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Two different *Oenococcus oeni* lineages are associated to either red or white wines in Burgundy: genomics and metabolomics insights

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Abstract

Oenococcus oeni is the bacterium most often associated with spontaneous malolactic fermentation (MLF) of wine. During MLF, malic acid is transformed into lactic acid and several metabolites are modified, modulating wine's total acidity and improving its sensory properties. Previous works have suggested that certain genetic groups of *O. oeni* strains are associated to different kinds of products. In the present study we have spotted two groups of strains isolated mainly from Burgundy wines, one associated to red wines and the other to white wines. Sequencing 14 genomes of red and white wine strains revealed that they share a common ancestor that probably colonised two different substrates –red and white wine-associated environments–, diverging over time and disseminating to various regions. Their capacity to perform MLF and modify the volatile profile of wine was determined by fermenting a chardonnay wine and analysing its volatile fraction with a non-targeted metabolomics approach by GC-MS. The strains had a different impact on the volatile composition depending on their group of origin. These results show for the first time a correspondence between the product of origin of the strains and the volatile profile of the wines they produce. Furthermore, the genetic features that might be implied in these different phenotypes are examined.

Keywords : *Oenococcus oeni*, malolactic fermentation, comparative genomics, metabolomics

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OENO One, 2017, **51**, 3, 309-322

Introduction

Microorganisms have, for millennia, played a central role in the discovery and development of fermented food by humans. It has been observed that the biogeography of microorganisms is influenced by human practices, as microorganisms have been domesticated to different food matrices that are produced in different regions (Legras *et al.*, 2007; Douglas and Klaenhammer, 2010). Even for foods and beverages that are made nearly worldwide such as bread and wine, in which species are not always specific to a region or product, local variations in the biogeography of microorganisms have been observed in the form of genomic traces (Legras *et al.*, 2007; Almeida *et al.*, 2015). Even if *Saccharomyces cerevisiae* is the main yeast species responsible for the fermentation of wine, the contribution of the microbiological signature of non-*Saccharomyces* genera to the development of typical wine aroma has already been studied (Capozzi *et al.*, 2015). The complexity of the ecosystem associated to oenological environments leads to a discussion about the possible existence of the so-called microbial terroir (Gilbert *et al.*, 2014). Evidence suggests, at least for wine, that soil microbiome influences the grapevine-associated microbiota and that this microbial signature might be partially responsible for differential wine phenotypes (Bokulich *et al.*, 2014; Zarraonaindia *et al.*, 2015; Knight *et al.*, 2015).

Oenococcus oeni is the main species responsible for the malolactic fermentation (MLF) of wine, which normally follows the alcoholic fermentation (AF) produced by yeasts (Davis *et al.*, 1986). Multilocus sequence typing analyses have revealed that the population of *O. oeni* is composed of a great diversity of strains that cluster in at least three main genetic groups, named A, B and C and a number of subgroups that sometimes correlate with their region (Chile, South Africa, Eastern France) or product of origin (cider, red and white wines, champagne) (Bilhère *et al.*, 2009; Bridier *et al.*, 2010). Genomic studies based on sequences of a few tens of strains have confirmed these genetic groups and they have suggested that ancestral *O. oeni* strains associated with fruits have been progressively domesticated to develop in cider and in wine, the strains of group A being presumably the best-adapted to wine (Borneman *et al.*, 2012; Campbell-Sills *et al.*, 2015). Recently, analysis of nearly 200 genomes, mostly from Australian isolates, showed that more than 60% of Australian isolates cluster in a closely-related group (a subgroup of A), suggesting that strains of this group may out-compete the other strains during fermentation or that they are well suited to Australian winemaking conditions (Sternes and Borneman, 2016).

During MLF *O. oeni* converts malic acid into lactic acid and CO_2, which makes wine softer in taste (Lonvaud-Funel, 1999). It also produces or degrades numerous secondary metabolites that can modify the fruity, vegetal or smoked aromas and contribute to the overall complexity of wine aroma (de Revel, 1999; Bartowsky, 2005; Vallet *et al.*, 2008; Antalick *et al.*, 2010; Antalick *et al.*, 2012). Several studies have been made regarding the impact of different strains of *O. oeni* and other LAB in the composition of wine after MLF, both in primary and secondary metabolites (Pozo-Gayón *et al.*, 2005; Ugliano and Moio, 2005; Lee *et al.*, 2009a; Lee *et al.*, 2009b; Hernandez-Orte *et al.*, 2009; Costello *et al.*, 2013; Sumby *et al.*, 2013; Malherbe *et al.*, 2013). However, it is still unknown whether strains of the same group have similar impacts on the quality of wines.

In a recent survey of lactic acid bacteria present during MLF in wines and ciders produced in diverse regions of France, we have collected nearly 3000 isolates of *O. oeni*, whose genotyping revealed 514 strains clustered into 43 genetic groups (El Khoury *et al.*, 2017). Most groups contain strains collected in different regions or different types of wines. Conversely, each regional wine, including those of Burgundy, contains strains that cluster in several different genetic groups. Nevertheless, two peculiar groups were detected from Burgundy wines. Group A5 contained 17 strains isolated almost exclusively from white wines of this region and group A2.8 included 28 strains from the region's red wines. The present study aimed at analysing the specificities of these groups. We have analysed strains of both groups at the genomic level in order to confirm their clustering and to determine their phylogenetic position within the *O. oeni* species. We have also compared their capacity to perform MLF in both types of wines and their impact on the volatile compounds. The results suggest that these two groups of strains have evolved to adapt to the two types of wines found in Burgundy and other regions and that the presence of strains from one or the other group during MLF modulates differently the quality of wines.

