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Regulation of stress response in *Oenococcus oeni* as a function of environmental changes and growth phase

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Abstract

Oenococcus oeni is a lactic acid bacterium which is able to grow in wine and perform malolactic fermentation. To survive and grow in such a harsh environment as wine, *O. oeni* uses several mechanisms of resistance including stress protein synthesis. The molecular characterisation of three stress genes *hsp18*, *clpX*, *trxA* encoding for a small heat shock protein, an ATPase regulation component of ClpP protease and a thioredoxin, respectively, allow us to suggest the existence in *O. oeni* of multiple regulation mechanisms as is the case in *Bacillus subtilis*. One common feature of these genes is that they are expressed under the control of housekeeping promoters. The expression of these genes as a function of growth is significantly different. Surprisingly, the *clpX* gene, which is induced by heat shock, was highly expressed in the early phase of growth. In addition to stress protein synthesis, adaptation to the acid pH of wine requires efficient cellular systems to extrude protons. Using inhibitors specific for different types of ATPases, we demonstrated the existence of H^+-ATPase and P-type ATPase.

Keywords: *Oenococcus*; Malolactic fermentation; Stress gene; ATPase

1. Introduction

Oenococcus oeni is a lactic acid bacterium which shares an acidophilic behaviour. This bacterium is able to grow in wine at an acidic pH, in the presence of ethanol and sulphite. *O. oeni* is most often responsible for malolactic fermentation (MLF) in wine. MLF is a secondary fermentation occurring in wine after completion of alcoholic fermentation performed by yeast. This step of wine fermentation is crucial for many vinification processes (Kunkee, 1991). It is the bacterial conversion of L-malate into L-lactate and CO_2 and leads to a natural decrease of acidity together with an enhancement of stability and quality of wine.

In winemaking processes, MLF is a step that is not completely controlled. The hostile environment of wine can lead to a delay in starting of MLF. One of

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our aims is to understand the genetic and physiological processes involved in the stress response from this bacterium. The results obtained may contribute to control the conditions of *O. oeni* preadaptated starter culture production to optimise direct inoculation in wine. Our work also contributes to knowledge of stress response in lactic acid bacteria.

Among the cellular mechanisms involved for *O. oeni* survival in wine, three appear to play a key part:

1. MLF which is involved in proton motive force generation and in the maintenance of internal pH by proton consumption during the L-malate decarboxylation step. MLF has been extensively studied in different ways as L-malate transport (Salema et al., 1994), energetic of the reaction (Olsen et al., 1991), biochemical aspects of the malolactic enzyme (Caspritz and Radler, 1983) and molecular characterisation of the *mle* locus (Labarre et al., 1996a,b).
2. The ATPase systems that function as proton extruding pumps. Little information is available on the implication of ATPase in acid tolerance of *O. oeni*. Higher ATPase activity at low pH was observed within an acid-resistant mutant of *O. oeni* (Drici-Cachon et al., 1996). In *Streptococcus mutans* and *Enterococcus hirae*, acid tolerance could be related directly to levels of H^+-ATPase activity (Belli and Marquis, 1991).
3. The stress protein synthesis is part of a typical heat shock like response induced by the wine medium. Survival of *O. oeni* in wine and its ability to perform MLF was improved by pretreatment of cells at 42°C (Guzzo et al., 1994). This temperature is known to induce synthesis of stress proteins in *O. oeni* (Guzzo et al., 1997). Many of these proteins function as molecular chaperones or proteases that could participate in the refolding or degradation processes of denatured proteins in the cell (Craig et al., 1993).

In this paper, we focused on the regulation of stress genes expression in *O. oeni*. The three genes studied are the following: *hsp18* that encodes for a small heat shock protein called Lo18 (Horwitz, 1992; Guzzo et al., 1994); *clpX* that encodes for an ATPase regulation component of ClpP protease (Gottesman et al., 1993); *trxA* that encodes for a thioredoxin (Holmgren, 1985; Jobin et al., 1999). In a second part, recent results concerning ATPase activities are described and their involvement in the *O. oeni* acid-tolerance are discussed.

2. Transcriptional analysis of stress genes

Total RNA was prepared from *O. oeni* cells submitted or not to heat shock at 42°C. Northern blot analysis of the *hsp18* gene expression revealed a single transcript of 600 nucleotides that was detectable only in heat-shocked cells (Jobin et al., 1997). In contrast, *trxA* and *clpX* genes were faintly expressed in normal growth conditions and their expression appears largely enhanced by heat shock (Fig. 1). So, the expression of these genes was temperature dependent and appeared to be regulated at the transcriptional level. Maximal rate of induction was observed after 5 min of incubation at 42°C for *clpX* and *hsp18* and after 60 min for *trxA*. *hsp18* which is not expressed under normal growth conditions may have no essential housekeeping function.

