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Exploration of phenomena contributing to the diversity of *Oenococcus oeni* exopolysaccharides

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ABSTRACT

Many food-grade bacteria produce exopolysaccharides (EPS) that may modify the food texture or affect their survival rate during food processing. This is the case of *O. oeni*, a bacterial species who drives malolactic fermentation in wine. The five strains analyzed in the present study all display both isolated genes dedicated to homopolysaccharide synthesis and gene clusters potentially associated with heteropolysaccharide synthesis. The number of isolated glycosyltransferase gene present and the gene composition of one of the operons change from one strain to the other. The soluble EPS yields and the EPS monomer composition vary depending on the strain and or the medium composition. *O. oeni* appears as a bacterium able to synthesize both homo and heteropolysaccharides. This unique property has rarely been described. Moreover, the abundance of the genetic determinants associated with EPS metabolism suggests that it is very important for the adaptation of the bacteria to wine.

1. Introduction

Polysaccharide-producing microorganisms can be isolated from diverse ecological niches, including wine. Even though the presence of yeasts mannoproteins in wines was long ago regarded as beneficial, all other microbial polysaccharides were considered as detrimental for the beverage quality (Pasteur, 1866; Luthi, 1957; Dubourdieu et al., 1981; Dubourdieu and Moine, 2000; Ribéreau-Gayon et al., 2000). Bacterial polysaccharides were particularly dreaded and were often associated with "ropiness", a wine spoilage phenomenon. Ropiness is an alteration, caused most of the time due to the production of a β-glucan by "ropy" lactic acid bacteria displaying a glycosyltransferase gene named *gtf* (Ribéreau-Gayon et al., 2000; Dols-Lafargue and Lonvaud-Funel, 2009). This genetic determinant of ropiness is the most studied and most frequent one, even though other should also exist (Walling et al., 2005a).

Among the ropy bacteria displaying the *gtf* gene, *Oenococcus oeni* specific strains are found (Ibarburu et al., 2007; Dols-Lafargue et al., 2008). *O. oeni* is the species that drives malolactic fermentation (MLF) in most wines in temperate regions. Most *O. oeni* strains do not display the *gtf* gene and do not induce ropiness (Werning et al., 2006; Dols-Lafargue et al., 2008). However, an extensive study of wines before and after spontaneous or induced malolactic fermentation (MLF) clearly showed that *O. oeni*, the dominant bacterial species at this stage of winemaking, is capable to modify the wine polysaccharide content (Dols-Lafargue et al., 2007). In addition, nonropy *O. oeni* strains were shown to produce exopolysaccharides (EPS) in model media (Ciezack et al., 2009; Bazin et al., 2011). These results open-up new research areas concerning the impact of bacterial polysaccharides on wine quality. The concentrations of EPS produced are quite low (50–200 mg/L) but similar to those formed by ropy bacteria, even though no ropy characteristic is to be deplored (Ciezack et al., 2009). If the polysaccharides produced display appropriate structures, such EPS concentrations seem sufficient to modulate the wine colloidal equilibrium, with subsequent effects on beverage stability and palatability. Moreover, these EPS may enhance *O. oeni* survival in stressful conditions such as those prevailing in wine at the time of MLF.

The aim of the present work is to identify the EPS produced and the phenomena contributing to the diversity of the EPS phenotype in the species *O. oeni*. Particular attention will be paid on the genetic equipment dedicated to EPS synthesis and on the importance of microbial physiology in the modulation of the EPS synthesis. For this, five representative strains will be studied:

- *O. oeni* ATCC BAA-1163, AWRIB429 and PSU-1, three nonropy *O. oeni* strains which produce nonsignificant amounts to moderate quantities of EPS in model media and whose genome sequences are publicly available,
- *O. oeni* IOEB Sarco 277, a nonropy strain, commercialized as a malolactic starter (Lactoenos SB3) which produces significant amounts of soluble EPS according to Ciezack et al. (2009),
- and *O. oeni* IOEB IOEB 0205, a ropy strain which displays the *gtf* gene and produces β-glucan but also heteropolysaccharides according to previous work (Dols-Lafargue et al., 2008).

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2. Materials and methods

2.1. Strains and origin

The nature and origin of the bacteria used in this study are presented in Table 1.

2.2. Culture media

Bacteria were propagated either in MRS derived dialyzed medium (Dols-Lafargue et al., 2008) or in a semi defined (SMD) medium specifically developed for EPS production by *O. oeni*. The SMD medium contained: (base) casamino acids 10 g/L, sodium acetate 3,4 g/L, KH_2PO_4 1 g/L, $MgSO_4$, 7 H_2O 0.1 g/L, $MnSO_4$, 4 H_2O 0.1 g/L, ammonium citrate 2.7 g/L, bactotryptone 5 g/L, malate 3 g/L, and yeast nitrogen base 6.7 g/L, adenine, uracyl, thymine, guanine 5 mg/L each, and a carbohydrate (either glucose 20 g/L or glucose and sucrose, 10 g/L each). The carbohydrate solutions were prepared as 10× solutions and were sterilized 20 min at 121 °C, while the base was prepared as a 2× solution and sterilized by filtration (0.2 μm cut off). Before sterilization, the pH was adjusted to 5.0.

2.3. PCR

Total genomic DNA of lactic acid bacteria was purified using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). Primers were designed according to the nucleotide sequences of *O. oeni* ATCC BAA-1163, AWRIB429 or PSU-1 genomes (GenBank accession number AAUV00000000, ACSE00000000 and CP000411), or according to *gtf* sequence (GenBank EU556433) and are listed in pairs in Table S1 in supplemental data. All the *eps* genes or groups of genes identified in the study were screened by PCR. Thirty PCR cycles (30 s at 95 °C, 30 s at 55 °C, 2 min at 72 °C) were performed before agarose gel electrophoresis followed by ethidium bromide staining.

