Expression of the *Oenococcus oeni* *trxA* gene is induced by hydrogen peroxide and heat shock

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Sequencing of the DNA region located upstream of the α -acetolactate synthase and decarboxylase (*alsS*–*alsD*) cluster of *Oenococcus oeni* allowed identification of an ORF, named *trxA*. This encodes a protein of 104 amino acids very similar to known thioredoxins. The protein encoded by the cloned fragment was able to complement *Escherichia coli* strains lacking a functional thioredoxin. Considering the results of protein sequence comparisons and complementation experiments, it was concluded that the *trxA* gene encodes a functional thioredoxin. Studies of *trxA* expression showed that the abundance of *trxA* mRNA was similar during all growth stages. A significant increase in *trxA* mRNA levels was observed in the presence of hydrogen peroxide in the medium or after heat shock. A single transcriptional start site was determined with total RNA isolated from cells subjected or not subjected to oxidative stress or heat shock. In each case the same promoter region was identified and shown to have a high similarity to the consensus promoter sequence of Gram-positive bacteria, as well as to that of *E. coli* and the previously mapped promoters from *O. oeni*.

Keywords: *Oenococcus oeni*, thioredoxin, oxidative stress, heat shock

INTRODUCTION

Thioredoxins have been isolated and characterized from many micro-organisms, plants and animals (Holmgren, 1985). Such proteins are involved in a wide variety of biochemical processes in living cells. Reduced thioredoxin serves as an efficient hydrogen donor for many reductase enzymes and protein disulfide isomerases (Holmgren, 1989). The active site of thioredoxin (Trp-Cys-Gly-Pro-Cys) is highly conserved and contains two redox-active cysteine residues, which form either a disulfide (oxidized form) or a dithiol (reduced form). The S–S bond of oxidized thioredoxin is reduced by NADPH and a specific enzyme called thioredoxin reductase (Moore *et al.*, 1964).

Thioredoxin appears to be an essential protein for many organisms, such as *Bacillus subtilis* (Scharf *et al.*, 1998), *Synechocystis* (Navarro & Florencio, 1996) and *Rhodobacter sphaeroides* (Pasternak *et al.*, 1997), in contrast to

Escherichia coli (Holmgren *et al.*, 1978). A variety of stresses, including heat, salt stress or ethanol treatment, strongly enhance the synthesis of thioredoxin in *B. subtilis* (Scharf *et al.*, 1998). In this species, a double control of stress induction at two different promoters has been demonstrated: the first promoter is σ^B -dependant, whilst the second is presumably σ^A -dependant and might require activation by some other unknown mechanism.

Oenococcus oeni is a lactic acid bacterium which is able to grow in wine and to perform the malolactic fermentation (Dicks *et al.*, 1995; Kunkee, 1967; Labarre *et al.*, 1996a). No information is available on thioredoxin synthesis and activity in this heterofermentative bacterium. In the course of our investigations on the stress response of *O. oeni*, we previously studied an 18 kDa small heat-shock protein (Lo18), highly induced by multiple stresses such as heat shock, acid shock, the presence of ethanol or sulfite, and in stationary phase (Guzzo *et al.*, 1997, 1998). The *hsp18* gene encoding this protein was cloned and characterized (Jobin *et al.*, 1997). After purification of the protein, it was shown that Lo18 could form oligomers and prevent citrate

The GenBank accession number for the sequence reported in this paper is XT93091.

synthase from thermal aggregation (F. Delmas, personal communication).

We report here the nucleotide sequence of a chromosomal DNA fragment encoding the thioredoxin of *O. oeni*. The size of the transcript and the transcriptional start site were determined and the expression of the gene in response to oxidative and heat stresses was investigated.

METHODS

Bacterial strains, plasmids and media. The strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) broth or LB agar at 37 °C. In the selective medium the antibiotic erythromycin was used at a concentration of 200 µg ml⁻¹. *O. oeni* strain Lo84.13 was grown at 30 °C in FT80 medium (pH 5.3) (Cavin *et al.*, 1989), modified by adding meat extract (5 g l⁻¹) instead of Casamino acids.

