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Identification of variable genomic regions related to stress response in *Oenococcus oeni*

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ABSTRACT

The lactic acid bacterium *Oenococcus oeni* is the most important species involved in malolactic fermentation due to its capability to survive in presence of ethanol and in the acidic environment of wine. In order to identify novel genes involved in adaptation to wine, a new approach using genome-wide analysis based on stress-related genes was performed in strain *O. oeni* PSU-1, and 106 annotated stress genes were identified. The *in silico* analysis revealed the high similarity of all those genes through 57 *O. oeni* genomes; however, seven variable regions of genomic plasticity could be determined for their different presence observed among these strains. Regions 3 and 5 had the typical hallmarks of horizontal transfer, suggesting that the strategy of acquiring genes from other bacteria enhanced the fitness of *O. oeni* strains. Certain genes related to stress resistance were described in these regions, and similarities of putative acquired regions with other lactic acid bacteria species were found. Some genomic fragments present in all the strains were described and another new genomic island harbouring a threonine dehydrogenase was found. The association of selected sequences with adaptation to wine was assessed by screening 31 *O. oeni* strains using PCR of single genes, but no sequences were found to be exclusive to highly performing malolactic fermentation strains. This study provides new information about the genomic variability of *O. oeni* strains contributing to a further understanding of this species and the relationship of its genomic traits with the ability to adapt to stress conditions.

1. Introduction

Oenococcus oeni is a Gram-positive lactic acid bacterium (LAB) isolated mainly from winemaking process (Bordas et al., 2013; González-Arenzana, Santamaría, López, & López-Alfaro, 2013; Wibowo, Eschenbruch, Davis, Fleet, & Lee, 1985) and it is responsible for the malolactic fermentation (MLF) in red wines. Since *O. oeni* is the LAB species most resistant to wine conditions, its physiological and molecular responses to wine stress have been widely studied (Bartowsky, 2005; Beltramo, Desroche, Tourdot-Maréchal, Grandvalet, & Guzzo, 2006; Bordas, Araque, Bordons, & Reguant, 2015; Bourdineaud, Nehmé, Tesse, & Lonvaud-Funel, 2003; Guzzo et al., 2000; Olguín, Bordons, & Reguant, 2010; Renouf, Delaherche, Claisse, & Lonvaud-Funel, 2008) in order to unveil strain-specific phenotypes linked to genomic features (Sumby, Grbin, & Jiranek, 2014). Recently, combined "omics" approaches have elucidated specific gene activation and protein regulation during stressful wine conditions (Costantini et al., 2015; Margalef-Català, Araque, Bordons, Reguant, & Bautista-Gallego, 2016; Olguín et al., 2015).

Genome analyses have indicated *O. oeni* has a compact genome of 1.8 Mb which presumably reflects its adaptation to the ecologically restricted niche of fermenting grape juice and wine (Borneman, Bartowsky, McCarthy, & Chambers, 2010). The hypermutable status of the genus *Oenococcus* due to the lack of mismatch repair (MMR) – *i.e.*, the genes *mutS* and *mutL* – explains the observed high level of allelic polymorphism among known *O. oeni* isolates and likely contributes to the unique adaptation of this genus to acidic and alcoholic environments (Marcobal, Sela, Wolf, Makarova, & Mills, 2008). Moreover, recent genomic analyses have explored the molecular mechanisms involved in the fitness and genomic diversity of this bacterium (Borneman, McCarthy, Chambers, & Bartowsky, 2012; Campbell-Sills et al., 2015; El Gharniti et al., 2012; Sternes & Borneman, 2016). Mobile genetic elements, like plasmids, transposable bacteriophage DNAs, transposable elements, genomic islands (GI), and many other specialized genetic elements face bacteria with continuous challenges to genomic stability, promoting evolution through horizontal gene transfer (HGT) (Abby & Daubin, 2007; Darmon & Leach, 2014). GIs are

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typically recognized as DNA segments found in some closely related strains. It is thought that their formation contributes to the diversification and adaptation of microorganisms, thus having a significant impact on the genome plasticity and evolution (Juhas et al., 2009), and some plasmids are possibly related with the performance of strains in wine (Favier, Bilhère, Lonvaud-Funel, Moine, & Lucas, 2012).

Renouf et al. (2008) and Bon et al. (2009) used genomic subtractive hybridization between two isolates of *O. oeni* with differing efficiency in MLF to elucidate the genetic bases of this intra-strain diversity and it was suggested that the selection of *O. oeni* industrial starters could be based on the presence of specific genetic markers (Renouf et al., 2008). In particular, six regions of genomic plasticity were revealed and the presence of eight genes involved in adaptation to wine could be associated to high performing strains (Bon et al., 2009). A large proportion of these ORFs resembled genes involved in carbohydrate transport and metabolism, cell wall/membrane/envelope biogenesis, and replication, recombination and repair. More approaches to identify the genomic diversity of *O. oeni* strains and mobile elements have been used to look up for specific homolog sequences related to stress (Athané et al., 2008), insertion sequences (IS) (El Gharniti et al., 2012) and transposases (Stefanelli, 2014).

