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Real-time PCR for characterizing the stress response of *Oenococcus oeni* in a wine-like medium

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Abstract

The tolerance of the lactic acid bacterium *Oenococcus oeni* to hostile wine conditions is essential for the success of malolactic fermentation (MLF). In this study, reverse transcription quantitative PCR (RT-qPCR) was used to quantify the transcript level of 13 genes that could play a role in adaptation of *O. oeni* in wine. To optimize survival and growth in wine, cells were adapted during growth at low pH (3.5) prior to inoculation into wine. The level of gene expression was analyzed after growth at pH 3.5 in a rich medium and during MLF in a wine-like medium. RT-qPCR analyses exhibited different expression ratios of stress genes. The data obtained showed that determination of mRNA levels could constitute a new approach to studying the stress response of *O. oeni* after adaptation at low pH and during growth in a wine-like medium.

Keywords: Lactic acid bacteria; *Oenococcus oeni*; Malolactic fermentation; Real-time RT-PCR; Stress; Transcription; Wine

1. Introduction

During the process of winemaking, malolactic fermentation (MLF) follows alcoholic fermentation, carried out by yeasts. MLF consists of the decarboxylation of L-malic acid into L-lactic acid. This conversion of a di-acid into a mono-acid deacidifies wine and results in a softer feeling in the mouth [26]. MLF also improves microbiological stability and organoleptic characteristics [23]. For many years, MLF has been recognized as an indispensable step in the elaboration of most wines. Generally, MLF is carried out by indigenous lactic acid bacteria, mainly *Oenococcus oeni* strains, and can occur spontaneously. However, this step can start randomly, and any delay could lead to an alteration of wine quality. These delays are due to very harsh environmental conditions in the wine for bacterial survival and growth, such as low pH, high alcohol content, high concentration of SO_2 and low temperature [32].

Several mechanisms enable *O. oeni* to withstand stress conditions: (i) activation of MLF to generate a protonmotive force so as to maintain intracellular pH (pH_i) [27,28]; (ii) activation of membrane-bound H^+-ATPases [3]; (iii) stress protein synthesis [16]; and (iv) modification of membrane fluidity [6,30]. More information about the mechanisms involved in the adaptation of *O. oeni* to stress conditions is required, particularly under winemaking conditions. A better knowledge of stress physiology may be useful to optimize survival of starter cultures of *O. oeni*. Indeed, winery practices recommend commercial freeze-dried bacterial cultures of *O. oeni* for direct inoculation into wines to improve the control of MLF [26]. However, induction of MLF by inoculation with malolactic starters is not effective in "difficult wines" (for example, in wines having a pH inferior to 3.2) because of significant cell mortality. To overcome this problem, adaptation processes have been shown to enhance the survival of *O. oeni* cells to stress conditions in wine: heat-shock at 42 °C [17] or growth in the presence of ethanol (8%, vol/vol) [12]. The increase in cell survival is linked to stress response mechanisms: heat treatment has been shown to increase the synthesis of heat shock proteins (HSPs), notably a small HSP named Lo18 [15], and growth of *O. oeni* in the

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presence of ethanol leads to a modification in membrane composition [6]. 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 resisting an upcoming stress condition [29].

Until now, in order to characterize the *O. oeni* stress response, cells were submitted to only one kind of stress for a short time to perform a shock. In wine, the situation is more complex because cells are submitted to multiple stresses. The aim of this work was to monitor the expression level of stress genes from *O. oeni* inoculated into wine using the reverse transcription quantitative polymerase chain reaction (RT-qPCR) [5,9,11,31]. Wine medium was inoculated with cells grown under sublethal stress conditions to enhance survival. The level of gene expression was compared under three conditions: in a laboratory medium adjusted to pH 5.3 or 3.5, and in a wine-like medium.

2. Materials and methods

2.1. Bacterial and yeast strains, growth and stress conditions

O. oeni ATCC BAA1163 (formerly known as IOB 8413) was grown at 30 °C in FT80 medium (pH 5.3 or 3.5), which was modified by the addition of meat extract instead of casamino acids (mFT80 medium) [4]. This strain was used because its genome had been sequenced by our laboratory in collaboration with the Laboratoire de Biotechnologie et Microbiologie appliquée (UMR 1219, INRA-Université Victor Segalen Bordeaux 2), the Centre de Bioinformatique de Bordeaux (CbiB, Université Victor Segalen Bordeaux 2) and GENOME Express (Grenoble).

For the acidic adaptation of *O. oeni*, cells collected in mid-exponential phase after growth in mFT80 medium at pH 5.3 were inoculated ($OD_{600\,nm} = 0.2$) into mFT80 medium at pH 3.5 and incubated at 30 °C for 16 h. When cells reached an OD of 0.6, they were directly inoculated into wine. For ethanol adaptation, cells collected in mid-exponential phase after growth in mFT80 medium at pH 5.3 were inoculated ($OD_{600\,nm} = 0.2$) into mFT80 medium at pH 5.3 in the presence of 10% (vol/vol) ethanol and incubated at 30 °C for 10 h. When cells reached an OD of 0.6, they were directly inoculated into wine.

Saccharomyces cerevisiae commercial strain BRG (Oeno-France, Rueil-Malmaison, France) was grown at 25 °C under agitation in YPD broth (20 $g\,l^{-1}$ bactopeptone, 10 $g\,l^{-1}$ yeast extract and 20 $g\,l^{-1}$ glucose, pH 5.3).

