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A survey of oenophages during wine making reveals a novel group with unusual genomic characteristics

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ABSTRACT

Oenophages have so far been mostly isolated from red wines under malolactic fermentation (MLF), and correspond to temperate or ex-temperate phages of Oenococcus oeni. Their genomes are clustered into 4 integrase gene sequence groups, which are also related to the chromosomal integration site. Our aims were to survey the occurrence of oenophages in a broader and more diverse collection of samples than those previously explored. Active phages were isolated from 33 out of 166 samples, which mostly originated from must and MLF. Seventy one phage lysates were produced and 30% were assigned to a novel group with unusual genomic characteristics, called unk. All unk members produced similar RAPD and DNA restriction patterns, were negative by PCR for the signature sequences previously identified in the integrase and endolysin genes of oenophages, and lacked any BamHI restriction site in their genome. The data support that development of additional and novel *signature* genes for assessing oeno*phage* diversity is now required.

1. Introduction

There's a large consensus in the scientific community that quality and identity of wines will benefit from a better knowledge of the bacterial, yeast and fungal communities associated with soils, grapes and products during fermentation. The unveiling of microbiote and its dynamics during wine making is expected to help in the identification of the ecological factors and farming systems that explain the biodiversity, and guide practices to avoid wine quality depreciation. Culture-dependent diversity surveys have been reported worldwide (Barata et al., 2012; Capece et al., 2013; Martins et al., 2012; Nisiotou et al., 2011; Renouf et al., 2007) and recent metagenomic analyses now offer a more complete picture of these communities (Bokulich et al., 2012; Piao et al., 2015; Pinto et al., 2014; Portillo et al., 2016; Zarraonaindia et al., 2015). Wine microbial consortia can be viewed as a dynamic world of interactions and some links have been recently observed between vineyard environmental conditions and microbial inhabitation patterns (Bokulich et al., 2014; Liu et al., 2017).

Molecular inventories of the grape-surface microbiota have recently demonstrated an unexpected diversity of eubacterial communities. Among them, discriminant populations of *Acetobacteraceae* and *Lactobacillales* represent sources of quality variation, being important organisms in wine spoilage (Bartowsky, 2009) and the latter in malolactic wine fermentations (MLF) (Bartowsky and Borneman, 2011; Lonvaud-Funel, 1999). Apart from these characterizations, very few data do consider phages are actors of the community, although they are an ubiquitous feature of prokaryotic existence (Clokie et al., 2011). Yet, examples from other ecosystems show that natural bacteriophage populations may play a role in shaping bacterial populations and communities as obligate parasites, vectors of horizontal gene transfer, drivers of bacterial evolution and mediators of competition among species (Koskella and Brockhurst, 2014). The magnitude and variability of phage populations during wine making remain so far unexplored, and substantial progress is needed to adapt the experimental designs to capture the whole viral community.

The few available data on phages of oenological origin have been obtained in the 1980s and 1990s and are restricted to phages infecting lactic acid bacteria (LAB), in relation with the capacity of some species to conduct MLF. The process, which reduces acidity and increases microbial stability, is largely driven by *Oenococcus oeni*, which is the best

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adapted species to the harsh conditions prevailing in wine (Lonvaud-Funel, 1999). Several studies investigated the presence of phages infecting *O. oeni* (or oenophages) in wine as it could offer alternative explanations to the delayed or stuck fermentations that are often encountered by winemakers. Pioneering studies mostly targeted red wine samples under spontaneous MLF, and numerous temperate phages infecting *O. oeni* were isolated (Arendt et al., 1990, 1991; Arendt and Hammes, 1992; Cazelles and Gnaegi, 1982; Davis et al., 1985; Huang et al., 1996; Nel et al., 1987; Poblet-Icart et al., 1998; Santos et al., 1998; Sozzi et al., 1976, 1982). However, their genetic and biological diversity remained largely unexplored and most were not subsequently characterized beyond their description by electron microscopy, and lytic spectra. Interestingly, the sensitivity of some isolated oenophages to low pH, ethanol and other components added to wine (such as bentonite) was demonstrated suggesting a rapid inactivation of phage particles in wine (Henick-Kling et al., 1986). Other authors also pointed out the high diversity of indigenous strains associated with spontaneous fermentation that can carry on the malolactic conversion (Ribéreau-Gayon et al., 2005) and suggested that a natural bacterial strain rotation may occur. Phage lysis of one part of the population would therefore not impact the overall malolactic conversion, as other strains may compensate the destruction and accomplish this task. In the absence of known incidents involving bacteriophages in the wine industry, phage load was considered as not representing a significant risk of fermentation failure during wine making, and studies with oenophages were not pursued during the last 20 years.

Recently, there has been some renewed interest in the characterization of prophages following large-scale sequencing of isolate genomes. Lysogeny was confirmed to be rather frequent in the species (Borneman et al., 2012; Jaomanjaka et al., 2013), in agreement with previous studies showing a frequency of 45–60% of *O. oeni* strains reported to be lysogenic (Arendt et al., 1992; Poblet-Icart et al., 1998). Prophages in *O. oeni* were shown to be incredibly varied in their properties, from host range, genetic content to persistence capacities (Borneman et al., 2012; Doria et al., 2013; Jaomanjaka et al., 2013, 2016) making classification a complex task. Genome sequences of oenococcal temperate phages are clustered into four groups, which are related to the chromosomal integration site (Ballestra et al., 2011; Borneman et al., 2012; Jaomanjaka et al., 2013). Integrase gene polymorphism was observed, and conserved sequences were identified in the integrase genes from each group. A PCR typing method was proposed and proved to be helpful in our laboratory to rapidly assess the genetic diversity of oenophages isolated either from wine samples or following induction of lysogens (Jaomanjaka et al., 2013). The selected sequences were also recently amplified from phage lysates obtained after UV induction of lysogenic strains collected from Piemont typical wines (Costantini et al., 2017).