The antioxidant mechanisms of *O. oeni* have been defined as widely dispersed (Su et al., 2015), which indicates that antioxidant properties depend on the strain and culture medium. In the completely assembled strain of *O. oeni*, PSU-1, genes involved in the disulphide-reducing pathway, and systems that remove reactive oxygen (NADH-oxidase and NADH-peroxidase) were found, however no homolog to superoxide dismutase was identified (Mills, Rawsthorne, Parker, Tamir, & Makarova, 2005). Recent studies (Margalef-Català et al., 2016b; Margalef-Català et al., 2017b; Margalef-Catalàet et al., 2017a) have reported the possible main role of redox systems glutathione (GSH) and thioredoxin (TRX) in coping with wine related stress. These studies showed the increased transcriptional levels of some thioredoxin genes in response to low pH and high ethanol, and they also showed the improvement of *O. oeni* stress resistance due to GSH addition.

The major aim of this study was to identify putative variable genomic regions through a collection of *O. oeni* genomes starting from the stress gene annotation of PSU-1 strain and specifically the oxidative stress. Comparative genome analyses were used to identify genetic differences between the 57 *O. oeni* strain genomes isolated from different world areas, and the results showed seven plasticity regions, highlighting the role of some genes harboured in these fragments. We have confirmed some variable genomic regions and we have added the current genetic annotation based on the strain PSU-1. As a result, we report an *in silico* approach to detect possible genes and fragments linked to positive oenological characteristics.

2. Materials and methods

2.1. O. oeni strains and gene sequences

The *in silico* analysis of the presence of several genes related to stress was carried out with a collection of 57 different genomes of *O. oeni* sequenced strains (Table S1 in the Supplementary data). The nucleotide sequences of stress-related genes were obtained from the completely assembled genome of strain PSU-1 (NCBI Reference Sequence NC_008528.1, GenBank assembly accession GCA_000014385.1, National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov), by using the following key words related to stress and specifically to redox systems: stress, oxidative, redox, peroxidase, damage, shock, oxidase, redoxin and reductase.

Moreover, 31 *O. oeni* strains were used to deepen the analysis of some genomic regions (Table 1) *in vivo*. Among them, 19 strains were isolated from red wines from south Catalonia, in NE Spain. Other nine strains were commercial starters and three strains with available genome were chosen as controls. Stock cultures (kept frozen at − 80 °C) were grown in MRS broth medium (De Man, Rogosa, & Sharpe, 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 (OD_{600nm} = 1.6) and the harvested pellet was kept at − 20 °C in order to extract the DNA.

In order to find if there was some correlation between the presence of some genes related to stress response and their malolactic performance, these 31 strains were assayed in wine like media (WLM) (Bordas et al., 2013) with 2 g/L L-malic acid and ethanol (12–14%, v/v), adjusted to pH 3.4, and the time for complete consumption of L-malic acid was evaluated.

2.2. DNA extraction

The genomic DNA extraction was performed with Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's protocols. Then the DNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the samples were diluted to the concentration of 10 ng/μL.

2.3. Detection of specific genes by PCR amplification

Primers used in this study are reported in Table S2 (in the Supplementary data). PCR reaction for the amplification of single genes were performed with the GoTaq® DNA polymerase reagents (Promega), whereas the intergenic PCRs were conducted using GoTaq® Long PCR Master Mix (Promega) (Stefanelli, 2014). The amplifications were performed in a thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, Thermo Fisher Scientific). In the case of single genes, reactions mix were prepared in a final volume of 20 μL containing 4 μL buffer 5 ×, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, 0.025 U/μL of polymerase, and 10 ng of DNA. The amplifications were carried out with the following protocol: 5 min at 94 °C, 30 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and 72 °C for 7 min. For the intergenic PCRs, 0.2 μM of each primer and 10 ng of DNA were added to 10 μL of GoTaq® Long PCR Master Mix. The protocols of amplifications were: 2 min at 94 °C, 35 cycles at 94 °C for 30 s, 54–60 °C (depending on the genes) for 30 s, 65 °C for 1 min for each kbp, and 72 °C for 10 min. The PCR products were verified by electrophoresis on agarose gel at 0.8–1%, coloured with EuroSafe (Euroclone S.p.A., Pero, Italy), and using the marker O'GeneRuler™ DNA (Thermo Scientific).

2.4. Bioinformatics tools

BLAST (Basic Local Alignment Search Tool) programs (Altschul, Gish, Miller, Myers, & Lipman, 1990) -in particular, BLASTN and BLASTX- at the NCBI were used to evaluate sequence conservation and the presence or absence of genes in defined NCBI databases (using Whole Genome Shotgun) or local databases (created for CLC Genomics Workbench 7.0.4). After all BLAST analyses, hits with significant similarities (90–100% coverage and identity) were revised to avoid possible sequencing errors and gaps in the draft assemblies. Coverage and identity are referred in the text as *cov* and *ide*, respectively. Microbial genomic viewer (v.2.0) (Overmars, Kerkhoven, Siezen, & Francke, 2013) was used to extract the GC % of genes and to visualize the genomic regions. The CiVi (Circular genome Visualization) program (Overmars, van Hijum, Siezen, & Francke, 2015) was used to construct the circular map of PSU-1.

2.5. Phylogenetic tree analysis

All protein sequences used for phylogenetic analysis were obtained from the NCBI database. Each dataset was aligned using the Muscle computer program (Edgar, 2004) and was manually adjusted with Jalview 2.6.1 (Waterhouse, Procter, Martin, Clamp, & Barton, 2009).