2.2. Wine-like medium and malolatic fermentation

It is difficult to use a real wine because of the presence of phenols, which prevent RNA extraction. The composition of the synthetic must and winemaking conditions were based on the work of Alexandre et al. [1]. The synthetic must contained glucose (75 $g\,l^{-1}$), fructose (85 $g\,l^{-1}$), DL-malic acid (10 $g\,l^{-1}$), L-tartaric acid (2 $g\,l^{-1}$), ammonium chloride (0.2 $g\,l^{-1}$) and yeast carbon base (11.7 $g\,l^{-1}$); pH was adjusted to 3.5 with NaOH. The must (10 l) was inoculated at the rate of 10^6 CFU ml^{-1} with a 24-h-old *S. cerevisiae* strain BRG culture, and alcoholic fermentation was performed at 20–22 °C for 16 days. Then, the wine was clarified by filtration through a 0.22 μm pore size filter (Millipore, France) to remove yeasts. After filtration, the physico-chemical properties of the wine-like medium were as follows: 10.5% (vol/vol) ethanol, 4.6 g of L-malic acid per liter and pH 3.5. The wine was stored at 4 °C.

MLF was initiated by direct inoculation with *O. oeni* ATCC BAA1163 grown at pH 3.5 for 16 h ($OD_{600\,nm} = 0.6$) to a final concentration of 2.10^6 CFU ml^{-1} (1 unit $OD_{600\,nm} = 5.10^8$ CFU ml^{-1}) in 2 l of wine. A control without inoculation was performed to verify any spontaneous MLF. The glass containers were incubated at 18 °C.

2.3. Bacterial numeration and consumption of L-malic acid in wine

Bacterial numeration was performed by counting cells (CFU ml^{-1}) spread on agar plates of FT80 agar medium, pH 5.3 and incubated at 30 °C for five days. The concentration of L-malic acid was determined with the Boehringer enzymatic kit (Mannheim, Germany) according to the manufacturer.

2.4. RNA extraction

Total RNA was extracted under seven conditions: (i) mid-exponential phase in mFT80 at pH 5.3; (ii) mid-exponential phase in mFT80 at pH 3.5; (iii) two days after inoculation into wine (T2); (iv) six days after inoculation into wine (T6); (v) ten days after inoculation into wine (T10); and (vi) sixteen days after inoculation into wine (T16). Total RNA extractions were performed using the RNeasy kit according to the manufacturer's instructions (Qiagen). *O. oeni* cells were harvested by centrifugation. They were disrupted with glass beads 0–50 μm in a FastPrep FP120 Instrument (Thermo Savant-BIO101) at 4 °C for 6 × 30 s at 6000 *g*. Samples were then treated as recommended by the manufacturer (Sigma). Purified RNAs were suspended in 30 μl of DMPC 0.1% (dimethylpyrocarbonate)-treated water. RNA concentrations were calculated by measuring absorbance at 260 nm using a SmartSpec Plus Spectrophotometer (Bio-Rad).

2.5. Reverse transcription and real-time PCR

Prior to reverse-transcription, RNA (2 μg of total RNA) was treated with 2 units of DNase (Invitrogen) as described by the manufacturer. Then, the cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad) as recommended. Absence of chromosomal DNA contamination was confirmed by real-time PCR. Primers were designed in order to have a length of about 20–25 bases, a G/C content over 50% and a T_m of about 60 °C. The length of the PCR products ranged from 90 to 160 bp. Primer3 primer design software was used to select primer sequences. Secondary structures and dimer formation were controlled with Oligo Analyzer 1.0.3 Software. Primers were purchased from Invitrogen (Paisley, Scotland).

Table 1
Function of genes and primer sequences used in this study

Target gene	Function of gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Amplicon length (bp)
ldhD	D-lactate dehydrogenase	GCCGCAGTAAAGAACTTGATG	TGCCGACAACACCAACTGTTT	102
hsp18	Stress protein Lo18	CGGTATCAGGAGTTTTGAGTTC	CGTAGTAACTGCGGGAGTAATTC	102
clpP	ClpP protease	CGGTACCAAAGGCAAGCGTTTTAT	CTCTTCCGAGTCTTCAAAAGTTGAT	131
clpX	Clp ATPase protein	GGGTGTTTATATTTGTGACGAATG	GGGTGTTTATATTTGTGACGAATG	110
trxA	Thioredoxin	GCCACTTGGTGTACCCCTTGT	TCCATTTGCCGTTTCCTGGTTT	120
grpE	GrpE, heat shock chaperone class II	CGCAGGCAGAAAAGAACAATC	GCTGAAGACGAAGCAGTTGC	126
groES	GroES, heat shock chaperone class I	GCCACAACAGAACCCATCACTGGTT	GGCGATCGAATTGTTCTTAGTAT	106
ctsR	CtsR, heat shock transcriptional regulator	GGGCCATGGCAGAAGCTAATATTTCAG	AAACGGGTGTTGATTACATAATT	147
cfa	Cyclopropane fatty acid synthase	TGGTATTACATTGAGCGAGGAG	CGTCTTTGAGATCACGATAATCC	113
clpL1	Clp ATPase protein	ATTATAATGACGATCCCTTCGT	GGATCCCTGAACCGTTATTTGCTTGTTG	163
clpL2	Clp ATPase protein	ATTATAATGACGATCCCTTCGT	GGATCCCTGAACCGTTATTTGCTTGTTG	100
hdcA	Histidine decarboxylase	GGGACGGTAGTGAACTTCCAA	GAGCAGGATAGTGACATATC	99
mleA	Malolactic enzyme	CCGACAATTGCTGATACAATTGAA	GGCATCAGAAACGACCAGCAG	156
atpB	ATPase F_1F_0 β-subunit	ATACTGATCCGGCTCCGGC	CAGCGGGATAAATACCTTG	93