All such recent advances now open new doors toward a better assessment of phage diversity and ways in which oenophages may influence their host's evolution and population dynamics. More studies are also needed when considering the growing trends of using MLF starters. The question as how phage predation may be integrated during selection, stabilization and use of commercial strains is still far from being straightforward. Because of their repeated use, bacteriophage attack could become problematic and could delay MLF and/or open the way for less desired, indigenous populations to dominate (Mills et al., 2005). On another hand, several commercial strains were recently shown to harbor prophages, which may be a source of phages during fermentation. However, as observed in other environments, lysogenic strains may not always result in detrimental consequences and the possibility that prophages have impact on the properties of their host may be of great interest for strain design.

The objective of our study was to extend the search of oenophages to different types of wines, geographical origins and steps during wine making. Interestingly, some newly-isolated phages did not respond to any of the signature sequences previously identified in the integrase and endolysin genes of oenophages and we posit that they form a new group. Together, our results reveal that diversity is higher than previously expected and that additional signature genes may be required for examining phage diversity in *O. oeni*.

2. Materials and methods

2.1. Bacterial strains

The strain *O. oeni* IOEB-SARCO 277 was used in this study. It was previously identified as not containing endogenous phage (Jaomanjaka et al., 2013). The strain was grown in liquid or solid MRS (Man Rogosa and Sharpe) (Difco, Fischer Bioblock Scientific, Illkirch, France) adjusted to pH 4.8 at 25 °C.

2.2. Collecting of wine samples

A total of 166 samples of oenological origin were collected from different wine estates located in the Bordeaux area, including the Institute of Vine and Wine Sciences of Bordeaux (ISVV) where an in-house vinification trial was conducted. Our survey encompassed 11 distinct appellations. Samples were collected during the vinification of red, dry white and sweet Bordeaux (Sauternes) wines during the 2013 and 2014 vintages, and corresponded to distinct grape varieties. It should be noted that sweet wines from Sauternes (botrytized wines) do not naturally undergo MLF. Detailed information is provided in Table 1 and in supporting online material (Table S1).

Total samples were taken during alcoholic fermentation, or AF (n = 91), malolactic fermentation, or MLF (n = 24) and also during ageing or in bottles (n = 21). The early steps of wine making also received a particular attention with the collecting of samples from must (n = 17), “pieds de cuve” (PDC, see below for details) or juices (n = 13).

The AF steps were driven using powdered or wild yeasts. When indicated, the traditional method of ‘pied de cuve’ (PDC) was used. It consists in collecting some riped and healthy grapes a few days before harvest in order to propagate indigenous or selected yeasts. The PDC is then inoculated in the vat to promote alcoholic fermentation. MLF were mostly driven by endogenous LAB, and the few cases where starters were used are indicated in Table S1. Different vats were occasionally collected at a same step in a given estate. Such samples corresponded to different conditions (aeration; spontaneous *versus* inoculated fermentation). Details are given in Table S1.

A total of 47 samples were punctual samples and traceability was only available at the level of the appellation. They included some samples collected during ageing as well as from bottles (Table 1). For the remaining 119 samples, traceability information was provided and included the identity of the estate (numbered A to S), type of wine, vinification step (must; PDC; AF or MLF) and presence of sulfites on grapes post-harvest. Fermentation progress was daily followed by density determinations.

A total of 114 of 119 samples were provided by 15 estates (A to O) which were chosen for kinetic analyses. Five additional estates (P to S) provided a unique sample.

Of note, information collected from winemakers indicated that all MLF were successful except for samples 148 and 149, which showed a delayed fermentation, and sample 163, for which a stuck fermentation was observed despite the inoculation of a commercial starter to trigger MLF.

All samples were stored at 4 °C before use.

2.3. Isolation of phages and purification

We used two distinct protocols in parallel. In protocol 1, samples were centrifuged (5000 × g, 10 min) and filtered. The 0.2 μm membrane filters made of polyether sulfone allowed most free phages to

Table 1
Global survey of oenophages in 166 samples of oenological origin.

Estate[a]		Step[b]											
A) Traceability to the estate													
Sam-ple	Appellation/grape var.	Juice	PDC	Must	AF				MLF		Ageing	Bottle	Pos/tot
					Start	Mid	End	PRO	Start	End			
A	Bordeaux/Sauvignon white	1$				4,5,6,7,8,9,10,11							0/9
B	Entre-deux mers/Merlot			61			122						0/2
C	Bordeaux/Merlot						132		133,134,135,136				1/5
D	Lussac Saint Emilion/Merlot	17$	22$,23$ 24$,25$				78,79,80,81,82,83						0/11
E	Lussac Saint Emilion/Sauvignon /Merlot		31	30	34,35,36,37,38,39		108,109,110,111, 112,113						0/14
F	Lussac Saint Emilion/Merlot				44,45,46,47,48,49,50	51,52,53,54,55,56	72,73,74,75,76,77						0/19
G	Saint Emilion/Merlot			62			124			161			0/3
H	Pomerol/ Merlot / Cabernet Sauvignon						85,86,87,88 89	139,140,141,142	145,146,147,144 143				0/12 1/2
I	Sauternes/Sémillon			67,68 69,70			119,121,126,138						4/8
J	ISVV/Cabernet Sévignon				84	114	120						0/3
K	Bordeaux white/Sauvignon white	2	26		27,28,29	64,65,43							1/8
L	Bordeaux/Merlot			115,116			117,118						0/4
M	Saint Emilion/Merlot	3	16	18	19,20	40,32	71,57						0/9
N	Sauternes/sémillon dry		12				13						1/2
O	Sauternes/Sauvignon white		14		15	21							0/3
P	Sauternes/Sémillon Muscatelle			162									1/1
Q	ISVV/Carménère									163*			1/1
R	Sauternes/Sauvignon			164									1/1
	Sauternes/Sémillon			165									1/1
S	Bordeaux/Merlot			166									1/1
B) Traceability to the appellation and grape variety													
na	Saint Emilion/Cab Franc			125		33	41,42,123,131					127,128, 129	5/9
na	Margaux			63			58,59,60						0/4
na	Pauillac/Cab Sauv-Merlot						66, 130				90,91,92,93,94,95, 96,97,98,99,100, 101,102,103,104, 105,106,107		2/20
na	Sauternes-Sauvignon			137									1/1
na	Gers / Gamay /Merlot								148* 149*				2/2
na	Médoc/Cab Sauv-Merlot								150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160				10/11
	Total		30				91		24		18	3	

