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Genetic and transcriptional study of glutathione metabolism in *Oenococcus oeni*

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ABSTRACT

Although *Oenococcus oeni* is the main species that is responsible for malolactic fermentation (MLF), harsh wine conditions can limit its performance. Although several mechanisms underlying the response to stress have been studied in this species, little is known regarding the cellular systems that protect against oxidative stress in other bacteria, such as glutathione (GSH). *O. oeni* cannot synthesize GSH but contains several genes related to its utilization. In this study, the relative expression (RE) of the seven genes involved in the GSH redox system found in *O. oeni* PSU-1 (*gshR*, *gpo*, three glutaredoxin-like genes and two subunits of an hypothetical transporter) has been measured. The study was performed using three strains, with each exhibiting a different GSH uptake capacity. The strains were grown in a stress-adaptation medium supplemented with 5 mM GSH and under different adaptation stress conditions (pH 4 and 6% ethanol). The RE showed that only some of these genes, including one for a possible glutaredoxin (OEOE_RS04215) and *cydC* for a subunit of a putative GSH transporter (OEOE_RS1995), responded to the addition of GSH. The presence of ethanol had a relevant effect on gene expression. Among the studied genes, the one for a NrdH-redoxin (OEOE_RS00645) showed a common response to ethanol in the strains, being over-expressed when grown with GSH. In most cases, the transcriptional changes were more evident for the strain with a higher capacity of GSH uptake. Malolactic performance of the three strains after pre-adaptation was evaluated in wine-like media (12% ethanol and pH 3.4). It was observed that the addition of GSH during pre-adaptation growth had a protective role in the cells exposed to low pH and ethanol, resulting in a quicker MLF.

1. Introduction

The stress conditions encountered by lactic acid bacteria (LAB) in different niches have led them to evolve and improve their survival capacity under harsh conditions (Mills et al., 2011). LAB have different response systems involved in redox balance that avoid oxidation by ROS (Reactive Oxygen Species) accumulated in the cell. An important compound engaged in oxidative stress protection is glutathione (GSH). This thiol is ubiquitous in eukaryotes and in Gram-negative bacteria. It has been proposed that some Gram-positive organisms possess glutathione synthesis capacity and/or utilization machinery (Fernándes and Steele, 1993). This antioxidant is made-up of three amino acids (Glu-Cys-Gly) and due to the thiol group of cysteine it can reduce ROS. Moreover, GSH has numerous metabolic functions reducing disulphide bonds to cysteine in proteins (Masip et al., 2006). To that end, GSH is oxidized to GSH disulphide (GSSG) by glutaredoxin (Grx) in order to eliminate peroxides or by glutathione peroxidase (Gpo) in order to reduce disulphide bonds. Glutathione reductase (GshR) reduces GSSG into GSH with the use of NAPDH. On the other hand, glutaredoxins utilize the reducing power of GSH to maintain and regulate the cellular redox state and redox-dependent signalling pathways (Lillig et al., 2008). Another GSH-associated function is the glutathione S-transferase (Gst). This large family of enzymes catalyse the transfer of the tripeptide GSH to a xenobiotic substrate for the purpose of detoxification (Vuilleumier and Pagni, 2002). For many organisms the ratio of GSH/GSSG works as a cellular redox hint (Bianucci et al., 2012; Ilyas and Rehman, 2014; Iurlo et al., 2015).

Although *Oenococcus oeni* is the main species responsible for malolactic fermentation (MLF), harsh wine conditions can limit its performance (Versari et al., 1999). Despite several mechanisms of response to stress have been studied in this species (Beltramo et al., 2006), little is known regarding the cellular systems that protect against oxidative stress such as GSH. Even though *O. oeni* cannot synthesize GSH, it has been previously reported that this bacterium is able to uptake it from the medium (Margalef-Català et al., 2016b). Pophaly et al. (2012) described the presence of several GSH related genes in *O. oeni* and other LAB, according to an *in silico* analysis of the genomes published at that time. However, little is known about the physiological effects of GSH in LAB. In *Lactococcus lactis*, GshR activity has been detected in all of the studied strains, and it has been shown that GSH protects against oxidative stress during aerobic growth (Li et al., 2003). The protective role

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of GSH against acidic stress at pH 2.5 (Zhang et al., 2007) and osmotic stress (Zhang et al., 2010) was also observed in *L. lactis*. In *Lactobacillus sanfranciscensis*, a higher tolerance to oxygen in presence of GSH has been observed and is associated with a higher GshR activity (Jänsch et al., 2007). Regarding *O. oeni*, its metabolism in relation to redox balance has barely been studied. Three proteomic studies have suggested the possible role of GshR in the stress response of *O. oeni* (Silveira et al., 2004; Cecconi et al., 2009; Margalef-Català et al., 2016a). A transcriptional study of the *gshR* gene corroborated the response to wine related stress (Bordas et al., 2015). Therefore, *O. oeni* could use the GSH available in wine. It has been reported that the GSH content in wine depends on the grape variety, but mainly, it depends on the yeast strain responsible for alcoholic fermentation (Lavigne et al., 2007). As in other LAB, GSH could play a protective role against stress in *O. oeni*. This antioxidant compound could be used as an additive to improve the fitness of MLF starter cultures before inoculation. A maximum GSH uptake rate has been observed during exponential growth in *O. oeni* (Margalef-Català et al., 2016b). Therefore, the addition of GSH would be more effective in the preparation of starter cultures than during wine MLF, where cells barely grow.