Materials and methods

1. *O. oeni* strains and culture conditions

O. oeni strains were obtained from the Biological Resources Center Oenology (CRBO) of ISVV (Villenave d'Ornon, France). Strains CRBO_14194, CRBO_14195, CRBO_14196, CRBO_14198,

CRBO_14200, CRBO_14202 and CRBO_14203 were isolated from Chardonnay wines of Burgundy and strains CRBO_14205, CRBO_14206, CRBO_14207, CRBO_14210, CRBO_14211, CRBO_14212 and CRBO_14213 from Pinot noir wines of Burgundy. Strain CRBO_11105 was isolated from a red wine of Aquitaine and strain CRBO_14214 from red wine of Val de Loire. All the strains were propagated at 26 °C in a grape juice medium containing 25% commercial grape juice, 5 g/L of yeast extract and 0.1% tween80, adjusted to pH 4.8 with KOH. Commercial strains PN4™ and VP41™ were obtained from Lallemand SAS in their commercial format.

2. Wine and malolactic fermentation conditions

MLF trials were performed in a Pinot noir (12.6% alcohol, pH 3.15, titratable acidity 4.90 g/L and malic acid 3.6 g/L) and a Chardonnay wine (12.8% alcohol, pH 3.02, titratable acidity 5.10 g/L and malic acid 3.1 g/L) from Burgundy region. They were filter sterilised progressively at 3 µm, 0.8 µm and 0.2 µm. Filtered wines were stocked in 70 mL tubes at 4 ºC until inoculation. Four strains of each group A5 and A2.8 were propagated in grape juice medium, collected by centrifugation and inoculated to $2 \cdot 10^6$ cells/mL in wine to start MLF. Lyophilised commercial strains were used according to the manufacturer's instructions (Lallemand SAS) and were inoculated at $2 \cdot 10^6$ cells/mL. MLF were carried out at 20 °C in 20 mL flasks with a minimum of contact with air. Trials were performed in triplicate and MLF progression was followed twice per week in only one of the replicates in order to limit the contacts with air for the two other replicates. MLF progression was monitored by determining malate concentration using the Roche L-Malic acid kit according to the manufacturer's recommendations (r-Biopharm).

3. Genomic DNA purification, DNA sequencing and assembly

Microbial DNAs used for genome sequencing were extracted using the wizard genomic DNA purification kit according to manufacturer's recommendations (Promega). The genomic DNAs were sequenced by Illumina MiSeq technology with paired-end reads and read length of 250bp at the Genomic and Transcriptomic Facility of Bordeaux. The obtained reads were cleaned with trim_galore v. 0.4.0 and extended with FLASH v1.2.11 (Magoc and Salzberg, 2011). Genomes were assembled *de novo* with Minia v. 1.0.6 (Chikhi and Rizk, 2013). Each genome was assembled either from the clean reads, either from the clean and extended reads, with kmer lengths of 25, 37 and 49, giving a total of 6 independent assemblies per genome. Assembly statistics were calculated using homemade scripts and the best of the six assemblies for each genome was kept based on their assembly statistics (N50, N90, largest contig size, shortest contig size).

4. Phylogenomic trees

The distances between genomes were calculated using ANIm algorithm with JSpecies v. 1.2.1 software (Richter and Rosselló-Móra, 2009). The obtained similarity matrix was transformed into a distance matrix and parsed into the format required by MEGA using homemade scripts. Phylogenomic trees were reconstructed by the neighbour joining method with MEGA v. 6.06 (Tamura *et al.*, 2013).

5. Genomes annotation and subsystems classification

Genomes were annotated on the RAST platform with Classic RAST annotation scheme, RAST gene caller and FIGfam Release70 (Aziz *et al.*, 2008). Frame shifts fixing was turned on. The features of the genomes annotated by RAST were systematically classified in subsystems as part of the annotation pipeline and data mining was facilitated through the SEED environment (Overbeek *et al.*, 2014). A matrix containing the quantity of genomic features classed into each subsystem category was built for each strain. For cluster analysis, the matrix was normalised with the formula log1p(x-min(x)), where x represents the number of features. The clusterisation was performed using Canberra distances and Ward clustering method using pheatmap R package. Since Canberra distances computation does not admit vectors composed of only 0's, the normalised categories composed of only 0's were replaced by 1's; it doesn't have any effect in the clusterisation given that they represent non-informative categories (i.e. all the strains have the same number of features for the same category, hence they do not contribute to their discrimination).

6. Chemicals

Ethanol (≥99.9%) was obtained from Merck (Damstadt, Germany). Ethyl butyrate-4,4,4-d_3 (>99%), ethyl hexanoate-d_{11} (>98%), ethyl octanoate-d_{15} (>98%) and ethyl *trans*-cinnamate-d_5 (phenyl-d_5) (>99%) were obtained from Cluzeau (Sainte-Foy-la-Grande, France). Sodium chloride (norma pure) was purchased from VWR Chemicals (Fontenay-sous-Bois, France).

