Microbiología, Sevilla 2007. Poster.

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Estudio transcripcional sobre el metabolismo del citrato en *Oenococcus oeni* durante la fermentación maloláctica

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Introducción

O. oeni es la principal bacteria encargada de la fermentación maloláctica (FML), la cual es deseada en muchos vinos debido al aporte en las características organolépticas. Si bien *O. oeni* puede desarrollarse y llevar a cabo la FML de forma espontánea, un mejor control de la misma se obtiene mediante el uso de cultivos estáters. No obstante, la inducción de la FML sigue siendo difícil debido a la perdida de viabilidad celular causada por las características estresantes del vino (Marcas y col., 2000). Estudios basados en biología molecular pueden llevar a nuevas técnicas de selección de cepas donde no sólo podrá tenerse en cuenta la respuesta a estrés, sino también el tipo de metabolitos producidos durante la FML.

Objetivo

Puesta a punto y aplicación de las técnicas moleculares basadas en PCR cuantitativa a tiempo real (Real Time PCR) para estudiar el cambio en la expresión de los genes relacionados al metabolismo del citrato en *O. oeni* durante la FML y en condiciones de estrés acídico.

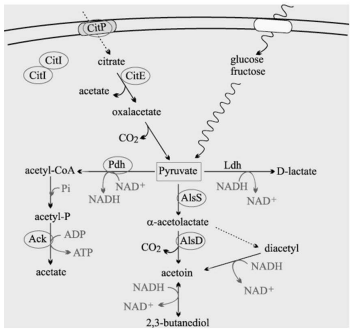

Figura 1. Rutas principales del metabolismo del citrato y el piruvato en *O. oeni*. Los genes investigados en este estudio corresponden a: CitI – activador transcripcional; CitP – citrato permeasa; CitE – citrato liasa; Pdh – piruvato decarboxilasa; AckA – acetato quinasa; Ldh – lactato deshydrogenasa; AlsS – α-acetolactato sintetasa; AlsD - α-acetolactato decarboxilasa. Las líneas discontinuas hacia el diacetil denotan un camino no enzimático.

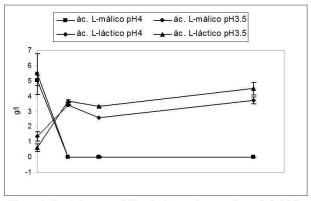

Figura 3. Seguimiento metabólico de *O. oeni* durante y luego de la FML en el medio de cultivo mFT80.

Materiales y Métodos

Para determinar los patrones de expresión, se diseñaron cebadores correspondientes a los genes *citP* (transportador de citrato) (Ramos y col., 1994), *citE* (subunidad β de citrato liasa) (Hugenholtz, 1993), *citI* (regualdor de citrato liasa) (Martin y col., 2005), *pdh* (piruvato deshidrogenasa) (Wagner y col., 2005), *ackA* (acetato kinasa) (Wagner y col., 2005), *alsS* (α-acetolactato sintasa) (Garmyn y col., 1996), *alsD* (α-acetolactato deshidrogenasa) (Garmyn y col., 1996) (Figura 1). El gen *ldhD* (lactato deshidrogenasa), se utilizó como control interno de la transcripción. El método crítico del umbral comparativo ($2^{\Delta\Delta Ct}$) fue aplicado para cuantificar el nivel de expresión de los genes (Beltramo y col., 2006). Las muestras a analizar se tomaron al inicio (T0), durante (T1), al final (T2) y 5 días luego de finalizada la FML (T3). T0 fue definido como condición calibradora para comparar las diferencias de a cuerdo al primer nivel de expresión. El ARNm total se extrajo a partir de células cultivadas en vino sintético pH4 (control) y pH3,5.

Resultados y Discusión

En las condiciones de estudio ensayadas, los datos obtenidos sugieren que el pH afecta la expresión de algunos de los genes correspondientes a la ruta metabólica del citrato. Estas diferencias podrían estar en algunos casos, vinculadas a la fase de crecimiento.

- Al inicio de la FML (T0-T1) hay un aumento en la expresión de *citE* sólo a pH3.5 (Figura 2a), la cual disminuye durante T1-T2 para mantenerse hacia el final de la FML. *citI* no presentó diferencias de expresión en ninguno de los periodos estudiados. *citP* fue sobre-expresado durante T2-T3, especialmente a pH4.

- El gen *ackA* presentó una sobre-expresión general durante todas las fases de crecimiento, aunque más marcadas durante T0-T1 y T2-T3. Este último aumento podría estar vinculado con la sobre-expresión de *pdh* también a T2-T3, ya que los dos genes están relacionados en la producción de ATP y balance redox (Wagner y col., 2005) (Figura 1). Ambos genes han tenido una mayor respuesta a pH3.5.

-El gen *alsD* fue sobre-expresado durante toda la fase de crecimiento, aunque mucho mayor durante T2-T3. El aumento en los niveles de expresión de *alsD* durante la última fase de crecimiento podría estar vinculado al aumento en los niveles de transcripto de *alsS* a pH3.5 (Figura 2e), ya que ambos están metabólicamente vinculados (Figura 1). Los dos genes mostraron una mayor respuesta a pH3.5.

El seguimiento metabólico realizado a través del consumo del ácido L-málico no denota diferencias en cuanto al cambio de pH (Figura 3).

Conclusiones

Las diferencias de expresión observadas sugieren que algunos de los genes vinculados al metabolismo del citrato podrían estar respondiendo tanto al cambio de pH como a la fase de crecimiento. En este sentido, vale la pena remarcar la sobre-expresión de los genes vinculados indirectamente a la formación de diacetilo (*alsS* y *alsD*) y directamente a la formación de ácido acético (*pdh* y *ackA*), los que finalmente, tendrán mayor repercusión en las características organolépticas del vino.

