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Polysaccharide Production by Wine Lactic Acid Bacteria: Negative Trait or Potential Advantage? A Review

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Abstract

Several wine lactic acid bacteria, especially *Pediococcus parvulus* and *Oenococcus oeni*, have been described exopolysaccharides producers. *O. oeni* is the bacterium that most frequently performs malolactic fermentation in temperate areas, while *P. parvulus* is rather considered a spoilage agent, because many strains cause wine ropiness. This alteration is linked to the accumulation of β-glucan in the wine. The polymer synthesis is catalyzed by a glucosyltransferase Gtf, encoded by the *gtf* gene. A detailed study has shown that this gene was much more common than could have been expected from the ropy phenotype prevalence among wine lactic acid bacteria. In addition, many other genetic determinants associated with the production of exopolysaccharides have been identified and associated with more "discrete" phenotypes, especially in *O. oeni*. Instead being detrimental to wine quality, these polymers may be valued in several ways in the future, as their presence is clearly correlated with better survival of bacteria in several situations. This could allow the development of more robust malolactic starters.

Keywords: Exopolysaccharide; Wine; *Pediococcus*; *Oenococcus*; Malolactic starter; Ropiness

Introduction

Winemaking requires a succession of transformations that must be well managed by the winemaker. Some involve the naturally occurring microbial flora but also, often, selected starters, added to the must or wine at the appropriate time [1,2].

After crushing, the must is naturally contaminated by a very diverse microbial consortium, initially present on the surface of the grape berry. Yeasts grow first and drive alcoholic fermentation. *Saccharomyces cerevisiae* is often the dominant yeast species. Nevertheless, many other native yeast species are present and active. At the end of alcoholic fermentation, at the yeast lysis, only certain microbial species have resisted, mainly lactic and acetic bacteria. The lactic acid bacteria found in musts and wine belong to the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Weissella* and *Oenococcus* [3]. The associated species have a fermentative metabolism (facultative aerobe) with lactic acid as their main product. These bacteria usually multiply after the alcoholic fermentation and consume the elements of the medium (sugars, acids) left by the yeasts [4]. Nevertheless, at this stage of winemaking, the main visible transformation carried out by the lactic acid bacteria is the conversion of the L-malic acid present in L-lactic acid and CO_2. Although it is not a real fermentation, this reaction is called malolactic fermentation (MLF). MLF is essential for producing red wines and many white wines [5,6].

Its success depends on the ability of native lactic acid bacteria to survive and grow. Most often, *Oenococcus oeni* becomes the dominant species and the leader of MLF in wines of temperate climates [7]. Lactic bacteria other than *O. oeni* are more often found after MLF, during the aging or the storage of wines, at winemaking stages where their presence is undesirable because associated with spoilage [3,5,8].

Despite the accumulation of knowledge, the MLF is still an imperfectly controlled stage of winemaking. The indigenous flora does not multiply sufficiently to lead the conversion of malic acid to an end and, sometimes, the strains that develop during MLF are responsible for wine spoilage. On the model of yeast starters used to drive alcoholic fermentation, malolactic starters have been developed [2]. Depending on their characteristics, they can be introduced into the wine at the end or during alcoholic fermentation, but implantation failures are not rare: MLF can be initiated by the starter and then stopped or driven by indigenous microorganisms, with the risk that dominant strain metabolism produces alteration [5,9].

Understanding the mechanisms of bacteria adaptation to wine is therefore essential, for both the development of high-performance starters and a better protection of wine against spoilage agents.

Lactic acid bacteria in wine: struggle for life and major contribution to wine quality and wine depreciation

As mentioned above, lactic acid bacteria can be found at all winemaking stages, but their activity is mainly observed at the latest stages (MLF and aging) (Figure 1). At these stages, the wine is a very hostile environment: the pH is low (2.9-4.0), the medium contains few nutrients and, in particular, little carbohydrates (which are essential for the production of energy by lactic acid bacteria), little oxygen and several inhibitors (alcohol>12%, sulfur dioxide, polyphenols) of microbial growth [9].