[a]Estates are named from A to S; na, non-available;

[b]166 samples were collected at different steps and positive samples are underlined. Last column indicates the number of positive samples/total number analyzed; PDC, pied de cuve; AF, alcoholic fermentation; MLF, malolactic fermentation; PRO, post running-off; *delayed or stuck MLF; $ presence of sulfites. Wine processes where MLF does not naturally occur are indicated with a slash mark.

pass, while the temperate phages in lysogenic bacteria were removed (Muniesa et al., 2005). In protocol 2, unfiltered samples were inoculated into 10 mL of MRS broth (10% v/v) supplemented with 0.1 mg/mL pimaricin, an antimycotic compound killing yeasts, moulds and other fungi. Assays were incubated for 5 days at 25 °C, centrifuged and filtered. Since MRS was not seeded by *O. oeni* sensitive strains, incubation only allowed the growth of indigenous strains present in the samples. Among them, sensitive strains could be infected by free phages while natural lysogens were likely to release prophages. Both phenomena were expected to contribute to an increase in phage density.

All samples were stored at 4 °C. The phages were enumerated on the indicator strain *O. oeni* IOEB-SARCO 277 using the classical double-layer plating technique described by Jaomanjaka et al. (2013, 2016), on MRSΦ agar (MRS supplemented with $MgSO_4$ 3.75 g/L and $CaCl_2$ 2.375 g/L). The top agar solution was similar to regular agar medium except that the agar concentration was 6 g/L rather than the usual 20 g/L used for bottom agar plates. A mixture of a mid-log culture of the indicator strain (0.2 mL at OD_{600} 0.2–0.3) and 100 μL of samples (or dilutions thereof) were added in 5 mL of molten soft-agar and poured on a bottom MRSΦ plate. Detection limit of direct plaque assays was 10 plaque-forming units per mL (PFU/mL). Plates were incubated at 25 °C for 4 to 7 days before examination of plaques. Plaque morphology types were established on the basis of size (small, medium, large), clarity (turbid, clear), and edges (sharp or diffuse) of plaques. Isolated plaques on plates of each sample were examined, and the maximum number of plaque morphological classes produced by the sample was determined. Plaques were picked, suspended in 0.5 mL of sterile MRSΦ medium and stored at 4 °C. They were propagated on the same strain and plaques picked again. This step was repeated twice to ensure purity.

To obtain high-titers phage lysates, the soft-agar method described by Fortier and Moineau (2009) was used. Briefly, enumeration of the initial phage suspension was done as previously described. The dilution of phage yielding confluent lysis was determined and ten confluent lysis plates were prepared using this specific dilution of lysate. After incubation, soft agars were scraped off the plates with a spatula and transferred to a sterile tube and let stand for 30 min at room temperature to allow phages to elute from the soft-agar. The sample was centrifuged, and the supernatant was filtered. Phage titers ranging from 10^6 to 10^{10} PFU/mL were obtained and stored at 4 °C until use.

2.4. Concentration of phage and transmission electron microscopy

The lysate (10^9 PFU/mL) was treated with DNase I (1 μg/mL; Invitrogen, Illkirch, France) and RNase A (1 μg/mL; Promega, Charbonnières, France) at 37 °C for 30 min. Polyethylene glycol (PEG) precipitation was carried out as follows. Sodium chloride (final concentration, 1 M) and PEG 6000 (10% w/v) were added to the treated lysate. The sample was stored overnight at 4 °C and sedimented 10 min at 11000 × *g* at 4 °C. The pellet was carefully washed twice in TM buffer (50 mM Tris-HCl, $MgS0_4$ 10 mM), and resuspended in a small volume of the same buffer resulting in a 25 fold concentration. The sample was next washed with chloroform/isoamyl alcohol (24:1) (v/v). Phages (10 μL; ~10^{10}/mL) were deposited on carbon-coated copper grids for 30 s, and colored with uranyl acetate (saturated in water, pH 4.5 for 30 s). Stained particles were examined with a Hitachi H7650 electron microscope operated at 80 kV.

2.5. Extraction of phage DNA and restriction digestion

A purified lysate (12 mL) was subjected to ultracentrifugation at 20000 × *g* for 2 h (Beckman ultracentrifuge, SW32 rotor). The phage pellet was resuspended in 500 μL of TM buffer. Phage DNA was purified using the phenol chloroform method described by Sambrook and Russell (2001) and resuspended in 50 μL of Tris-EDTA buffer (pH 7.6). Phage DNA was quantified by optical density at 260/280 nm with a Biospec-nano spectrophotometer (Shimadzu, Columbia, USA). DNA was digested with the restriction endonucleases *Eco*RI, *Hin*dIII, *Nde*I and *Bam*HI under the conditions recommended by the manufacturer (New England Biolabs, Evry, France). Restricted DNA was electrophoresed on 0.8% (w/v) agarose gels in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA), and visualized by UV photography after staining with ethidium bromide. The 100 bp DNA ladder (M1), Lambda DNA digested with *Hin*dIII (M2) and 2-Log DNA ladder (M3) (New England Biolabs, Evry, France) were used as markers. The size of the phage genome was estimated by summing up of the lengths of restriction fragments.