Los resultados obtenidos en este estudio son un primer paso para futuros análisis donde se incluyan condiciones más similares a las del vino así como también, un seguimiento más detallado de la evolución de los metabolitos intermediarios en la ruta del citrato durante la FML. En conjunto, el análisis transcripcional podría aplicarse como criterio adicional en la selección de cepas de *O. oeni* como cultivos iniciadores.

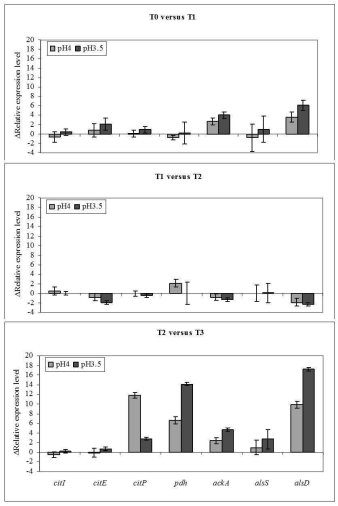

Figura 2. Diferencias en los niveles de expresión relativa de siete genes de *O. oeni* entre dos condiciones (pH4 y pH3.5) y a diferente fase de crecimiento.

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Expression of *Oenococcus oeni* genes related to cellular adaptation and metabolic development in wine conditions

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Introduction

It is known that *Oenococcus oeni* is the main responsible for malolactic fermentation (MLF) in wine. MLF is desirable in most red wines due to the enrichment of organoleptic characteristics. Even though MLF occurs spontaneously in wines, it starts randomly and any delay can lead to an alteration of wine quality. In order to have a better control over this process, winemakers can resort to the use of commercially available strains of *O. oeni.* However, induction of MLF still remains difficult and it is not always successful. To overcome this problem, it is necessary to understand the possible effects of wine conditions over the adaptation process and metabolic development of the population.

Aim

To evaluate the difference in the relative expression level (REL) of several genes of *O. oeni* that might be implicated in both wine adaptation and metabolic development.

Materials and Methods

MLF was initiated by direct inoculation of *O. oeni* PSU-1 grown at pH 4.0 in cFT80 medium, based on Cavin et al. (1989) modified by the addition of 0.5 g l^{-1} of citrate and meat extract (5 g l^{-1}) instead of casamino acids. Cells were collected at the end of the exponential phase ($OD_{600\,nm}$ = 1) and inoculated to a final concentration of 10^7 CFU ml^{-1} in cFT80 medium at pH 3.5 without ethanol (referred as pH3.5) and pH 3.5 with 10% (vol/vol) ethanol (referred as pH3.50H) . For all assays total RNA was extracted from cells of three samples during MLF: at the time of inoculation (T0); one day after inoculation (T1); and once MLF is finished (T2). MLF was monitored by measuring L-malic acid, citric acid, acetic acid and OD_{600nm}. The REL was calculated by monitoring mRNA with Real Time RT-qPCR during MLF and amplifications were carried out with specific primers (Table 1). Results were analyzed using the comparative critical threshold ($\Delta\Delta C_T$). T0 was used as the calibrator condition and *ldhD* gene as internal control.

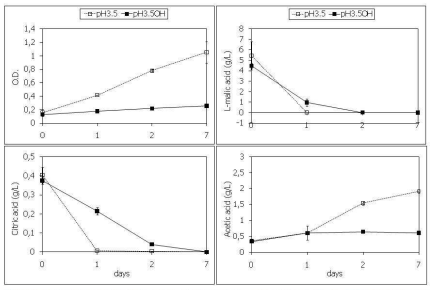

Figure 2. Growth and metabolic monitoring of *O. oeni* during MLF in cFT80 medium, at pH3.5 in presence (solid lines) and absence (dashed lines) of 10% of ethanol.

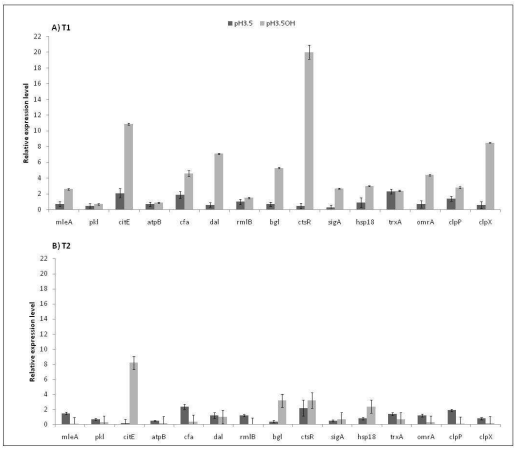

Figure 1. Relative expression level (REL) of fifteen *O. oeni* genes in cFT80 pH3.5 along time: A) T1 and B) T2. The calibrator conditions used was pH4 at the time of inoculation (T0). Data shown are mean values with standard deviations (n=3).