OENO One, 2017, **51**, 3, 309-322

7. Chromatography conditions (HS-SPME-GC/MS)

A method was adapted from Antalick *et al.* (2010). 5 µL of internal standard solution (ethyl butyrate-4,4,4-d_3, ethyl hexanoate-d_{11}, ethyl octanoate-d_{15} and ethyl *trans*-cinnamate-d_5 (phenyl-d_5) at 200 mg/L in ethanol) was added to 5 mL of wine, then introduced into a 20 mL standard headspace vial filled with 3.5 g of sodium chloride. The solution was homogenized with a vortex shaker and then loaded onto a Gerstel autosampling device. The program consisted of swirling the vial at 500 rpm for 2 min at 40 °C, then inserting the fibre into the headspace for 30 min at 40 °C as the solution was swirled again, then transferring the fibre to the injector for desorption at 250°C for 15 min. The fibre used was polydimethylsiloxane 100 µm (PDMS-100) (Supelco, Bellefonte, PA, USA). It was conditioned before use as recommended by the manufacturer.

Gas chromatographic analyses were carried out on an Agilent 7890A GC system coupled to an Agilent 5975C quadrupole mass spectrometer and equipped with a Gerstel MPS2 autosampler. Injections were in the splitless mode for 0.75 min, using a 2 mm I.D. non-deactivated direct liner. A BP21 capillary column (50 m x 0.32 mm, 0.25 µm film thickness, SGE, Courtaboeuf, France) was used and the carrier gas was helium N55 with a column-head pressure of 8 psi. The oven temperature was programmed at 40 °C for 5 min then raised to 220 °C at 3 °C/min and then held at that temperature for 30 min. The mass spectrometer was operated in electron ionization mode at 70 eV with selected-ion-monitoring (SIM) and SCAN mode.

8. Untargeted metabolomics analysis by PARAFAC

All raw chromatogram files were exported from Agilent Chemstation version D.03.00.611 (Agilent Technologies) as netCDF-files and imported into MATLAB version 8.0 (The MathWorks Inc., Natick, MA, USA) using built-in functions. In-house written and PLS-Toolbox functions have been used for further data processing in MATLAB. Preprocessing of the multi-way array was done using the nprocess.m function of the N-way toolbox. Prior to the mathematical transformations useless parts of the chromatogram at the beginning and at the end were removed. The data analysis approach has been reported recently (Vestner *et al.*, 2016). The methodology consists of the segmentation of full scan GC-MS chromatograms along the retention time axis (corrected by an internal standard) and mathematical transformations including the calculation of sums of squares and cross product (SSCP) matrices of segments. The result of the segmentation and mathematical transformation is a three-way array with the dimensions *number of samples* × *number of samples* × *number of segments* (first and second mode are identical) which can be decomposed using parallel factor analysis (PARAFAC). Loadings of the first and second mode (sample mode) of the PARAFAC model can be interpreted in the same way as PCA scores, while the loadings of the third mode (segment mode) are represented as congruence loadings which represent the contribution ('correlation') of a segment on the corresponding PARAFAC component. Segments with high congruence loadings (> 0.75) are considered to 'highly correlate' with the corresponding component and therefore, as important to explain systematic differences among samples which are represented by this component in the sample mode loadings ('scores'). Important segments are deconvoluted and peak profiles are integrated using AMDIS (Stein, 1999) and corrected by an internal standard. All peaks that are significantly different (Student's *t*-test, alpha = 0.5) between the two groups of lactic acid bacteria are compiled in a peak table. The identification of peaks is done by comparing their spectra against the NIST database.

Results

1. Genomic features and phylogenomic distribution of strains

Six and eight strains of groups A5 (white wine) and A2.8 (red wine), respectively, were selected to produce and compare their genomes. They were sequenced by the Illumina method and assembled to produce drafts of 127 to 287 contigs (table 1).

All the reported genomes have a size of around 1.8 Mb, which is consistent with previous reports for *O. oeni* (Mills *et al.*, 2005; Borneman *et al.*, 2010; Borneman *et al.*, 2012; Campbell-Sills *et al.*, 2015). The number of protein encoding genes (PEG) that were detected and annotated by RAST fall in the order of ~1800, which is also comparable with data reported in the literature (Mills *et al.*, 2005; Borneman *et al.*, 2010; Borneman *et al.*, 2012; Campbell-Sills *et al.*, 2015; Sternes and Borneman, 2016). To determine their phylogenetic distribution, a phylogenomic tree was reconstructed with these 14 newly sequenced genomes and 50 additional ones reported on NCBI (Borneman *et al.*, 2012, Campbell-Sills *et al.*, 2015). The tree was calculated from ANIm distances and reconstructed by the