The survival of the species/strains present relies on their ability to overcome these difficulties. Many mechanisms complement each other specifically in each species and strain and, since the wine is different from one cellar to the other and from one year to the next, the "performance" of each strains should be considered as relative [2]. Nevertheless, many candidate metabolic tools have been identified: stress proteins, metabolic tools to overcome acidic pH (malolactic enzyme, biogenic

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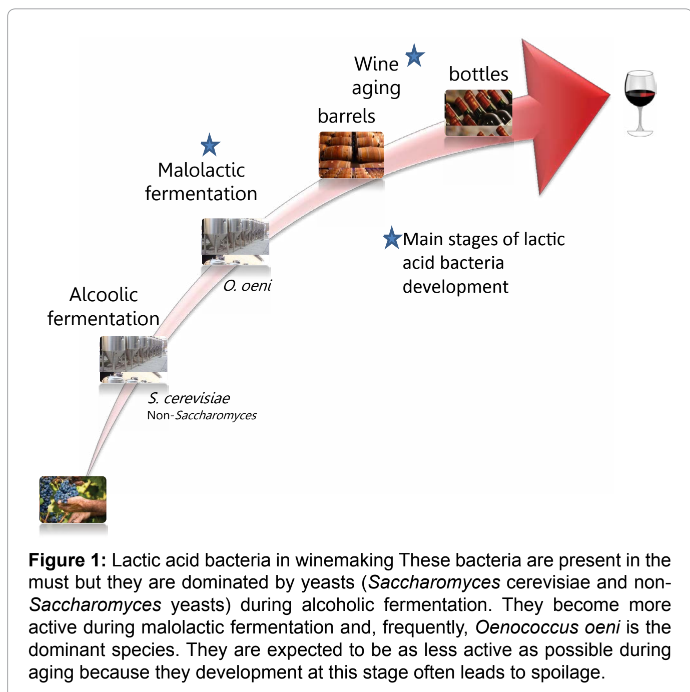

Figure 1: Lactic acid bacteria in winemaking These bacteria are present in the must but they are dominated by yeasts (*Saccharomyces* cerevisiae and non-*Saccharomyces* yeasts) during alcoholic fermentation. They become more active during malolactic fermentation and, frequently, *Oenococcus oeni* is the dominant species. They are expected to be as less active as possible during aging because they development at this stage often leads to spoilage.

amine formation pathways...), ability to grow in a deficient medium (high affinity transport routes and rare substrate metabolism), highly energetic pathways, production of exopolysaccharides [10-16]. Some of these metabolic pathways contribute to the quality of wine and others to its deterioration. The distinction between good and bad bacteria must therefore be considered at the level of strains rather than species, according to their metabolic equipment.

We will focus specifically on the production of exopolysaccharides, i.e. exocellular polysaccharides, and the adaptation of lactic acid bacteria to wine. Exopolysaccharides are ubiquitous components of lactic acid bacteria surfaces [17]. They can be classified in 3 major groups, according to their exact external location:

- WPS or wall polysaccharides, attached to the cell, covalently or not, but without forming a capsule,
- CPS (or capsular polysaccharides), most of the time bound to the peptidoglycan and which form a thick and cohesive (capsule) or fine and cohesive (film) outer layer,
- And the exocellular polysaccharides released into the cell environment, called EPS (Figure 2).

The distinction between these polysaccharides is sometimes controversial: the capsules are observable in negative staining in conventional microscopy, but some polymers can form a dense layer, visible in electron microscopy but not thick enough to be visible in negative staining [18]. In addition, CPS can be released depending on growth conditions or because of unstable cell binding and can be mistaken for EPS. Conversely, some EPS may be loosely bound to the cell [19].

