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RNA fingerprinting analysis of *Oenococcus oeni* strains under wine conditions

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ABSTRACT

Oenococcus oeni is a lactic acid bacterium of economic interest used in winemaking. This bacterium is the preferred species for malolactic fermentation (MLF) due its adaptability to the chemically harsh wine environment. MLF enhances the organoleptic properties and ensures deacidification of wines.

The aim of this work was the transcriptional characterization of six *O. oeni* strains, four of them selected from distinct winemaking regions of Portugal, as candidates to malolactic starters, and two commercial malolactic starters. Using crossed assays with wines from different Portuguese winemaking regions, strain characteristic transcriptional patterns induced by each wine were analyzed based on Random Arbitrarily Primed PCR (RAP-PCR).

The obtained results suggest that the starter strains showed more constrained and limited transcription profiles, whereas a high variation on the distribution of the transcription profiles was observed for the regional strains in each wine.

According with our results, RAP-PCR is a useful technique for a preliminary investigation of strain behavior under different wine environmental conditions, which can be applied in field studies to monitor differential patterns of global gene expression and to select markers for the surveillance of malolactic starters performance in winemaking, as well as for quality and safety control.

1. Introduction

Malolactic fermentation (MLF) is one of the most difficult steps to control in winemaking. MLF, especially important for high acidic wines, decreases its total acidity, and enhances organoleptic properties. *Oenococcus oeni* is the lactic acid bacteria (LAB) mainly responsible by MLF. This species is normally well adapted to the harsh environmental conditions of wine (Lonvaud-Funel, 1999). In addition to LAB occurring naturally in wine, malolactic starter strains of *O. oeni* are often used during the winemaking process to improve the efficiency of the MLF. Differences between malolactic starter strains are related to their inherent stress resistance (Guzzo et al., 1998).

Molecular techniques known in general as RNA fingerprinting include differential-display PCR (DD-PCR) (Liang and Pardee, 1992; McClelland et al., 1995; Chia et al., 2001), fluorescent differential display (FDD) (Ripamonte et al., 2005; Sico et al., 2009; Bonomo et al., 2009) and random arbitrarily primed PCR (RAP-PCR) (Welsh et al., 1992; Wong and McClelland, 1994; Shepard and Gilmore, 1999; Du and Kolenbrander, 2000; Frias-Lopez et al., 2004; Papadimitriou et al., 2008) and have become routine to examine changes in gene expression. These molecular methods could be useful tools to identify differently expressed transcripts and to compare the difference of cDNA fingerprints when certain environmental conditions and time periods are involved, using small amounts of RNA (Collin and Olsén, 2001; Lockyer et al., 2004).

RAP-PCR utilizes an arbitrary primer at a low annealing temperature for both the first- and second-strand cDNA synthesis reactions (Welsh et al., 1992; Shepard and Gilmore, 1999). Differences in gene expression could be detected from the obtained fingerprinting pattern by observing the presence or absence of specific products obtained from different populations of cells (Shepard and Gilmore, 1999). In spite of its intrinsic variability, common to other random priming methods, this technique has been successfully applied to several prokaryotic systems (Wong and McClelland, 1994; Shepard and Gilmore, 1999; Papadimitriou et al., 2008; Garbeva and de Boer, 2009; Ferraz et al., 2010).

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In the present study, the transcriptional profiles of *O. oeni* strains were characterized by RAP-PCR under environmental wine conditions, in order to analyze the effect of distinct wine matrices and to investigate the potential of this approach in the selection of malolactic starter strains.

2. Materials and methods

2.1. Wine samples

Four finished wines from different winemaking regions (Douro, Dão, Ribatejo and Alentejo) of Portugal were used in this study. The finished wines from Douro, Dão, Ribatejo and Alentejo region presented 1.43 g/l, 1.35 g/l, 1.47 g/l and 0.92 g/l of malic acid, respectively. Malic acid was added to each wine sample in order to obtain a final concentration of 3 g/l, approximately. pH values were 3.65 for Douro wine, 3.57 for Dão wine, 3.29 for Ribatejo wine and 3.53 for Alentejo wine.

The four wine samples were previously sterilized by filtration through a 0.2 μm pore size membrane. The efficiency of the sterilization method was confirmed by plate medium inoculation (MRSm agar) followed by 8 days incubation at 30 °C.

2.2. Bacterial strains and growth conditions

Six *O. oeni* strains were selected, based on their enological characteristics (unpublished results), including four strains previosouly isolated from wines of different Portuguese winemaking regions, namely Douro (AI202), Dão (ID58), Ribatejo (Agro1) and Alentejo (IAL7) (Marques et al., 2011) and two commercial malolactic starters (VP41 and PSU-1).

Strains were cultivated until the end of exponential phase in MRSm (MRS, Merck; pH 5.5 supplied with 0.5% malic acid) medium at 30 °C. The cells were harvested by centrifugation, washed with sterile distilled water and resuspended in sterile distilled water to approximately 10^8 cfu/ml. The bacterial suspension of each *O. oeni* strain was used to inoculate the experimental media (wine from Douro, Dão, Ribatejo and Alentejo region, previously sterilized by filtration, and also MRSm culture medium) at a rate of 2% (v/v). Incubation was carried at 20 °C during two, four and eight days.