Table 1. Assembly and annotation statistics of the sequenced strains

Group	Strain	Sequence coverage (X)	Accession	Genome size (bp)	Number of contigs	N50	L50	N90	L90	PEGs
A5	CRBO_14194	38	SAMN04122363	1786610	196	27411	18	4263	82	1847
	CRBO_14195	71	SAMN04122364	1789621	127	49436	13	7354	49	1853
	CRBO_14196	48	SAMN04122365	1798795	208	27547	23	5901	77	1862
	CRBO_14198	88	SAMN04122366	1789795	174	28822	19	6019	73	1850
	CRBO_14200	90	SAMN04122367	1789801	167	39836	13	5457	64	1847
	CRBO_14203	48	SAMN04122369	1807672	131	40244	15	7105	55	1874
A2.8	CRBO_11105	48	SAMN04122350	1793882	200	28533	23	4638	81	1830
	CRBO_14205	66	SAMN04122370	1729210	225	23427	21	3884	93	1772
	CRBO_14206	63	SAMN04122371	1738384	202	25660	21	4438	86	1790
	CRBO_14207	40	SAMN04122372	1779011	251	24022	23	4989	81	1806
	CRBO_14210	64	SAMN04122373	1830066	202	28303	19	5172	81	1893
	CRBO_14211	46	SAMN04122374	1775057	287	13491	39	3274	139	1822
	CRBO_14213	102	SAMN04122375	1814591	137	38947	15	7291	55	1901
	CRBO_14214	50	SAMN04122376	1754584	271	15632	33	3074	130	1786

neighbour joining method. Figure 1 confirms that all the new strains belong to the genetic group A as suggested previously (El Khoury *et al.*, 2017). They cluster in two separate groups that most likely represent subgroups A5 and A2.8. Surprisingly, the tree also revealed that the two groups are closely related and that they are more distant from all other genomes. This suggests that they have diverged from a common ancestor strain fairly recently, at least more recently than their separation from all other groups of strains. In addition, four strains isolated from champagne (IOEB_B16, IOEB_0205, AWRIB422 and AWRIB548) cluster together with white wine strains of group A5 and group A2.8 has two strains isolated from red wines of Aquitaine (CRBO_11105) and Val de Loire (CRBO_14214). The clustering of these strains is not inconsistent because although they come from different regions, they were isolated from wines that have comparable physicochemical properties as those of the white and red wines of Burgundy.

The newly sequenced strains have been placed in the phylogenomic tree reported by Campbell-Sills *et al.* (2015) by the ANIm method. The cluster composed of mainly Burgundy strains is shown, strains isolated from red wine (A2.8) are highlighted in red, strains from white wine (A5) are highlighted in yellow. The distance is expressed in dissimilarity percentage.

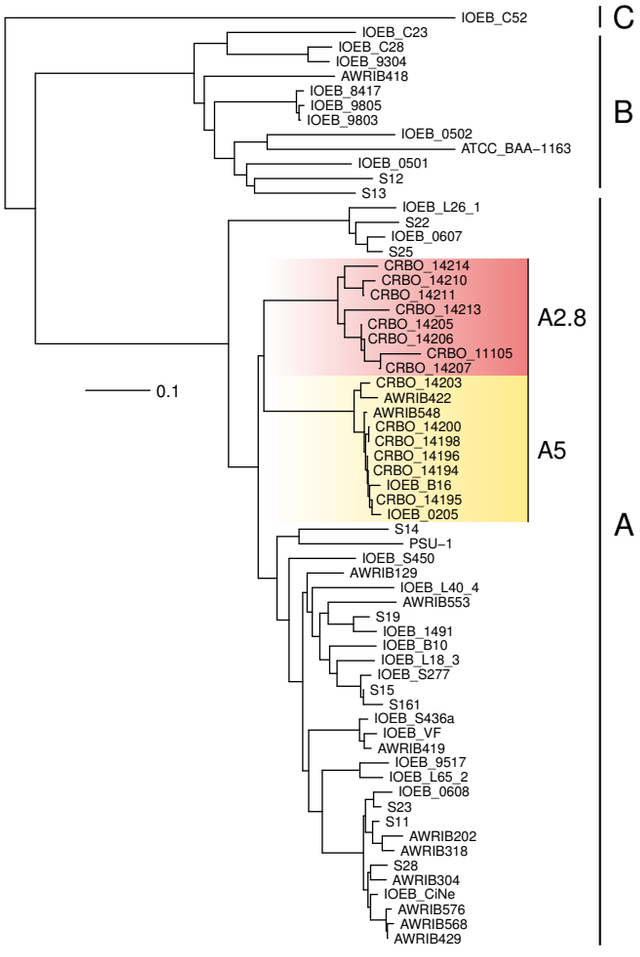

Figure 1. Phylogenomic tree of the sequenced strains.