The main objective of this work was to investigate the genes involved in GSH metabolism in *O. oeni* and to determine whether GSH addition and wine related stress factors can modulate their expression. The potential of GSH addition to improve the preadaptation of *O. oeni* to stress conditions before inoculation was also evaluated. An *in silico* analysis was performed to elucidate the genetic composition associated with GSH utilization in *O. oeni*. The seven GSH related genes found in PSU-1 were analysed by real-time qPCR in three strains under different preadaptation conditions. These strains were chosen due to their differences in GSH uptake capacity (Margalef-Català et al., 2016b). Finally, the malolactic performance of the three strains was evaluated after preadaptation under different stress conditions, with and without added GSH.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Three strains were used in this study according to their GSH uptake capacity (Margalef-Català et al., 2016b): (1) PSU-1 (ATCC BAA-331) whose genome was the first in this genus to be fully sequenced (Mills et al., 2005) and which has a medium capacity to import GSH, (2) the type strain (CECT217 = ATCC 23279^T), which was named in this study as 217^T, and which has a poor GSH import ability, and (3) strain 3P2, which was isolated from a Grenache wine (Bordas et al., 2013) and is the strain with the highest uptake of reduced GSH in a screening of 30 *O. oeni* strains. The stock cultures (kept frozen at −80 °C) were grown in MRS broth medium (De Man et al., 1960) supplemented with 4 g/L L-malic acid and 5 g/L fructose at pH 5.0 at a constant 28 °C in a CO_2 incubator. Cells were collected at the end of the exponential phase and inoculated (0.1%) into the assay medium as described below. The growth was controlled by measuring the optical density at 600 nm.

2.2. Evaluation of the effect of GSH on gene expression and malolactic fermentation

The three strains were assayed under different preadaptation conditions using PC-MRS (Peptone Casaminoacids MRS) described by Margalef-Català et al. (2016b). The conditions assayed were as follows: pH 5 as the control; pH 5 and 6% ethanol (v/v); pH 4; and pH 4 and 6% ethanol. For each assay condition, there was one culture grown with added GSH (5 mM) and another without GSH. The cells were harvested at the mid-exponential phase. Afterwards, the cells were directly inoculated in flasks containing 50 mL of wine-like medium (WLM) plus 12% (v/v) ethanol, pH 3.4 and maintained at 20 °C. The inoculation and WLM composition were as previously described by Bordas et al. (2013) but diminishing yeast extract content to 2.5 g/L- in order to increase the harsh conditions of WLM. All of the assays were performed in duplicate and the inoculum growth was monitored by measuring the absorbance (OD_{600nm}) and counting plates in MRS agar medium supplemented with 4 g/L L-malic acid and 5 g/L fructose at pH 5.0. The measurements of L-malic acid consumption were performed with the multianalyser Miura One (TDI SL, Barcelona, Spain) and the enzymatic kit ready to use from the same company in order to determine the end of MLF.

2.3. Gene sequences and primer design

Nucleotide sequences of *O. oeni* were obtained from the National Center for Biotechnology Information (NCBI). The abbreviations used in this work and the code for the genes from *O. oeni* strain PSU-1 (accession number in NCBI NC_008528) are shown in Table 1. The primer design was performed according to Olguín et al. (2009).

2.4. RNA extraction

Total RNA was extracted using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) from cells harvested by centrifugation at 4500*g* for 15 min, washed with cold PBSx1, frozen in liquid nitrogen and kept at −80 °C until analysis. RNA was treated with Turbo DNA-free kit (Life Technologies, USA). The total nucleic acid concentrations were calculated using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany).

2.5. Real-time quantitative PCR

The reverse transcription and the Real-time qPCR (RT-qPCR) were performed according to Olguín et al. (2009). The absence of chromosomal DNA contamination was confirmed by RT-qPCR. For the selection of the most suitable internal control for qPCR, five housekeeping genes (*dnaG*, *dpoIII*, *gyrA*, *gyrB*, and *ldhD*) were tested. On the basis of primer efficiency and Ct values of different samples, *dnaG* and *gyrA* were chosen for the analysis as they showed the fewest differences between all of the strains in all the samples. The efficiencies of amplifications were calculated using the formula $E = [10^{(1/-s)} - 1] \times 100$, where s is the slope of the standard curve with several dilutions of cDNA (Beltramo et al., 2006). In this study, the threshold value was automatically determined by the instrument. The amplification efficiency was calculated from the raw data using LinRegPCR software (Ruijter et al., 2009; Tuomi et al., 2010). The relative expression value was calculated using the Ct values of *dnaG* and *gyrA* and the final result is the mean of both results. The analysis was made from biologically duplicated independent assays and for each sample technical triplicates were analysed by qPCR.

2.6. Bioinformatics tools

BLAST (Basic Local Alignment Search Tool, NCBI) programs, in particular, BLASTN and BLASTX, were used to evaluate the sequence conservation and the presence or absence of GSH genes and proteins in the different species.

All of the protein sequences used for phylogenetic analysis were obtained from the NCBI database. Each dataset was aligned using Muscle (Edgar, 2004) and was manually adjusted with Jalview 2.6.1 (Waterhouse et al., 2009). The phylogenetic analyses were performed using the MEGA v6.0 software package (Tamura et al., 2011), and the neighbour-joining method was used for tree reconstruction (Saitou and Nei, 1987). The statistical reliability of phylogenetic tree topology was evaluated by bootstrapping with 1000 replicates (Felsenstein, 1985).