2.3. DNA and RNA extraction, quantification and DNase treatment

After growth for a period of two, four and eight days in the different wines and also in the culture medium, the cells from *O. oeni* strains were harvested by centrifugation. The pellets were washed twice with phosphate buffer saline (PBS) pH 7.0 at 10 mmol/l, resuspended in 250 μL Tris–EDTA with 10 mg/ml lysozyme and incubated at 37 °C for 1 h.

DNA was extracted using an UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). RNA was extracted using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cells were homogenized in 1 ml of Trizol® reagent and centrifuged at 12 000 *g* for 10 min at 4 °C. Samples were incubated at room temperature for 5 min followed by the addition of 200 μl chloroform, incubation at room temperature during 3 min and centrifugation at 12 000 *g* for 15 min at 4 °C. Aqueous phase was collected to a new diethylpyrocarbonate (DEPC) treated tube and the RNA was precipitated by the addition of 500 μl isopropyl alcohol followed by incubation at room temperature for 10 min and centrifugation at 12 000 *g* for 10 min at 4 °C. RNA was washed by gentle mixing with 1 ml of 75% ethanol followed by centrifugation at 7400 *g* for 5 min at 4 °C. After air-drying, the purified RNA was resuspended in 50 μl of DEPC-treated water. The RNA samples were quantified by measuring the absorbance at 260 nm with Anthos Zenyth 3100 (Anthos Labtec Instruments, Salzburg, Austria). RNA samples of known concentration were used for calibration.

To avoid false positive amplification in reverse transcriptase PCR (RT-PCR), the residual contaminating DNA was removed by DNase treatment. For each sample, 3 μg of RNA were treated with 50 U DNase I (Invitrogen) and incubated at 37 °C for 45 min. The reaction was stopped, after the addition of EDTA to a final concentration of 25 mmol/l, by heating at 65 °C for 15 min. The efficiency of the treatment was confirmed by negative PCR amplification of the housekeeping gene *rpoB*. After DNase treatment, RNA integrity was assessed by agarose gel electrophoresis; 1 μg of RNA of each DNase-treated sample were loaded on a 1% agarose gel in 0.5% TBE buffer at a voltage of 90 V for 2 h. The remaining RNA was stored at −80 °C and further used for cDNA synthesis.

2.4. RNA fingerprinting

RNA fingerprinting analysis was performed by random arbitrarily primed PCR (RAP-PCR).

In order to select 3 primers for RAP-PCR experiments, an initial screening was performed with three RNA and three DNA samples using ten different primers: csM13 (Huey and Hall, 1989); 1281 and 1290 (Akopyanz et al., 1992); UBC275 (University of British Columbia, Canada); OPC19 (Operon Technology, Alameda, CA, USA); $(GACA)_4$ and $(GTG)_5$ (Meyer et al., 1993); pA and pH (Ulrike et al., 1989); and JV17HC (Le Jeune et al., 1995). The selected primers for the RAP-PCR analysis were csM13, $(GTG)_5$ and pH. Primers, dNTPs and reagents used for RT-PCR were purchased from Invitrogen (UK) and reagents used for PCR amplifications were purchased from Bioron (Ludwigshafen, Germany), with the exception of primers and dNTPs that were also from Invitrogen.

cDNA synthesis was performed using SuperScript™ III reverse transcriptase (RT) in accordance with the manufacturer's instructions, but with some modifications. Briefly, 210 ng of random hexamers primers (these short primers provided cDNA for a large number of different genes), 0.1 mmol/l deoxynucleoside triphosphates (dNTPs) and sterile DEPC-treated water were added to 400 ng of total RNA to a final volume of 13 μl. The mixture was heated to 65 °C for 10 min, followed by incubation on ice during 1 min. Subsequently, 4 μl of 5× First-Strand buffer, 1 μl 0.1 mol/l dithiothreitol (DTT), 1 μl RNaseOUT™ recombinant RNase inhibitor (40 U/μl) and 0.5 μl of SuperScript™ III RT (200 U/μl) were added to a final volume of 20 μl. RT-PCR reactions were performed in a T Personal Themocycler (Biometra, Goettingen, Germany) as follows: incubation at 25 °C for 5 min and at 50 °C for 45 min. Upon completion of first-strand cDNA synthesis, the reaction was heated to 70 °C for 15 min to inactivate RT and then immediately stored at −20 °C.

DNA and cDNA were amplified using a reaction mixture containing 2.5 μl of 1× PCR buffer, 0.65 μl of $MgCl_2$, 0.5 μl of dNTPs (10 mmol/l), 1 μl of one random primer (csM13 or $(GTG)_5$ or pH) (50 μM), 2.5 μl BSA (10×), 1.25 μl DMSO, 0.2 μl of DFS Taq DNA polymerase (5 U/μL) and 1 μl of cDNA or DNA in a final volume of 25 μl. The reaction was incubated at 95 °C for 5 min and the following parameters were used for 40 cycles of PCR: 94 °C 1 min, 40 °C 1 min, 72 °C 2 min; a final extension was performed at 72 °C for 10 min. Upon completion, the reaction was stored at 4 °C.

In order to select the best method for RAP-PCR products visualization, three samples were visualized on 1% agarose gel stained with ethidium bromide and also on a 6% polyacrylamide gel stained with silver nitrate.

The RAP-PCR products were firstly visualized on 1% agarose gel (to confirm the presence of amplification products) and then resolved on a 6% polyacrylamide gel prepared in TBE buffer. Electrophoresis was performed at 60 V during 3 h. The agarose and