CPS and EPS are polymers of variable size (a few 10 to 10000 kDa). They may be made of a single type of ose (homopolysaccharides) or of several different types of monosaccharides (heteropolysaccharides). They can be made of neutral or of charged monosaccharides, and may or may not comprise non-glucidic substituents. And finally, they can be linear or branched [20]. The

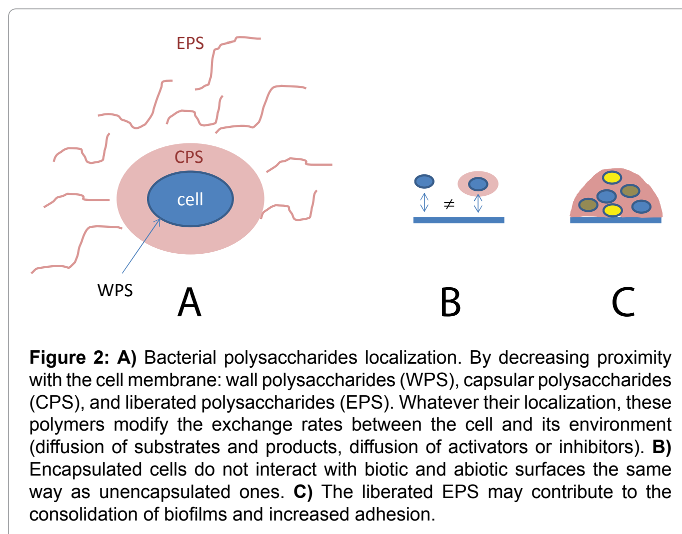

Figure 2: A) Bacterial polysaccharides localization. By decreasing proximity with the cell membrane: wall polysaccharides (WPS), capsular polysaccharides (CPS), and liberated polysaccharides (EPS). Whatever their localization, these polymers modify the exchange rates between the cell and its environment (diffusion of substrates and products, diffusion of activators or inhibitors). **B)** Encapsulated cells do not interact with biotic and abiotic surfaces the same way as unencapsulated ones. **C)** The liberated EPS may contribute to the consolidation of biofilms and increased adhesion.

heteropolysaccharides released into the medium by lactic acid bacteria are composed of repeating units containing at least two different types of monomers and from 3 to 8 residues. The production levels of released EPS vary from 20 $mg.L^{-1}$ to 2.3 $g.L^{-1}$ at the most [21]. The most common homopolysaccharides in lactic acid bacteria are α-glucans (dextran, mutan, alternan, reuteran), β-fructans (levan or inulin), but also β-glucans. Depending on the polymers, on the producing bacteria and on the biosynthetic pathways, production levels vary from a few tens of $mg.L^{-1}$ to several tens of $g.L^{-1}$ [22].

EPS generally do not constitute an energy reserve for the cell producing them. But, by modifying cell accessibility and diffusion in the environment, EPS could protect bacteria from desiccation, phagocytosis, predation by protozoa, antibiotics and other toxic compounds, as well as from osmotic or cold stress [20,21,23-25]. EPS could also play a role in adhesion, by modulating cell interactions with biotic and abiotic surfaces, and in the formation of biofilms, thus facilitating the colonization of diverse ecosystems [26]. Nevertheless, EPS contribute more to biofilm consolidation than to cell primary adhesion (Figure 2) [27].

EPS could therefore contribute to the adaptation of certain strains of lactic acid bacteria to the wine environment, but their formation can also lead to wine spoilage, as previously shown for other ecological niches [28].

Ropiness, a well understood but uncommon microbial disease of wine, cause of bacterial polysaccharides bad reputation among winemaking and cidermaking people.

