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Characterization of a new virulent phage infecting the lactic acid bacterium *Oenococcus oeni*

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ABSTRACT

ΦOE33PA is a new virulent siphophage infecting *Oenococcus oeni* that was isolated from a red wine collected in Pauillac (France). Although the phage could not lysogenize its host, a conserved sequence within the integrase genes harbored by oenophages could be detected, and corresponded to a B-type integrase. The phage host range encompassed ten out of the 38 *O. oeni* strains tested. One-step growth kinetics revealed latent and burst periods of 4 and 5 h, respectively, with a burst size of about 45 plaque-forming units per infected cell. The phage had a distinctive restriction profile when compared with Φ10 MC, another B-type oenophage previously isolated in our laboratory. Incubation in wine could inactivate high-titer suspensions of ΦOE33PA in a short time at room temperature. However, encapsidated phage DNA could still be detected by real time PCR, and these non-infectious viruses dominated the wine samples showing that direct enumeration of phages in wine samples using the double-layer agar technique only informs about the quantity of residual infectious phages. Kinetics studies throughout the fermentation using both qPCR as well as plating should now provide reliable understanding of the phage dynamics during wine making.

1. Introduction

During winemaking, a major source of concern is the progress of malolactic fermentation (MLF) which reduces acidity and increases microbial stability, and is largely driven by the lactic acid bacterium (LAB) *Oenococcus oeni*. The MLF process can be spontaneous, relying on indigenous *O. oeni* strains, but winemakers often prefer to control MLF with predictable starters, available under ready-to-use concentrated cultures. The manufacture (of wine) requires the inoculation of 10^6 selected bacterial cells per mL of wine to control the fermentation and to obtain high-quality end-products.

The specialized industries which produce and sell microbial cultures for wine use specific criteria of selection including technological performances, safety and stress resistance (Coucheney et al., 2005; Solieri et al., 2010; Torriani et al., 2010). Assessment of the possible interactions of strains with their cognate bacteriophages is also recommended (Torriani et al., 2010). Practices may differ among starter culture suppliers and phage relationship of MLF cultures is not always one of the top criteria during selection of appropriate strains (Jaomanjaka et al., 2013). In contrast, this requirement is more strict regarding the LAB used in the milk fermentation industry, which correspond to a limited number of defined strains, chosen for direct inoculation. As a consequence of the limited number of strains used, a phage infection may frequently cause the disruption of lactic acid fermentations in dairy products. Accordingly, starter culture suppliers will carefully test their strains for the presence of prophages and their spontaneous induction upon inoculation in milk. Besides the risk in induction, prophages may also represent a source of recombination with free replicating lytic phages, leading to the emergence of problematic lytic phages with expanded host range. When possible, dairy strains sensitive to lytic phages and lysogenic strains carrying easily inducible prophages should not find their way into commercial

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products. Strains with natural resistance to phage are preferred and associated in rotation cultures.

During wine making presence of bacteriophages active against *O. oeni* (oenophages) has been often demonstrated (Sozzi et al., 1976; Cazelles and Gnaegi, 1982; Sozzi et al., 1982; Davis et al., 1985; Nel et al., 1987; Arendt et al., 1990, 1991; Arendt and Hammes, 1992; Huang et al., 1996; Santos et al., 1998). Pioneering studies have been performed on wines from the 1981 vintage in the experimental winery at the Federal Agricultural Research Station of Changins (Sozzi et al., 1982). Eight different wines were inoculated with strains of *O. oeni* and it was shown that the four samples with delayed MLF contained phages that were active against the strain used for inoculation. Similar observations were also made during spontaneous fermentations in different estates in Switzerland Romandy and wines showing slow and stuck fermentations were shown to contain phages (Cazelles and Gnaegi, 1982). Later investigations by Henick-Kling et al. (1986a, b) strengthened the conclusions of Sozzi and collaborators and also showed that some factors (pH, total SO_2 concentration, presence of fining agents) were not conducive to the survival and occurrence of phages in wines. Despite the scientific quality of such studies, warning of the consequences of phage predation during wine making, the genetic and biological diversity of oenophages has remained largely unexplored and most isolated oenophage particles have not been subsequently characterized beyond their description by electron microscopy, and lytic spectra.

