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Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *Oenococcus oeni*

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Abstract

The expression gene pattern reflects, in part, mechanisms involved in adaptation to environmental conditions. Thus, we established and validated a method that enables relative transcript quantification in different conditions in the lactic acid bacteria *Oenococcus oeni*, notably in a technological medium. First, we determined an internal control in our conditions by reverse transcription quantitative polymerase chain reaction (RT-qPCR) using the SYBR® Green I technology. Among the seven presumed housekeeping tested genes, the *ldhD* gene was retained for further experiments. Then, the PCR reproducibility was verified in our conditions and the comparative critical threshold ($2^{\Delta\Delta C_T}$) method was applied to quantify the transcript level of genes. The quantification of transcript levels of several stress genes already studied in our laboratory by Northern blot after a heat shock and at the entry of stationary phase allowed us to validate this method. RT-qPCR appeared as a powerful tool to study *O. oeni* response in stress conditions and wine mimetic conditions.

Keywords: Lactic acid bacteria; *Oenococcus oeni*; Wine; Internal control; Quantitative PCR; Transcription; Stress

1. Introduction

The expression pattern reflects, in part, mechanisms involved in adaptation to environmental conditions (Bustin, 2000). All adaptive responses imply several genetic switches that control metabolic changes that take place (Abee and Wouters, 1999). Thus, the ability to quantify transcriptional levels of specific genes could be useful to characterize the physiological state and stress response of bacteria. Current methods used to study gene expression, such as Northern hybridization, quantitative competitive polymerase chain reaction (PCR), and RNase protection assays, have a small dynamic range and a lack of sensitivity (Vandecasteele et al., 2001). The reverse transcription quantitative polymerase chain

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reaction (RT-qPCR) is a powerful technique, which allows the quantification of steady-state mRNA levels in bacteria (Goerke et al., 2000; Corbella and Puyet, 2003; Vandecasteele et al., 2003; Devers et al., 2004).

Two quantifications could be used: relative quantification, or absolute quantification (Bustin, 2000; Bustin, 2002). Relative quantification is the analytic method of choice for many real-time PCR studies (Ginzinger, 2002) and it is best applied when there many genes to be tested in many conditions (Freeman et al., 1999). This method requires the use of internal control for data normalisation. Indeed, variations such as the amount of starting material, enzymatic efficiencies, and differences between cultures of overall transcriptional activity can interfere with the final quantification of cDNA levels (Vandesompele et al., 2002). Presumed housekeeping genes are used as internal controls in eukaryotic organisms. The expression of these genes is often considered to fluctuate very little in comparison to others genes. However, in given conditions, their expression can vary considerably (Thellin et al., 1999; Vandecasteele et al., 2001; Vandesompele et al., 2002). There is no consensus for internal control in bacteria. The most frequent strategy implies the determination of an internal control gene to normalize mRNA fractions for each study.

In this work, we presented a method to define an internal control for relative quantification of transcript levels in stress conditions and in wine mimetic conditions in the lactic acid bacterium *Oenococcus oeni*. This bacterium is mainly responsible for malolactic fermentation (MLF). During winemaking, the MLF follows alcoholic fermentation carried out by yeasts. This step decreases the total acidity of wine and also improves microbiological stability and organoleptic characteristics (Versari et al., 1999). Wine is a medium with very harsh environmental conditions: low pH, high alcohol content, and high concentrations of SO_2 (Versari et al., 1999). The capacity of *O. oeni* to survive and grow in wine is essential for MLF, and requires several mechanisms involving the generation of a proton motrice force (Salema et al., 1996), activation of membrane-bound H^+-ATPases (Carreté et al., 2002), modification of membrane fluidity (Tourdot-Maréchal et al., 2000; da Silveira et al., 2003), and stress protein synthesis (Guzzo et al., 2000). Previous studies concerning *O. oeni* stress responses and MLF have used physiological and biochemical techniques. This approach consists in validation of a molecular method to characterize *O. oeni* transcripts levels in several stress conditions and technological medium.

The aim of this work is to explore transcription of presumed housekeeping genes to determine a reference for further studies. The validation of the RT-qPCR method using the SYBR® Green I technology to study relative transcript level after a heat shock and at the entry of stationary phase was performed.

