Use of AFLP analysis for the selection of *Oenococcus oeni* strains as starters in wine malolactic fermentation

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RIASSUNTO 1ST Italiano

La tecnica molecolare AFLP è stata utilizzata per genotipizzare una popolazione di *Oenococcus oeni*. L'analisi AFLP ha permesso di sviluppare profili molecolari che discriminavano singoli ceppi. Su alcuni ceppi selezionati è stato studiato anche il metabolismo del malato. Dai risultati ottenuti si evince che alcuni ceppi possono essere considerati potenziali candidati da utilizzarsi come starters per fermentazioni malolattiche industriali.

ABSTRACT 2ND English

A molecular assay based on the Amplified Fragment Length Polymorphism (AFLP) technique was performed for genotyping strains of *Oenococcus oeni*. The AFLP technique discriminated at strain level and proved to be a valid alternative to other molecular methods. The malate metabolism was also tested in several selected strains based on AFLP analysis. According to genotyping and malate metabolism analyses some selected strains may be considered potential candidates as starters in industrial malolactic fermentation.

INTRODUCTION

Malolactic fermentation (MLF) is a fundamental biochemical reaction occurring in wine at the end of alcoholic fermentation. During MLF, bacteria convert malic acid into lactic acid and carbon dioxide (Ribéreau-Gayon et al., 2006) contributing to the organoleptic quality of wines through the production of flavour compounds. (Lonvaud-Funel, 1999).

This is a spontaneous process induced by the indigenous flora or could be induced artificially through injection of starter cultures. The last process is a commonly practice used in the cellar and fermentations and consists in the injection of bacterial strains selected to ensure the quality of the product. Various bacterial genera have been reported to be able to conclude the MLF and, among them, *Oenococcus oeni* is the most important species involved.

Many strains of this species are well adapted to survive and grow in wine (Bartowsky, 2005). The spontaneous development of *O. oeni* strains in wine is related to their different ability to use such energy sources as sugars, L-malate and amino acids. (Ribéreau-Gayon et al., 2006).

Molecular tools were widely used to study microbial diversity of grapes and wine and they have proved to be particularly useful in identifying strains within species that are homogeneous at genome level, such as for *O. oeni* species. Genotyping diversity among

strains of this species was performed using DNA fingerprinting based on Restriction analysis of genomic DNA, as Restriction Endonucleases Analysis-Pulsed-Field Gel Electrophoresis, Polymerase Chain Reaction-based methods, as Random Amplified Polymorphic DNA, *16S-23S rDNA* intergenic spacer region analysis-PCR (Kelly et al., 1993; Viti et al., 1996; Zavaleta et al., 1997; Sato et al., 2000; Zapparoli et al., 2000; Bartowsky et al., 2003). It has been demonstrated that genotyping, besides having a taxonomic value, has practice relevance for the individuation of strains of interest for biotechnological processes (Rodas et al., 2005). Several strains of *O. oeni* were identified by distinct genomic fingerprinting according to the geographical origin (Zapparoli et al., 2000, Guerrini et al., 2003).

Recently, a high genotypic heterogeneity of wild *O. oeni* strains, isolated in wines undergoing spontaneous MLF, was demonstrated (Lòpez et al., 2007). Strains discriminated by molecular tools can be characterized by biochemical and technological assays in order to verify their aptitude to represent a possible fermentation starter (Coucheney et al., 2005). Thus, the availability of a reliable tool able to explore genetic source represented by malolactic microflora harbouring in grapes and wines, would be recommended.

Our objective in this study was to use Amplified Fragment Length Polymorphism (AFLP) analysis as tool for molecular characterization of *O. oeni* population ,.with the perspectives to select strains for using as a MLF starter in wine. This technique which has proved to be the most useful tool so far for discriminating *O. oeni* among strains of bacteria, *Lactobacillus* (LAB) included (Gancheva et al., 1999; Rico et al., 2004; Hong et al., 2005) Finally, malate metabolisms was tested in several strains in order to individuate possible candidates to be used as a MLF starter.

MATERIAL AND METHODS

Bacteria DNA extraction

Lactic acid bacteria cells at the exponential growth phase were harvested by centrifugation and suspended in 1ml of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) solution containing 10mg/ml of lysozyme (Sigma, Germany). Cells were then lysed by adding 100µl of 10% SDS (w/v) and 10µl of proteinase K (20 mg/ml) (Sigma). Crude DNA preparation was purified by performing two phenol/chloroform/isoamyl alcohol (25:24:1) and one chloroform/isoamyl alcohol (25:1) extractions. Genomic DNA was precipitated by adding two volume of cold ethanol. The precipitated DNA was washed with 70% ethanol and left to air dry. The DNA pellet was dissolved in an appropriate volume of TE buffer. Concentration of genomic DNA was spectrophotometrically quantified using the Nanodrop ND-1000 Apparatus (Celbio, Italy).