Until 2007, the majority of published work on polysaccharides production by wine bacteria dealt with wine ropiness. Ropiness is one of the 4 major bacterial alterations of the wines described by Pasteur in 1866 [1]. The spoiled wines have an oily consistency: the wines are thick and flow without noise, sometimes forming a string (Figure 3A). The increase in thickness is related to the production, by the bacteria, of a sticky polymer, whose presence can be detected by picking bacterial colonies (Figure 3B). Several types of microorganisms can lead to this type of spoilage, with the production of polymers with probably a wide variety of structures (homo and heteroglycans) in wine, beer or cider [29-35]. Nevertheless, the polymer most frequently encountered and the only one whose structure has been elucidated is an β-glucan. It has a high molecular weight, between 500 and 2000 kDa; it is composed of

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trisaccharide repeat units, with a main chain of β-1,3-linked D-glucoses and a branch, consisting of a single β-1,2-linked D-glucose residue (Figures 3C and 3D) [30]. This polymer strongly resembles the capsular β-glucan produced by *Streptococcus pneumoniae* type 37, except that it is half as branched [36].

The microorganism most frequently implicated and mostly studied as the agent for ropiness belongs to the genus *Pediococcus* [37]. Different strains were isolated from white and red wines from the Bordeaux region or from Basque ciders and then proved to induce the spoilage at laboratory scale. First classified in the species *Pediococcus cerevisiae* on the basis of phenotypic tests, then in the species *Pediococcus damnosus* by DNA hybridizations [33,37,38], these bacteria have been definitively identified as *Pediococcus parvulus* on the basis of the 16S RNA sequence [39].

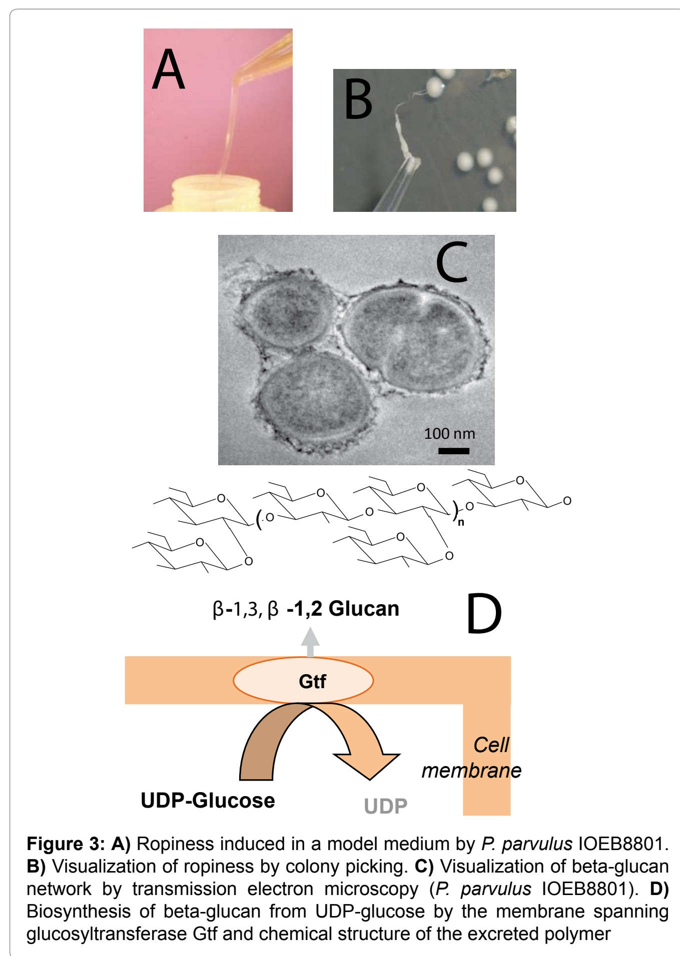

Figure 3: A) Ropiness induced in a model medium by *P. parvulus* IOEB8801. **B)** Visualization of ropiness by colony picking. **C)** Visualization of beta-glucan network by transmission electron microscopy (*P. parvulus* IOEB8801). **D)** Biosynthesis of beta-glucan from UDP-glucose by the membrane spanning glucosyltransferase Gtf and chemical structure of the excreted polymer