2. Materials and methods

2.1. Bacterial yeast strains, growth conditions, and stress conditions

Oenococcus oeni IOB 8413 was grown at 30 °C in FT80 medium (pH 5.3), modified by the addition of meat extract instead of casamino acids. The stationary phase ($OD_{600\ nm}$=1.8) was obtained after 24 h of growth. For stress applications, when the cells were in midexponential phase ($OD_{600\ nm}$=0.6), absolute ethanol was added to 11% vol/vol, or 1 N HCl was added until pH 3.5, or cells were incubated at 18 °C, or cells were incubated at 42 °C, respectively, for ethanolic shock, acidic shock, cold shock, and heat shock. Each treatment was applied for 30 min.

Before inoculation in wine, the midexponential phase cells were inoculated ($OD_{600\ nm}$=0.2) in FT80 medium at pH 3.5 and incubated at 30 °C until cells reached an OD of 0.6.

The *Saccharomyces cerevisiae* strain BRG 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. Synthetic wine and malolatic fermentation

Wine used for the experiments was prepared using synthetic musts, which 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}); the pH was adjusted to 3.5 with NaOH. Must was inoculated at a rate of

10^6 CFU ml^{-1} with a 24-h-old *S. cerevisiae* strain BRG and the alcoholic fermentation was performed at 20–22 °C for 16 days. Then, wine was clarified by filtration through a 0.22-μm pore size filter (Millipore, France) to remove yeasts. After filtration, the physicochemical properties of wine were as follows: 10.5% (vol/vol) ethanol, 4.6 g l^{-1} L-malic acid, pH 3.5. The wine was stored at 4 °C.

MLF was initiated by direct inoculation with *O. oeni* IOB 8413 to a final concentration of 2×10^6 CFU ml^{-1} (1 UDO=5×10^8 CFU ml^{-1}). A control without inoculation was performed to verify the absence of spontaneous MLF starts. Glass containers were incubated at 18 °C—a temperature similar to cellar temperature.

2.3. RNA extraction

Total RNAs were extracted using 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 0.1% dimethylpyrocarbonate (DMPC)-treated water. RNA concentrations were calculated by measuring absorbance at 260 nm using a SmartSpec Plus Spectrophotometer (Bio-Rad).

2.4. Reverse transcription and real-time PCR

Before the reverse transcription, RNAs (2 μg of total RNA) were treated with 2 U of DNase (Invitrogen) as described by the manufacturer. Then, cDNAs were synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) as recommended. The absence of chromosomal DNA contamination was checked by real-time PCR.

Primers for real-time PCR were designed in order to have a length of about 20–25 bases, a G/C content of over 50%, and a T_m of about 60 °C. The length of the PCR products ranged between 90 and 160 bp. Primer design software (Primer3) was used to select primer sequences. Secondary structures and dimers formation were controlled with the Oligo Analyzer 1.0.3 Software. Primers were purchased from Invitrogen.

Real-time PCRs were 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

Table 1
Primers sequences

Symbol	Forward primer (5′ →3′)	Reverse primer (5′ →3′)	Amplicon length (bp)
ldhD	GCCGCAGTAAAGAACTTGATG	TGCCGACAACACCAACTGTTT	102
gyrA	CGCCCGACAAACCGCATAAA	CAAGGACTCATAGATTGCCGAA	95
gyrB	GAGGATGTCCGAGAAGGAATTA	GCCTGCTGGGCATCTGTATTA	107
rpoD	CGTGATCCAATGCCTGAAGAAA	GGAGTTTCCAAAGAGACAGGTT	104
dnaG	TGTGGACGGAGTGGCAATGT	CGGTATTTTCTGTATATTTACTATCG	127
glk	GATGGTTCTCATATTGTTCCT	ACCAGGCGTTCCCATTCCAAT	108
rrs	TCGTAAAGCACTGTTGTAAGG	GGATAACGCTCGGGACATACG	132
groES	GCCACAACAGAACCCATCACTGGTT	GGCGATCGAATTGTTCTTAGTAT	106
grpE	CGCAGGCAGAAAAGAACAATC	GCTGAAGACGAAGCAGTTGC	126
ctsR	GGGCCATGGCAGAAGCTAATATTTCAG	AAACGGGTGTTGATTACATAATT	147
clpL2	ATTATAATGACGATCCCTTCGT	GGATCCCTGAACCGTTATTTGCTTGTTG	100
clpP	CGGTACCAAAGGCAAGCGTTTTAT	CTCTTCCGAGTCTTCAAAAGTTGAT	131
clpX	GGGTGTTTATATTTGTGACGAATG	GGGTGTTTATATTTGTGACGAATG	110
clpL1	ATTATAATGACGATCCCTTCGT	GGATCCCTGAACCGTTATTTGCTTGTTG	163
hsp18	CGGTATCAGGAGTTTTGAGTTC	CGTAGTAACTGCGGGAGTAATTC	102

