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Variability in gene content and expression of the thioredoxin system in *Oenococcus oeni*

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ABSTRACT

The thioredoxin system protects against oxidative stress through the reversible oxidation of the thioredoxin active center dithiol to a disulphide. The genome of *Oenococcus oeni* PSU-1 contains three thioredoxin genes (*trxA1*, *trxA2*, *trxA3*), one thioredoxin reductase (*trxB*) and one ferredoxin reductase (*fdr*) which, until recently, was annotated as a second thioredoxin reductase. For the first time, the entire thioredoxin system in several *O. oeni* strains isolated from wine has been analysed. Comparisons at the DNA and protein levels have been undertaken between sequences from *O. oeni* and other genera and species, and the genera *Leuconostoc* and *Lactobacillus* were found to present the highest similarities. The gene most frequently absent from a collection of 34 strains and the sequences annotated in the NCBI database was *trxA1.* Moreover, phylogenetic analysis suggested that this gene was horizontally transferred from *Lactobacillus* to *O. oeni*. Strain-dependent expression profiles were determined in rich and in wine-like media. General over-expression was detected after inoculation into wine-like medium, with *trxA3* being the most highly expressed gene. The increased transcriptional levels of the thioredoxin genes are indicative of the crucial role of this system in the *O. oeni* response to wine harsh conditions.

1. Introduction

Thioredoxin (Trx) is a small protein (10–12 kDa) with two cysteine redox-active sites (Holmgren, 1985) that was first discovered in *Escherichia coli* as a natural hydrogen donor for ribonucleotide reductase (Laurent et al., 1964). The catalytic activity of these proteins (containing a -CXXC- motif) and the Trx fold comprised of five β-strands surrounded by four short α-helices is evolutionarily conserved from archaea and bacteria to man (Kumar et al., 2004; Lu and Holmgren, 2014). This protein, together with thioredoxin reductase (TrxR) and the NADPH cofactor, constitute the Trx system, a key antioxidant system in the defence against oxidative stress. Through the Trx system's disulphide reductase activity, this system regulates the protein dithiol/disulphide balance (Mostertz et al., 2008; Lu and Holmgren, 2014).

In addition to the system's conserved role as a high-capacity hydrogen donor system for reductive enzymes, the Trx-TrxR system exhibits other specific functions, including enzymatic reactions (Tsang and Schiff, 1978), H_2O_2 reduction (Das and Das, 2000), DNA synthesis (Holmgren, 1989), energy transduction (Lindahl and Florencio, 2003), phage T7 DNA replication (Huber et al., 1987), filamentous phage assembly (Feng et al., 1999), and redox sensing (Kumar et al., 2004).

The diversity of bacterial environments has resulted in the evolution of diverse types of antioxidant systems. The Trx-TrxR system, glutathione-glutathione reductase, and catalase are the major antioxidant systems. While Trx is ubiquitous in bacteria, the glutathione system or catalase are lacking in certain bacteria. Indeed, the glutathione system is absent in some Gram-negative bacteria and in most Gram-positive bacteria, including *Bacillus subtilis*, *Lactococcus lactis* and *Streptococcus aureus* (Scharf et al., 1998; Uziel et al., 2004; Serata et al., 2012; Lu and Holmgren, 2014), while catalase is found in most Gram-negative bacteria.

In *E. coli* the regulation of thioredoxin expression has been studied using different mutants and conditions. The expression of the *trxA* gene has been found to increase during the stationary phase cultures and is inversely proportional to generation time (Lim et al., 2000).

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Lactic acid bacteria (LAB) exhibit different patterns of Trx gene expression. Six ORFs for the Trx system have been described in the *Lactobacillus plantarum* WCFS1 genome (NCBI accession PRJNA62911): four genes encoding thioredoxins (*trxA1*, *trxA2*, *trxA3* and *trxH*), a thioredoxin reductase (*trxB*), and a ferredoxin NAD(P) reductase, which was annotated as thioredoxin reductase until February 2015. These six genes are dispersed throughout the genome and are highly conserved within *Lb. plantarum* strains regardless of ecological niche (Serrano et al., 2007). In contrast, the *Lactobacillus casei* Shirota strain has four thioredoxin genes (*trxA1*, *trxA2*, *trxA3* and *trxA4*) and one putative thioredoxin reductase gene designated *trxB* (Serata et al., 2012). In these studies, the importance of the Trx systems for these bacteria has become clear. In particular, overproduction of *trxB1* (encoding TrxR) improves oxidative stress tolerance in *Lb. plantarum* (Serrano et al., 2007). The generation of *Lb. casei* gene disruption mutants for different *trx* genes revealed that the Trx-TrxR system is essential for aerobic growth and is the major system used to maintain the intracellular thiol/disulphide balance (Serata et al., 2012).

This research focuses on *Oenococcus oeni*, the main LAB responsible for malolactic fermentation (MLF). This fermentation is crucial for many vinification processes (Wibowo et al., 1985; Davis et al., 1988; Kunkee, 1991; Lonvaud-Funel, 1999; Bartowsky, 2005). Little is known regarding the Trx-TrxR system and its function in this species, which can grow in wine - a hostile environment containing ethanol and sulphite and with a low pH (Guzzo et al., 2000). In the first sequenced *O. oeni* genome, that of strain PSU-1, three genes were annotated as thiol-disulphide isomerase (*trxA1*, *trxA2*, *trxA3*) and two genes as thioredoxin reductase (*trxB1*, *trxB2*). Nevertheless, the *trxB2* gene was recently designated as a ferredoxin NADPH reductase (OEOE_RS00770).