Results and Discussion

Results show that the main differences in REL were present at the middle of MLF (Figure 1A). Genes associated to malate (*mleA*) and citrate (*citE*) metabolisms had a higher REL in presence of ethanol. This could be related to stress response since the decarboxylation of these acids is associated to ATPase activity, which is involved in the maintenance of intracellular pH (Fortier et al. 2003). Nevertheless, *atpB* gene expression was not increased in response to ethanol. These results are in agreement with Beltramo et al. (2006). Consumption of L-malate and citrate was slower in the presence of ethanol; nevertheless differences were not relevant indicating a good adaptation of cells to ethanol content (Fig. 2).
As previously described (Bartowsky et al., 2004), there is no clear relationship between sugar and acids consumption. Moreover, previous work has shown the inhibition of sugar consumption in presence of ethanol whereas L-malic and citric acids were simultaneously consumed (Olguín et al., submitted). Therefore, the transcriptional response of *pkl* was chosen in order to elucidate if sugar consumption played a role in this behavior. Nevertheless, no REL differences were found for this gene.
Two genes related to membrane and cell wall biosynthesis (*cfa* and *dal*) were also over-expressed in presence of ethanol at the middle of MLF (5-fold and 7-fold respectively). This is in agreement with previous studies of REL under stress conditions (Beltramo et al., 2006) and the increase in the number of membrane-associated proteins in response to ethanol (Silveira et al., 2004). Although it has been described an increase of dTDP-glucose dehydratase protein in response to ethanol (Silveira et al., 2004), *rmlB* was not induced by ethanol.
The REL of *bgl* was also increased in presence of ethanol especially at the middle of MLF (7-fold) (Figure 1AB). This is in accordance with the reported enhancement of β-glucosidase activity when ethanol is present in the medium (Grimaldi et al., 2000)
Among genes previously related to stress response (Bourdineaud et al., 2004; Arvik et al., 2005; Beltramo et al., 2006), the mayor REL differences were observed in presence of ethanol in *ctsR* (20-fold) and *clpX* (7-fold) (Figure 1A). The same situation but with a minor REL difference was present in *sigA* (2-fold), *hsp18* (3-fold), *omrA* (4-fold) and *clpP* (2-fold). On the other hand, *trxA* showed no REL difference in absence or presence of ethanol although its REL was slightly increased at the middle of MLF (2-fold).

Conclusion

The study of the transcriptional profile of these genes will be useful to find the relation between the genetic response and the cellular adaptation to wine conditions in different *O. oeni* strains.

Target gene	Description	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon length (bp)	References
mleA	Malolactic enzyme	CCGACAATTGCTGATACAATTGAA	GGCATCAGAAACGACCAGCAG	156	Beltramo et al., 2006
pkl	Phosphoketolase	GGTTACCTTGTCTGGGTTCTTG	CACTGACGAGGTGTCTTATC	126	This work
citE	Citrate lyase β subunit	CCGCACGATGATGTTTGTTCC	GCTCAAAGAAACGGCATCTTCC	108	Previous work
atpB	ATPase F_1F_0 β-subunit	ATACTGATCCGGCTCCGGC	CAGCGGGATAAATACCTTG	93	Beltramo et al., 2006
cfa	Cyclopropane fatty acid synthase	TGGTATTACATTGAGCGAGGAG	CGTCTTTGAGATCACGATAATCC	113	Beltramo et al., 2006
dal	D-alanine-D-alanine ligase	CCTAAGCGAGCCTAATACAC	CCTGTTGGCTTCGATTCATTCC	92	This work
rmlB	dtdP-glucose-4,6-dehydratase	TATCCGCAATGCGCAATTGG	GAACGGTCAACATGCGATTCAG	93	This work
bgl	β glucosidase	GACGAGGATCTTTCCCAATG	GGTTCGATACCGTACTTGTG	102	This work
ctsR	Master regulator of stress response	GGGCCATGGCAGAAGCTAATATTTCAG	AAACGGGTGTTGATTACATAATT	147	Desroche et al., 2005
sigA	RNA polymerase sigma subunit	GGATCTTGGACGTGATCCAATG	CGATCGGAGTTTCCAAAGAGAC	119	This work
hsp18	Heat shock protein Lo18	CGGTATCAGGAGTTTTGAGTTC	CGTAGTAACTGCGGGAGTAATTC	102	Beltramo et al., 2006
trxA	Thioredoxin	GCCACTTGGTGTACCCCTTGT	TCCATTTGCCGTTTCCTGGTTT	120	Beltramo et al., 2006
omrA	ABC transporter	TCAGTTATTGGCGTGGTC	CGCTATGGTCGTGCTATC	114	This work
clpP	ClpP protease	CGGTACCAAAGGCAAGCGTTTTAT	CTCTTCCGAGTCTTCAAAAGTTGAT	131	Beltramo et al., 2006
clpX	Clp ATPase protein	GAAGCGTGTTAACGAGTCC	CAGCGACTGAGCCAAATAAG	112	This work
ldhD	D-lactate deshydrogenase	GCCGCAGTAAAGAACTTGATG	TGCCGACAACACCAACTGTTT	102	Desroche et al., 2005

Table 1. Gene descriptions and primer sequences.