2.4. Growth, substrate and product measurements

Cell growth was estimated through the measurement of the cell suspension absorbance (600 nm, OD_{600}).The whole culture medium was centrifuged (10,000×*g*, 15 min, 4 °C), and the substrates and products in the supernatant were analyzed. Glucose concentration in the culture supernatant was measured by anion exchange chromatography (Aminex HPX87H column, Bio-Rad, Marnes La Coquette, France) using a Waters (Milford, USA) system consisting of a pump (Waters 600), an injector (Waters 717) and a refractometer (Waters 2414). The eluant (H_2SO_4 5 mM) had a constant flow rate (0.5 mL/min) and separation was performed at room temperature. For EPS concentration measurements, 5 volumes of ethanol–HCl 1 N (95-5) were added to the supernatant to precipitate the polysaccharides. The tubes were let to stand for 24 h at 4 °C. Then, they were centrifuged (10,000×*g*, 15 min, 4 °C), and the pellet was washed with ethanol (80 vol.%), centrifuged again, dried for 20 min at 65 °C and dissolved in distilled water. The amount of neutral polysaccharides was determined by the phenol sulfuric acid method (Dubois et al., 1956), using glucose as the standard. For each sample, the polymer precipitation and assays were done in triplicate.

DNA concentration was assessed by staining 100 μL of supernatant with 100 μL of 12 μM Syto9 (Invitrogen, France). After 15 min in the dark at room temperature, the plate was analyzed through a Biotek (USA) Synergy microplate reader, calibrated with stained genomic DNA. The background signal due to the culture medium was taken into account.

Table 1
Origin of the strains used in the study.

Strain name	Species	Characteristics
IOEB Sarco 277	*Oenococcus oeni*	Commercial starter, Laffort Oenologie France (Lactoenos SB3)
AWRIB429	*Oenococcus oeni*	Commercial starter (Lalvin VP41), Lallemand, Canada
ATCC_BAA-1163	*Oenococcus oeni*	Isolated from nonropy red wine
PSU-1	*Oenococcus oeni*	Commercial starter
IOEB 0205	*Oenococcus oeni*	Laboratory ropy strain
NRLL B-1299	*Leuconostoc mesenteroides*	Dextran producer (Bozonnet et al., 2002)

2.5. Assessment of viability

The bacteria were harvested by centrifugation (8000×*g*, 5 min, 4 °C) and re-suspended in NaCl (0.9%) to obtain approximately 10^8 cells/mL. For counts of live and dead cells, 25 μl of cellular suspension was mixed with 25 μL of an aqueous solution containing the following concentration of nucleic acid stains: 12 μM of Syto9 and 60 μM of propidium iodide (Invitrogen, France). After 15 min in the dark at room temperature, 10 μL of stained sample was examined under Olympus BX51 epifluorescence microscope. All green cells were considered alive while red cells were considered as dead.

2.6. Capsule detection

For capsule detection, the cellular suspension was mixed (vol/vol) with a Nigrosine (20%) aqueous solution (Sigma-Aldrich, L'Isle d'Abeau, France) and then, 10 μL of the negatively stained sample was examined under Olympus BX51 microscope. The capsule appeared as a white halo around the cells.

2.7. Polysaccharide monomer composition

A volume of 300 to 500 mL of culture supernatant was filtered on a 10 kDa cut-off Amicon membrane, followed by a second ultrafiltration step, in order to retain the fraction with molecular weight >100 kDa. The monomer composition of the high molecular weight fraction obtained was determined after acid hydrolysis (H_2SO_4 2 N, 6 h, 100 °C). The neutral monomer composition was determined by gas liquid chromatography of alditol acetate derivatives using inositol as the internal standard. Sugar analysis was performed with an Agilent 6850 series GC system equipped with an ESP2380 macrobore column (25 m × 0.53 mm). ^{1}H NMR analysis was performed as previously described (Dols et al., 1998).

The EPS produced on SMD containing glucose and sucrose were precipitated in the presence of 5 volumes of ethanol–HCl 1 N (95–5), centrifuged (10,000×*g*, 15 min, 4 °C), and the pellet was washed three times with ethanol (80%), dried for 20 min at 65 °C and dissolved in distilled water. After acid hydrolysis (HCl 2 N, 6 h, 100 °C), the solution was neutralized with NaOH 6 N. The glucose and fructose content was measured by anion exchange chromatography as mentioned above.

2.8. Revelation of dextransucrase activity

SDS-PAGE was carried out by the method of Laemmli (1970) with 10% (w/v) acrylamide gels. After growth on SMD glucose–sucrose medium, *O. oeni* cells were harvested by centrifugation (10,000×*g*, 4 °C, 5 min) and re-suspended in sample buffer (Tris–HCl 50 mM pH 6.8, SDS 1%, Glycerol 30%, Bromophenol blue, 0.01%, β-mercaptoethanol 1%). The preparation was deposited on minigels (Miniprotean, Biorad, Marnes la Coquette, France). After migration, the gel lanes displaying the molecular weight markers were stained with Coomassie brilliant blue R-250. Prior to in situ detection of dextransucrase activity, enzyme renaturation was performed: the gel was washed three times with 20 mM sodium acetate buffer (pH 5.4) containing 0.05 g/L $CaCl_2$ and 0.1% (v/v) Triton X-100 at 4 °C, to eliminate the SDS. Then, it was