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. This was used to verify the specificity of real-time PCR reaction for each primer pair. Efficiency of amplifications was 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. The efficiency E can be calculated by the formula $E=[10^{(1/-s)}-1]\times 100$, where s is the slope of standard curve.

For each measurement, a threshold cycle value (C_T) was determined. This was defined as the number of cycles necessary 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 (RFU).

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 (internal target RNA). The following equations were used ("calibrator" refers to reference environmental conditions and "sample" defines tested environmental conditions):

$$\Delta C_T = C_T \text{ of internal control} - C_T \text{ of gene of interest}$$

$$\Delta\Delta C_T = \Delta C_T \text{ of sample} - \Delta C_T \text{ of calibrator}$$

Relative expression level=$2^{\Delta\Delta C_T}$.

In order to determine an internal control, the fold changes were calculated using the $2^{\Delta C_T}$ method as previously described (Livak and Schmittgen, 2001).

2.5. Nucleotide sequence accession numbers

The nucleotide sequences have been submitted to the nucleotide sequence database/EMBL under accession numbers: AJ606044 (*clpP*, *clpL1*), Y15953 (*clpX*), X99468 (*hsp18*), AJ831540 (*ldhD*), AJ831541 (*gyrA*), AJ831542 (*gyrB*), AJ831543 (*rpoD*), AJ831544 (*dnaG*), AJ831545 (*glk*), AJ831548 (*rrs*), AJ831549 (*ctsR*), AJ831550 (*grpE*), AJ831551 (*groES*), and AJ831552 (*clpL2*).

3. Results

3.1. Determination of an internal control for RT-qPCR experiments in O. oeni

Previous studies have highlighted the difficulty of finding a good internal control for experiments and its importance (Vandecasteele et al., 2001; Bustin, 2002). The determination of such a control requires several steps: choose presumed housekeeping genes, verify that their expression was not affected by the experimental conditions, and ensure that the PCR efficiencies of the internal control and all tested genes are similar and above 90% (Ginzinger, 2002).

In order to determine a reference for further gene expression experiments with *O. oeni*, seven genes were chosen: (i) two genes encoding enzymes of sugar metabolism (the *ldhD* gene encoding the D-lactate deshydrogenase and the *glk* gene encoding the glucose kinase); (ii) four genes of two loci-encoding enzymes necessary for cell division or DNA transcription, namely *gyrA* and *gyrB* encoding gyrase subunits, *rpoD* encoding the transcriptional regulator sigma, and *dnaG* encoding the primase; and (iii) the *rrs* gene encoding the 16*S* RNA (Table 2).

Their transcript levels were measured in exponential phase, in stationary phase, and in different stress conditions: heat shock (42 °C) that is the reference for stress application, cold shock (18 °C), ethanolic shock (11% vol/vol), and acidic shock (pH 3.5). These three conditions corresponded to wine stress.

Table 2
Functions of genes used for the determination of an internal control

Gene	Product	Known essential functions
ldhD	D-lactate dehydrogenase	Sugar catabolism pathway
gyrA	Gyrase subunit A	DNA replication, supercoiling of chromosomal DNA
gyrB	Gyrase subunit B	DNA replication, supercoiling of chromosomal DNA
rpoD	Sigma δ factor	Binding of RNA polymerase and initiation of transcription
dnaG	Primase	Initiation of chromosomal DNA replication
glk	Glucokinase	Kinase involved in maintenance of the pool of glucose
rrs	16*S* RNA ribosomal subunit	Structural component of the 16*S* ribosomal subunit

In addition, their transcript levels were measured in synthetic wine to determine the effect of multiple stresses medium. After conduction of alcoholic fermentation on synthetic must, wine was directly inoculated with 2×10^6 cells ml^{-1} from culture in FT80 at pH 3.5. No loss of viability was observed. *O. oeni* started to grow on the first day and MLF was completed in 16 days. After 6 days of MLF (when the population reached 2×10^7 CFU ml^{-1}), RNA extractions were realized.