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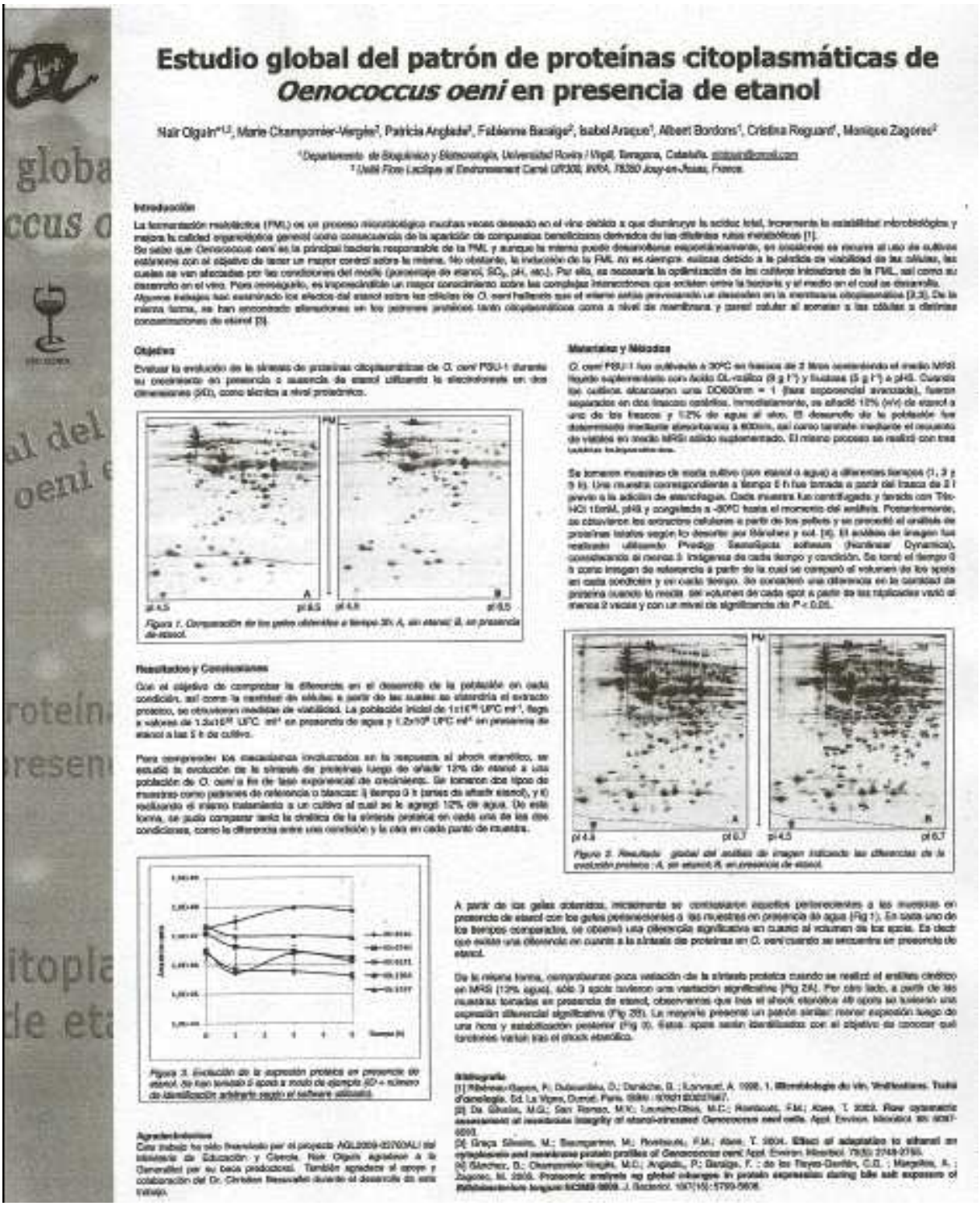

Estudio global del patrón de proteínas citoplasmáticas de *Oenococcus oeni* en presencia de etanol

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Introducción

La fermentación maloláctica (FML) es un proceso microbiológico muchas veces deseado en el vino debido a que disminuye la acidez total, incrementa la estabilidad microbiológica y mejora la calidad organoléptica general como consecuencia de la aparición de compuestos beneficiosos derivados de las diferentes rutas metabólicas [1].

Se sabe que *Oenococcus oeni* es la principal bacteria responsable de la FML y aunque la misma puede desarrollarse espontáneamente, en ocasiones es necesario el uso de cultivos starters con el objetivo de tener un mayor control sobre la misma. No obstante, la inducción de la FML no es siempre exitosa debido a la pérdida de viabilidad de las células, las cuales se van afectadas por las condiciones del medio (porcentaje de etanol, SO_2, pH, etc.). Por ello, es necesaria la optimización de los cultivos iniciadores de la FML, así como su crecimiento en el vino. Para conseguirlo, es imprescindible un mayor conocimiento sobre las complejas interacciones que existen entre la bacteria y el medio en el cual se desarrolla.

Algunos trabajos han examinado los efectos del etanol sobre las células de *O. oeni* hallando que el mismo actúa provocando un desorden en la membrana citoplasmática [2,3]. De la misma forma, se han encontrado alteraciones en los patrones proteicos tanto citoplasmáticos como a nivel de membrana y pared celular al someter a las células a distintas concentraciones de etanol [3].

Objetivo

Evaluar la evolución de la síntesis de proteínas citoplasmáticas de *O. oeni* PSU-1 durante su crecimiento en presencia o ausencia de etanol utilizando la electroforesis en dos dimensiones (2D), como técnica a nivel proteómico.

Materiales y Métodos

O. oeni PSU-1 fue cultivado a 30ºC en frascos de 2 litros conteniendo el medio MRS líquido suplementado con ácido DL-málico (6 g l^{-1}) y fructosa (5 g l^{-1}) a pH5. Cuando los cultivos alcanzaron una DO650nm = 1 (fase exponencial avanzada), fueron separados en dos frascos control. Inmediatamente, se añadió 12% (v/v) de etanol a uno de los frascos y 12% de agua al otro. El desarrollo de la población fue determinado mediante absorbancia a 600nm, así como también mediante el recuento de viables en medio MRS sólido suplementado. El mismo proceso se realizó con tres [illegible].

Se tomaron muestras de cada cultivo (con etanol o agua) a diferentes tiempos (1, 3 y 5 h). Una muestra correspondiente a tiempo 0 h fue tomada a partir del frasco de 2 l previo a la adición de etanol/agua. Cada muestra fue centrifugada y lavada con Tris-HCl 10mM, pH8 y congelada a -80ºC hasta el momento del análisis. Posteriormente, se obtuvieron los extractos celulares a partir de los pellets y se procedió al análisis de proteínas totales según lo descrito por Sánchez y col. [4]. El análisis de imagen fue realizado utilizando Prodigy SameSpots software (Nonlinear Dynamics), considerando al menos 3 imágenes de cada tiempo y condición. Se tomó el tiempo 0 h como imagen de referencia a partir de la cual se comparó el volumen de los spots en cada condición y en cada tiempo. Se consideró una diferencia en la cantidad de proteína cuando la media del volumen de cada spot a partir de las triplicadas varió al menos 2 veces y con un nivel de significancia de $P < 0.05$.