Real-time PCR was carried out on a Biorad-I-Cycler with the IQ™ SYBR® Green Supermix (Bio-Rad) in 96-well plates. After dilution of cDNA, 5 µl was added to 20 µl of PCR mixture (12.5 µl of IQ™ SYBR® Green Supermix, 2.0 µl of each primer at 7.0 pmol μl^{-1} and 5.5 µl of RNase-free water). Four dilutions of cDNA were performed. Specific cDNAs were amplified by real-time PCR with specific primers (Table 1). In each run, a negative control was included. Thermal cycling conditions were designated as follows: initial denaturation at 95 °C for 3 min, followed by 38 cycles of 95 °C for 15 s and 60 °C for 30 s. Fluorescence measurements were recorded during each annealing step. An additional step starting from 90 to 60 °C (0.05 °C s^{-1}) was performed to establish a melting curve, and was used to verify the specificity of the real-time PCR reaction for each primer pair. Efficiencies of amplifications were determined by running a standard curve with serial dilutions of cDNA. A PCR that amplifies the target sequence with 100% efficiency (E) can double the amount of PCR products in each cycle. Efficiency E can be calculated by the formula $E = [10^{(1/-s)} - 1] \times 100$, where s is the slope of standard curve. Primers were designed to amplify a specific internal region of each gene (Table 1). For each measurement, a threshold cycle value (C_T) was determined. This was defined as the number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background. In this study, the threshold value was determined with a baseline set manually at 100 relative fluorescence units.

Results were analyzed using the comparative critical threshold ($\Delta\Delta C_T$) method in which the amount of target RNA is adjusted to a reference relative to an internal calibrated target RNA as described by Desroche et al. [8]. The results were normalized by using the *O. oeni ldhD* gene coding for lactate dehydrogenase. In order to confirm the use of the *ldhD* gene as the internal control, *ldhD* expression was routinely determined from RNA extracted at 2, 6, 10 and 16 days of malolactic fermentation (T2, T6, T10, T16). All ΔC_T between two samples were less than one, which validates our internal control in these conditions (data not shown). Measures were taken for each condition from cDNA synthesized from RNA extracted from three independent cultures and performed in triplicate for each gene.

To compare the results obtained under the three conditions (mid-exponential-phase-cells growing at pH 5.3 in comparison with mid-exponential-phase-cells growing at pH 3.5, or two days after inoculation into wine in comparison with adaptation in mFT80 medium at pH 3.5), we considered that genes were significantly down- or upregulated if their relative expression level was found to be at least twofold lower or higher than the calibrator condition as previously described [8].

2.6. Statistical analysis

A one-way analysis of variance (ANOVA) was performed to test whether there was a significant evolution in gene expression levels during MLF. When one-way ANOVA was significant, the Student–Newman–Keuls method ($n = 3$, $P < 0.05$) was used to locate significant differences. SigmaStat® Version 3.0.1 software (SPSS Inc.) was used to perform statistical analysis.

2.7. Nucleotide sequence accession numbers

The nucleotide sequences were submitted to the nucleotide sequence database/EMBL under the following accession numbers: AJ606044 (*clpP*, *clpL1*), Y15953 (*clpX*), X82326 (*mleA*), X99468 (*hsp18*), XT93091 (*trxA*), AJ491851 (*atpB*), AJ831540 (*ldhD*), AJ831546 (*cfa*), AJ831547 (*hdcA*), AJ831549 (*ctsR*), AJ831550 (*grpE*), AJ831551 (*groES*) and AJ831552 (*clpL2*).

3. Results

3.1. Adaptation of O. oeni cells improved survival in wine

Survival and growth of *O. oeni* was analyzed in wine-like medium (see Section 2). Before performing MLF, cells grown under three conditions were used to inoculate the wine. Survival rates were then calculated after two days of incubation. The results showed that when non-adapted *O. oeni* cells were inoculated into wine (cells harvested in mid-exponential phase in mFT80, pH 5.3), a drastic decrease in the bacterial population was seen, i.e., a survival rate of less than 1% (data not shown). In contrast, the survival rates of adapted cells grown in