Studies of oxidative stress in *O. oeni* have described the overexpression of one of the *trxA* genes (OEOE_RS08215) in response to hydrogen peroxide and heat shock (Jobin et al., 1999; Guzzo et al., 2000), as reported for *B. subtilis* (Mostertz et al., 2008). Another *trxA* gene (OEOE_RS01675) has been described as a genetic marker for stress resistance (Renouf et al., 2008).

The purpose of this research was to study the complete thioredoxin system in *O. oeni*. To this end, the presence of the *trx* genes was assessed experimentally in a collection of *O. oeni* strains and *in silico* using published genomes. A phylogenetic study of protein sequences was carried out in order to understand the relationship of *O. oeni* with other LAB species. Finally, to evaluate the role of each *trx* gene in the stress response, the transcriptional responses of the *trx* genes were studied under optimal growth conditions in rich medium and during MLF in a wine-like medium.

2. Materials and methods

2.1. Oenococcus oeni strains and culture conditions

Most strains used in this study were isolated from 21 red wines of southern Catalonia of the 2008 vintage, from two different appellations of the origin DOQ Priorat and DO Tarragona. Moreover, five strains were isolated from Italian wines. Other strains used were obtained from commercial companies, and one was the type strain. All strains are described in Table 1.

Stock cultures were thawed from −80 °C and grown in modified MRS broth medium (MRSmf), which is MRS (De Man et al., 1960; Difco Laboratories, Detroit, MI, USA) supplemented with 4 g l^{-1} L-malic acid and 5 g l^{-1} fructose at pH 5.0, at 28 °C in a CO_2 incubator. Cells were collected at the end of the exponential phase and inoculated (0.1%) into MRSmf for DNA extraction or to act as the inoculum of an MLF.

2.2. Malolactic fermentation (MLF) in wine-like medium

Subcultures of the *O. oeni* strains were directly inoculated into flasks containing 100 ml of wine-like medium (WLM) containing 12% (v/v) ethanol at pH 3.4. Inoculation was performed as previously described (Bordas et al., 2013), but we reduced the protein content by 50% and did not add L-cysteine to increase the harshness of the WLM. All assays were performed in duplicate, and inoculum growth was monitored by measuring the absorbance (OD_{600nm}) and counting colonies grown on plates made with MRSmf agar (20 g l^{-1} agar). One hour after inoculation in WLM, the cells were collected (1 h point), and the L-malic acid content was measured, then MLF was monitored at periodic intervals, including the midpoint (i.e., 50% consumption of L-malic acid) and end of MLF. Other cell samples were collected at four experimental points (0 h, 1 h, mid-MLF, and end-MLF). L-malic acid consumption was measured using the multianalyser Miura One (I.S.E. S.r.l, Guidonia, Italy) and an enzymatic kit (TDI SL, Barcelona, Spain).

2.3. Gene sequences

O. oeni nucleotide sequences were obtained from the National Center for Biotechnology Information (NCBI). The abbreviations used in this research and the codes of genes from the *O. oeni* strain PSU-1 (accession number in NCBI NC_008528) are shown in Table 2.

2.4. DNA and RNA extraction

Harvested cells were incubated with lysozyme (50 mg ml^{-1}) for 30 min at 37 °C, after which genomic DNA was extracted using a High Pure PCR template kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Total RNA extractions were performed on cells harvested by centrifugation, frozen in liquid nitrogen and stored at −80 °C. The extractions were performed using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany) and the RNA was treated with Turbo DNA-free (Life Technologies, USA). Total nucleic acid concentrations were calculated using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany).

2.5. Thioredoxin gene detection by specific PCRs

To assess the presence of thioredoxin genes in the genomes of *O. oeni* strains, each pair of primers was tested by PCR (Table 2). PCR amplifications were conducted with a BIOTAQ PCR Kit (Bioline, UK) in a final volume of 20 μl, containing 2 μl 10× buffer, 0.2 mM dNTPs, 3.5 mM MgCl2, 0.2 μM of each primer, 0.025 U μl^{-1} DNA polymerase, and approximately 10 ng of DNA. The amplifications were performed in a Thermal Cycler 2720 (Applied Biosystems, USA) with the following cycling conditions: 5 min at 94 °C; 30 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s; and 72 °C for 7 min. The amplification products were resolved by electrophoresis on 1.4% (w/v) agarose gels run at 100 V for 2 h 45 min and stained with ethidium bromide. DNA molecular weight markers II and VI (proportion 1:2) from Roche Diagnostics (Basel, Switzerland) were used as references.

2.6. Real time quantitative PCR

Reverse transcription, real-time quantitative PCR (qPCR) and primer design were performed according to Olguín et al. (2009). The absence of chromosomal DNA contamination was confirmed by qPCR. The primers designed for this study are listed in Table 2. To select the most suitable internal control for Reverse Transcription