2.6. Phage typing

A Biorad i-Cycler was used for the amplification reactions, which were achieved in a 25 μL volume using the Taq 5 × Master Mix kit (New England, Biolabs, Evry, France) and 0.2 μM of each primer. The phage DNA released by heat lysing of particles (10^3 PFU per reaction) served as template for PCR (Jaomanjaka et al., 2013). All 71 lysates were first submitted to a control PCR using malolactic enzyme gene targeted primers (Divol et al., 2003). Absence of amplicon was considered as an absence of contaminating bacterial DNA in the phage sample. Each lysate was subsequently typed using four PCR tests that distinguish the A, B, C and D groups among oenophages, based on their integrase (*int*) sequence. Phage lysates were also tested using two PCR assays targeting a short conserved sequence in the endolysin (*endo*) gene (sequences have been reported in Jaomanjaka et al., 2013). To date, all inducible prophages as well as the free replicating oenophages isolated from enological samples by Jaomanjaka et al. (2013, 2016) have yielded an amplicon with the primer couple endoF/R (447 bp), or alternately with the couple endoF/R′ (449 bp).

When a sample produced a negative signal with *int* and *endo*-primers, confirmation was obtained through a second PCR amplification where particles were replaced with 0.5 μL of template DNA (50 ng). Phage DNAs were also analyzed by RAPD-PCR, using the OPA9 oligonucleotide primer (5′-GGGTAACGCC-3′) at a final concentration of 10 μM. The cycling program consisted of a single step of 2 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 20 s at 45 °C and 2 min at 68 °C, and a final elongation of 10 min at 68 °C. Chromosomal DNA of strain IOEB277 used to propagate the phages was used as a negative control.

All oligonucleotides were purchased from Eurofins MWG-Operon (Munich, Germany).

3. Results

3.1. Phage recovery from samples of enological origin

A total of 166 samples were obtained from local wineries located in different geographic areas of Aquitaine between August 2013 and November 2014. The strategy for collection of samples was based on sampling red, sweet or dry white wines at relevant steps (must, AF, MLF) of the fermentation process. Our objectives were to recover the free phages infecting *O. oeni* present in the samples, as well as those resulting from the spontaneous induction from endogenous strains. To this purpose, samples were screened for the presence of phages after filtration (protocol 1) and following incubation in MRS and then filtration (protocol 2). Strain IOEB277 was used as the indicator strain due to its sensitivity to all oenophages so far isolated in our laboratory (Jaomanjaka et al., 2013, 2016).

The proportion of samples that tested positive for phage was 20% (33/166) (Table 2). Only one third of the samples (*n = 13*) yielded plaques with the two protocols used. We also observed that 45% (*n = 15*) yielded plaques with the direct isolation protocol only, while incubation of the sample in MRS before phage screening was necessary to visualize plaques for 5 additional positive samples. Oenophages were associated with different types of wines (red, dry white or sweet) (Table 2). No phages were found in samples collected from wineries located in Lussac Saint Emilion (*n* = 44; 3 estates), Entre-Deux-Mers

Table 2
Isolation and molecular typing of oenophages from 33 positive samples

Characteristics of the 33 samples (N, number; wine type; appellation; grape variety; estate and step) are indicated: PDC, pied de cuve; AF, alcoholic fermentation; MLF, malolactic fermentation; Sauv, Sauvignon; Cab, Cabernet; na, identity of the estate non available. The presence of oenophages was assessed by using two protocols (1 and 2). Samples which failed to yield plaques with one protocol (1 or 2) are boxed in grey. Numbers of distinct plaque morphotypes (PM) observed during isolation are given (none, 1, 2, 3 or 4). A total of 71 plaques was collected and purified. Concentrated phages lysates were produced and the newly-isolated phages were typed using a PCR protocol that distinguishes four different groups (Int groups A, B, C or D) among oenophages, based on their integrase gene sequences (Jaomanjaka et al., 2013). Phages yielding no amplicon were called unk, for unknown.

*The lysate initially contained a C- and B-type phage.

Characteristics of the samples					Characteristics of the isolated phages				
N	Wine	Appellation - grape variety	Estate	Step	Total number of PM isolated	Protocol 1		Protocol 2	
						N of distinct PM	Int group	N of distinct PM	Int group
67	Sweet white	Sauternes - Sémillon	I	Must	2	1	unk	1	unk
68					1	1	unk	none	-
69					3	2	unk	1	unk
70					3	2	unk	1	unk
137		Sauternes-SauvBlanc	na		1	1	unk	none	-
164					1	1	D	none	-
162		Sauternes-Sémillon/Muscatelle	P		1	1	unk	none	-
165		Sauternes-Sémillon	R		1	1	D	none	-
125	Red	Saint-Emilion-CabFranc	na		4	1	A	3	A
166		Bordeaux-Merlot	S		1	none	-	1	A
2	Dry	Bordeaux-SauvBlanc	K	Juice for PDC	2	none	-	2	D
12	White	Bordeaux-Sémillon	N	PDC	2	2	unk	none	-
135	Red	Bordeaux - Merlot	C	MLF	1	none	-	1	A
143		Pomerol - CabSauv	H		1	none	-	1	A
148		Gers-Gamay	na		4	none	-	4	A (2), B (2)
149		Gers-Merlot	na		2	none	-	2	B; C*
150		Médoc-CabSauv/Merlot	na		1	none	-	1	unk
151					3	2	unk ; A	1	A
152					1	1	A	none	-
153					3	2	unk, A	1	unk
154					2	2	A	none	-
155					3	1	unk	2	A
156					2	1	unk	1	A
157					3	3	unk, A, D	none	-
158					2	1	unk	1	unk
160					3	3	unk (1); A (2)	none	-
163		Bordeaux-Carménère	Q		2	1	A	1	A
131		Saint-Emilion-CabSauv	na	AF	5	2	unk; A	3	unk (1); A (2)
130		Pauillac- CabSauv/Merlot	na		2	2	unk; A	none	-
107				Ageing	2	2	unk	none	-
127		Saint-Emilion-CabSauv/Merlot	na	Bottle (2000)	3	3	Unk, A, D	none	-
128				Bottle (2011)	2	2	A; unk	none	-
129				Bottle (2012)	2	2	A; unk	none	-

(n = 2; one estate), Margaux (n = 4; one estate), and Pomerol (n = 12; one estate). Absence of phages may be related to the reduced sampling size, and a broader set of samples from distinct estates is now needed to draw any accurate conclusion.