The ropy pediococci isolated around Bordeaux display a 5.5 kb plasmid, named pF8801 [38]. The plasmid sequence includes genes involved in the maintenance and transfer of the plasmid (rep and mob) and a 1701bp gene, named *gtf* and encoding a glucosyltransferase. The Gtf protein (566 amino acids) has strong homologies (32% protein identity) with Tts, the glucosyltransferase involved in the synthesis of β-glucan type 37, which is very similar to the ropy β-glucan [35,40,41]. On the Tts model, β-glucan production is controlled by a single, processive transmembrane glucosyltransferase, Gtf, catalyzing the polymerization of glucosyl residues, from UDP-glucose [42,43]. This enzyme is multifunctional because it achieves both the synthesis of β-1,3 and β-1,2 bonds and the export of the polymer (Figure 3). The polymer accumulates around the cells, where it forms a hairy halo (Figure 3C) and then in the medium to which it confers, under certain conditions, a ropy character (Figures 3A and 3B) [31,37,44]. This envelope is weakly linked to the cell because washes allow eliminating it [45]. Glucan production is inseparable from the growth of the ropy *Pediococcus*. Nevertheless, this synthesis appears rather like a metabolic leak, because it occurs essentially when the bacterial growth slows down. In addition, the portion of glucose consumed by the bacteria and used to synthesize the glucan is less than 1% [46].

In order to evaluate whether β-glucan accumulation conferred a selective advantage to the producing bacteria, we compared the behavior of *P. parvulus* IOEB 8801 (*gtf*+with a ropy phenotype) and its non-ropy isogenic mutant IOEB 0206 (having lost the plasmid carrying the *gtf* gene, *gtf*-). The cells, isolated at the end of the exponential growth phase, were subjected to unique stresses, relevant in wine: acid pH, presence of ethanol or sulfur dioxide (Figure 4A). The survival of the *gtf*+ strains is 1 to 2 log higher, in particular in an acid medium. Compared with its non-ropy mutant, the *gtf*+ strain has also an increased survival rate, when it is introduced into wine and, in particular, in white wine:

