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Bio-molecular characterisation of indigenous *Oenococcus oeni* strains from Negroamaro wine

Maria Stella Cappello [a,*], Stefania De Domenico [a], Antonio Logrieco [b], Giacomo Zapparoli [c]

[a] Institute of Science of Food Production, CNR, 73100 Lecce, Italy
[b] Institute of Science of Food Production, CNR, 70126 Bari, Italy
[c] Department of Biotechnology, University of Verona, 37134 Verona, Italy

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ABSTRACT

The variation in the coding capacity within *Oenococcus oeni* can have a significant impact on wine quality. The detection of several genes involved in important metabolic pathways (i.e. citrate, sulphur and arginine metabolisms) was performed on 10 indigenous *O. oeni* strains from Negroamaro wine, a red table wine (Apulia, Italy). These strains were selected from 95 isolates, collected during spontaneous malolactic fermentation, according to the results of an Amplified Fragment Length Polymorphism (AFLP) analysis. A total of 16 genes were screened, most (11) of which had never previously been assayed on *O. oeni*. All strains possessed 10 genes encoding enzymes such as malolactic enzyme (*mle*A), esterase (*est*A), citrate lyase (*cit*D, *cit*E and *cit*F), citrate transporter (*mae*P), α-acetolactate decarboxylase (*als*D), α-acetolactate synthase (*als*S), S-adenosylmethionine synthase (*met*K) and cystathionine β-lyase (*met*C) and resulted negative in the detection of genes encoding cystathionine γ-lyase (*met*B), ornithine transcarbamylase (*arc*B) and carbamate kinase (*arc*C). The sequence of PCR fragments of 11 genes of a representative strain (ITEM 15929) was compared to those of three reference *O. oeni* strains. The indigenous strain was phylogenetically more similar to PSU-1 and ATCC BAA1163 than AWRI B429. This study describes new genetic markers useful for detecting the genetic potential of *O. oeni* strains to contribute to aroma production and for investigating the population structure of the species.

1. Introduction

Malolactic fermentation (MLF) is an important secondary process in winemaking and generally occurs after alcoholic fermentation has been completed and plays a significant role in the elaboration of the majority of wines, in particular red ones.

Oenococcus oeni is the main lactic acid bacterium involved in this biological process, due to its excellent adaptation capacity to wineries (Marques et al., 2011). Extensive studies over several years have furnished a considerable amount of information on the genetic, physiology and metabolism of this bacterium (Lonvaud-Funel, 1999; Liu, 2002; Bartowsky and Borneman, 2011; Borneman et al., 2012; Abrahamse and Bartowsky, 2012). Secondary metabolic activities exhibited by *O. oeni* have great impact the sensory properties of wine (Lonvaud-Funel, 1999; Liu, 2002; Bartowsky and Borneman, 2011). The increasing acquisition of data on these activities stimulated our investigations into variability within indigenous populations isolated from various winemaking environments. Indeed, strains can modify wine flavour in different ways, according to their metabolic diversity and great interest has been shown in the study of intraspecific heterogeneity in *O. oeni* (Bartowsky et al., 2003).

Analysis of the structure of *O. oeni* populations, by genetic characterisation of representative strains, is recognised to be a necessary step to investigate MLF microbiology in a certain winemaking area (Borneman et al., 2012). High intraspecific variability among *O. oeni* strains has been ascertained by different non-PCR and PCR based approaches, such as Pulse Field Electrophoresis Gel (PFGE) and Random Amplified Polymorphism DNA (RAPD-PCR) (Ruiz et al., 2008; Vigentini et al., 2009; Bridier et al., 2010; Solieri et al., 2010; Bartowsky and Borneman, 2011; Borneman et al., 2012). Moreover, Amplified Fragment Length Polymorphism (AFLP), that is a technique based on the combination of both approaches, has proven to be a powerful tool for *O. oeni* typing (Cappello et al., 2008, 2010). Intraspecific diversity of *O. oeni* was also investigated by a

Abbreviations: MLF, Malolactic fermentation; LAB, Lactic acid bacteria; PCR, Polymerase Chain Reaction; PFGE, Pulse Field Electrophoresis Gel; RAPD-PCR, Random Amplified Polymorphism DNA; AFLP, Amplified Fragment Length Polymorphism; MLST, Multi-Locus Sequence Typing.

* Corresponding author. Tel.: +39 0832 422607; fax: +39 0832 422620.
E-mail address: maristella.cappello@ispa.cnr.it (M.S. Cappello).

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sequencing-based approach such as Multi-Locus Sequence Typing (MLST) or comparative genomic analysis (De las Rivas et al., 2004; Delaherche et al., 2006; Bilhère et al., 2009; Borneman et al., 2010; Bordas et al., 2013; Gonzáles-Arenzana et al., 2013). This approach carried out on target housekeeping genes described perfectly the *O. oeni* strain variability within different populations and provides information on the correlation between the phenotype and the genotype (Renouf et al., 2008, 2009).

In this work, we examined indigenous *O. oeni* isolates from Negroamaro wine (Apulia, Italy) undergoing spontaneous MLF with different molecular techniques. Strain discrimination was carried out by AFLP technology that, despite being used widely for several bacteria species, is not so common for *O. oeni*. Moreover, we also used PCR-based detection to investigate the isolates for the presence of genes coding for enzymes of relevance in winemaking, such as those encoding proteins involved in the citrate, sulphur and arginine metabolisms. Finally, genetic relatedness between a representative Negroamaro wine strain and reference *O. oeni* strains was examined by gene sequence fragments.