2. Cluster analysis of subsystems

Genome annotations were analysed in order to investigate the main genetic differences between the

OENO One, 2017, **51**, 3, 309-322

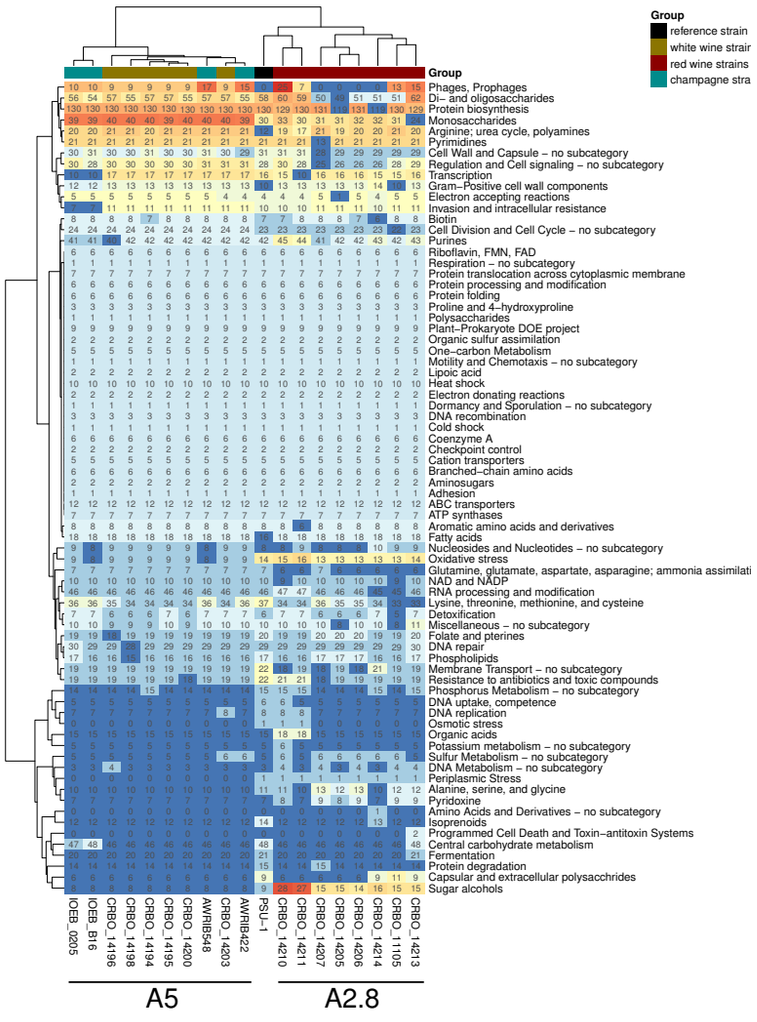

Figure 2. Cluster analysis of the subsystems of the annotated strains.

two groups of strains. The hierarchy of the functional roles of genes permits to classify the genetic functions into four levels: categories, subcategories, subsystems and roles, starting from the most general up to the most specific (Overbeek *et al.*, 2005). All the protein encoding genes (PEGs) of groups A5 and A2.8 strains, as well as those of the reference strain PSU-1, were classified according to this hierarchy, making a total of 22 categories, 74 subcategories, 241 subsystems and 796 roles.

A cluster analysis based on the 74 subcategories confirmed that the strains form two different groups and revealed the functional categories that contribute to distinguish each group (figure 2). The cluster analysis revealed that genes of the subcategories "phages", "di- and oligosaccharides", "monosaccharides", "cell wall and capsule" are more represented in group A5 strains. In exchange, genes of the "sugar alcohols", "oxidative stress" and "periplasmic stress" subcategories are more abundant in group A2.8 strains (t-test p-val < 0.0001). A preliminary analysis of the roles in these subcategories indicated the presence of fructose specific components and absence of mannitol specific components in group A5 strains, which is consistent with the same observation made for two of the analysed strains of champagne (AWRIB422 and AWRIB548) (Borneman *et al.*, 2012; Cibrario *et al.*, 2016). All the strains of this also group also contain a glucan synthesis gene producing a free or cell-bounded extracellular dextran, which was previously detected in champagne strains (Dimopoulou *et al.*, 2014). Among the specific genes of group A2.8 strains, we have detected those for mannitol and ß-glucoside utilisation and several genes involved in the stress response such as an organic hydroperoxide resistance protein, a ferroxidase and an iron-binding ferritin-like antioxidant protein that were previously reported in a variable genomic region present in different strains such as *O. oeni* PSU-1 (Bon *et al.*, 2009).

The numbers inside the cells indicate the number of features that fall into each subsystem category. Colour scale indicates from less abundant features (blue) to more abundant (red) in each category. Colour boxes in the upper dendrogram indicate the group of strains as indicated in the legend.

3. Metabolomics analysis

Strains of both groups were used to carry out MLF in a red wine of Pinot noir and a white wine of Chardonnay in order to determine if the resulting wines differ according to the type of strains. Four strains from each group were propagated in a laboratory medium and inoculated in wines to $2 \cdot 10^6$ cells/mL. Two commercial strains were used as positive control and a non-inoculated wine used as negative control. MLF was monitored during sixty days after inoculation. Strains of group A2.8 could achieve MLF in the Pinot noir wine with performances comparable to that of commercial strains, but A5 strains failed to start MLF in the same matrix (not shown). It is likely that these strains have a low tolerance for red wine as they also failed to start MLF in other trials performed in Pinot noir and Gamay wines (not shown). In the Chardonnay wine, group A5 strains were able to completely deplete malic acid (table 2) and so did the commercial strains used as positive control, while A2.8 strains had an heterogeneous behaviour.

The A2.8-group strains had an opposite behaviour. While they all completed MLF in red wine, only one completely degraded malic acid in the white wine and the other three had consumed only 10 to 80%. After the same period, malic acid was not degraded in a non-inoculated control wine.