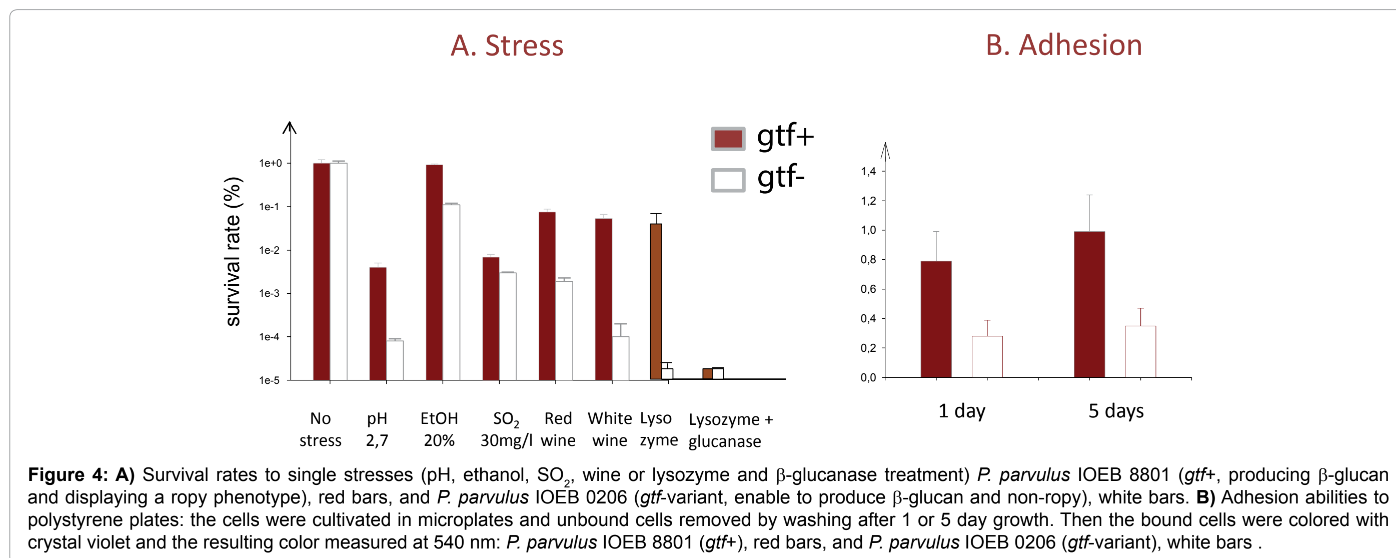

Figure 4: A) Survival rates to single stresses (pH, ethanol, SO_2, wine or lysozyme and β-glucanase treatment) *P. parvulus* IOEB 8801 (*gtf*+, producing β-glucan and displaying a ropy phenotype), red bars, and *P. parvulus* IOEB 0206 (*gtf*-variant, enable to produce β-glucan and non-ropy), white bars. **B)** Adhesion abilities to polystyrene plates: the cells were cultivated in microplates and unbound cells removed by washing after 1 or 5 day growth. Then the bound cells were colored with crystal violet and the resulting color measured at 540 nm: *P. parvulus* IOEB 8801 (*gtf*+), red bars, and *P. parvulus* IOEB 0206 (*gtf*-variant), white bars .

white wine would be a stronger stress than red wine for the non-ropy *Pediococcus* but not for the ropy one [43]. In addition, the ropy strain better resists to lysozyme treatment. By combining lysozyme with a fungal β-1,3, β-1,6 glucanase, known to hydrolyze wine β-glucans, it is possible to considerably improve the bactericidal efficacy of lysozyme (Figure 4) [30,47]. However, β-glucanase alone does not show significant bactericidal activity, but the treated cells lose their glucan envelope which may therefore constitute a protection against the action of lysozyme [44]. Finally, the *gtf+* strain exhibits substantially increased adhesion capacities relative to their *gtf*-mutant, both in the early phase of biofilm setup (1 day) and later (5 days) (Figure 4B).

The synthesis of β-glucan therefore appears to be a tool for bacteria to better survive in wine and, in particular, in white wine and also as a factor favoring the persistence of undesired bacteria on the winemaking material.

LAB genome surveys suggest that EPS production by wine lactic bacteria is almost the norm. New perspectives for these molecules?

O. oeni is the lactic acid bacterial species best suited to acidic and alcoholic environments. Thus, it is the species that generally drives MLF, even though other species can catalyze this transformation, particularly in less acidic wines. In 2007, a study carried out on red wines during malolactic fermentation showed that the development of *O. oeni* induced significant changes in the wine polysaccharide composition, whatever the strain involved. Degradation of grapes polysaccharides phenomena superimposed with release of new polymers. Most of the time, these changes go unnoticed, because the monomer composition of the polysaccharides released is close to that of those initially present in the wines. Moreover, the polysaccharide release in wine most often does not induce any change in wine viscosity [48].

By growing various *O. oeni* strains in model media, it appeared that:

- The maximum EPS concentration obtained with the selected strains was low (25 to 205 $mg.l^{-1}$), but significant; it was markedly higher when cells were cultured in the presence of sucrose [49,50]
- The released EPS did not constitute a carbon reserve, as none of the strain studied consume them after growth substrate exhaustion [50].
- With the exception of some strains that induce a thick appearance (ropiness), the accumulation of EPS is not visible to the naked eye. A silky halo (silkiness) is sometimes observed during *O. oeni* growth but it is not associated with the formation of EPS [49].
- The composition of the culture medium (nature of the carbon source, presence of ethanol) modulates EPS biosynthesis differently, depending on the strain considered. The production of EPS is very low, when the medium induces a limitation of the bacterial growth (synthetic medium deficient, for example). Growth is therefore essential for the production of EPS. However, EPS production appears to be partly decoupled from growth for most strains because EPS accumulate mainly at the end of growth and at the beginning of the stationary phase [50,51].
- The size distribution of EPS was variable according to strains, with 1 to 4 peaks observed in exclusion chromatography and polymers with a molecular mass ranging from 8 to more than 500 kDa, and more rarely 10^6 kDa. In most cases, a significant fraction of the EPS produced has a molecular weight of less than 100 kDa [50,52].