3. Promoter regions

Primer extension analysis allowed us to identify the putative promoter regions of the three genes. All the promoters identified showed a high similarity to the consensus sequence found in vegetative promoter from gram positive bacteria as well as housekeeping promoter from *E. coli* (Fig. 2). This result goes against the hypothesis of the control of expression by an alternative sigma factor, known to occur for heat shock regulon of *E. coli* (Mager and de Kruijff, 1995) or the genes of class II of *B. subtilis* (Hecker and Völker, 1998). Moreover no CIRCE element (Hecker et al., 1996) was present in the region of these promoters. However, the *hsp18* promoter region presents two directly repeated sequences that overlap the -35 and -10 housekeeping promoter sequence. These repeated heptanucleotide sequences have been identified as potential CtsR binding sites (Derré et al., 1999). This sequence analysis suggests that *hsp18* could be regulated by a repression mechanism involving a CtsR-like regulator.

In the case of *trxA*, the regulator elements allow-

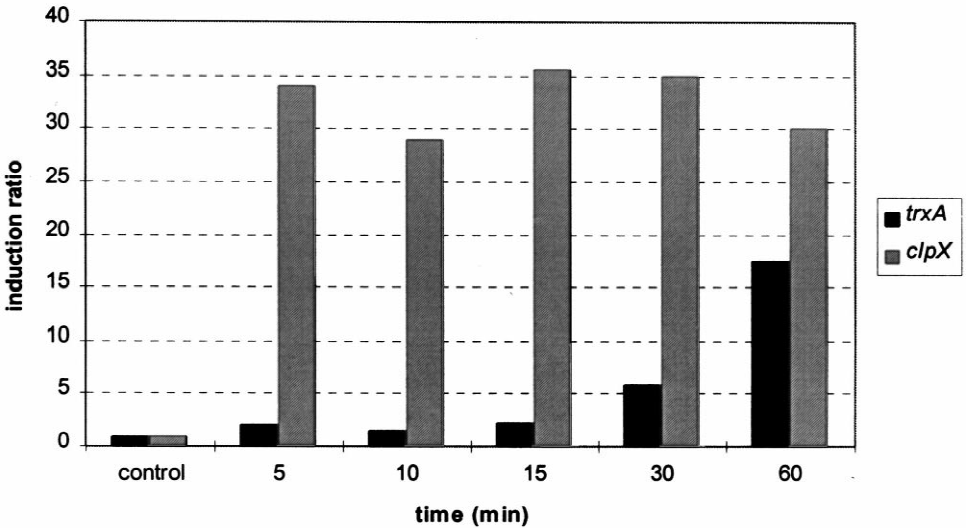

Fig. 1. *clpX* and *trxA* mRNAs induction during heat shock. Northern-blot hybridisations were performed with total RNA isolated before (control) and at different times (5, 10, 15, 30 and 60 min) of exposure at 42°C. The diagram shows the relative amounts of detected mRNAs with respect to control levels.

	-35		-10
consensus	TTGACA	-17-	TATAAT
hsp18	TTGACT	-17-	TATTAT
clpX	TTCTCG	-18-	AATAAT
trxA	TTGCAT	-17-	TACAAT

Fig. 2. Alignment of promoter regions from *O. oeni* stress genes. The putative −35 and −10 promoter sequences determined for *trxA*, *clpX* and *hsp18* genes are compared with the consensus promoter sequence of housekeeping genes from gram-positive bacteria as well as *E. coli*. Identical nucleotides are shaded.

ing induction at high temperature are unknown. Finally, as *trxA*, *clpX* could be regulated at the transcriptional level by a regulatory mechanism not yet identified. But the 5′ untranslated region (5′-UTR) of *clpX* mRNA is very long (408 nucleotides). Such mRNA 5′-UTR has been shown to interfere with the stability of mRNA in a growth dependent manner (Emory and Belasco, 1990) and after cold shock (Fang et al., 1998). Consequently, the increase of *clpX* mRNA detection by Northern blot after heat shock may involve a post-transcriptional regulation occurring at the long 5′-UTR *clpX* mRNA.

4. Stress gene expression as a function of growth

Detection of specific mRNA allowed us to evaluate the expression rate of these genes during the different stages of growth (Fig. 3). *clpX* appeared to be highly expressed at the early phase of growth and during the exponential growth phase. Then at the beginning of the stationary growth phase the quantity of messenger decreased until it became undetectable at the late stationary growth phase. On the other hand, *hsp18* mRNA was only detected at the end of the exponential phase but not at the beginning of the growth. Then the signal reached a maximum during the late stationary phase. Concerning *trxA*, the messenger was detected at a significant level at all stages of growth without significant difference in the intensity of the signal. Clearly, these three heat shock genes were expressed in different ways as a function of growth.