Samples were collected from different wineries at relevant steps of the process: must (n = 30), AF (n = 91), MLF (n = 24), ageing (n = 18) or in bottles (n = 3). Some noteworthy points follow. First a total of 12 out of the 30 samples collected at the very beginning of the wine-making process (must, juice, PDC) were positive for plaques (Table 1). Most of these samples did not contain sulfites and this may explain the success of the recovery. Accordingly, we could demonstrate that no phage was recovered after one of the samples (166) was added with sulfites (5 g/hL) in the winery, thus confirming the antiviral activity of SO_2 (Henick-Kling et al., 1986). Next, although sampling sizes were different at each step, we observed that phage recovery was less successful during AF and ageing. In particular, despite the large number of samples collected during AF, the diversity of the estates providing them, and the choice of several sampling times during AF, only two samples collected during red wine making (130 and 131) were found positive. Of note, they were taken at the end of AF, and the possibility that MLF had already started may not be excluded.

3.2. Enumeration of oenophage populations

Population densities of oenophages were measured in the 33 positive samples. Phages were found at low levels in 28 samples, with values ranging from 10 to 80 PFU/mL, regardless of the protocol used and origin of the samples. Populations were slightly higher in two must samples collected during the process of sweet (5.5×10^3 PFU/mL) and dry white wine (8×10^3 PFU/mL) (Table S1).

Information given by winemakers indicated that all the 30 above-mentioned samples were associated with fermentation processes which did not suffer from any delay, or development of undesirable microbiota. In contrast, phage titers were found to be significantly higher in the three remaining positive samples (148, 149 and 163). They all corresponded to red wines which were sent to our laboratory for analysis because of a significant delay in MLF. Sample 163 was obtained from a stuck MLF during an in-house trial conducted at ISVV Bordeaux, and yielded titers of 2.2×10^4 PFU/mL and 5×10^8 PFU/mL with protocols 1 and 2, respectively (Table S1). MLF did not proceed even-though a commercial starter had been inoculated, and a second curative inoculation also failed. The phage lysate obtained from the tank was shown to be active against the commercial strain used. Samples 148 and 149 were made from two distinct grape varieties (Gamay and Merlot, respectively) from the Gers appellation. Phage populations were significantly higher (8×10^6 PFU/mL and 1.3×10^7 PFU/mL, protocol 2) compared to other phage-containing-red wines which completed MLF (< 10 PFU/mL). Enumeration of LAB demonstrated that their concentration was below 10^4 CFU/mL and was therefore not sufficient to trigger MLF, since the bacterial concentration required to achieve optimal degradation of malic acid is usually around 10^6 – 10^7 CFU/mL (Lonvaud-Funel, 1999).

3.3. Isolation and molecular typing of oenophages

All 33 positive samples were examined for plaque morphology. Ten samples yielded a unique plaque morphotype. For the 23 remaining samples, 2 to 5 plaques that clearly differed in size and aspect were readily distinguished on the initial isolation plates obtained with protocol 1 and/or 2 (Fig. 1; Table 2). All plaque morphotypes were recovered to account for the possibility that different oenophages were present in a single sample. A total of 71 lysates were produced and phage particles PCR reactivity with int-specific primers was assessed according to Jaomanjaka et al. (2013). As seen in Table 2, 40 out of the 71 newly-isolated phages were classified as members of one of the four previously described int groups: A (n = 30), D (n = 6), B (n = 3) and C (n = 1). Interestingly, the lysate containing the unique member of the C group was also found to contain a B-type phage. Following an additional subculture, we could produce a pure lysate of the B-type phage deprived of the C one. In contrast, repeated subcultures were needed to obtain a pure lysate containing the C phage.

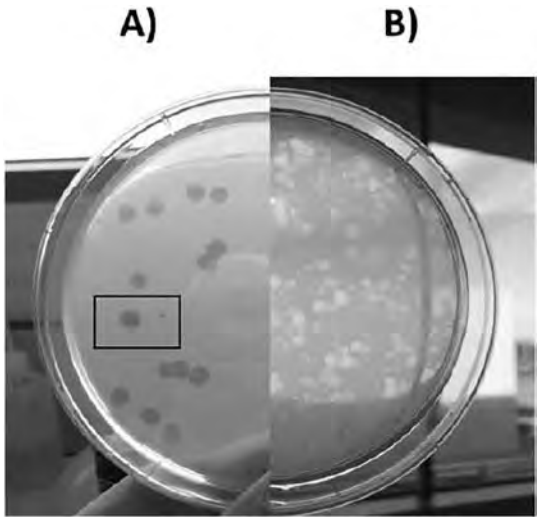

Fig. 1. Examples of plaque morphotypes observed on *O. oeni* IOEB277
Distinct morphotypes observed in sample 70 (sweet wine, Sauternes) with protocol 1 (**A**) and sample 148 (red wine, Gers) with protocol 2 (**B**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Surprisingly, the 31 remaining newly-isolated phages had unusual characteristics, since their viral DNA did not contain any of the identifiable sequences conserved in the integrase, nor in the endolysin genes of oenophages (Jaomanjaka et al., 2013, 2016). Same results were obtained when phage particles were replaced with extracted DNA as the template for PCR amplification. The newly isolated phages which are sufficiently divergent to prevent recognition by PCR were further called unk, for unknown.