For each condition, cDNAs were normalized with equal amounts of total RNA and real-time PCRs were performed. Then, C_T values were determined from two independent cultures. Averages of C_T were given in Fig. 1. The specificity of each primers pair was controlled by melt curves, and the mean of efficiencies for the seven primers pairs used was $93\pm4\%$. In order to determine if transcript level was affected by the experimental conditions, the fold change in transcript level was calculated using the $2^{\Delta C_T}$ method.

As shown in Table 3, all transcripts levels but one (*rrs*) presented the same evolution: they decreased in stationary phase. The highest variation was obtained for *dnaG*; its transcript level was 76-fold fewer than in exponential phase and 934-fold fewer than after heat shock. The growth fold changes calculated for *glk*, *ldhD*, *gyrA*, *gyrB*, and *rpoD* were similar (approximately 17-fold change). Variations obtained ($2^{\Delta C_T}$) for *rrs* transcript levels in the different conditions are weak (five as maximal fold change). However, the transcript level of this gene was 1000-fold higher than the level of other genes (Fig. 1). Even if this gene seemed to have a relatively constant transcript level, it is not representative of mRNA fraction (see Section 4).

For others genes, effects of stress application were variable. The *dnaG* and *rpoD* transcript levels presented the highest variations; their stress fold changes were, respectively, 83 and 50. The *gyrA* and *gyrB* transcripts levels presented the same evolution whatever the stress conditions were. Their fold changes were similar to those obtained for *glk* gene (13- to 20-fold), but were weaker than those of *dnaG* and *rpoD*.

Among the seven genes, the *ldhD* transcript level was weakly affected by tested conditions (31 as maximal fold change), particularly by stress conditions (fivefold change). This gene has a relatively constant transcript level compared to other genes. The *ldhD* gene was chosen as internal control for our further experiments. In addition, the *ldhD* transcript C_T was between 20 and 30—values recommended to avoid primer–dimer formation and nonspecific amplifications (Peters et al., 2004). It could be noted that the *gyrA* gene could also be used for studies with multiple internal controls in stress conditions.