Since strains of group A5 failed to initiate MLF in red wine, the comparison of volatile compounds produced by the strains of the two groups during MLF was only performed in white wines. The resulting MLF samples were characterised by HS-SPME-GC and analysed under an untargeted metabolomics pipeline based on a PARAFAC model following the method proposed by Vestner *et al.* (2016). One sample (CRBO_14212A) was discarded since it represented an outlier according to the algorithm of Filzmoser, Maronna and Werner. Segmentation of the chromatograms resulted in a total of 86 segments. 24 segments containing only baseline or artefact peaks such as siloxane peaks from column bleeding were excluded from the data set. The three-way array obtained from mathematical transformations of the remaining 61 segments had the dimensions 19 × 19 × 61 (number of samples × number of samples × number of segments) including duplicates of each sample. PARAFAC models with 2 to 15 components were built to examine the optimal number of components. Core consistency diagnostic, residuals, captured variance and interpretability of loadings were examined to find an appropriate PARAFAC model which explains the variation among samples the best. An 11 component PARAFAC model gave the best interpretable results by explaining 81.6 % of the total variation in the dataset. PARAFAC components two (16.1 % explained variation), seven (6.6 % explained variation) and eleven (4.5% explained variation) contain information on systematic differences between the two groups of samples (figure 3), while the other components reflect only unsystematic differences in the chromatograms. The segments 48 and 57 on component 2, the segments 15, 23 and 39 on component 7 and the segments 23 and 39 on component 11 are responsible for the differentiation of the two groups of samples. These segments are considered to be 'highly correlated' with the raw data

Table 2. Quantitation of malic acid at day 60 after inoculation

Group	Strain-repetition	Malic acid (mg/L)
A5	CRBO_14194-A	<0.01
	CRBO_14194-B	<0.01
	CRBO_14195-A	<0.01
	CRBO_14195-B	<0.01
	CRBO_14196-A	<0.01
	CRBO_14196-B	<0.01
	CRBO_14202-A	<0.01
	CRBO_14202-B	<0.01
A2.8	CRBO_14206-A	<0.01
	CRBO_14206-B	<0.01
	CRBO_14208-A	0.553
	CRBO_14208-B	0.52
	CRBO_14210-A	0.066
	CRBO_14210-B	1.597
	CRBO_14212-A	1.805
	CRBO_14212-B	2.051
Commercial	PN4™-A	0.009
	PN4™-B	0.137
	Lalvin VP41™-A	<0.01
	Lalvin VP41™-B	<0.01
Control	Control-A	2.363
	Control-B	2.24

OENO One, 2017, **51**, 3, 309-322

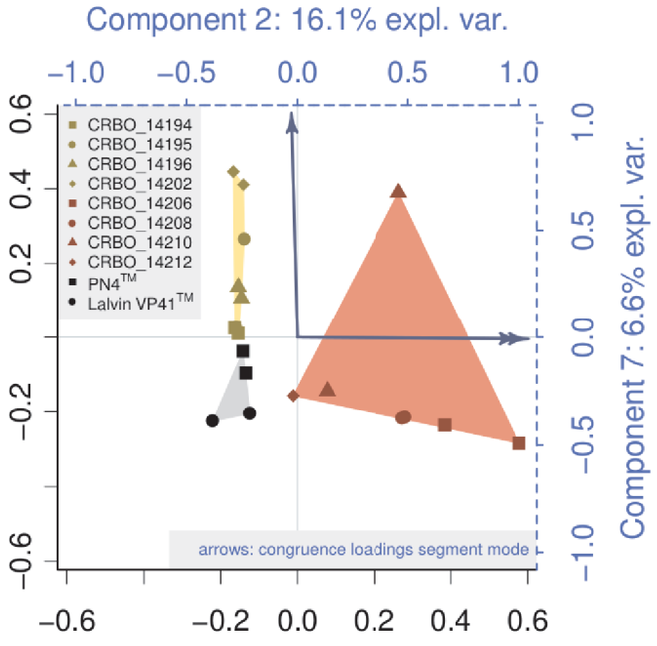

Figure 3. PARAFAC model of the MLF wine samples.

(congruence loadings > 0.75). Only peaks from these 5 segments were deconvoluted and integrated using AMDIS. All integrated peaks were checked for differences between mean values of the two groups of samples using Student's t-test with alpha = 5 %. Five peaks showed significant differences between the two groups of samples.

Two modes of PARAFAC are superposed: the samples mode (dots) and the loadings mode (arrows). The colours of the points and polygons indicate the group of the strains, either 'white wine' (yellow), 'red wine' (red) or commercial strains (black).

Of the five significant peaks identified by PARAFAC, two could be identified: they correspond to diethyl succinate and butyl ethyl succinate. A comparison of the peak areas of these compounds reveals that they are present at comparable concentrations in the wines fermented with white wine strains and the control wine, while it is present at about twice the concentration in wines fermented with red wine strains (table 3).