The analysis of 50 genomic sequences of *O. oeni* provided an inventory of the genes potentially involved in exopolysaccharide (EPS) biosynthesis (Figures 5 and 6). Several chromosome loci were identified: two loci where complex clusters comprising from 3 to 15 genes were inserted. These were named *eps1* and *eps2*. Three isolated glycoside-hydrolase genes named *dsrO*, *dsrV* and *levO* were also found, always at the same insertion site, and three isolated glycosyltransferase genes named *gtf*, *it3*, *it4*. The insertion site of *gtf*, the glucosyltransferase gene associated with ropiness in Pediococci (see the above section), varied from one strain of *O. oeni* to the other (Figure 5A).

The *eps* gene distribution on the phylogenetic tree was examined [52]. All the studied genomes (50) possessed several genes dedicated to EPS metabolism. The isolated genes were present or absent depending on the strain and the *eps* gene clusters composition diverged from one strain to another. Three different models were found for the *eps1* cluster; the *eps2* cluster was much more variable: among 43 strains of the 50 strains studied, 15 models were encountered of which one strongly truncated. The *eps2* cluster was absent in the remaining 7 strains (Figure 5B).

The soluble and capsular EPS production capacity of several strains was examined after growth in different culture media and the EPS structure was determined. Genotype to phenotype correlations showed that several EPS biosynthetic pathways were active and complementary in *O. oeni* (Figures 5B and 6). Can be distinguished:

(i) A Wzy-dependent synthetic pathway, involving UDP-osyl precursors, a priming glycosyltransferase and several other non-processive glycosyltransferases, a flippase to externalize the repeating unit of the polymer and a polymerase. This allows the production of heteropolysaccharides made of glucose, galactose and rhamnose, mainly in a capsular form [52,53].

(ii) Homopolysaccharides can be synthesized from sucrose: dextran (α-glucan) production is catalyzed by the dextransucrase DsrO, a glycoside-hydrolase of the GH70 family, and levan (β-fructan) can be produced by strains bearing a non-truncated *levO* gene. LevO is a levansucrase, a glycoside-hydrolase of the GH68 family [52,54].

(iii) The glucan synthase pathway (Gtf) described in ropy *pediococci* is also active in specific *O. oeni* strains, and drives the synthesis β-glucan in a free and a cell-associated form, giving a ropy phenotype to model growth media [43,52].

(iv) It3 is able to transfer glucose and galactose but its contribution to EPS biosynthesis remains unclear, and the activity of It4 was not explored [55].

All *O. oeni* strains studied thus present the genetic material necessary to synthesize both homo and heteropolysaccharides. The genome of *O. oeni* has a limited size (1.7-1.8 Mb) [56,57]. In addition, this genome is hypermutable suggesting that useless genes have been eliminated throughout evolutions [58]. The presence of multiple genes and large gene clusters associated with EPS biosynthesis suggests that this metabolism is important for the adaptation of the bacterium to its ecological niche, wine. The presence of two *eps* gene clusters is a trait common to most strains of the species. The production of the EPS capsule seems directly correlated to the presence of a non-truncated *eps2* gene cluster, while the function of cluster *eps1* remains unclear.