DNA manipulations, sequencing and analysis. Molecular techniques were carried out using standard methods (Sambrook *et al.*, 1989). Double-stranded plasmid DNA was purified with a Qiagen plasmid kit and sequenced using the dideoxy chain-termination method (Sanger *et al.*, 1977). Computer analyses of nucleotide and amino acid sequences were carried out with the BLAST (Altschul *et al.*, 1990) and CLUSTAL W programs (Higgins *et al.*, 1994).

Isolation of total RNA and Northern blot analysis. *O. oeni* total RNA was purified as previously described (Labarre *et al.*, 1996b) from control cells (grown at 30 °C), or cultivated in the

Fig. 1. *O. oeni* *trxA-alsSD* cluster. The positions of *trxA*, *alsS* and *alsD* are indicated by boxes. The direction of transcription is indicated by arrows. *Scal* restriction sites are shown. The probe used for Northern blotting is indicated by the bold line below the map.

presence of hydrogen peroxide (10 mg l⁻¹), or after addition of hydrogen peroxide (8–20 mg l⁻¹) for 30 or 60 min, or submitted to heat shock for 5–30 min. RNA (30 µg) was separated in a 1.2% (w/v) agarose/6.6% (v/v) formaldehyde gel and then transferred to Hybond-N+ membranes (Amersham). The *trxA* probe (Fig. 1) was radiolabelled with [α -³²P]dATP, using a Random Primer DNA Labelling System (Gibco-BRL). All the Northern blot analyses were repeated at least twice.

Primer extension analysis. Oligonucleotides (TRX1, 5'-CCG GAT TTG GTT GCT TCC TC-3'; TRX2, 5'-TCA GTC ACA GTC ACG CCG GA-3'; and TRX3, 5'-TTG CCG AAT TTA ACC CTC GA-3') were used to map the 5' terminus of *trxA* mRNA in *O. oeni*. Total RNA (5 µg) was mixed with 2 pmol of each primer. After a 5 min denaturation step at 100 °C, the mixture was cooled in ice for annealing. Reverse transcription was carried out as recommended by the manufacturer with the Superscript II RNase H⁻ Reverse Transcriptase (Gibco-BRL). Analysis of the extension products was performed as previously described (Jobin *et al.*, 1997).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference
Strains		
<i>O. oeni</i>		
Lo84.13	MLE ⁺	Oenological Institute of Bordeaux
<i>E. coli</i>		
TG1	<i>supE hsdΔ5 thi Δ(lac-proAB) F'(traD36 proA⁺B⁺ lacI^q lacZ ΔM15)</i>	Gibson (1984)
AR1062	<i>F⁻ thr leu ara azi fhuA lacY tsx minA gal rspL xyl mtl thi hsdR</i>	D'Enfert <i>et al.</i> (1987)
BH2012	<i>F⁻ araD139 ? galU galK hsdR rpsL metE46 argH1 trxA2 ilvC::Tn5</i>	Lim <i>et al.</i> (1985)
A312	<i>metE::Tn10</i>	Russel & Model (1986)
A313	<i>trxA::kan metE::Tn10</i>	Russel & Model (1986)
Plasmids		
pGID023	Shuttle vector for <i>E. coli</i> and <i>L. plantarum</i> , derivative of pJDC9 containing pE194 replication functions; Em ^R	Hols <i>et al.</i> (1994)
pGID600	pJDC9 containing a 7 kbp <i>Sau3A</i> fragment from <i>O. oeni</i> DNA	Garmyn <i>et al.</i> (1996)
pGID608	pGID023 containing a 863 bp <i>Scal</i> fragment from pGID600	This study

RESULTS

Nucleotide sequence of *trxA*

In a previous publication, we reported the isolation of a recombinant clone (pGID600) carrying the *O. oeni alsSD* cluster (Fig. 1) and the corresponding nucleotide sequence (Garmyn *et al.*, 1996). We found an ORF in the same orientation as *alsSD* that terminated 115 bp upstream of *alsSD*. We have now determined the nucleotide sequence upstream of this region by primer-walking using pGID600 as a template. An ORF of 104 codons is preceded by a putative ribosome-binding site (AAGGAGG) complementary to the sequence 3'-UUUCCUCCA (complementary bases underlined) at the 3' end of *Leuconostoc mesenteroides* 16S rRNA (Yang & Woese, 1989). *L. mesenteroides* was taken as reference because the 3' end of *O. oeni* 16S rRNA has not been determined, and it is known that the sequence is conserved at the 3' ends of different Gram-positive bacteria (Chassy & Murphy, 1993). Furthermore, the genus *Leuconostoc* is closely related to *Oenococcus* (Dicks *et al.*, 1995). Inspection of the 3' noncoding sequence showed an inverted repeat sequence that could form a stem-and-loop structure in mRNA with a calculated ΔG_f of -68 kJ mol^{-1} . The deduced primary structure of the encoded TrxA is composed of 104 amino acids, with a predicted molecular mass of 11.5 kDa. The *O. oeni* TrxA exhibits sequence similarity to other bacterial TrxA (Fig. 2), with a particularity high sequence identity (48%) to *B. subtilis* (Chen *et al.*, 1989). The primary structure of *O. oeni* thioredoxin revealed