Discussion

In this study we delved into the biological and oenological significance of a monophyletic clade composed of two groups of *O. oeni* strains previously detected in red and white wines of Burgundy, throughout a genomics/metabolomics analysis. The two groups do not contain all the strains that develop in the red and white wines produced in this region, but they are remarkable because each of them contains strains that have been isolated from a single type of wine, suggesting that they are specifically adapted to develop either in red or in white wines (El Khoury *et al.*, 2017). Our phylogenomics analysis of 14 new genome sequences produced from these strains confirms their clustering and also reveals two unanticipated features. First, strains derived from champagne wines cluster together with strains of group A5 isolated from white wines. The group of champagne strains was previously detected by multi-locus sequence typing (Bridier *et al.*, 2010) and comparative genomics (Campbell-Sills *et al.*, 2015). Although it was initially thought that these strains were specific to champagne wines, their new position in group A5 clearly indicates that they are present in wines of different regions. Nevertheless, champagne wines are white wines and they share some physicochemical properties with those produced in Burgundy, especially a low pH. Therefore, it is not surprising that these strains group together. On the contrary, this supports the hypothesis that group A5 strains are specifically adapted to this type of wine. The second new information revealed by the phylogenomic analysis is that the two groups of strains A5 and A2.8 are phylogenetically very close. This was unanticipated because all the *O. oeni* strains sequenced to date split into a large number of lineages which are more or less close to each other. The most distant wine strains

Table 3. Significant peaks areas.

Strain \ RT (min)	15.256	19.201	28.633	32.934	37.184
CRBO_14194	1.3E-03	8.8E-03	3.5E-03	1.8E-01	1.9E-03
CRBO_14195	1.4E-03	1.0E-02	4.2E-03	1.8E-01	2.0E-03
CRBO_14196	1.5E-03	9.0E-03	4.0E-03	1.9E-01	2.3E-03
CRBO_14202	2.1E-03	1.1E-02	4.6E-03	2.1E-01	1.9E-03
CRBO_14206	5.3E-04	7.5E-03	3.5E-03	5.2E-01	5.6E-03
CRBO_14208	5.7E-04	5.6E-03	2.5E-03	5.1E-01	4.6E-03
CRBO_14210	1.4E-03	4.9E-03	2.3E-03	4.3E-01	4.2E-03
CRBO_14212	7.9E-04	1.8E-03	1.3E-03	2.7E-01	2.9E-03
PN4	1.1E-03	7.7E-03	3.6E-03	1.9E-01	2.1E-03
VP41	4.5E-04	8.3E-03	3.0E-03	1.5E-01	1.7E-03
C^-	0.0E+00	0.0E+00	9.6E-04	2.0E-01	2.5E-03
Tentative identification	-	-	-	Diethyl succinate	Butyl ethylsuccinate

reported to date are those of groups A and B, but even in group A the diversity is very important (Campbell-Sills *et al.*, 2015; Sternes and Borneman, 2016). Therefore the close proximity of two groups of strains that are supposedly associated with either red or white wines might have a special significance. It is likely that these two groups derive from a common ancestor and it is tempting to speculate that they split in the region of Burgundy, since the vast majority of strains were isolated from wines of this region (El Khoury *et al.*, 2017). However, a different scenario is possible. The two groups could have split in another region and, because the strains have capacities to develop in white or red wines, they were able to colonise those produced in Burgundy.

Although the objective of this study was not to compare the fermentation capacities of the two groups of bacteria, they were tested in different types of wines (red and white) to perform MLF. The strains of group A5 proved unable of initiating MLF in the red wine, whereas they performed well in white wine and conversely, those in group A2.8 achieved MLF in the red wine, but not always completely in the white wine. The results suggest that the origin of the strains correlates well with their fermentation capacities. However, the wines used in these trials were sterile filtered before inoculating the bacteria, which is not normal winemaking conditions and could have made the wines more difficult to ferment. Interestingly, even if the strains performed MLF in the white wine more or less efficiently, they produced wines whose volatile fractions were different according to the group of strains. Previous studies have shown that different strains have different impacts on the volatile fraction of wines (Pozo-Gayón *et al.*, 2005; Ugliano and Moio, 2005; Lee *et al.*, 2009a; Lee *et al.*, 2009b; Hernandez-Orte *et al.*, 2009; Costello *et al.*, 2013; Sumby *et al.*, 2013; Malherbe *et al.*, 2013), but this is the first time that this impact is correlated to the genetic proximity of the strains. The fact that the two groups of strains show differences in the volatile fraction of the fermented wines shed a new light on the existence of microbiological component associated with given wines and on the possible repercussions of the highlighted microbial diversity on the typical quality traits of regional wines, which is a field of considerable economic importance (Capozzi and Spano, 2011).