3.4. Unk phages form a novel group with unusual genomic characteristics

To compare the 31 newly-isolated unk bacteriophages, various restriction endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII, *Nde*I) previously shown to restrict the genomes of oenophages were used. On the first hand, we observed that none of the unk genomes was cleaved by *Bam*HI (Fig. S1). On the other hand, all unk phages yielded profiles similar to each other using *Eco*RI (Fig. S1), *Hin*dIII and *Nde*I (data not shown). Of note, all 31 phage DNAs also produced closely related DNA restriction patterns in RAPD amplification (Fig. S1). Altogether, our results suggest that the 31 isolated unk phages are genetically very similar, and belong to a single group.

We next assessed whether unk and other oenophages could also be distinguished by their genome size and particle morphology. Since no oenophage has been isolated from grape must before, phage unk67.1 was chosen as the representative of its group, and characterized in more details. Based on the sizes of the fragments produced by digestion with *Eco*RI, *Nde*I and *Hin*dIII, unk67.1 DNA was estimated to have 42.5 ± 0.2 kb, which is consistent with data by Jaomanjaka et al. (2013) (Fig. 2). Transmission electron microscopy revealed an icosahedral capsid with a long, flexible and non-contractile tail, indicating that this phage belongs to the *Siphoviridae* family, a characteristic shared with all oenophages described so far (Fig. 2). The fact that unk67.1 (like other unk phages) produced clear plaques prompted us to assess its ability to lysogenize *O. oeni* IOEB277 by using the protocol described by Jaomanjaka et al. (2016). All our attempts to isolate lysogenic derivatives were unsuccessful suggesting that unk67.1 is a lytic oenophage.

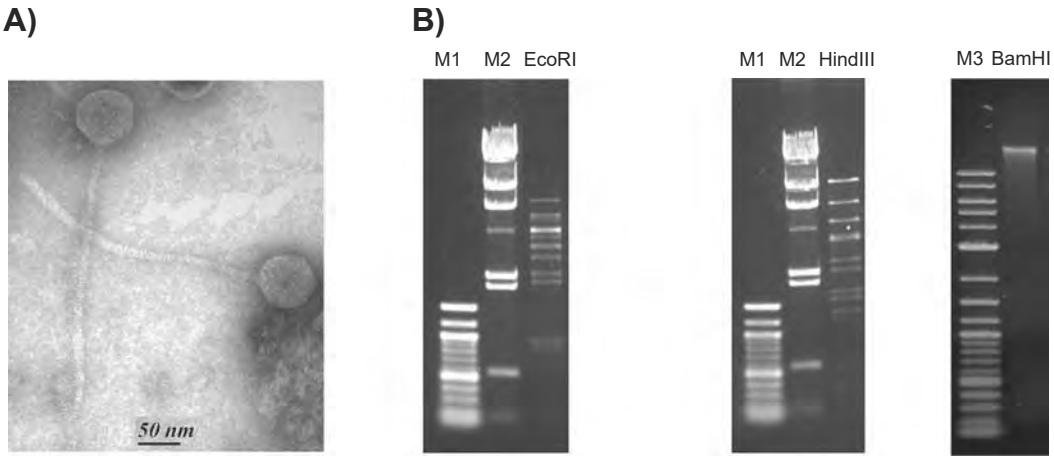

Fig. 2. Preliminary characterization of phage unk67 isolated from Sauternes must.
A) Transmission electron microscope of phage unk67. Phage displayed a head diameter of c. 70 nm with long tails of c. 300 nm.
B) Agarose gel show the DNA fragments obtained by digestion of viral DNA with the restriction enzymes *Eco*RI, *Hin*dIII and *Bam*HI. M1, 100 bp DNA ladder; M2, λ DNA-*Hin*dIII digest; M3, 2-Log DNA Ladder (New England Biolabs, Evry, France).

3.5. Phage richness in samples and prevalence of the O. oeni phage-types

Through molecular typing of oenophages, we could demonstrate the co-existence of distinct phages belonging to at least 2 to 3 genetic groups in 13 samples (Table 2). The samples with highest number of phage types (richness) were all associated with red wine making processes, and harbored distinct combinations: unk and A (9 samples), unk, A and D (3 samples), A and B (1 sample) and B and C (1 sample). Our findings also shed some light on the prevalence of unk phages, being detected in 64% of the positive samples (21 out of 33). In comparison, A-, D- and B- related phages were isolated from 57%, 15% and 3% of the positive samples (19 out of 33), respectively. It is both striking and surprising that A and B group phages were associated with red wine making in our study, while unk and D phages were associated with all types of processes (sweet, white and red). Unk, A and D phages were isolated during most steps along fermentation (must, PDC, MLF, AF and ageing).

4. Discussion

The overall objective of the study was to uncover the abundance and diversity of phages infecting the LAB *O. oeni* during wine making. Our data provide some clues as to how the choice of the protocol affects phage recovery. The isolation of numerous oenophages also provided the opportunity to better characterize phage diversity and to investigate the reliability of the existing PCR classification scheme based on integrase gene polymorphism in their typing (Jaomanjaka et al., 2013). This work also allowed a first insight into the distribution of phages along fermentation in wineries. Each of these three specific points will be discussed in the following paragraphs.