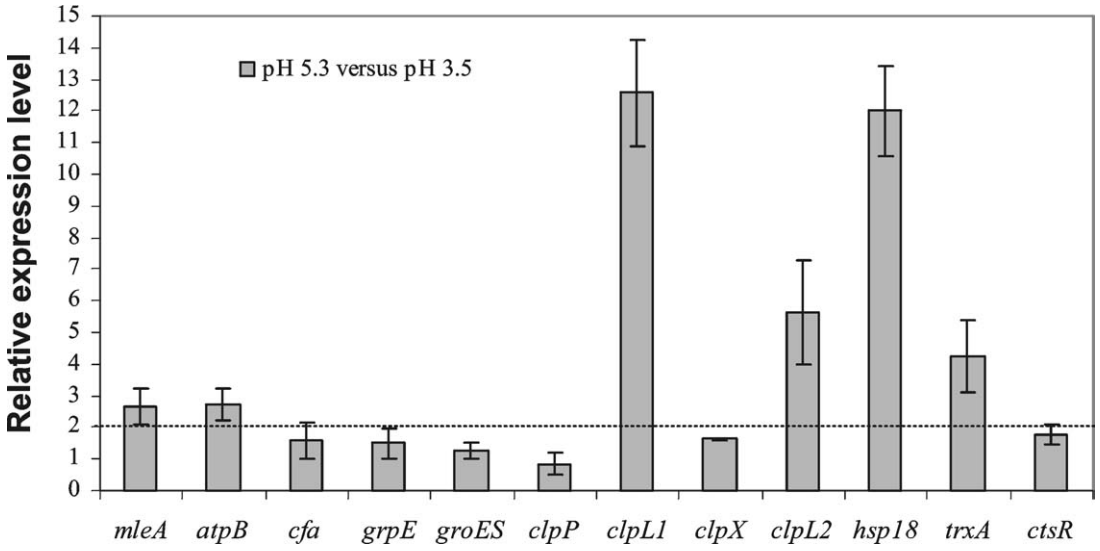

Fig. 1. Comparison of the relative expression levels of several genes of *O. oeni* between mid-exponential-phase-cells growing in mFT80 at pH 5.3 and mid-exponential-phase-cells growing in mFT80 at pH 3.5. Gene expression was quantified using RT-qPCR and the comparative critical threshold ($\Delta\Delta C_T$) method. The growth condition at pH 5.3 was defined as the calibrator.

the presence of ethanol or at acidic pH were respectively 15 and 100% (data not shown). The acidic adaptation, which generated the greatest survival in wine, was retained to perform the MLF experiments.

3.2. Choice of loci to characterize the stress response of O. oeni *by RT-qPCR*

Previous studies concerning the stress response [2,8,18–20] and analysis of the genome enabled us to select 13 genes for our experiments. The chosen genes encoded: (i) enzymes involved in energetic metabolism: the *mleA* gene encoding the malolactic enzyme [21,22], the *atpB* gene encoding the β-subunit of the F_1F_0 ATPase [10], the *hdc* gene encoding a histidine decarboxylase, for its role in histamine production, a biogenic amine [24]; (ii) the *cfa* gene encoding CFA synthase (cyclopropan fatty acyl synthase), which changes the configuration of the fatty acids in the membrane [14]; and (iii) molecular chaperones and protease: two loci contain *grpE–dnaK–cbpA* genes and *groES–groEL* genes, which encode both universal chaperone complexes, five members of the Clp family, i.e., the two loci *clpP–clpL1* and *ctsR–clpC*, the *clpX* gene, the *clpL2* gene, the *hsp18* gene, encoding the Lo18 smHSP and the *trxA* gene, encoding a thioredoxin. The *ctsR* gene encodes the transcriptional regulator of *grpE*, *groES*, *clpP*, *clpL2*, *ctsR* and *hsp18* genes [13]. For RT-qPCR, the transcript level of the first gene of each locus was quantified except for *clpP–clpL1*, since an internal promoter *pclpL1* had been identified in a previous study [2]. It is worth mentioning that the *hdcA* gene was not expressed under our conditions.

3.3. Molecular characterization of the acid adaptive response

Relative expression levels for each gene were calculated for mid-exponential-phase-cells growing at pH 5.3 and at 3.5. These experimental conditions enabled us to identify genes with higher expression during growth at low pH. As previously determined [8], a relative expression level higher than two is considered significant. Analysis of the data indicated that expression of the *grpE*, *groES*, *ctsR*, *clpP*, *clpX* and *cfa* genes was not affected by growth at pH 3.5 in mFT80 medium (Fig. 1). However, the relative expression levels of the other genes increased more than twofold after acidic adaptation. *mleA* and *atpB* levels increased about threefold. *clpL2* and *trxA* expression increased between four- and sixfold, while *hsp18* and *clpL1* expression increased about twelvefold in comparison with growth at pH 5.3.

3.4. Malolactic fermentation monitoring

Three independent MLFs were conducted with *O. oeni* cells previously adapted at pH 3.5. Results of cell enumeration and L-malic acid degradation are shown in Fig. 2. Neither degradation of L-malic acid nor spontaneous growth was observed with the control wine (data not shown). No loss of viability was observed after inoculation with acid-adapted cells. *O. oeni* cells started to grow the first day and MLF was complete after 16 days. Growth stopped on the tenth day. At the end of MLF, the population of *O. oeni* was about 4.10^7 CFU ml^{-1}. The final pH was 3.63 for the three independent experiments. During MLF, samples were taken at several time points to follow gene expression. Four time points were selected: the second day (when cells were affected by the multiple stresses of wine with around 4.5 $g\,l^{-1}$ of L-malic acid), the sixth day (exponential growth phase, with around 3.5 $g\,l^{-1}$ of L-malic acid), the tenth day (stationary growth phase, with around 2.0 $g\,l^{-1}$ of L-malic acid) and the sixteenth day (end of MLF) (respectively T2, T6, T10, T16).

