Food Microbiology 38 (2014) 295–302

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Oenococcus oeni strain typification by combination of Multilocus Sequence Typing and Pulsed Field Gel Electrophoresis analysis

Lucía González-Arenzana, Pilar Santamaría, Rosa López, Isabel López-Alfaro*

ICVV, Instituto de Ciencias de la Vid y del Vino (Gobierno de La Rioja, Universidad de La Rioja and CSIC), C/Madre de Dios 51, 26006 Logroño, La Rioja, Spain

ARTICLE INFO

Article history:
Received 24 January 2013
Received in revised form
26 July 2013
Accepted 30 July 2013
Available online 14 August 2013

Keywords:
PFGE
MLST
Oenococcus oeni

ABSTRACT

Oenococcus oeni is usually the main lactic acid bacteria (LAB) responsible for conducting malolactic fermentation (MLF) in wines. Pulsed Field Gel Electrophoresis (PFGE) is one of the most common methods used to identify different genotypes among the wine LAB populations. Although PFGE is a powerful typing tool, it is time-consuming and its results are not easily exchangeable between laboratories so typing methods such as Multilocus Sequence Typing (MLST) have been developed. In this study, thirty *O. oeni* isolates from Rioja Tempranillo wines were characterized performing *Sfi*I and *Apa*I PFGE and MLST with eight housekeeping genes. Using the latter technique, six new alleles have been described for five genes. PFGE was slightly more efficient than MLST because of the number of genotypes and of the index of diversity (ID) that each technique discriminated. This has been the first time that PFGE and MLST results have been combined to shape a unique dendrogram. Thus, the combination of results from both typing methods allowed the discrimination of twenty-two PFGE-ST genotypes showing the highest ID of these research (0.947). According to these results, the future application of the combination of PFGE and MLST results could be successful for reliable *O. oeni* strain typification.

1. Introduction

The species *Oenococcus oeni* has been shown to be the best adapted lactic acid bacteria (LAB) to the pH and ethanol of wine, and so it is the most frequently detected species during malolactic fermentation (MLF) (López et al., 2007; Pramateftaki et al., 2012).

Several studies have been conducted so far focussing on the investigation of *O. oeni* biodiversity to gain insight into the complex ecosystem of wine, to select and to prepare well-defined starters of biotechnological interest in winemaking and to study the contribution of certain strains to wine composition (González-Arenzana et al., 2013; Izquierdo et al., 2004; Vigentini et al., 2009). All these studies have used efficient and precise molecular methods to identify and to discriminate strains. The genetical and phylogenetical homogeneous *O. oeni* characteristic makes strain differentiation only possible through high resolution techniques such as those based on DNA analysis (Le Jeune & Lonvaud- Funel, 1997). Several typing methods have been employed to identify *O. oeni* strains, among which macrorestriction analysis of DNA by Pulsed Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST) have been found to be the most efficient (Bilhère et al., 2009; Bridier et al., 2010; de las Rivas et al., 2004). The two methods are also interesting since they target different genetic variations: MLST reveals punctual mutations and also longer deletions and mutations in a few genes, whereas PFGE is more sensitive to large-scale genomic rearrangements (Bilhère et al., 2009). Moreover, MLST targeting housekeeping genes has the advantage of generating portable and comparable data between laboratories that are used, not only for strain identification, but also for evolutionary and population studies (Maiden et al., 1998).

In addition, some authors have reported that only the combination of results from different techniques is able to provide a complete picture, especially when the aim is to study the ecology of natural microbial populations (Nigatu, 2000). Other authors achieved better discrimination after combining numerical analysis of the patterns obtained from PFGE and randomly amplified polymorphic DNA (RAPD) (Ruiz et al., 2008; Sánchez et al., 2004). However, RAPD-PCR method has been critisized for lack of reproducibility and efficacy even when two primers were used at the same time (López et al., 2008).

Therefore, this study was designed with the aim of establishing a method that completes the current way of typing *O. oeni*. For this purpose, PFGE with *Sfi*I and *Apa*I endonucleases was performed along with the MLST of eight of the most informative housekeeping

* Corresponding author. ICVV, Servicio de Investigación y Desarrollo Tecnológico Agroalimentario, Ctra. de Mendavia-Logroño (NA 134, km. 88), 26071 Logroño, La Rioja, Spain. Tel.: +34 941 291383; fax: +34 941 291392.
E-mail addresses: isabel.lopez@larioja.org, isabel.lopez@icvv.es, ilopezalfaro@yahoo.es (I. López-Alfaro).

0740-0020/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fm.2013.07.014

Table 1
Genes and primers employed for MLST analysis.

Gene	Enzyme function	Primer	Sequence (5′-3′)	Amplicon size (bp)
ddl	D-Ala-D-Ala ligase	*ddl-1*	CGATGTTAGCAAGCGTTCG	911[a]
		ddl-2	TTCGTATTTCCCGGTAGTG	
gyrB	Gyrase, β subunit	*gyrB-1*	TGGGCTTCATGGTGTTGGC	947[a]
		gyrB-2	CCCTCGACGATAAACAATTC	
rpoB	RNA polymerase, β subunit	*rpoB-1*	CGATATTCTCCTTTCTCCAATG	665[b]
		rpoB-2	CTTTAGCGATCTGTTCCAATG	
purK	Phosphoribosylamino-imidazole carboxylase	*purK-1*	TGGTTATCATGTTGGTATTTTGG	597[b]
		purK-2	GAAGCAGGAGCATAGGAAAGA	
g6pd	Glucose-6-phosphate dehydrogenase	*g6pd-1*	TTATATGTCTGTTGCTCCTCGT	669[b]
		g6pd-2	CCGGTTCTGATGTAAAAAGG	
pgm	Phosphoglucomutase	*pgm-1*	ATATCTGCCGAAGTGCTAAGAG	654[b]
		pgm-2	AGCAGCAATTTGATTTCCAG	
dnaE	DNA polymerase III, α subunit	*dnaE-1*	CGTATATAGAGCGCTTTGCC	714[b]
		dnaE-2	CGTTCTTATCGCGAGTTGTAC	
recP	Transketolase	*recP-1*	AGCGACAAACCATCCTTTATC	676[b]
		recP-2	CGACAGCTAAGGAATCATGAG	