We used the traditional means of characterizing phage environmental diversity which involve phage culturing of plaque assays, and consequently, only infectious phages were captured in our study. Of 166 samples tested, 33 yielded *Oenococcus* phage isolates that could be further propagated. Oenophages are therefore components of the enological ecosystem, and in most samples analyzed in our study, had no deleterious effects on MLF (in wines requesting this conversion). As reported for other environments, the sampling of phages appears to be limited by the procedures used to propagate them, and it may be argued that a first limitation is the use of a single indicator strain in our study, which may have biased the selection. Another point of great importance concerns sample preparation prior to analysis. Hence, the choice of the protocol (1, direct or 2, including growth in MRS) was shown to influence phage recovery. It is therefore advisable to use both protocols to isolate phages from the enological environment in future studies. Only 11 out of 33 samples tested positive for plaques with both protocols used. In contrast, the direct isolation protocol was shown to fail for 7 samples, and their incubation in MRS was necessary to visualize plaques. There may be several explanations for these results; first, the original samples were free of active phages, or had an initial phage count below levels of detection (10 PFU/mL). It is also reasonable to consider the possibility of an aggregation of phage particles in wine, leading to the retention of phage aggregates by the filter matrix during preparation of the sample in protocol 1. In all such instances, the fact that we observed plaques with protocol 2 suggests that release and/or enrichment during subsequent incubation in MRS has occurred, producing a lysate with a concentration exceeding the threshold. However it is difficult to establish how this was achieved. It is likely that the production of particles resulted from the lytic propagation of a small quantity of phages on indigenous strains. In MRS, the lysis time is 10 h for ΦOE33PA, while burst sizes of 25 and 45 have been measured for Φ1002 (Huang et al., 1996) and ΦOE33PA (Jaomanjaka et al., 2016), respectively. Alternately, the release and possible subsequent propagation of phages during the growth of indigenous lysogens, pseudolysogens, or cells having established a relationship with their hosts typical of the carrier state life cycle is also worth considering (Arendt et al., 1990). Spontaneous phage release occurs obviously in *O. oeni* and phage titers ranging from 10^3 to 10^5 PFU/mL have been reported in MRS cultures (Poblet-Icart et al., 1998; Tenreiro et al., 1993).

More intriguingly, although active phages were contained in 15 filtered samples, their presence could not be detected any more after inoculation and subsequent incubation of the sample in MRS broth (Table 2). Phages could have dropped below the detection limit because of the dilution rate in MRS (10^{-1}), or may correspond to low virulence bacteriophages that do not compete, or even grow in liquid culture. Alternately, oenophages could have lost activity during incubation in MRS, and two factors are here worth considering. First the impact on phage infectivity of a change in osmotic pressure due to the tenfold dilution of the sample in MRS may be questioned. Some data have shown that when phages are diluted from high salt concentration to low concentration solutions, phage DNA extrudes from the tail or their heads to break (Jonczyk et al., 2011). This phenomenon may be of particular relevance for must samples used to produce sweet wines. In this particular wine making process, grapes are infected by the mold *Botrytis cinerea* during extended ripening time prior to harvest, dehydrating the grape berries, which leads to elevated sugar concentrations in the must (300–500 g/L). Another possible factor leading to a loss of activity of oenophages may be the progressive acidification of the MRS

medium during incubation (Jaomanjaka et al., 2016). Such a modification can result from the growth and metabolic activity of endogenous microorganisms other than *O. oeni*, such as acetic bacteria, which are not inhibited by the presence of pimaricin in the MRS medium. All above mentioned possibilities would also imply that phage enrichment did not occur in MRS, and could therefore not counterbalance the dilution effect and/or phage decay.

Despite the obvious limitations inherent in our protocol, our first goal was reached and several oenophages were isolated from several wineries producing different types of wines. It should be reminded that oenophages were first isolated from white wines in Switzerland (Cazelles and Gnaegi, 1982; Sozzi et al., 1976). However, subsequent molecular characterization studies mostly focused on phages isolated from red wines (Davis et al., 1985; Henick-Kling et al., 1986; Jaomanjaka et al., 2013). Our newly-isolated phages associated with white wine-making are therefore interesting candidates for molecular characterization including sequence analysis and comparison in the future. As a first step toward exploring these issues, we decided to type the newly-isolated phages using our scheme based on integrase polymorphism. To this purpose, each purified phage lysate was submitted to four PCR assays (intA, intB, intC and intD). A total of 40 out of 71 lysates could be categorized into one of the four described int groups. The finding that these newly isolated oenophages harbor a single integrase type sequence in their genome is in agreement with (i) our previous characterization of oenophages isolated from wine samples (Jaomanjaka et al., 2013, 2016) and (ii) *in-silico* examination of whole genome sequences of lysogens performed at the Australian Wine Research Institute (Borneman et al., 2012) and in our laboratory (Jaomanjaka et al., 2013). A recent paper by Costantini et al. (2017) gives a different picture and suggests that some oenophages induced from lysogenic strains associated with Piemont wines might bear 2 or 3 types of integrase genes. The large collection of phages isolated from enological samples in our study now provides a powerful resource for performing whole genome sequencing. These data will add to the current repository of knowledge regarding oenophage genome architecture and evolution.

Among the 40 phages whose membership was determined, a majority were of the A and D-type, and this is in agreement with our first survey on red wines from Pauillac (Jaomanjaka et al., 2016). The rarity of C-type oenophages also confirmed our previous data. Compared to A, B and D phages, the purification of the single C-type phage was also more technically demanding. We presume that this phage may have a different fitness in the conditions prevailing during isolation. Since poly-lysogeny has been established in the *O. oeni* species (Borneman et al., 2012; Jaomanjaka et al., 2013), the C-type phage may also display a weak within-host competitive ability (Refardt, 2011).

