International Journal of Food Microbiology 166 (2013) 331–340

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Expanding the diversity of oenococcal bacteriophages: Insights into a novel group based on the integrase sequence

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ARTICLE INFO

Article history:
Received 3 February 2013
Received in revised form 14 June 2013
Accepted 25 June 2013
Available online 7 July 2013

Keywords:
Oenococcus oeni
Malolactic fermentation
Lysogeny
Prophage induction

ABSTRACT

Temperate bacteriophages are a contributor of the genetic diversity in the lactic acid bacterium *Oenococcus oeni*. We used a classification scheme for oenococcal prophages based on integrase gene polymorphism, to analyze a collection of *Oenococcus* strains mostly isolated in the area of Bordeaux, which represented the major lineages identified through MLST schemes in the species. Genome sequences of oenococcal prophages were clustered into four integrase groups (A to D) which were related to the chromosomal integration site. The prevalence of each group was determined and we could show that members of the int_B- and int_C-prophage groups were rare in our panel of strains. Our study focused on the so far uncharacterized members of the int_D-group. Various int_D viruses could be easily isolated from wine samples, while int_D lysogens could be induced to produce phages active against two permissive *O. oeni* isolates. These data support the role of this prophage group in the biology of *O. oeni*. Global alignment of three relevant int_D-prophages revealed significant conservation and highlighted a number of unique ORFs that may contribute to phage and lysogen fitness.

1. Introduction

In the context of an increasing worldwide interest in improving the quality and safety of wines, significant effort has been made to provide exhaustive inventories of the complex microbial communities associated with grapes and wine. Grapes were reported to have a complex microbial ecology including filamentous fungi, yeasts and bacteria with different physiological characteristics and effects upon wine production. These communities were found to be particularly prone to fluctuations in composition, and a complexity and diversity of the consortium have been found at all stages of the winemaking process: on grape berries, in must during fermentation, and in wine during aging (Renouf et al., 2007; Barata et al., 2012; Martins et al., 2012). Presence of viruses infecting bacteria (called bacteriophages) in this changing ecosystem has been widely neglected, and it remains unknown whether they may play a significant role in shaping the microbial communities, and influence the dynamics of fermenting bacteria, or spoilage microorganisms which lead to depreciation of wine. Yet, increasing evidence is being found in other ecosystems (such as aquatic systems, and mammalian gut microbiota) indicating that propagation of bacteriophages not only controls host diversity and abundance, but can also in some cases benefit bacterial host fitness under specific environmental conditions (Weinbauer and Rassoulzadegan, 2004; Mills et al., 2012).

During winemaking, a major source of concern is the progress of malolactic fermentation (MLF) which reduces acidity and develops aromas in the final product, and is largely driven by the lactic acid bacterium *Oenococcus oeni*. The process can be spontaneous, relying on endogenous *O. oeni* strains, but winemakers often prefer to control MLF with predictable starters, available under ready-to-use concentrated cultures. Pioneering studies have focused on the possible predation of *O. oeni* by specific viruses, and diverse oenococcal phages have been successfully isolated from wines with sluggish or delayed MLF (Sozzi et al., 1982; Tenreiro et al., 1993; Davis et al., 1985; Henick-Kling et al., 1986a, 1986b). Some have been characterized in terms of morphology, lytic spectra, restriction enzyme analysis of the genome, DNA homology, genome size and protein structure (Nel et al., 1987; Arendt et al., 1991; Boizet et al., 1992; Tenreiro et al., 1993; Davey et al., 1995; Santos et al., 1998). The lack of characterization at the molecular level has considerably limited the assessment of oenococcal phage diversity. On the other hand, a high prevalence of lysogeny has been largely documented in *O. oeni* (Cavin et al., 1991; Tenreiro et al., 1993; Poblet-Icart et al., 1998) and lysogenic strains have been proposed to serve as a reservoir for phages, protecting them from the aggressive conditions prevailing in wine (Gindreau, 1998; Henick-Kling et al., 1986b). More recent comparisons of complete genome data confirmed the presence of numerous phage-related sequences in the genomes of strains, making prophages a major contributor of the genetic diversity in the *O. oeni* species (Zé-Zé et al., 1998; Bon et al., 2009; Borneman et al., 2012a, 2012b).

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http://dx.doi.org/10.1016/j.ijfoodmicro.2013.06.032

In order to gain insights in the significance of lysogeny in *O. oeni*, the integration modules comprising the integrase-encoding genes (*int*) and the phage attachment sites from different temperate oenococcal phages, namely fog30, fog44, fogPSU1 and Φ10MC have been identified and sequenced (Gindreau et al., 1997; Santos et al., 1998; Parreira et al., 1999; São-José et al., 2004). Phages segregated in three groups regarding the tRNA site used for site-specific recombination. In addition, a linking of these tRNA sites and *int* sequences has been evidenced (Ballestra et al., 2011). These data were recently confirmed in an *in silico* evaluation of prophages carriage in strains of *O. oeni* (Borneman et al., 2012b). Interestingly, this study also revealed the existence of putative complete prophages harboring a distinct tRNA integration site and cognate *int* sequence, corresponding to a probable fourth group. However, whether these sequences produce active phage on induction has not been documented. The present study was conducted to gain more information on this so far unexplored group of prophages. Their prevalence was analyzed in a set of 28 strains of *O. oeni* mostly collected in the Bordeaux area and reflecting the overall genetic diversity in the species. We assessed whether (i) these prophages were able to be induced for self-replication and to be excised from the genome, and (ii) free bacteriophages belonging to this group were present in wine samples. Global alignments of three active prophages belonging to this novel group are presented.