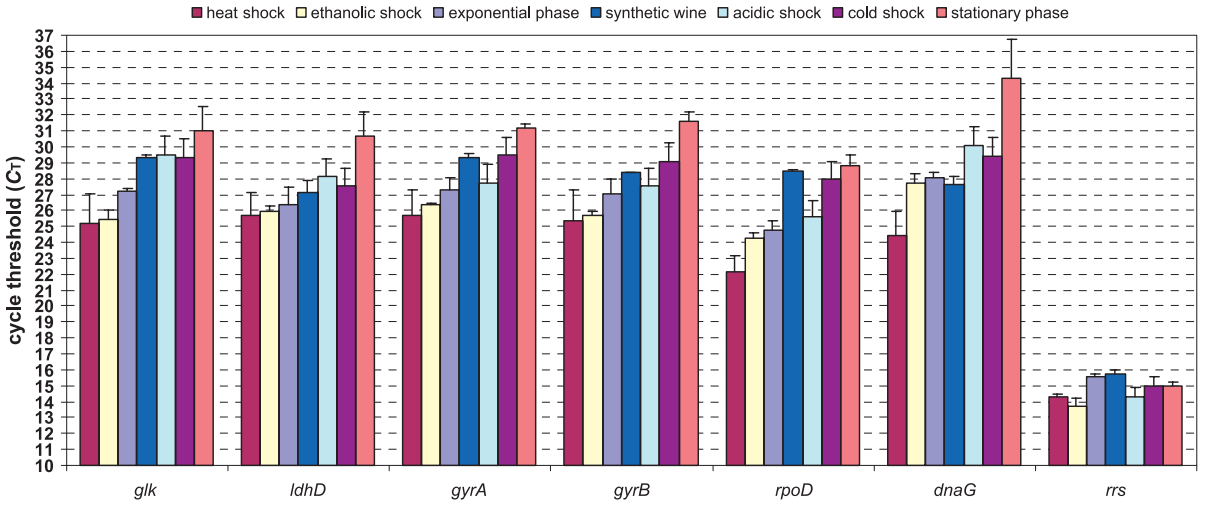

Fig. 1. Histogram of the cycle threshold (C_T) of seven presumed housekeeping genes determined in seven different conditions: exponential phase, heat shock (42 °C), ethanolic shock (11% vol/vol), acidic shock (pH 3.5), cold shock (18 °C), synthetic wine, and stationary phase. For each condition, C_T was measured from two independent cDNA; the means are represented in the histogram.

Table 3
Effect of stress application on the seven presumed housekeeping genes used for the determination of an internal standard

Gene	*glk*	*ldhD*	*gyrA*	*gyrB*	*rpoD*	*dnaG*	*rrs*
Maximal fold change	54	31	45	77	100	934	5
Growth fold change	13	19	14	24	16	76	1
Stress fold change	20	5	15	13	83	50	5

Maximal fold change in gene expression ($2^{\Delta C_T}$) was calculated from the highest variations obtained for all conditions. Growth fold change was calculated from the variations obtained between exponential and stationary phases. Stress fold change was calculated from the maximal variations obtained between following conditions: heat shock (42 °C), ethanolic shock (11% vol/vol), exponential phase, synthetic wine, acidic shock (pH 3.5,) and cold shock (18 °C).

3.2. Establishment of comparative critical threshold method ($\Delta\Delta C_T$) in a technological medium (synthetic wine)

Few studies have reported variations between different quantitative PCRs realized with cDNAs extracted from independent cultures. In order to test reproducibility between RT-qPCRs in our conditions, we used cDNA synthesized from RNA extracted from three independent cultures. The application and reproducibility of the comparative critical threshold method ($\Delta\Delta C_T$) were tested in a synthetic wine. Changes in *gyrA* transcript level normalized to the *ldhD* gene were measured on cDNA obtained from cells grown during 6 days in synthetic wine. Real-time PCRs were performed in triplicate for each sample of cDNA. The relative transcript level was normalized to *ldhD* gene and was calibrated to the weaker expression (cDNA 2; see Table 4).

The mean of C_T, standard deviations (S.D.), and coefficients of variation (CV) were calculated. The results are given in Table 4. For the three cDNA, intraassay and interassay CVs of C_T were about 3%. These values allow us to validate the reproducibility of the method under our conditions. Concerning the ΔC_T, intraassay CVs ranged between 2.4% and 6.6%, and interassay CVs were about 9.1%. Not surprisingly, intraassay CVs calculated from the relative expression level ($\Delta\Delta C_T$) are higher than CVs obtained from the ΔC_T. They were comprised between 14% and 20%. Thus, CVs increased at each step of calculation of the relative expression level. For the relative transcript level, CV of interassay (three independent experiments) was about 26%.

These results permitted us to determine a criterion to consider a variation as physiological or due to experiment. We observed that S.D. of C_T obtained for the three cDNA was less than one (Table 4). Moreover, the difference ($2^{\Delta\Delta C_T}$) between *ldhD* level and *gyrA* level (two genes susceptible for use as internal control) was less than twofold (1.353 ± 0.355). Thus, in our experiments, we considered that a ΔC_T higher than one was a physiological change. In other words, genes were considered downregulated or upregulated if their level was found to be at least twofold (relative expression level=2^1 or 2^{-1}) fewer or higher than the calibrator condition.

3.3. Application of the RT-qPCR to study the response of *O. oeni* to heat shock and during stationary phase

The aim of the development of an RT-qPCR method in *O. oeni* was to study the stress response. Analysis of genome and previous studies allows us to select several stress genes to validate the method. They were as follows: (i) the *grpE* and *groES* genes, which encoded well-known universal cochaperonins; (ii) five members of the Clp family: the two genes of the locus *clpP-clpL1*, the *clpX* gene, the *ctsR* gene, and the *clpL2* gene; and (iii) the *hsp18* gene encoding a small HSP.