Figura 1. Comparación de los geles obtenidos a tiempo 3h: A, sin etanol; B, en presencia de etanol.

Resultados y Conclusiones

Con el objetivo de comprobar la diferencia en el desarrollo de la población en cada condición, así como la cantidad de células a partir de las cuales se obtendría el extracto proteico, se obtuvieron medidas de viabilidad. La población inicial de 1x10^{08} UFC ml^{-1}, llega a valores de 1.2x10^{09} UFC ml^{-1} en presencia de agua y 1.2x10^{8} UFC ml^{-1} en presencia de etanol a las 5 h de cultivo.

Para comprender los mecanismos involucrados en la respuesta al shock etanólico, se estudió la evolución de la síntesis de proteínas luego de añadir 12% de etanol a una población de *O. oeni* a fin de fase exponencial de crecimiento. Se tomaron dos tipos de muestras como patrones de referencia o blancos: i) tiempo 0 h (antes de añadir etanol), y ii) realizando el mismo tratamiento a un cultivo al cual se le agregó 12% de agua. De esta forma, se pudo comparar tanto la cinética de la síntesis proteica en cada una de las dos condiciones, como la diferencia entre una condición y la otra en cada punto de muestra.

Figura 3. Evolución de la expresión proteica en presencia de etanol. Se han tomado 5 spots a modo de ejemplo (ID = número de identificación arbitrario según el software utilizado).

Figura 2. Resultado global del análisis de imagen indicando las diferencias de la evolución proteica: A, sin etanol; B, en presencia de etanol.

A partir de los geles obtenidos, inicialmente se compararon aquellos pertenecientes a las muestras en presencia de etanol con los geles pertenecientes a las muestras en presencia de agua (Fig 1). En cada uno de los tiempos comparados, se observó una diferencia significativa en cuanto al volumen de los spots. Es decir que existe una diferencia en cuanto a la síntesis de proteínas en *O. oeni* cuando se encuentra en presencia de etanol.

De la misma forma, comparamos poca variación de la síntesis proteica cuando se realizó el análisis cinético en MRS (12% agua), sólo 3 spots tuvieron una variación significativa (Fig 2A). Por otro lado, a partir de las muestras tomadas en presencia de etanol, observamos que tres el shock etanólico 48 spots se tuvieron una expresión diferencial significativa (Fig 2B). La mayoría presentó un patrón similar: menor expresión luego de una hora y estabilización posterior (Fig 3). Estos spots serán identificados con el objetivo de conocer qué funciones varían tras el shock etanólico.

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Estudio de la capacidad de captación de glutatión por *Oenococcus oeni* en diferentes fases de crecimiento y su efecto sobre la respuesta al estrés oxidativo

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Introducción

El glutatión es un antioxidante celular. En el mosto se han descrito niveles de glutatión, sobretodo en su forma reducida (GSH), de hasta 300 mM. El contenido de glutatión en vino es más bajo y variable en función de la cepa de *Saccharomyces cerevisiae* que lleva a cabo la fermentación alcohólica. El glutatión jugaría un papel relevante en el vino protegiendo tioles volátiles durante el envejecimiento en botella. A pesar de la potencial importancia de los niveles de glutatión en el perfil aromático del vino, nunca ha sido estudiada la capacidad de utilización de este compuesto por parte de *Oenococcus oeni*, bacteria láctica responsable de la fermentación maloláctica. Esta especie es incapaz de sintetizar glutatión, pero podría incorporarlo del medio como se ha descrito para otras bacterias lácticas [1].

Objetivos

Los objetivos de este trabajo eran evaluar la capacidad de *O. oeni* para incorporar el glutatión intracelularmente y comprobar si dicha captación podría modificar la respuesta al estrés oxidativo.

Materiales y métodos

Estudio de la captación de glutatión y efecto sobre el crecimiento de O. oeni

Para este estudio se utilizó la cepa de *O. oeni* PSU-1 (ATCC BAA311). La capacidad de captación de GSH por parte de dicha cepa se evaluó utilizando el medio de cultivo MRS (Difco) modificado con 4 g/l de L-málico y 6 g/l fructosa a pH 5, al que se le adicionaron diferentes concentraciones de GSH. El GSH/GSSG intracelular fue analizado utilizando el kit GT30 (Oxford Biomedical) a partir de extractos celulares obtenidos por lisis con perlas de vidrio en un Mini BeadBeater (Biospec Products). La cantidad de proteína total de los extractos celulares se analizó por el método de Bradford [2].

Estudio del efecto de la presencia de glutatión en la respuesta al estrés oxidativo

Se evaluó el efecto de la presencia de GSH en el medio de cultivo sobre dos factores causantes de estrés oxidativo: presencia de 5 mM de H_2O_2 y de etanol al 12% (v/v), ambos durante 1 hora de incubación. La viabilidad se midió por contaje de células mediante microscopía de fluorescencia utilizando el kit Live/Dead BacLight™ (Invitrogen). Para medir la expresión génica de la glutatión reductasa la extracción de RNA, síntesis de cDNA y PCR a tiempo real se realizaron como se describe en trabajos previos [3], utilizando los primers gsh-F: 5'-GGCATTATCACCGAGCTGTT-3' y gsh-R:5'-TCCCGAAGAAGCAAAGAAGA-3'. La expresión relativa se calculó según el método de ΔΔCt utilizando como gen de referencia la lactato deshidrogenasa [4]. Todos los ensayos fueron realizados por duplicado.