The more important outcome of the study was the finding of a new group of 31 related phages, called unk. All members share similar RAPD and restriction profiles suggesting that they are strongly related at the genome level. Preliminary characterization of one of those unk phages called unk67.1, revealed that both phage morphotype and size of the genome are consistent with data reported for members of the four established groups, A to D (Arendt et al., 1991; Arendt and Hammes, 1992; Davis et al., 1985; Huang et al., 1996; Jaomanjaka et al., 2016; Santos et al., 1998). In contrast, unk phages did not exhibit several key genomic features, which are globally applicable to previously described oenophages (Borneman et al., 2012; Jaomanjaka et al., 2013, 2016). First their genomes did not contain the typical integrase sequences found in all temperate and ex-temperate oenophages. This unusual genomic feature suggests that they have a distinct and so far uncharacterized integrase, or that they are deprived of any integrase sequence, being true virulent phages. Accordingly, all our attempts to lysogenize *O. oeni* with unk67.1 failed. Unk phages also lacked a second conserved sequence located in the endolysin gene, previously associated with 90% of the oenophages previously described (Jaomanjaka et al., 2013, 2016). Another peculiar feature of unk phages was the absence of *Bam*HI sites in their genomes. This contrasts with previous data showing that free active oenophages (Arendt et al., 1991; Boizet et al., 1992; Huang et al., 1996; Jaomanjaka et al., 2016) as well as resident prophages in publicly available sequenced bacterial genomes (belonging to A, B, C and D groups) (Borneman et al., 2012; Jaomanjaka et al., 2013) are all restricted by this enzyme. Nucleotide sequence information (preferably whole genome sequence) is now required to study diversity within the unk group. Differences might be expected since we observed that several unk phages associated with the same samples could be distinguished based on their distinct plaque morphotypes. Next-generation sequencing applied to unk phage will also give essential data to understand the evolutionary relationships with other groups, offering new tools to monitor and assess the ecological significance of bacteriophages in the oenological environment, including wine making equipments.

Our last objective was to provide a first insight in the distribution of oenophages along the vinification process, which is poorly understood. The isolation of phages from must (from red and white grapes) drew our attention as a previous trial by Nel and coworkers proved unsuccessful (Nel et al., 1987). This may be indicative of their presence on grapes, either as free particles, or following their release after spontaneous prophage induction of lysogenic strains. The incidence of *O. oeni* at the early stages of the vinification is known to be low, and the reservoir of *O. oeni* is currently questioned as the species has so far been seldom isolated from grape surfaces in the vineyard (Bae et al., 2006; Bokulich et al., 2012; Garijo et al., 2009; Nel et al., 1987). The use of non-culture based methods also supports the same conclusions and DNA of *O. oeni* has only been rarely detected from grapes (Bae et al., 2006; Renouf et al., 2005, 2007). Recent metagenomics data also failed to identify the species in the highly diverse microbiome associated with freshly crushed grapes collected from portuguese appellations (Pinto et al., 2015) and Dolce wines (botrytized wine) (Bokulich et al., 2012). Portillo et al. (2016) were recently successful in finding members of *Oenococcus* in grape musts from the Priorat region in Spain, which accounted for 5.5% (on average) of the total bacterial communities. Considering a burst size of 50 oenophages produced per infected bacterial cell, a value recently determined for ΦOE33PA (Jaomanjaka et al., 2016), phage monitoring is likely to lower the detection limits of *O. oeni*.

The question as to whether phages exclusively enter wine from vineyard has also to be raised. The low concentrations in phage measured in musts could also result from their presence in the winery (Bokulich et al., 2013). Free phages and/or lysogens are likely to shelter in equipments which often involve difficult to clean, porous surfaces. Accordingly, the ability of *O. oeni* to colonize different surfaces such as stainless steel tanks and oak barrels was recently demonstrated (Bastard et al., 2016).

Among the 15 wineries where sampling was done at two distinct steps (must/AF or AF/MLF), only five contained phages. In this small number of cases, we observed that oenophages were detected only in one step. With this caveat of small sampling size in mind, it is likely that contamination by free phages/lysogens present in equipments (pumps, pipes, tanks, barrels, filters) or in MLF starters may also explain why active oenophages were detected sporadically along wine making.

Irrespective of the source of oenophages (wineyards and/or winery equipements), their detection also depends on many factors including the ability of free phages to survive and persist in the temporally fluctuating enological environment, the kinetics of phage action, the proportion of lysogenic strains in the population and the kinetics of prophage excision during cell growth. Free oenophages are known to be progressively inactivated in wine. However, we still lack information about the physical and chemical factors inactivating phages at each step (acids, ethanol, sulfites or other so far unidentified components) and the kinetic patterns of inactivation. In the present study, the isolation of unk phages reinforces the view that oenophage diversity is higher than expected, and may suggest a degree of heterogeneity among phages

with respect to persistence.

On the other hand, presence of active phages should also probably account for the particular dynamics of the *O. oeni* population (see below), as well as for possible transitions between optimal virulent, and temperate (and possibly dormant) strategies along winemaking. In red wines, strains of *O. oeni* do not replicate during AF and populations usually remain at low titers ($\sim 10^3$ CFU/mL). Owing to the fact that occurrences of lysogeny correlate well with conditions unfavorable for rapid host growth (Paul, 2008), the low concentration of indigenous *O. oeni* strains during AF may represent a reservoir for phages through stable lysogenization. Lysogeny may benefit the phage, as free particles would be otherwise rapidly inactivated in the surrounding medium. Lysogeny may also benefit the bacterial host. Prophages, by modulating substrate utilization capability and/or by providing new functions may help bacteria cope with harsh environments, especially during AF. Following AF, bacteria resume growth, and the population of *O. oeni* can reach $\sim 10^8$ CFU/mL after completion of MLF (Lonvaud-Funel, 1999). This increase in the population has been shown to result in the spontaneous release of phages by lysogens during growth (Ribéreau-Gayon et al., 2005), reaching a minimal threshold to enable detection in our studies.

In conclusion, we surveyed the occurrence of oenophages along wine making and isolated 71 phages. Proportions were assigned to a new group called unk with unusual genomic characteristics. Diversity is therefore higher than previously expected and further characterization of unk phages will now assess whether they are true lytic phages and provide additional signature genes for examining phage diversity in *O. oeni*. Our study also provides a great starting point for future studies on the persistence of phages in winery facilities in relation with hygienic practices.

Conflict of interest

Authors declare that no conflicts of interest exist.

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