2. Materials and methods

2.1. Strain isolation and culture conditions

Lactic acid bacteria were isolated from Negroamaro wine produced during spontaneous must fermentation in the experimental cellar Conti Guarini (Spongano Italy) during the 2010 vintage. A total of 250 kg of grapes, from five different vineyards in the Negroamaro wine area, were vinified. At the devatting, the wine had a pH value of 3.60 and ethanol 12.7% (v/v), total acidity 3.77 g/L as tartaric acid, residual sugars 5.8 g/L, volatile acidity 0.39 g/L as acetic acid, L-malic acid 1.40 g/L, L-lactic acid 0.43 g/L. During the AF L-malic acid did not undergo to depletion. In order to collect malolactic bacteria sampling was carried out during MLF, which started about a week after the devatting and lasted for 49 days. Wine samples were weekly collected during the MLF. The isolates were grouped according the three different phases of MLF, beginning, middle and end which corresponded to the L-malic acid depletion up to 20%, 21–80% over 81%. In particular the strain ITEM16023 was isolated during the initially phase of the malolactic fermentation process. The strains ITEM15929, ITEM15930 and ITEM15992 were isolated in the middle phase where the highest L-malate consumption rate was measured. Lastly the strains ITEM15933, ITEM15956, ITEM16009, ITEM16021and ITEM16018 were isolated to the end of the fermentation process. To prevent any contamination source, all trials were conducted in an area of new construction within of the winery. Five millilitres of each wine was sampled, to which was added 1 volume of sterile glycerol (100% v/v) and stored at −80 °C. Appropriate dilutions of must and wine were also plated on MRS supplemented with 2% tomato juice pH 4.8 (MRS-tj), to which was added 0.1 mg/ml cycloheximide to suppress yeast growth.

The bacterial strains, deposited in the culture collection at the Institute of Sciences of Food Production, Bari, Italy (ISPA) (http://www.ispa.cnr.it/Collection), were grown at 28 °C for 7–10 days in (MRS-tj), pH 4.8 in a 5% CO_2 atmosphere conditions, using the Anaerogen™ 2.5 L System (Oxoid, UK).

2.2. Identification of O. oeni and PCR amplification of 16S rDNA

A total of 240 lactic acid bacteria isolated during MLF were morphologically analysed by optical microscopy and those having cocci cells were analysed for *O. oeni* by species-specific PCR according to Zapparoli et al. (1998). DNA extraction from pure culture was carried out as described by Cappello et al. (2008). Species attribution was confirmed after 16S rDNA gene sequencing, amplified by PCR as previously described (Klijn et al., 1995).

2.3. Fluorescent AFLP analysis

AFLP fingerprinting was performed using the AFLP Microbial Fingerprinting Kit (Applied Biosystems-PE Corporation, Foster City, CA.), according to the manufacturer's instructions. Approximately 10 μg of genomic DNA was digested with EcoRI and MseI (New England Biolabs, Hitchin, Hertfordshire, UK) and the DNA fragments were bonded to double-stranded restriction site-specific adaptors from the kit. The pre-selective and selective PCR reactions were carried out as previously described (Cappello et al., 2008). For clustering, fragments of between 50 and 500 bp were analysed with NTSYS software using the Dice similarity coefficient, based on the presence/absence of the bands and then clustered by the unweighted pair group method with arithmetic mean (UPGMA). Using the Dice product–moment correlation coefficient. The repeatability of the AFLP technique was determined by separate experiments using the genomic DNA of three strains, and the primer pairs A/C (E2-M1 FAM) and C/G (E2-M3 JOE) that produced the highest number of fragments.

2.3.1. Biodiversity indices

Simpson index (*D*) (Simpson, 1949) and Shannon–Wiener index (*H*) (Whittaker, 1972) were calculated to have further information on strain composition in the *O. oeni* population. The taxon strain was established at 98% of similarity according to the AFLP dendrogram in this study and Cappello et al. (2008, 2010). The index *D* was calculated as $\sum(n_i(n_i-1))/(N(N-1))$, and the index *H* as $-\sum(n_i/N \ln(n_i/N))$ where n_i is the number of isolates of $_i$th strain and *N* is the total number of isolates. Shannon–Wiener equitability index was calculated as $E = H/\ln S$ where *S* is the total number of strains. Simpson diversity index (SID) is expressed as $1 - D$ ranges from 0 to 1 (1 − 1/number of strains) approaching 1 as the number of strains increases; *H* ranges from 0 to ln (1/number of strains) (generally between 1.5 and 3.5), while *E* ranges between 0 and 1 with 1 being complete evenness. Evenness expresses how evenly individuals in a population are distributed over the different strains. When *E* is close to 0, it indicates that most of individuals belongs to one or few strains, when *E* is close to 1, it indicates that each strain consists of the same number of individuals.

2.4. DNA sequencing

The PCR products of the isolates and the genotypes were sequenced using an ABI PRISM™ GeneticAnalyzer (Applied Biosystems, Foster City, CA, U.S.A.). Sequence similarity searches were carried out using basic local alignment search (BLAST) (Altschul et al., 1997) on the GeneBank databases.

2.5. PCR detection of enzyme-encoding genes and malate metabolism assay

The presence of 16 genes was detected in *O. oeni* by PCR amplification (Table 1). Ten of them (*mle*A, *bgl*, *est*A, *mae*P, *als*D, *als*S, *met*K, *met*C, *met*B and *gsh*R) were analysed using new primers developed in this study, deduced from nucleotide sequences of *O. oeni* PSU-1 (locus_tag:OEOE_1569). PCR reactions were also carried out using primers obtained with sequences available in the literature in these genes (Mtshali et al., 2012) (see Table 2).

By contrast, in order to detect the genes encoding proteins involved in arginine metabolism (*arcA*, *arcB* and *arc*C) the primers described by Araque et al. (2009) were used, while those encoding enzymes involved in citrate metabolism (*cit*D, *cit*E and *cit*F) were