Resultados y discusión

La adición de GSH al medio en concentraciones hasta 1 mM no afectó al crecimiento de *O. oeni* PSU-1 (Figura 1). Sin embargo se pudo confirmar la captación de glutatión por parte de *O. oeni* en presencia de concentraciones de al menos 0,2 mM de GSH añadido (Tabla 1). Los valores de GSH intracelular incorporados por *O. oeni* son comparables a los descritos para otras bacterias lácticas [1]. Cabe destacar que la captación de glutatión aumentó de forma proporcional al GSH añadido hasta una concentración de 0,5 mM, pero se multiplicó considerablemente respecto al aumento de la concentración de GSH añadido cuando ésta última era de 1 mM. Este fenómeno podría estar asociado a una activación de los sistemas de transporte de péptidos a partir de determinadas concentraciones de GSH. Se apreciaron también diferencias importantes en la proporción de la forma reducida y oxidada del glutatión intracelular en las dos fases de crecimiento estudiadas. En este sentido, el ratio GSH/GSSG fue mucho menor en fase estacionaria que en la exponencial indicando un mayor estado de oxidación celular, lo cual resulta coherente con la oxidación progresiva asociada al envejecimiento celular. Cabe destacar el aumento del ratio GSH/GSSG intracelular asociado al aumento de la concentración de GSH añadido en fase exponencial. Este hecho apuntaría al posible efecto protector del GSH ante la oxidación celular durante el crecimiento, si se considera el ratio GSH/GSSG como un posible indicador del estado de oxidación.

Tabla 1. Contenido medio intracelular de GSH (forma reducida) y GSSG en nmol/mg proteína total y ratio GSH/GSSG en *O. oeni* PSU-1.

GSH (mM)	Fase exponencial			Fase estacionaria		
	GSH	GSSG	GSH/GSSG	GSH	GSSG	GSH/GSSG
0	ND	ND	-	ND	ND	-
0,001	ND	ND	-	ND	ND	-
0,01	ND	ND	-	ND	ND	-
0,2	0,12	0,43	0,28	0,12	0,45	0,25
0,5	0,39	0,81	0,48	0,32	1,23	0,26
1	5,61	8,68	0,65	ND	11,26	-

ND: No detectado

Posteriormente, se evaluó el potencial efecto protector del GSH en células en fase exponencial de crecimiento ante dos tipos de estrés asociados a la oxidación, como son la presencia de H_2O_2 y de etanol, siendo este último uno de los principales inhibidores a los que se enfrenta *O. oeni* en el vino. Se observó la duplicación del % de supervivencia después del choque con 12% de etanol en presencia de 1 mM de GSH respecto al cultivo control sin GSH añadido (Figura 2). Este efecto no pudo ser observado en la respuesta a la adición de H_2O_2. En este caso, se apreció poco descenso de la población por efecto del choque, lo que podría haber enmascarado el posible efecto del GSH.

Respecto al gen de la glutatión reductasa (*gsh*), se apreció una destacable inhibición de su expresión en presencia de GSH por efecto del estrés oxidativo, tanto del etanol como de H_2O_2 (Figura 3). Este hecho indicaría que la la presencia de GSH intracelular procedente del medio inhibe la expresión de gsh. Por otra parte, la transcripción de *gsh* no varía de forma significativa (valores de expresión relativa cercanos al rango 0,5 -2) después del choque oxidativo en ausencia de GSH, lo que indicaría que dicho gen no responde de forma directa al estrés oxidativo, si no que sería la presencia de GSH intracelular captado del medio la que podría jugar un papel protector ante la oxidación.

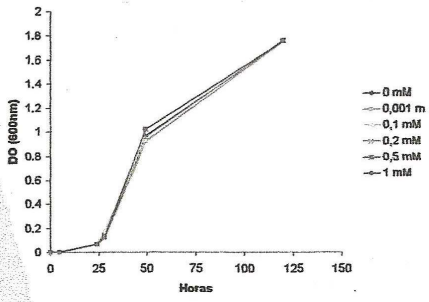

Figura 1. Crecimiento de *O. oeni* PSU-1 en presencia de diversas concentraciones de GSH añadido al medio.

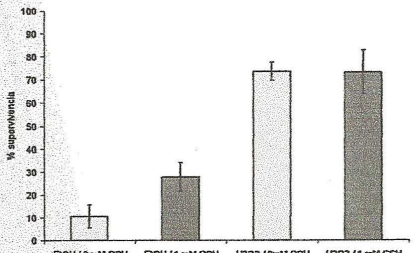

Figura 2. Porcentaje de supervivencia de *O. oeni* PSU-1 despues del choque oxidativo (etanol o H_2O_2) en células crecidas en presencia de GSH (1 mM) o en ausencia del mismo.

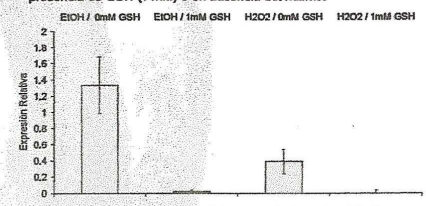

Figura 3. Expresión relativa del gen *gsh* después del choque oxidativo respecto a la expresión inicial en presencia o ausencia de GSH.

Conclusiones

- La presencia de GSH en el medio no afecta al crecimiento de la cepa *O. oeni* PSU-1 en las condiciones estudiadas.
- Se ha demostrado la capacidad de *O. oeni* PSU-1 de captar GSH del medio.
- La incorporación de GSH protege a la célula frente al choque etanólico, aumentando el % de supervivencia.
- Se observa una destacable inhibición de la expresión génica de la glutatión reductasa debido a la captación de GSH del medio.

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Factores que afectan el desarrollo de *Oenococcus oeni* y nuevas herramientas genéticas para la selección de cepas

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1. Introducción

Las bacterias lácticas (BL) han sido ampliamente estudiadas por su habilidad en llevar a cabo la segunda fermentación o fermentación maloláctica (FML) en el vino. Esta fermentación es en realidad una decarboxilación, donde el ácido L-málico (dicarboxílico) es transformado en ácido L-láctico (monocarboxílico). Este proceso es deseado por diversas razones, de las cuales se destacan tres: i) disminuye la acidez, especialmente en los vinos de zonas frías o blancos de elevada acidez; ii) mejora la calidad organoléptica general debido a la aparición de compuestos beneficiosos derivados de las distintas rutas metabólicas; iii) incrementa la estabilidad microbiológica del vino debido al consumo de substratos potenciales para microorganismos no deseados [20].