The transcript levels of these eight genes were measured by RT-qPCR during the exponential phase, during the stationary phase, and after heat shock (42 °C). The *ldhD* gene and the expression during exponential phase were used, respectively, as internal control and as calibrator used to calculate the relative expression levels (Fig. 2).

For all genes, as expected, we observed an increase of the transcript level after heat shock. The *clpX* transcript level was increased only twofold. The *groES*, *grpE*, and *ctsR* transcript level increases (sevenfold) were weaker than those obtained for *clpL1* (39-fold), *clpP* (14-fold), *clpL2* (30-fold), and *hsp18*. The highest increase was obtained for the latter; its level after heat shock was approximately 100-fold higher than in exponential phase.

Table 4
Reproducibility of RT-qPCR and the comparative critical threshold ($\Delta\Delta C_T$) methods

Sample	C_T *ldhD*	C_T *gyrA*	ΔC_T (C_T *ldhD*− C_T *gyrA*)	$\Delta\Delta C_T$ (ΔC_T assay− ΔC_T calibrator)	Relative expression level
cDNA 1	21.91	26.00	−4.08	0.14	1.10
	23.01	26.98	−3.96	0.71	1.63
	23.02	27.44	−4.41	0.39	1.31
Mean	22.65±0.63	26.81±0.73	−4.15±0.23		1.35±0.26
Intraassay CV (%)	2.82	2.75	5.61		19.72
cDNA 2	21.55	25.79	−4.23	0.000	1.00
	21.97	26.65	−4.68	0.000	1.00
	23.21	28.03	−4.81	0.000	1.00
Mean	22.24±0.86	26.82±1.13	−4.57±0.30		1.00±0.00
Intraassay CV (%)	3.89	4.22	6.62		
cDNA 3	22.40	26.13	−3.72	0.50	1.42
	22.91	26.73	−3.81	0.86	1.81
	23.75	27.66	−3.91	0.90	1.87
Mean	23.02±0.68	26.84±0.77	−3.82±0.09		1.70±0.24
Intraassay CV (%)	2.95	2.87	2.41		14.3
Interassay mean	22.64±0.72	26.82±0.77	−4.186±0.383		1.353±0.355
Interassay CV (%)	3.18	2.90	9.15		26.21

cDNAs were synthesized from RNA extracted from wine. Three independents real-time PCRs were performed with each sample of cDNA. C_T data were calculated with iCycler™ IQ Optical System Software Version 3.0a. The relative expression level of *gyrA* gene was normalized to *ldhD* gene and the weaker expression of the three cDNAs was used as the calibrator.

In stationary phase, profiles obtained for the eight genes were different. Four groups can be distinguished: (i) the *clpX* level was repressed twofold; (ii) the *groES*, *grpE*, *ctsR*, and *clpP* levels were not, or weakly, affected by this physiological state; (iii) the *clpL2* level increased eightfold higher than in exponential phase;

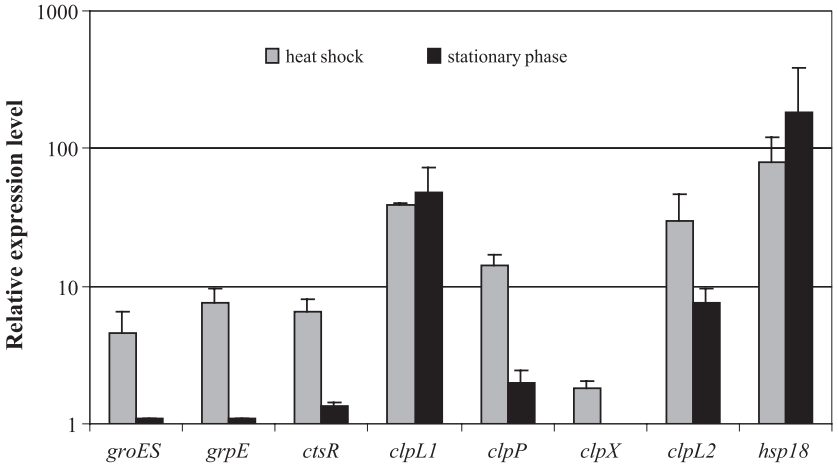

Fig. 2. Relative expression levels of several stress genes of *O. oeni* after heat shock (42 °C) and during stationary growth phase. Gene expression is quantified using RT-qPCR and the comparative critical threshold ($\Delta\Delta C_T$) method. The *ldhD* gene was used as the internal control, and expression during exponential phase was used as the calibrator. Two independent experiments were performed; vertical bars indicate standard deviations.

and (iv) the *clpL1* and *hsp18* levels were strongly increased, respectively, 50-fold and 180-fold.