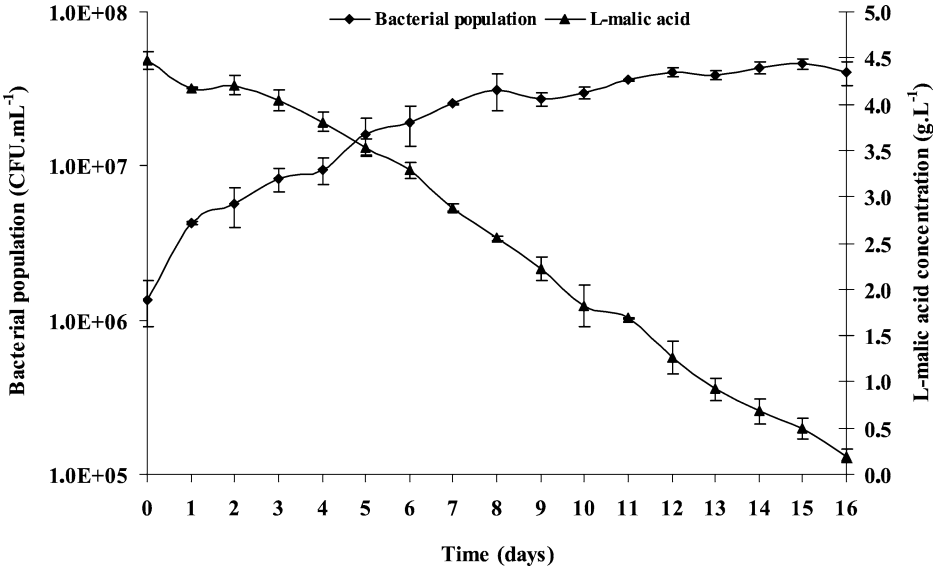

Fig. 2. Evolution of the bacterial population and L-malic acid consumption during MLF in a wine-like medium. MLF conduction was described in Section 2.

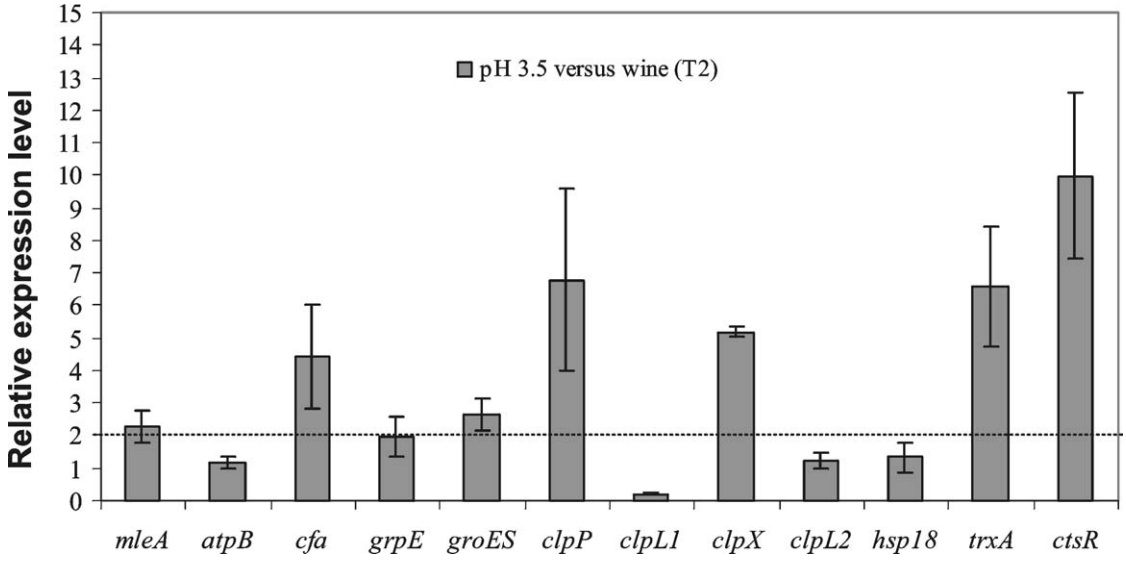

Fig. 3. Comparison of the relative expression levels of several *O. oeni* genes in cells adapted to growth in mFT80 at pH 3.5 for 16 h and cells after two days in wine (10% of ethanol and pH 3.5). Relative expression levels were calculated using the comparative critical threshold ($\Delta\Delta C_T$) method. The growth condition in mFT80 at pH 3.5 was defined as the calibrator.

3.5. Molecular characterization of the stress response after inoculation into wine

In order to study the effect of inoculation into wine on the stress response, relative expression levels were calculated two days after inoculation (11% ethanol (vol/vol), 18 °C, pH 3.5) in comparison with adaptation in mFT80 medium at pH 3.5 (no ethanol, 30 °C) (Fig. 3). The gene expression profiles obtained for growing cells at low pH and after two days of incubation in wine were substantially different (compare Figs. 1 and 3). The *atpB*, *clpL2* and *hsp18* genes had a steady transcript level (fold change equal to one). It is noteworthy that the *clpL2* and *hsp18* genes, the expression of which was not induced at T2, showed the highest relative expression levels in acidic adaptation in mFT80. Their level stayed at their maximal rate obtained in acidic conditions. The expression level of *clpL1* decreased after inoculation into wine (Fig. 2). The other genes had a higher expression level in wine at T2 compared to mFT80 medium at pH 3.5. The *groES*, *grpE*, *mleA* and *cfa* levels increased between twofold and fourfold. *ctsR*, *clpP*, *clpX* and *trxA* levels increased more than fivefold.

3.6. Temporal expression of tested genes during MLF

In order to follow temporal gene expression during MLF, we calculated fold changes between two periods of MLF (T6 versus T2, T10 versus T6, T16 versus T10) (Fig. 4). Statistical analyses showed that the transcript levels of four genes, i.e., *grpE*, *ctsR*, *clpP*, and *cfa,* did not significantly evolve during MLF; their transcript levels did not change during MLF.