[a] de la Rivas et al. (2006).
[b] Bilhère et al. (2009).

genes (Bilhère et al., 2009; de las Rivas et al., 2004). Results have been analyzed to compare and to combine the discriminatory ability of these two typing techniques.

2. Materials and methods

2.1. O. oeni isolates

Thirty randomly selected *O. oeni* isolates were included in this study. They are part of the strain collection of CIDA Research Centre of the Spanish northern region of La Rioja and they were obtained in a previous study of LAB ecology carried out in 2006, 2007 and 2008 vintages in several wineries in this region (González-Arenzana et al., 2013).

O. oeni isolates were grown in MRS agar (Scharlau Chemie S.A., Barcelona. Spain) modified with tomato juice (10% vv^{-1}), fructose (6 gL^{-1}), cysteine-HCl (0.5 gL^{-1}) and D,L-malic acid (5 gL^{-1}). The plates were incubated at 30 °C under anaerobic atmosphere (Gas Pak System, Oxoid Ltd., Basingstoke, England).

2.2. PFGE analysis

PFGE was carried out according to the method described by Birren and Lai (1993), with some modifications for the agarose block preparation (2007). Macrorestriction analysis was performed with two endonucleases: *Sfi*I, following the method reported by López et al. (2007), and *Apa*I, according to the method reported by Larisika et al. (2008) with the following modifications: 1.2% (wv^{-1}) agarose gels were submitted to 24 h with a pulse ramping between 0.5 and 20 s at 14 °C and 6 Vcm^{-1} in a CHEF DRII apparatus (Bio-Rad Laboratories, Hercules, CA).

2.3. MLST analysis

Genomic DNA was extracted from fresh culture plates following the method of rapid lysis described by López et al. (2008). According to recent literature on typing *O. oeni* by MLST, eight housekeeping genes encoding proteins were chosen for this analysis (Table 1): *ddl* (D-Ala-D-Ala ligase) and *gyr*B (Gyrase, β subunit) described by de las Rivas et al. (2004); and *rpoB* (RNA polymerase, β subunit), purK (Phosphoribosylamino-imidazole carboxylase), *g6pd* (Glucose-6-phosphate dehydrogenase), *pgm* (Phosphoglucomutase), *dnaE* (DNA polymerase III, α subunit) and *recP* (Transketolase) described by Bilhère et al. (2009).

PCR was performed in order to amplify these gene fragments from DNA of *O. oeni* strains by using the oligonucleotides included in Table 1. Each 50 μL- amplification reaction mixture contained 20 ng template DNA, $MgCl_2$ 2.5 mM, 20 μM of each deoxynucleotide triphosphate, 500 mM of each primer and 2.5 U BIOTAQ™ DNA polymerase (Bioline, London, UK). PCR program was: 95 °C for 2 min; followed by 30 cycles of 95 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min; followed by a final extension of 10 min at 72 °C. PCR was carried out with a Perkin Elmer Thermal GeneAmp PCR system 2700 and the obtained amplicons were purified and sequenced in Macrogen Inc. (Seoul, South Korea). Nucleotide sequences of the eight housekeeping genes were deposited in GenBank under the following accession numbers: JX240023–JX240052(*rpoB*), JX239993–JX240022 (*ddl*), JX240173–JX240202 (*purK*), JX240203–JX240232 (*g6pd*), JX240143–JX240172 (*pgm*), JX240053–JX240082 (*dnaE*), JX240083–JX240112 (*gyrB*) and JX240113–JX240142 (*recP*).

2.4. Numerical analysis of gel images and sequences

The conversion, normalization, and further processing of the stained gel images were carried out by InfoQuest™ software version 5.1 (Bio-Rad). Comparison of the pulse types obtained from the PFGE for *Sfi*I and *Apa*I was made by Composite Data combined comparison with average of experiment by Unweighted Pair Group Method using Arithmetic averages (UPGMA) (Ruiz et al., 2008). The weight of the results from each endonuclease was set at the same level.

The chromatograms and sequences obtained for the eight genes of the MLST scheme and the thirty bacterial isolates were analized by using InfoQuest™ 5.1. The sequences generated a consensus dendrogram by using the Composite Data combined comparison with average of experiment and UPGMA. The relevance of each gene was set at the same level to assess the dendrogram. Each different combination of allelic profiles was then determined as a sequence type (ST). Consequently, the allelic profiles that were 100% indistinguishable in the dendrogram shared the same ST.

Furthermore, each distinct gene sequence was compared with the *O. oeni* allelic sequences deposited to date in GenBank and an allele number was assigned. The identification number of alleles previously determined by de la Rivas et al. and Bilhère et al. was assigned to identical sequences (Bilhère et al., 2009; de las Rivas