Si bien diferentes especies de BL pueden intervenir en la FML, el mayor interés recae sobre Oenococcus oeni por ser la bacteria más tolerante a las duras condiciones del vino (bajo pH, concentración de etanol y sulfuroso). Cepas autóctonas de *O. oeni* presentes en el mosto/vino pueden desarrollarse espontáneamente y comenzar la FML. No obstante, cualquier retraso en el inicio de la misma podría llevar a una alteración de la calidad del vino. Por esta última razón, muchos procesos de vinificación actuales utilizan cultivos iniciadores de *O. oeni* para la inoculación directa. Por otro lado y a pesar del uso de cultivos iniciadores, la FML sigue siendo difícil de inducir debido a las condiciones del vino, las cuales resultan hostiles para estas bacterias. Por lo tanto, es necesaria la optimización de los cultivos iniciadores de la FML, así como su desarrollo en el vino. Para conseguirlo, es imprescindible un mayor conocimiento sobre las complejas interacciones que existen entre la bacteria y el medio en el cual se desarrolla.

2. Factores que afectan el desarrollo de *O. oeni* y la fermentación maloláctica

Además del estrés inherente a la composición química del vino, el desarrollo de *O. oeni* también depende de su interacción con las levaduras. Como ya se sabe, la población y diversidad de BL disminuye durante la fermentación alcohólica como consecuencia del metabolismo de las levaduras, es decir, la producción de etanol, ácidos grasos y SO2 [1, 16]. Además de estos compuestos, Comitini y col [5] han encontrado un factor proteínico producido por Saccharomyces cerevisiae, que puede inhibir el crecimiento de *O. oeni*. Más aún, mientras

que la mayoría de las cepas de S. cerevisiae producen menos de 30 mg/mL de SO2, otras cepas pueden producir hasta casi 100 mg/mL [1].

Por otro lado, la autólisis de las levaduras también favorece el desarrollo de las BL. Son dos las principales razones: la liberación de manoproteínas, las cuales adsorben ácidos grasos de cadena media que inhiben el desarrollo de las BL [1], y la liberación de aminoácidos, péptidos y poteínas que aumentan el contenido nutricional del medio. De hecho, se ha observado que el desarrollo de *O. oeni* y el inicio de la FML se ven favorecidos en medios que contienen una mezcla de aminoácidos y péptidos [18, 19]. Aunque la limitación de nitrógeno no es considerada como un factor de estrés, sino como un estimulador del crecimiento, se debe tener en cuenta que las condiciones estresantes del vino (pH y etanol) tendrán un efecto en los sistemas de transporte de aminoácidos hacia la célula. El etanol, pH y la presencia de ácidos grasos dan lugar a una respuesta de *O. oeni* aparentemente debido a un cambio en la permeabilidad y la composición de la membrana plasmática [7, 9].

Algunos trabajos realizados con el fin de estudiar el efecto de la co-inoculación de cultivos iniciadores de BL y levaduras seleccionadas han proporcionado información acerca del comportamiento de dichas poblaciones durante la vinificación. Es posible inocular el mosto con cultivos de *O. oeni* al inicio, durante o al final de la fermentación alcohólica. No obstante, el pH y el momento de la inoculación determinarán cuan rápido se iniciará la FML [21]. Cuando la inoculación de BL se realiza al final de la fermentación alcohólica, la presencia de levaduras parece favorecer la viabilidad bacteriana y la actividad maloláctica incluso en condiciones difíciles como a valores de pH cercanos a 3 [13, 21].

Otros compuestos que afectan el desarrollo de las BL y la FML son el cobre, componente de varios pesticidas utilizados en viticultura, y el SO2 que se agrega al vino por su acción como antioxidante e inhibidor de microorganismos perjudiciales. Un estudio conducido en microvinificaciones simuladas sugiere que cepas comerciales y cepas aisladas de la bodega serían más tolerantes y eficientes que las cepas tipo en las condiciones de estrés determinadas por la presencia de cobre, SO2 y ácido dodecanoico [4]. El último es uno de los ácidos de cadena corta producidos durante el metabolismo de las levaduras que, como se mencionó anteriormente, inhiben el desarrollo de las BL. Más aún, en el mismo estudio se demuestra que este ácido es la molécula de mayor poder inhibitorio. No obstante, la presencia de todos los compuestos estresantes llevó a un retraso en el desarrollo de la FML en las microvinificaciones realizadas [4].

Finalmente, vale la pena mencionar que los compuestos fenólicos presentes en el vino también ejercen un efecto, favorable o desfavorable, sobre las BL. Se ha observado que el ácido gálico y los antocianos libres activan el crecimiento celular y FML en *O. oeni* [23]. Por otro lado, altas concentraciones de ácidos hidroxicinámicos han mostrado tener un efecto inhibidor sobre el crecimiento de la población, mientras que la FML es estimulada en presencia de catequina y quercitina [17]. Otro punto relacionado con las características organolépticas del vino es que los compuestos fenólicos parecen incrementar el consumo del ácido cítrico en *O.*

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oeni, pero al mismo tiempo, esto puede llevar a un aumento de la acidez volátil del vino [22]. No obstante, la presencia de ácido gálico parece retrasar o inhibir la formación de ácido acético a partir del metabolismo del ácido cítrico [17].