the presence of a typical active centre. However, the sequence between the two vicinal Cys residues was different from that found in most thioredoxins, with the Gly residue replaced by a Thr residue.

Expression in *E. coli* of the *O. oeni trxA* gene

The predicted amino acid sequence provided strong evidence that the cloned DNA contained the *trxA* gene encoding the thioredoxin of *O. oeni*. To identify the *trxA* gene product, we used an *E. coli* minicell system which allowed specific labelling of plasmid-encoded proteins (D'Enfert *et al.*, 1987). The 863 bp *ScaI* DNA fragment of pGID600 (Fig. 1) was subcloned in pGID023. Minicells were prepared from *E. coli* AR1062, which was transformed with plasmid pGID023 and pGID608. Proteins were labelled with L-[^{35}S]methionine and analysed by SDS-PAGE (Labarre *et al.*, 1996a). Minicells carrying pGID608 expressed a protein that had a molecular mass of almost 12 kDa (data not shown). This apparent molecular mass is consistent with the calculated value deduced from the nucleotide sequence.

An *in vivo* assay for thioredoxin function was used to test whether the *O. oeni* TrxA protein can substitute for *E. coli* thioredoxin. *E. coli* contains the enzyme methionine sulfoxide reductase, which uses thioredoxin as a source of reducing power and thereby enables methionine-requiring strains to utilize methionine sulfoxide for growth (Russel & Model, 1986). Two different mutant strains, BH2012 and A313, with phenotype

Fig. 2. Comparison of the predicted amino acid sequence of *O. oeni* TrxA (OOENI) with those of TrxA from *B. subtilis* (BACSU) (Chen *et al.*, 1989), *Chlamydia psittaci* (CHLPS) (Rockey *et al.*, 1996), *Mycoplasma genitalium* (MYCGE) (Fraser *et al.*, 1995), *E. coli* (ECOLI) (Lim *et al.*, 1985), *R. sphaeroides* (RHOSH) (Pille *et al.*, 1990), *Synechocystis* sp. (SYNY3) (Kaneko *et al.*, 1995) and *Clostridium litorale* (CLOLI) (Kreimer & Andreessen, 1995). Amino acids identical to those of *O. oeni* TrxA are boxed. Asterisks indicate residues that are identical in all sequences. Black boxes indicate redox-active cysteines. Numbers on the right indicate the position of the last amino acid. Percentages in parentheses indicate the identity scores between TrxA from *O. oeni* and from other bacterial species.

Fig. 3. Detection of *trxA* mRNA as a function of growth stage. RNA samples were taken from cells during exponential phase (OD_{600} 0.6 and 0.9), at the beginning of stationary phase (OD_{600} 1.2) and during stationary phase (OD_{600} 1.6).

Fig. 4. Northern blot analysis of stress-induced *trxA* expression. (a) Effect of oxidative stress. RNA samples were isolated from exponentially growing *O. oeni* cells growing in the presence (lane 1) or absence (lane C) of a sublethal dose of hydrogen peroxide (10 mg l^{-1}). In the adjacent lanes, cells in exponential phase were submitted to treatment for 30 or 60 min with various doses (8, 10, 12 and 20 mg l^{-1}) of hydrogen peroxide, as indicated. (b) Effect of heat shock. RNA samples were isolated from *O. oeni* cells grown at 30°C (lane C) or from heat-shocked cells at 42°C . For heat-shocked cells, samples were submitted to a rapid temperature shift (less than 2 min). The end of the shift was considered as the start of heat shock, and cells were withdrawn at the time points (in minutes) indicated.