5. Characterisation of ATPase activities

The isolation of a spontaneous mutant of *O. oeni* defective in membrane-bound ATPase activity was

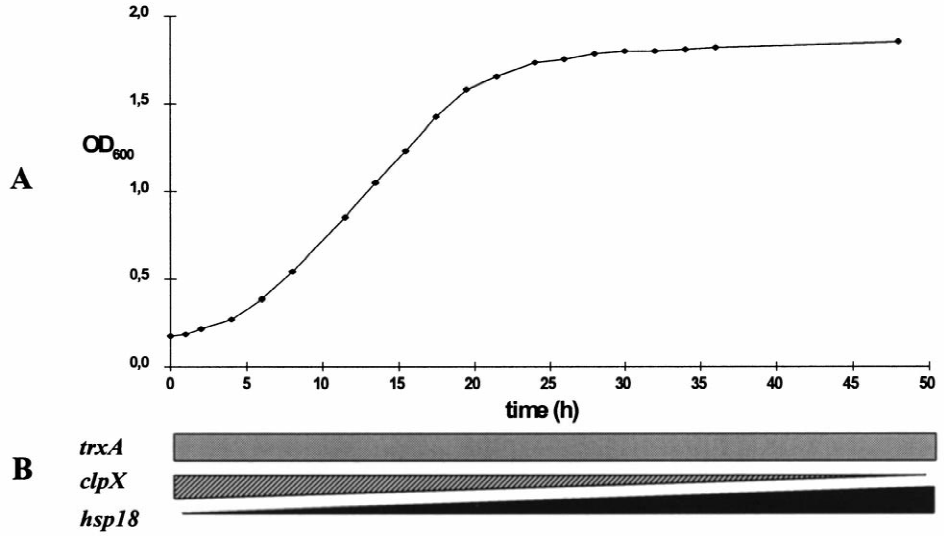

Fig. 3. Schematic representation of the expression of *trxA*, *clpX* and *hsp18* as a function of growth phase. The total RNA was extracted during the different stages of *O. oeni* growth (A) and submitted to Northern-blot analysis. The relative amounts of *trxA*, *clpX* and *hsp18* mRNAs are symbolised by the thickness of the bars (B).

performed using neomycin sulfate as selective marker. A rapid death of the mutant was observed at low pH suggesting a crucial role of ATPase in acid-tolerance of this strain (Tourdot-Maréchal et al., 1999). The same results were obtained for acid sensitivity of variants of lactic acid bacteria like *Lactococcus lactis* (Yokota et al., 1995) and *Lactobacillus helveticus* (Yamamoto et al., 1996) with reduced membrane-bound H^+-ATPase activities. Nevertheless, no significant increase of ATPase activity was observed in *O. oeni* cells incubated at low pH. This absence of induction could be explained by the existence of several ATPase systems whose maximal activities depend on the pH value of the medium. The use of specific inhibitor agents of different type of ATPase allowed us to suggest the existence of H^+-ATPase and P-type ATPase. Membrane fraction from *O. oeni* was purified as previously described (Tourdot-Maréchal et al., 1999) and ATPase activity was determined (Tourdot-Maréchal et al., 1999). The total activity was inhibited most efficiently by 1 mM *N,N′*-dicyclohexylcarbodiimide DCCD (80%) and to a lesser extent by 1 mM vanadate (20%). These results led us to conclude that the cell membrane of *O. oeni* harbour at least two ATPase activities which can be distinguished on the basis of their differential sensitivity to inhibitors. The major activity could be ascribed to the H^+-F_1F_0-ATPase which is known to be sensitive to DCCD. Little inhibition by vanadate may indicate the existence of P-type ATPases probably, a K^+-ATPase. The genes encoding the H^+-ATPase and the K^+-ATPase have been cloned from an *O. oeni* genomic bank and the molecular characterisation is currently being done.

6. Conclusion

We have identified for *O. oeni* three genes *hsp18*, *clpX* and *trxA* that all encode heat shock proteins. Theses genes are expressed in different ways as a function of growth. The detection of *hsp18* transcript at the entrance of stationary growth phase and after 24 h in stationary growth phase suggests that the product of this gene could be a good marker of this stage of growth. On the contrary, the high expression of *clpX* gene in the exponential growth phase may suggest a biological implication of ClpX protein in early growth stage.

Moreover, several regulation mechanisms allowing stress gene expression appear to exist in *O. oeni*. *hsp18* could involve CtsR mediated repression mechanism like genes of the class III of *B. subtilis*, whereas *clpX* regulation may occur at the post-transcriptional level. The mechanisms allowing *trxA*

gene expression are still unknown as the genes of the class IV of *B. subtilis*.

Acid pH is one of the major stresses inhibiting *O. oeni* growth in wine. Generally, bacteria should extrude H^+ at acidic pH (Booth, 1985). The high acid-sensitivity of a spontaneous mutant deficient in H^+-ATPase activity suggest that in *O. oeni*, H^+-ATPase could play a central role in the regulation of the intracellular pH. H^+ extrusion by ATP hydrolysis would be necessary to maintain a proton motive force and to regulate the intracellular pH. Nevertheless, other cation transport ATPases, such as K^+-translocating ATPase, could participate in the pH homeostasis mechanism (Kroll and Booth, 1983). Using inhibitors specific for both types of ATPase, we succeeded in demonstrating the existence of H^+-ATPase and P-type ATPase. This finding was confirmed by a molecular approach.

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