Forty years after the first observations made by Sozzi et al. (1976), our current knowledge of oenococcal phage biology is still limited, and lags somewhat behind that of phages infecting other industrially relevant LAB (see for review Mahony and van Sinderen, 2014; Kot et al., 2014). In light of this, the questions as whether phage predation can lead to product variability and reduce productivity during spontaneous fermentation, and how this may be integrated during selection, stabilization and use of MLF starters are still far from being straightforward. One of the principal questions that remain to be answered is the nature of the phage–host relationships. Lysogeny (Arendt et al., 1991; Cavin et al., 1991; Tenreiro et al., 1993; Poblet-Icart et al., 1998; Borneman et al., 2012; Jaomanjaka et al., 2013) and pseudo-lysogeny (Arendt et al., 1990) have been reported in the species, but a better understanding of the underlying mechanisms is needed. A series of temperate phages have been partially characterized (Borneman et al., 2012; Jaomanjaka et al., 2013) and basic scientific data should now also be generated regarding the occurrence of lytic phages. These are essential requirements since it is reasonable to assume that transitions between optimal virulent, temperate, and dormant strategies may occur as a function of environmental, ecological, and biophysical parameters of the oenological system.

The study described herein focuses on the efficacy of oenophages to kill their target hosts. Our aim was to establish whether virulent phages could be isolated from wines. We report the isolation of ΦOE33PA from a wine produced in Pauillac (France) and our result suggest that the phage has lost its lysogenic capacity. The paper describes its reproduction rate, host spectrum on strains of industrial significance, and persistence capacities in wine.

2. Material and methods

2.1. Bacterial strains

All strains were grown in liquid or solid MRS (Man Rogosa Sharpe) (Difco, Fischer Bioblock Scientific, Illkirch, France) or alternately in grape juice medium. This medium contained commercial red grape juice, 250 mL/L; yeast extract, 5 g/L; and Tween 80, 1 mL/L. Media were adjusted to pH 4.8 at 25 °C.

The characteristics of the different strains used are listed in Table 1. Strains were maintained as frozen stocks (−80 °C) in MRS broth, in the presence of 30% v/v glycerol.

2.2. Isolation, propagation and titration of phages

Phage ΦOE33PA was isolated from a red wine sample collected in Pauillac in 2008. Its isolation and propagation on strain *O. oeni* IOEB S 277 to obtain high titer lysates were done as previously described (Jaomanjaka et al., 2013). Φ10 MC was obtained after mitomycin C induction of strain *O. oeni* IOEB B10 (Gindreau et al., 1997), and further propagated on strain IOEB S 277. Phage lysates were filtered through a 0.2 μm-pore size sterile filter and stored at 4 °C. Phage counts were carried out using the classical double-layer plating technique, using MRS agar supplemented with $MgSO_4$ (3.75 g/L) and $CaCl_2$ (2.375 g/L) (MRSΦ). Incubations were carried out under anaerobic conditions at 25 °C for 4–7 days to allow plaque formation. Phage counts were expressed as plaque-forming units per mL (PFU/mL) (Jaomanjaka et al., 2013). The resistance level of bacterial strains to phages was expressed using the efficiency of plating (EOP) ratio. The EOP was defined as the ratio between PFU mL^{-1} obtained on each putative resistant strain and PFU mL^{-1} obtained on the strain initially used for the phage propagation (*O. oeni* IOEB S 277). Resistant strains were represented by EOP values < 1.

2.3. Transmission electron microscopy

The lysed culture (10^9 PFU/mL) was treated with DNase I (1 μg/mL; Invitrogen) and RNase A (1 μg/mL; Promega) at 37 °C for 30 min. Polyethylene glycol (PEG) precipitation was carried out as follows. Sodium chloride (final concentration, 1 M) was added to the treated lysate. Particles were recovered from the sample by precipitating with PEG 6000 (10%, wt/vol). The sample was stored overnight at 4 °C and sedimented 10 min at 11,000 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} particles/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.

2.4. Phage growth in microplates

Tests were conducted with strain IOEB S 277 and performed in 96-well microtiter plates. One milliliter of an overnight culture was transferred to 10 mL of fresh MRSΦ broth and incubated for 24 h, until the OD_{600} was ~0.2 (the viable cell counts were around 10^8 cells/mL). In all experiments, 500 μL of cell suspensions and 100 μL of phage lysate (or dilutions thereof) were mixed in a tube, in order to obtain a multiplicity of infection (MOI) varying from 10^{-1} to 10^{-6}. The sample was divided in three wells. The control experiments were performed with 100 μL of MRSΦ broth instead of phages. The plates were placed into a Synergy HT (BIO-TEK, Colmar, France) incubator at 25 °C and the optical density (OD_{600}) was regularly measured during 64 h. Two independent experiments were performed in triplicate.

2.5. Analysis of phage DNA and restriction

A starting volume of phage lysate of 20 mL (~10^9–10^{10} PFU/mL) was used to extract phage DNA using the phenol chloroform method described by Sambrook and Russell (2001). Purified DNA was digested with the restriction endonucleases *Bam*HI, *Bgl*II, *Eco*RI,