$\text{Met}^- \text{TrxA}^-$ and harbouring pGID023 were unable to use methionine sulfoxide to satisfy their methionine requirement. The control strain A312 ($\text{Met}^- \text{TrxA}^+$) harbouring pGID023, and both strains BH2012 and A313, carrying the recombinant plasmid pGID608 promoting *O. oeni* TrxA synthesis, grew equally well on minimal plates supplemented with L-methionine sulfoxide or L-methionine. This indicated that *O. oeni* thioredoxin serves as a cofactor for *E. coli* methionine sulfoxide reductase *in vivo*.

Transcription analysis of the *trxA* gene

Total RNA was isolated and used to prepare Northern blots from samples taken during exponential phase (OD_{600} 0.6 and 0.9), at the beginning of stationary phase (OD_{600} 1.2) and during stationary phase (OD_{600} 1.6). These samples were hybridized with the *trxA* probe which corresponds to the 550 kb fragment containing part of the *trxA* gene. Autoradiograms (Fig. 3) showed a single 0.7 kb transcript hybridizing with the probe. *trxA*

Fig. 5. Mapping of the 5' end of *trxA* mRNA by primer extension. Equal amounts of RNA isolated before (lane 1) and 60 min after (lane 2) the addition of hydrogen peroxide (10 mg l^{-1}), and 15 min after heat shock (lane 3) were hybridized with primers complementary to the 5' region of *trxA* (see Methods). The extension products obtained with primer TRX3 for each RNA sample were electrophoresed in parallel with the sequencing products (g, a, t and c) obtained with the same labelled primer on a sequencing gel. The arrow indicates the position of the deduced start site.

mRNA was detected at a significant level and was estimated to be at roughly the same level during every growth stage, except in late-exponential phase where the transcript appeared at a higher level.

To investigate the effect of oxidative stress, *O. oeni* cells were cultivated in the presence of a sublethal dose of hydrogen peroxide (10 mg l^{-1}). When the exponential phase was reached, cells were withdrawn and total RNA prepared as described in Methods. Under these growth conditions, a higher level of *trxA* mRNA was detected in comparison to the control (Fig. 4a, compare lanes 1 and C). Moreover, treatment of exponential cells with hydrogen peroxide at various concentrations (8 – 20 mg l^{-1}) for 30 or 60 min significantly enhanced the amount of mRNA detected. The maximum rate of induction was achieved after 60 min of incubation in the presence of hydrogen peroxide at every concentration tested. No significant increase in the signal was observed at longer incubation times.

In order to examine the effect of temperature increase on *trxA* expression, Northern blot experiments were performed with total RNA extracted from cells submitted to heat shock. The amount of *trxA* messenger was enhanced 5 min after temperature shift from 30°C to 42°C and reached a high level of induction after 30 min of heat shock (Fig. 4b). This temperature induction suggested that the product of *O. oeni trxA* is a heat-shock protein.

To map the transcription initiation site of the *trxA* gene, nested oligonucleotides TRX1, TRX2 and TRX3 (see Methods) were used in a primer extension assay. RNA samples were extracted from *O. oeni* cells in exponential phase as controls, after addition of hydrogen peroxide (10 mg l^{-1} for 60 min) and after heat shock (42°C for 15 min). In all culture conditions, a single signal was obtained with oligonucleotides TRX1, TRX2 and TRX3 (Fig. 5), allowing the location of the 5' end of *trxA* mRNA 17 nucleotides upstream of the initiation codon. This transcription start point allowed the identification of the promoter for *trxA* as TTGCAT-17 bp-TACAAT. This sequence shows close similarity to the consensus found in the housekeeping promoter of Gram-positive bacteria as well as in *E. coli* (Graves & Rabinowitz, 1986) and other *O. oeni* promoters mapped previously (Jobin *et al.*, 1997; Labarre *et al.*, 1996b).