It is still difficult and speculative to correlate the genetic differences observed between the genomes of the two groups of strains and their possible adaptation to a type of wine or their capacity to produce volatile compounds during MLF. Nevertheless, since the two groups of strains are phylogenetically close, the sub-systems analysis revealed a limited number of specific genes in each group of strains, whose functions are particularly interesting. For instance, white wine strains carry the fructose specific components of the PTS, while red wine strains have the mannitol specific components. The features of PTS provide bacteria a system to assure optimal utilisation of carbohydrates in complex environments (Kotrba *et al.*, 2001) and variations in the PTS enzyme II sugar transporters have already been observed for a large collection of *O. oeni* strains (Sternes and Borneman, 2016). Several sugars are present in wine after alcoholic fermentation, especially fructose and pentoses such

OENO One, 2017, **51**, 3, 309-322

as ribose, arabinose and xylose (Ribéreau-Gayon *et al.*, 2012). LAB can use fructose as an e^- acceptor to produce mannitol during heterolactic fermentation, which permits the generation of ATP (Hornsey, 2007; Lahtinen *et al.*, 2011). It has been reported that *O. oeni* can use the mannitol pathway in fructose fermentation due to limiting redox regeneration capacity of the ethanol pathway and that the choice of the fermentation pathway between mannitol and fructose is tightly regulated in *O. oeni* in order to maintain the equilibrium of NAD(P)H (Richter *et al.*, 2003a; Richter *et al.*, 2003b; Cibrario *et al.*, 2016). It is not surprising then that the presence of the mannitol specific PTS components in red wine strains correlate with the presence of genes of oxidative stress response, as there are specific stressors characterizing red wines with respect of white ones. This is not the only function found in this study that might be related to the stress adaptation of *O. oeni*: a Dps protein that is lost in white wine strains, but present in red wine strains, has been observed to correlate with fitness in red wine (Bon *et al.*, 2009). In effect, another study has shown that *E. coli* over-expressing this gene has gained resistance to wine, copper and ferric ions (Athané *et al.*, 2008).

Exopolysaccharydes are very important for the adaptation of *O. oeni* to its ecological niche (Dimopoulos *et al.*, 2014). All the white wine strains carry the *gtf* gene, which is absent in all the red wine strains. The presence of this gene is correlated to an increased resistance to several stresses occurring in wine (alcohol, pH, SO_2) (Dols-Lafargue *et al.*, 2008). In particular, among this stressors, in the case of champagne and white wines of Burgundy the acidity is higher when compared to other wines. In the study by Dols-Lafargue *et al.* (2008), 7 out of 8 strains carrying the *gtf* gene had been isolated from white wine or Champagne. Just as for the genes of sugar utilisation, the presence of the *gtf* gene is not only a matter of survival for *O. oeni*, but might also have consequences at the organoleptic level since it is sometimes associated to a ropiness phenotype in wine (Dols-Lafargue *et al.*, 2008; Dimopoulos *et al.*, 2014).

Succinate and its derived esters are normally present in wine (Ribéreau-Gayon *et al.*, 2012). The formation of diethyl succinate during MLF carried out by *O. oeni* has been reported several times (Pozo-Bayón *et al.*, 2005; Ugliano *et al.*, 2005; Izquierdo Cañas *et al.*, 2008). Succinate, one of the precursors of diethyl succinate, can be combined with L-homoserine by the enzyme homoserine O-succinyltransferase (HSST), coded by the gene *metA*, in the reversible reaction succinyl-CoA + L-homoserine ⇌ CoA+ O-succinyl-L-homoserine. The HSST enzyme is also the first step in one of the three possible pathways of L-methionine biosynthesis from L-homoserine (Liu *et al.*, 2008), with succinate being re-released in one of the intermediary reactions catalysed by the enzyme Cystathionine gamma synthase (CGS) (Rowbury and Woods, 1964a; Rowbury and Woods, 1964b; Liu *et al.*, 2008). Although *O. oeni* does not carry the CGS enzyme, it does carry the cystathionine gamma lyase (CGL) enzyme, that has been reported to be able to produce α-ketobutyrate and succinate from O-succinyl-L-homoserine (Knoll *et al.*, 2011). The transcription of the gene coding for HSST is repressed by L-methionine (Saint-Girons *et al.*, 1988). A comparison against the genomes reported in Campbell-Sills *et al.* (2015) shows that all the white wine strains carry a frameshift mutation on the *metA* gene coding for the HSST enzyme, probably inactivating it. The gene coding the CGL enzyme, in exchange, is intact in all the strains. Our results suggest a link between the mutation of this enzyme in all the strains from white wine and the low levels of diethyl succinate produced, although the exact mechanism remains unknown. The fact that white wine strains could achieve MLF suggests that they are most probably obtaining L-methionine by other means; this is not surprising, since previous studies on 4 *O. oeni* strains determined that they were auxotroph for methionine (Remize *et al.*, 2006).

This preliminary exploration of the genetic features of these groups of strains probably explains only part of their adaptability to white and red wines, as well as their differences in the production of volatile compounds during MLF. Further genomics and gene expression studies are in progress with the aim of identifying all their differences. Nevertheless, these results show that the diversity of strains encountered in different wines and regions is not entirely random. There are strains lineages that are genetically better suited to develop and perform MLF in certain types of wines, as they possess different enzymatic equipment that impacts on the volatile fraction of wines.

Conclusion

The study of two genetic groups of *O. oeni* strains associated with wines of Burgundy and other regions, throughout a genomics/metabolomics analysis offers biological insights on the possible genetic determinants of *O. oeni* adaptation to white and red wine associated environments, confirming the increasing interest in the examination of

microbial diversity associated with fermented foods as possible general models in microbiology. Furthermore, we shed a new light on the existence of microbiological component associated with a given terroir and on the possible implications on the typical quality traits of regional wines. Further studies, including other non-volatile important metabolites and more strains of distant genetic groups, will give more clues on the impact of these variations at the organoleptic quality of wine.

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