Fluorescent AFLP analysis

The AFLP fingerprinting (Vos et al., 1995) was performed using the AFLP Microbial Fingerprinting Kit (Applied Biosystems-PE Corporation, Foster City, California U.S.A) according to the manufacturer's instructions. Six separate primer combinations were used for the selective amplification: E1-M1 Fam (*EcoRI*CA - *MseI*AA); E2-M1 Fam (*EcoRI*CC - *MseI*CA); E3-M3 Fam (*EcoRI*CG - *MseI*AG); E1-M2 Joe (*EcoRI*CA – *MseI*AC); E2-M3 Joe (*EcoRI*CC – *MseI*AG); E2-M3 Joe (*EcoRI*CG - *MseI*AC). The Eco*RI* primers were labelled with fluorescent dye (Applied Biosystems-PE Corporation). The PCR cycling condition for these reactions was done according to the AFLP microbial fingerprinting protocol by using a model PCR Express CELBIO Italy. Gene Mapper version 4.0 software (PE Applied Biosystems) was used to automatically size and quantify individual fragments by using the

internal lane standard. For clustering, fragments between 50 and 500 bp were analysed with NTSYS software by using the Dice similarity coefficient based on presence/absence of the bands and clustered by the un weighed pair group method with arithmetic Mean (UPGMA).

Malate metabolism assays

To determine the malic acid consumption rate, strains were cultured in MRS–tj, to which were added 2.5 g/l L-malic acid, monitoring cell growth by a turbimetric assay. Concentration of L-malic acid was determined enzymatically using Boehringer kit L-Malic acid (Mannheim, Germany). Cultures were sampled and, after removing bacterial cells by centrifugation, malic acid depletion was enzymatically monitored.

Malolactic activity (MLA) was determined in whole *O. oeni* cells according to (Zapparoli et al. 2004). MLA was expressed as μmol L-malic acid decarboxylated per hour and per mg dry weight. One unit $O.D._{600}$ was equivalent to 0.4 g dry weight (dry litre)$^{-1}$. (Cavin et al., 1998)

Malate metabolism assays were carried out at least in triplicate and values were analysed statistically by Tukey's Multiple Comparison Test. Mean values were considered significantly different when $p < 0.05$.

RESULTS and DISCUSSION

Characterization of the 87 isolates belonging to *O. oeni* species was carried out by AFLP analysis (Cappello et al., 2008 submitted). Each primer combination used produced an average of 50.4 amplification product per strain (107 with AC/G to 22 with AC/A). M3-E2 JOE primer pair was the most selective primer combination, producing a fragments number ranging from 100 to 107, in the strains analyzed.

The data, obtained by the AFLP assay of 87 *O. oeni* isolates, were statistically analyzed by UPGMA cluster analysis and a dendrogram was produced, showing the genetic distance among each single genotype. The strains studied were divided into four different principal clusters (A, B, C and D).

The homogeneous cluster A, comprising 39 isolates, showed a high AFLP similarity, from 93% to 95%. An amount of 21 strains clustered at group B with an AFLP similarity of 64%, they clustered together with the reference strain (O. oeni IOB8413; Oenological Institute of Bordeaux France).

In the group C clustered 16 isolates, having an AFLP similarity of 63% together with the type strain. The cluster D resulted the most variable and showed the lowest AFLP similarity among the 11 isolates grouped (58%).

Our results indicate that AFLP is a highly discriminatory technique among strains belonging to other microbial species, as seen from previous investigations (Gancheva et al., 1999; Rico et al., 2004; Hong et al., 2005) and that it could represent an alternative tool in order to study genotypic variation in *O. oeni*. Moreover, the most precise level of inter-species discrimination of AFLP method, in respect to other molecular tools utilized in studying this bacterium, was useful for a reliable selection of strain to be assayed for malate metabolisms. The utilization of this acid was widely demonstrated as being beneficial for bacterial survival and growth in wine (Cox and Henich-Kling, 1995).

Twenty eight strains were selected from 87 *O. oeni* as representatives of the clusters A, B, C and D and used to assay the malate metabolism. Different capabilities to metabolise the malate in MRS-tj were observed according to the statistical analysis. In general, strains that consumed more malic acid in MRS-tj showed a higher specific L-malate consumption rate. In particular, strains clustered at AFLP cluster A were less effective in metabolizing malic acid

than strains clustered in the other AFLP group. On the contrary, the majority of the strains more effective in metabolizing malic acid, clustered at AFLP cluster B.

This strain collection constituted the starting point for the selection as possible starters for quality wine production and by AFLP analysis it was possible to recognize genotyping relationship among them.

CONCLUSION

The AFLP analysis evidenced that the cluster D showed a 58% AFLP similarity in respect to clusters A, B and C. Moreover in the 87 examined isolates, the AFLP analysis revealed a 31% of variability.

Data of the malic acid consumption linked to the specific L-malate consumption rate furnished relevant information concerning the fermenting capability of the selected strains. In order to select bacteria with high malate metabolism performances and genomically different, representative strains for each AFLP cluster could be individuated as suitable candidates for further selection assays. Within cluster A one strain showed the highest malic acid consumption rates.

In conclusion, this study indicated a valid approach to individuate *O. oeni* strains destined for a selection program for malolactic starter cultures. The exploitation of the high discriminatory power of AFLP among several isolates from *Primitivo* wine which underwent a spontaneous MLF allowed a reliable strain typing. By the analysis of genotyping and malate metabolism data good candidates were identified, which could be destined to a further selection program, after evaluating their performance by microvinification experiments. Apart from its applicative aim this study, also represents a contribution in understanding the population dynamics of *O. oeni* during wine malolactic fermentation.

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