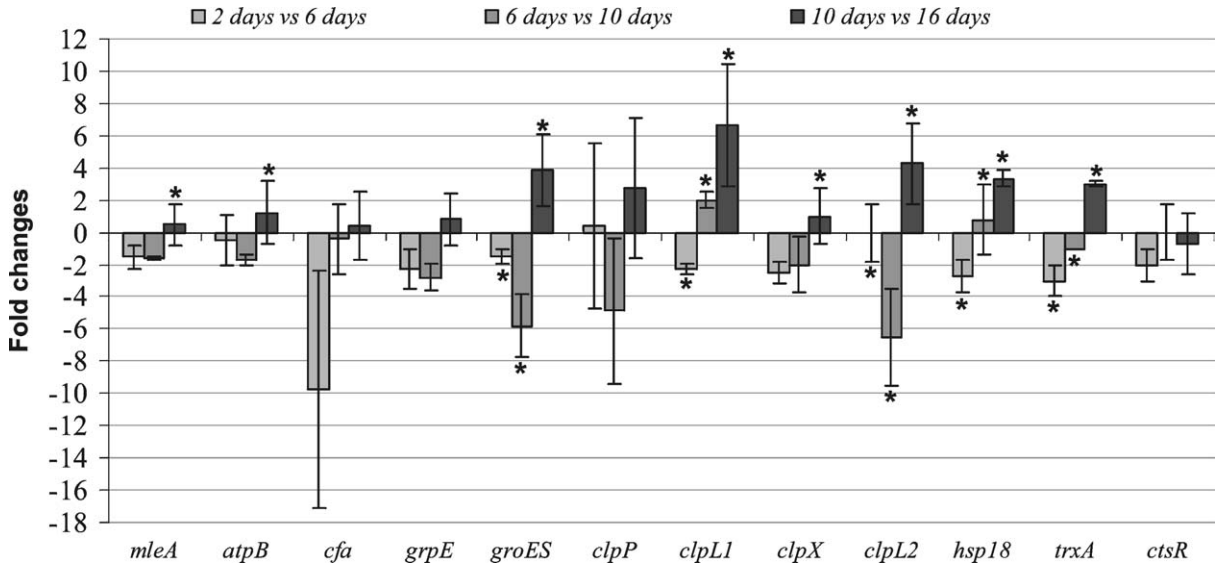

Fig. 4. Evolution of fold changes during malolatic fermentation. Gene expression was quantified using RT-qPCR and the comparative critical threshold ($\Delta\Delta C_T$) method. Fold changes represent ratios of the relative expression levels between two times of MLF. * indicates significant difference ($P < 0.05$).

Comparison of data obtained concerning expression of the other genes during vinification revealed a common trait. Between T2 and T10, mRNA levels decreased. The maximal levels were detected at T2. Between T2 and T6, transcript levels *of clpX, clpL1, hsp18 and trxA* more than halved. Between T6 and T10, unlike other genes, *clpL2 and groES* transcript levels more than halved. Between T10 and T16, increases were observed for *groES* (fourfold), *clpL2* (fourfold), *clpL1* (sixfold), *hsp18* (threefold) and *trxA* (threefold). This result can be explained by entry into the stationary phase. It should be noted that genes belonging to the CtsR regulon [13] presented different profiles, particularly *groES*, *clpL2* and *hsp18*, the levels of which increased at T16 contrary to *grpE*, *ctsR*, *clpP* levels. Observation of the evolution of transcript levels of the genes previously studied during growth in mFT80 [2,18–20] revealed that the highest transcript levels of *clpL1*, *trxA* and *hsp18* occurred in the stationary phase at T16. The highest transcript levels of *clpP* and *clpX* were observed respectively in mid-exponential phase (T6) and at the beginning of the growth (T2).

4. Discussion

The present work consisted of employing RT-qPCR as a sensitive method for study of the transcript level of *O. oeni* genes during MLF in a wine-like medium. The levels were measured with an internal control in order to calculate the relative amounts of mRNA as accurately as possible [8,25]. As previously detailed, standard deviation between the transcript levels of three independent experiments could be high due to several steps of calculations, but the interassay coefficients of variation were only about 3% [8].

To optimize survival and growth in wine, the strategy employed consisted of adapting cells by growing them at low pH (3.5) prior to inoculation. Our results show that *clpL2*, *clpL1*, *hsp18* and *trxA* expression were increased in the adaptive response after 16 h at low pH, and not only in the transient shock response as previously described [2,18,19]. After growth in a medium at pH 3.5, the mRNA levels of these four genes were at least four times higher than in the medium at pH 5.3. This result suggests that an improved basal level of gene expression can follow a transient increase, and can confer upon the cell a capacity for adaptation to stress. Indeed, with the challenge in wine, better cell viability was found for cells grown under acidic conditions. These data confirm the value of stress pre-adaptation for malolactic starter cells, particularly at low pH. Adapted cells were not submitted to different acidic conditions when they were inoculated into wine (the pH of the wine before inoculation was 3.5). However, even though acidity was the same, cells were subjected to a medium with ethanol (11%, v/v) at a temperature of 18 °C (versus 30 °C) and limited nutriments. Considering these multiple stresses taking place in wine, our results clearly confirm the existence of a cross-protection phenomenon induced by acid adaptation to various stresses. The survival of acid-adapted cells was up to two logs higher than that of non-adapted cells. This was not the case for cells adapted in the presence of ethanol.