DISCUSSION

This report presents the DNA sequence, characterization and mRNA analysis of the *trxA* gene of *O. oeni*. The primary sequence of the deduced protein revealed that TrxA has significant identity to a number of proteins belonging to the large family of thioredoxins, and in particular to TrxA from *B. subtilis* (48% identity) (Chen *et al.*, 1989), and that the Cys residues involved in catalysis are conserved. The C-X-X-C motif is critical in determining the exceptional reducing power of disulfide reductase (Konz *et al.*, 1998). Site-directed mutagenesis experiments showed that change of these two central residues can modulate the reducing power of DsbA, a member of the thioredoxin family (Grauschopf *et al.*, 1995). It has recently been reported that thioredoxin from anaerobic, amino-acid-utilizing bacteria possesses a modified consensus sequence at the active centre (Harms *et al.*, 1998). The thioredoxin of such bacteria did not react with thioredoxin reductase of *E. coli* and the authors suggested that these thioredoxins might form an evolutionarily separate group. One of the two amino acids between the cysteine residues is not conserved in *O. oeni* TrxA, in which a Thr residue is substituted for the highly conserved Gly residue. This substitution may alter the reducing power of the enzyme. Nevertheless, the protein encoded by the *O. oeni* *trxA* gene was able to complement *E. coli* mutant strains defective in thioredoxin activity. Taken together, these results led us to conclude that the *O. oeni* *trxA* gene encodes a functional thioredoxin.

Few transcriptional analyses of *trxA* bacterial genes have been reported so far. The only well-documented example concerns the *trxA* gene from *B. subtilis* (Scharf *et al.*, 1998). A variety of stresses, including heat and hydrogen peroxide, strongly enhanced the synthesis of thioredoxin in this bacterium. Transcription initiation of the monocistronic *trxA* gene occurs at two promoters with different profiles of induction in response to stress. One of these promoters is recognized by the RNA polymerase associated with an alternative σ^B factor (Hecker & Völker, 1998). In *Streptomyces coelicolor*,

the alternative sigma factor σ^R modulates expression of the *trxB* operon, encoding thioredoxin reductase and thioredoxin, in response to oxidative stress (Paget *et al.*, 1998).

The detection of an approximately equal level of *trxA* mRNA in growing cells and during stationary phase suggests that *trxA* expression is not growth-phase regulated. The level of *trxA* mRNA is strongly increased by hydrogen peroxide, indicating that thioredoxin is involved in response to oxidative stress, as in many other organisms (Fernando *et al.*, 1992; Kuge & Jones, 1994; Scharf *et al.*, 1998; Wiele *et al.*, 1997) whereas the *E. coli* and *Salmonella typhimurium* *trxA* genes are not known to be regulated (Farr & Kogoma, 1991). In addition to oxidative stress, *O. oeni* *trxA* is also induced by heat shock as is also the case in *B. subtilis* (Scharf *et al.*, 1998) and in human cells (Jacquier-Sarlin & Polla, 1996).

The 5' end of the *trxA* monocistronic transcript was identified in normal growth conditions and allowed us to identify a promoter with a consensus sequence typical for Gram-positive bacteria as well as for *E. coli* (Graves & Rabinowitz, 1986). An identical transcription initiation site was obtained with total RNA extracted from cells submitted to oxidative or heat stress. This result suggests that the same potential -35 and -10 regions are recognized by RNA polymerase in all growth conditions tested. During recent work in *B. subtilis*, at least four different classes of heat-inducible genes were distinguished. The genes of class I are controlled by a repressor which recognizes the CIRCE (controlling inverted repeat of chaperone expression) element (Hecker *et al.*, 1996). Class II genes are positively controlled by the alternative σ^B factor (Hecker & Völker, 1998). Recently, CtsR, a novel regulator of stress and heat shock response, has been shown to act as a repressor of stress genes belonging to class III (Derré *et al.*, 1999) and those of class IV are regulated by a still unknown mechanism. In lactic acid bacteria, heat-shock genes sharing characteristics of class I (Eaton *et al.*, 1993; Van Asseldonk *et al.*, 1993) and class III (Derré *et al.*, 1999) from *B. subtilis* have been reported. No CIRCE element was present in the vicinity of the *O. oeni* *trxA* start site of transcription. To our knowledge, no stress gene expressed under an unusual promoter sequence that may involve an alternative sigma factor has been reported so far in lactic acid bacteria. Moreover, the target consensus sequence corresponding to CtsR was not found in the promoter region of *O. oeni* *trxA*. Therefore, the induction of *O. oeni* *trxA* expression observed after heat or oxidative stresses may require another unknown regulator element, as has been suggested for the heat-shock genes belonging to class IV from *B. subtilis*.

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