4. Discussion

The aims of this present study were: (i) to adapt a simple, sensitive, and reproducible method for the determination of relative transcript level quantification in the lactic acid bacteria *O. Oeni*; and (ii) to study the transcript level of several stress genes during stationary phase and after heat shock.

The first step of the development of an RT-qPCR method was the determination of an internal control, which could be used to study the transcript level in different conditions. The induction rates were measured with an internal control in order to calculate the amounts of mRNA as accurately as possible (Moch et al., 2000). Previous studies clearly showed that there was no ideal and universal control gene (Vandecasteele et al., 2001; Bustin, 2002; Vandesompele et al., 2002). For eukaryotes, several genes are used as internal control, such as the gene encoding GADPH, actins, or 18*S* rRNAs (Thellin et al., 1999), but there is no consensus for prokaryotic internal control. Indeed, it has been shown that the expression of presumed housekeeping genes varied during cell growth and after heat shock (Vandecasteele et al., 2001; Corbella and Puyet, 2003). Thus, the transcript level of seven presumed housekeeping genes was measured in seven conditions (different stress conditions and cell growth). Previous studies have proposed amounts of 16*S* rRNA as internal control, but other studies have shown that the *rrs* transcript level was dependent on the state of bacteria (Condon et al., 1995). The RNA mass consists predominantly of rRNA molecules (95% quantity of all RNA) and is not always representative of mRNA fraction. The normalization of mRNA quantity with rRNA quantity could contain a bias. Indeed, our results have shown that the transcript levels of rRNA were 1000-fold higher than the transcript levels of mRNAs. Moreover, the rRNA pattern evolution was different compared to the other genes. For these reasons, even if the variations of the *rrs* transcript level were among the weakest, this gene was not chosen as internal control. Since the *ldhD* transcript levels were weakly affected by tested conditions, this gene was used as internal control. However, the *gyrA* gene could also be used. On the other hand, it could be noted that it is difficult to choose an internal control for the stationary phase (Vandecasteele et al., 2001) because global metabolism decreased. Indeed, our results have shown that the *rpoD* transcript level decreased in stationary phase. This led us to think that the global transcription is reduced in this condition. This phenomenon could explain the high transcript level differences found between the exponential phase and the stationary phase.

The results have shown that the RT-qPCR is reproducible under our conditions and can be adapted to study transcript level in a synthetic wine, close to technological medium. Indeed, cDNAs were synthesized from RNA extractions from this medium, and real-time PCR reactions were performed successfully with all the genes tested. The variations obtained from independent experiments were in accordance with previous studies (Livak and Schmittgen, 2001; Rajeevan et al., 2001). Moreover, the criterion defined to consider a variation as significant is in accordance with a previous study (Beenken et al., 2004).

The RT-qPCR method was used to study heat shock and stationary phase responses. The transcript levels of eight stress genes were analysed. Previous results obtained for *hsp18*, *clpX*, *clpP*, and *clpL1* genes were confirmed, which allow us to validate our method (Jobin et al., 1999; Beltramo et al., 2004). Their transcript levels increased greatly after heat shock. Moreover, *clpL1* and *hsp18* transcript levels increased at the entry of stationary phase contrary to *clpP* and *clpX* transcript levels. The *clpL1* and *hsp18* transcript levels, encoding, respectively, a small heat shock protein called Lo18 and a Clp ATPase ClpL, appeared as markers of the stationary phase. Moreover, when change folds were compared, it seemed that different mechanisms were implied in the regulation of stress genes. A regulation mechanism seemed to act for *clpL1*, *clpL2*, and *hsp18* transcripts levels in the stationary phase.

To conclude, RT-qPCR appeared as a rapid and sensitive method used to determine the relative transcript level of *O. oeni* during MLF in synthetic wine and could be used for studies of other lactic acid bacteria with industrial interests such as dairy starters.

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