3. Adaptación a las condiciones de estrés

Uno de los interrogantes que surge a la hora de elegir un cultivo iniciador o estárter, es si éste logrará desarrollarse en el vino de interés. Aparentemente, la diferencia entre las cepas utilizadas para la producción de dichos cultivos reside en su resistencia a las condiciones del vino. La exposición a un leve estrés puede resultar en una mejor resistencia a un segundo estrés más fuerte o a diferentes tipos de estrés [3].

Se ha observado que células de *O. oeni* adaptadas a condiciones ácidas, sobreviven mejor que las células no adaptadas en presencia de una alta concentración (30 mg/L) de SO2. De la misma forma, si se añade una baja dosis de SO2 (15 mg/L) durante el período de adaptación en condiciones ácidas, las células de *O. oeni* aumentan la tolerancia al SO2 [11]. La adaptación de células de *O. oeni* a las condiciones del vino también ha sido observada al someter las células a 42ºC [10] y a 8% de etanol [7]. Esta adaptación a las condiciones de estrés no sólo aumenta la supervivencia de las células cuando son inoculadas en el vino, sino que además mejora la habilidad de las mismas para llevar a cabo la FML.

Pero ¿qué es lo que sucede durante el proceso de adaptación? Desde hace algunos años, diversos investigadores se dedican a la búsqueda de respuestas para esta pregunta. Aparentemente, son varios los mecanismos involucrados en la adaptación, pero los principales son la variación en la fluidez y composición de la membrana plasmática y la síntesis de proteínas de respuesta a estrés [7, 10]. Actualmente, y al disponer de nuevas tecnologías basadas en biología molecular, el estudio de la adaptación y respuesta a estrés de *O. oeni* se basa en el cambio de la expresión genética. Es decir, se trata de conocer cómo la información contenida en los genes puede ser activada para sintetizar, o no, proteínas con diversas funciones por parte de la bacteria.

Los trabajos realizados con el fin de estudiar las diferencias de expresión genética vinculadas a las condiciones de crecimiento en *O. oeni* han permitido reconocer algunos genes que estarían vinculados con la respuesta a estrés. Entre ellos, se han identificado genes que codifican proteínas de respuesta a estrés como la Lo18 [10], genes involucrados en el metabolismo de ácidos como la enzima maloláctica [14], proteínas involucradas en el mantenimiento del pH en el interior celular como las ATPasas [8], etc. Muchos de estos genes han demostrado tener una diferencia de expresión en *O. oeni* dependiendo de las condiciones de cultivo [2]. Dado que las distintas rutas metabólicas están relacionadas con la respuesta a estrés, resulta interesante poder estudiar todos estos genes de forma conjunta.

4. Nuevas herramientas para la selección de cultivos iniciadores

Actualmente, la selección de cepas para la inoculación de vinos es llevada a cabo mediante estudios clásicos basados en la supervivencia de las células en el vino y el seguimiento de la FML. También se ha propuesto inducir los cultivos a leves condiciones de estrés, previo a la inoculación del vino, para obtener mejores resultados en el desarrollo de la población y la FML. No obstante, los últimos estudios basados en biología molecular podrían llevar a nuevas técnicas de selección donde no sólo podrá tenerse en cuenta la respuesta a estrés, sino también el tipo de metabolitos producidos durante el desarrollo de la población. A partir de la reciente secuenciación del genoma de *O. oeni* PSU-1 (Genbank NC_008528) y ATCC BAA-1163 (Genbank NZ_AAUV00000000), se tiene acceso a los genes de dichas cepas. No sólo se podrán estudiar los genes correspondientes a enzimas relacionadas con la respuesta a estrés, sino también aquellos genes relacionados con la modificación de las características organolépticas del vino, es decir, genes correspondientes a la FML y a la capacidad de utilizar el citrato [15]. El estudio de diversos genes en conjunto promete deducir qué cepas responderán mejor a las condiciones deseadas y qué tipo de metabolitos se producirán durante su desarrollo.

La expresión genética puede ser evaluada mediante el uso de la PCR cuantitativa o PCR a tiempo real. La técnica implica en primer lugar la extracción del ARN a partir de la célula, el cual equivale a aquellos genes que están siendo expresados. En segundo lugar, este ARN debe ser transformado en ADN complementario utilizando la enzima transcriptasa inversa en una reacción similar a la de PCR. Finalmente, la cuantificación de este ADN complementario da una idea aproximada de la expresión de un determinado gen, al ser comparado con la cuantificación de un gen que se expresa constitutivamente.

Esta técnica se ha utilizado para comparar tres cepas de *O. oeni* seleccionadas para la elaboración de cultivos estárter, donde el gen cuantificado fue el correspondiente a la proteína Lo18 de respuesta a estrés [6]. Los resultados de este estudio mostraron que el mayor nivel de expresión de dicho gen correspondía a la cepa que presentó una mayor actividad maloláctica a pH 3.

Si bien la rapidez de esta técnica es una gran ventaja, en comparación con las técnicas utilizadas hasta el momento, aún queda mucho trabajo por desarrollar. El mayor objetivo es poder diseñar un perfil de expresión que permita caracterizar las cepas en base a su capacidad de adaptación a los diferentes factores de estrés (pH, etanol, etc.) y la producción de metabolitos con impacto organoléptico (acetato, diacetilo, aminas biógenas, etc.).

5. Conclusiones

Aún se dispone de poca información en cuanto a los mecanismos de adaptación de *O. oeni* a las condiciones del vino. Actualmente se están llevando a cabo estudios que abordan de una forma más global la relación entre los mecanismos implicados en dicho proceso. El grupo de Biotecnología Enológica del Departamento de Bioquímica y Biotecnología de la Facultad de Enología (Universidad Rovira i Virgili de Tarragona) trabaja actualmente en la evaluación de nuevas técnicas que puedan ser utilizadas para este fin.

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