2. Material and methods

2.1. Bacterial strains and phages

All *O. oeni* strains were routinely grown in liquid or solid modified MRS (Difco, Fischer Bioblock Scientific, Illkirch, France) adjusted to pH 4.8 at 25 °C. Their characteristics have been described earlier (Bilhère et al., 2009; Favier et al., 2012). The strains were stored in 30% (vol/vol) glycerol at −80 °C.

2.2. Isolation and propagation of phages

Phages were isolated from 22 red wine samples collected at the beginning or at the end of MLF from diverse wineries (Medoc, Bordeaux region, Aquitaine, France). Wine samples were ten-fold diluted in MRS liquid medium, pH 4.8 and incubated for 5 days at 25 °C. Cultures were centrifuged and supernatants were filter-sterilized through 0.2-μm membranes. Wine samples were screened for the presence of phages 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). Eleven different indicator *O. oeni* strains mostly collected in the Bordeaux area were used. Eight strains have been isolated from red wine samples during spontaneous MLF (Sarco S14, S15, S24, S28, S51, S161, B10, IOEB 89006). One strain has been isolated from a white sparkling wine (Sarco S11). The others (IOEB-Sarco 277 and 450 PreAc®, Laffort) are commercial starters. Plates were incubated under anaerobic conditions at 25 °C for 4 to 7 days. Plaques (see Supplementary Fig. S1) were carefully excised, suspended in 0.5 ml of sterile MRSΦ medium and stored at 4 °C. Two successive rounds of purification were carried out for each phage.

2.3. High-titers phage lysates

For each purified phage/host, ten confluent lysis plates were prepared. Soft agars were collected, centrifuged, and the supernatant was filter-sterilized. Phage titers ranging from 10^6 to 10^{10} plaque-forming units per ml (PFU/ml) were obtained. Phage lysates were stored at 4 °C until use.

2.4. Host range determination

Bacteria were grown to exponential phase, and 200 μl of the bacterial culture was mixed in 5 ml of top MRS_Φ agar and poured onto MRS_Φ agar plates. Spot assays were performed using 8 μl of the purified phage. Plates were incubated at 25 °C 72h-96h, and presence of plaques was recorded as a positive test. The minimum detectable titer was 1.2×10^2 PFU/ml.

2.5. Induction with MC

For the induction of phages, mitomycin C was used as the inducing agent (0.5 to 1 μg/ml), with bacterial culture on modified MRS. Overnight cultures were diluted 10-fold in 10 ml of fresh broth, grown to an optical density at 600 nm (OD_{600}) of 0.2 to 0.3 prior to the addition of inducing agent, and incubated for 24 h. OD_{600} was measured periodically. Supernatants were filtered (0.2-μm membrane), and 100 μl of the filtrate (or of dilutions when necessary) was mixed with 200 μl of host cells (Sarco S25 and IOEB-Sarco 277) and added to 5 ml of top agar (0.6% [wt/vol] agar) at 40 to 45 °C and poured on agar plates. The protocol allowed the obtention of a functional phage lysate from the established lysogen B10 containing the integrated int_B-Φ10MC phage (Gindreau et al., 1997).

2.6. Comparative bioinformatic analysis of prophages

Raw sequences were assembled using Newbler and annotated using RAST (Rapid Annotation using Subsystem Technology) (Aziz et al., 2008). Homology searches were performed using NCBI BLAST2 software. Multiple alignments of protein sequences were constructed using COBALT (Papadopoulos, and Agarwala, 2007). Phylogenetic analyses of the phage proteins based on amino acid sequences were carried out using Neighbor–Joining (NJ) methods.

2.7. PCR and DNA sequencing of PCR products

Standard PCR reactions were performed in a 25 μl volume using the Biorad *Taq* PCR Master Mix kit, 0.2 μM of each primer, and 0.5 μl of template DNA (50 ng). Amplifications were also carried out on free phage particles. Diluted phage lysates (~10^3 PFU per reaction) 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. Primer design was achieved by using the eprimer3 and Oligo analyser 1.0.3 software. Oligonucleotides, purchased from Qiagen Operon, are listed in Table 1. Sequencing reactions were performed with 1 μl of purified PCR product, using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

2.8. Phage and prophage molecular typing

Based on the comparative genomic analysis of the available phage sequences, a PCR strategy for typing the four different integrase-type sequences was developed (Ballestra et al., 2011). A high level of nucleotide sequence identity was observed between *int* genes from group A (97 to 100% identity), and those from group D (99–100% identity). PCR primers amplified conserved 273-bp (Int_A f/r) and 343-bp fragments (Int_D f/r), respectively (Table 1). Group B contained a unique sequence, obtained from strain B10 (Φ10MC) (Gindreau et al., 1997). Group C comprised the integrase sequences from ΦFogPSU-1 and that of a prophage found in strain S28. The primer couples Int_B f/r and Int_C f/r were designed (Table 1) leading to the amplification of 443-bp and 406-bp, amplicons, respectively. All couples yielded amplicons with the expected size using lysogenic strains described by Borneman et al. (2012b).