In terms of the temporal expression of genes during growth in wine, several hypotheses are possible: (i) The high transcript level of some of the stress genes could be explained by the stress response of *O. oeni* and adaptation to multiple stresses. (ii) The decrease in the transcript levels occurring between T2 and T6 could be explained by the end of adaptation of cells to wine conditions, i.e., the capacity to restore the physiological non-stressed state in order to achieve growth. This fact has been defined as the necessary "equilibrium between stress response and growth efficiency" for yeast cells [34]. (iii) The increase in transcript levels between T10 and T16 could be explained by entry into the stationary phase, as most of the genes are induced by the growth stage under laboratory conditions in *O. oeni*. (iv) The patterns of transcript levels during growth in mFT80 and in the wine-like medium were similar. In previous works, differential expression patterns were found during

growth [2,16]. While *clpX* is more strongly expressed at the beginning of growth, *hsp18* and *clpL1* expression were highly induced upon entry into the stationary phase [2,16,20]. *clpP* and *trxA* were detected with a slight increase in the *clpP* transcript level at the beginning of growth and a slight increase in the *trxA* transcript level at the stationary phase [20]. The transcript pattern of these genes is comparable during growth in wine (Fig. 4). These five genes seemed to play a specific role during growth, and not only under stress conditions. However, although these genes presented a similar pattern, the quantities of transcripts were higher in wine. The transcript levels at T2 of these five genes were higher in the wine-like medium than in the mid-exponential phase in mFT80 (data not shown). This fact could be explained by an "adaptation state" of cells with high levels of molecular chaperones or proteases. In particular, the molecular chaperones Lo18 and ClpL, encoded respectively by *hsp18* and *clpL* genes, could constitute a protection against protein denaturation, when cells were inoculated into wine. These data suggest that cells maintained a high level of stress genes to survive in wine, as proposed for yeast cells by Zuzuarregui et al. [33].

Among stress genes, the *ctsR* gene encodes the transcriptional regulator of *clp*, *groES*, *grpE* and *hsp18* genes, whose products have chaperonin or proteolytic activities [7]. It is interesting to note that genes which have a CtsR box target (*ctsR*, *clpP*, *clpL2*, *hsp18*, *groES*, and *grpE*) [13] presented different patterns of induction. For example, expression of *hsp18* is induced at low pH and at T16 while *clpP* is not. Our results pointed out that the stationary and stress responses in wine of *O. oeni* appeared to involve regulation processes in addition to the CtsR-dependent regulation mechanism, as previously suggested [2,20]. These unknown regulation mechanisms may involve new transcriptional regulators or the stability of the transcript.

The results obtained provide information on the temporal expression of *O. oeni* genes during growth in a wine-like medium. This molecular approach enables a better understanding of the overall response of *O. oeni* to stress during MLF, and it would be of great interest to continue experiments on gene expression in several strains of different wines, and on other genes.

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References

[1] H. Alexandre, D. Heintz, D. Chassagne, M. Guilloux-Benatier, C. Charpentier, M. Feuillat, Protease A activity and nitrogen fractions released during alcoholic fermentation and autolysis in enological conditions, J. Indus. Microbiol. Biotechnol. 26 (2001) 235–240.

[2] C. Beltramo, C. Grandvalet, F. Pierre, J. Guzzo, Evidence for multiple levels of regulation of *Oenococcus oeni clpP–clpL* locus expression in response to stress, J. Bacteriol. 186 (2004) 2200–2205.

[3] R. Carreté, M.T. Vidal, A. Bordons, M. Constanti, Inhibitory effect of sulfur dioxide and other stress compounds in wine on the ATPase activity of *Oenococcus oeni*, FEMS Microbiol. Lett. 211 (2002) 155–159.

[4] J.F. Cavin, H. Prévost, J. Lin, P. Schmitt, C. Diviès, Medium for screening *Leuconostoc oenos* strains defective in malolactic fermentation, Appl. Environ. Microbiol. 55 (1989) 751–753.

[5] M.E. Corbella, A. Puyet, Real-time reverse transcription-PCR analysis of expression of halobenzoate and salicylate catabolism-associated operons in two strains of *Pseudomonas aeruginosa*, Appl. Environ. Microbiol. 69 (2003) 2269–2275.

[6] M.G. da Silveira, E.A. Golovina, F.A. Hoekstra, F.M. Rombouts, T. Abee, Membrane fluidity adjustments in ethanol-stressed *Oenococcus oeni* cells, Appl. Environ. Microbiol. 69 (2003) 5826–5832.

[7] I. Derré, G. Rapoport, T. Msadek, CtsR, a novel regulator of stress and heat shock response, controls *clp* and molecular chaperone gene expression in Gram-positive bacteria, Mol. Microbiol. 31 (1999) 117–131.

[8] N. Desroche, C. Beltramo, J. Guzzo, Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *Oenococcus oeni*, J. Microbiol. Methods 60 (2005) 325–333.

[9] M. Devers, G. Soulas, F. Martin-Laurent, Real-time reverse transcription PCR analysis of expression of atrazine catabolism genes in two bacterial strains isolated from soil, J. Microbiol. Methods 56 (2004) 3–15.

[10] L.C. Fortier, R. Tourdot-Maréchal, C. Diviès, B.H. Lee, J. Guzzo, Induction of *Oenococcus oeni* H^+-ATPase activity and mRNA transcription under acidic conditions, FEMS Microbiol. Lett. 222 (2003) 165–169.

[11] C. Goerke, S. Campana, M.G. Bayer, G. Doring, K. Botzenhart, C. Wolz, Direct quantitative transcript analysis of the *agr* regulon of *Staphylococcus aureus* during human infection in comparison to the expression profile in vitro, Infect. Immun. 68 (2000) 1304–1311.

[12] M. Graca da Silveira, M. Vitoria San Romao, M.C. Loureiro-Dias, F.M. Rombouts, T. Abee, Flow cytometric assessment of membrane integrity of ethanol-stressed *Oenococcus oeni* cells, Appl. Environ. Microbiol. 68 (2002) 6087–6093.

[13] C. Grandvalet, F. Coucheney, C. Beltramo, J. Guzzo, CtsR is the master regulator of stress response gene expression in *Oenococcus oeni*, J. Bacteriol. (2005), in press.

[14] D.W. Grogan, J.E. Cronan Jr., Cyclopropane ring formation in membrane lipids of bacteria, Microbiol. Mol. Biol. Rev. 61 (1997) 429–441.

[15] J. Guzzo, F. Delmas, F. Pierre, M.P. Jobin, B. Samyn, J. Van Beeumen, J.F. Cavin, C. Diviès, A small heat shock protein from *Leuconostoc oenos* induced by multiple stresses and during stationary growth phase, Lett. Appl. Microbiol. 24 (1997) 393–396.

[16] J. Guzzo, M.P. Jobin, F. Delmas, L.C. Fortier, D. Garmyn, R. Tourdot-Maréchal, B. Lee, C. Diviès, Regulation of stress response in *Oenococcus oeni* as a function of environmental changes and growth phase, Int. J. Food Microbiol. 55 (2000) 27–31.

[17] J. Guzzo, J.F. Cavin, C. Diviès, Induction of stress proteins in *Leuconostoc oenos* to perform direct inoculation of wine, Biotechnol. Lett. 16 (1994) 1189–1194.

[18] M.P. Jobin, F. Delmas, D. Garmyn, C. Diviés, J. Guzzo, Molecular characterization of the gene encoding an 18-kilodalton small heat shock protein associated with the membrane of *Leuconostoc oenos*, Appl. Environ. Microbiol. 63 (1997) 609–614.

[19] M.P. Jobin, D. Garmyn, C. Diviés, J. Guzzo, Expression of the *Oenococcus oeni trxA* gene is induced by hydrogen peroxide and heat shock, Microbiology 145 (1999) 1245–1251.

[20] M.P. Jobin, D. Garmyn, C. Diviès, J. Guzzo, The *Oenococcus oeni clpX* homologue is a heat shock gene preferentially expressed in exponential growth phase, J. Bacteriol. 181 (1999) 6634–6641.

[21] C. Labarre, C. Diviés, J. Guzzo, Genetic organization of the *mle* locus and identification of a *mleR*-like gene from *Leuconostoc oenos*, Appl. Environ. Microbiol. 62 (1996) 4493–4498.

[22] C. Labarre, J. Guzzo, J.F. Cavin, C. Diviés, Cloning and characterization of the genes encoding the malolactic enzyme and the malate permease of *Leuconostoc oenos*, Appl. Environ. Microbiol. 62 (1996) 1274–1282.

[23] A. Lonvaud-Funel, Lactic acid bacteria in the quality improvement and depreciation of wine, Antonie Van Leeuwenhoek 76 (1999) 317–331.
[24] A. Lonvaud-Funel, Biogenic amines in wines: Role of lactic acid bacteria, FEMS Microbiol. Lett. 199 (2001) 9–13.
[25] C. Moch, O. Schrogel, R. Allmansberger, Transcription of the *nfrA–ywcH* operon from *Bacillus subtilis* is specifically induced in response to heat, J. Bacteriol. 182 (2000) 4384–4393.
[26] J.C. Nielsen, C. Prahl, A. Lonvaud-Funel, Malolactic fermentation in wine by direct inoculation with freeze-dried *Leuconostoc oenos* cultures, Am. J. Enol. Vitic. 47 (1996) 42–48.
[27] M. Salema, I. Capucho, B. Poolman, M.V. San Romão, M.C. Dias, In vitro reassembly of the malolactic fermentation pathway of *Leuconostoc oenos* (*Oenococcus oeni*), J. Bacteriol. 178 (1996) 5537–5539.
[28] M. Salema, J.S. Lolkema, M.V. San Romão, M.C. Lourero Dias, The proton motive force generated in *Leuconostoc oenos* by L-malate fermentation, J. Bacteriol. 178 (1996) 3127–3132.
[29] J.W. Sanders, G. Venema, J. Kok, Environmental stress responses in *Lactococcus lactis*, FEMS Microbiol. Rev. 23 (1999) 483–501.
[30] R. Tourdot-Maréchal, D. Gaboriau, L. Beney, C. Diviès, Membrane fluidity of stressed cells of *Oenococcus oeni*, Int. J. Food Microbiol. 55 (2000) 269–273.
[31] S.J. Vandecasteele, W.E. Peetermans, R. Merckx, J. Van Eldere, Expression of biofilm-associated genes in *Staphylococcus epidermidis* during in vitro and in vivo foreign body infections, J. Infect. Dis. 188 (2003) 730–737.
[32] A. Versari, G.P. Parpinello, M. Cattaneo, *Leuconostoc oenos* and malolactic fermentation in wine: A review, J. Ind. Microbiol. Biot. 23 (1999) 447–455.
[33] A. Zuzuarregui, M. del Olmo, Analyses of stress resistance under laboratory conditions constitute a suitable criterion for wine yeast selection, Antonie Van Leeuwenhoek 85 (2004) 271–280.
[34] A. Zuzuarregui, M.L. del Olmo, Expression of stress response genes in wine strains with different fermentative behavior, FEMS Yeast Res. 4 (2004) 699–710.