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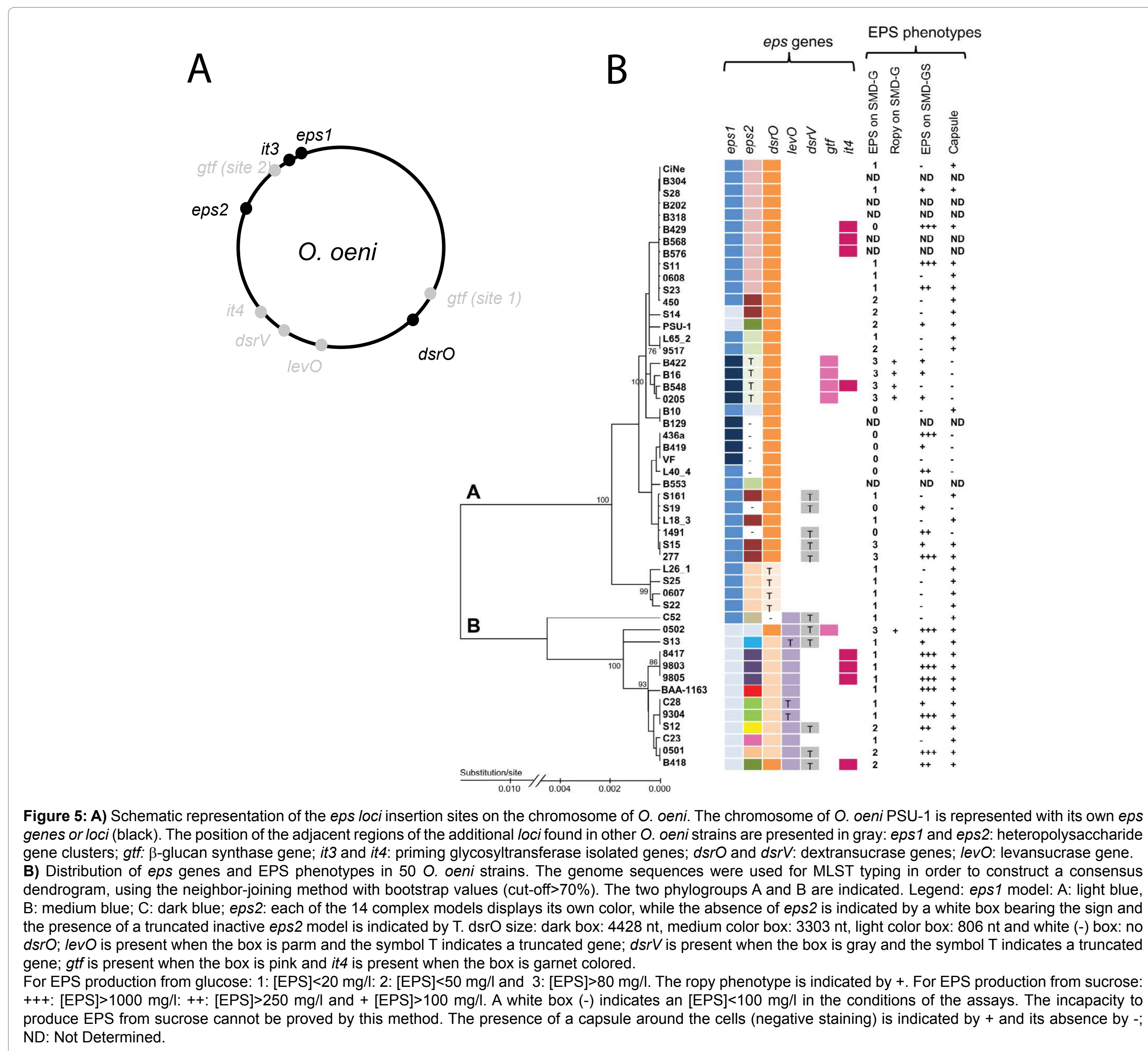

Figure 5: A) Schematic representation of the *eps loci* insertion sites on the chromosome of *O. oeni*. The chromosome of *O. oeni* PSU-1 is represented with its own *eps genes or loci* (black). The position of the adjacent regions of the additional *loci* found in other *O. oeni* strains are presented in gray: *eps1* and *eps2*: heteropolysaccharide gene clusters; *gtf:* β-glucan synthase gene; *it3* and *it4*: priming glycosyltransferase isolated genes; *dsrO* and *dsrV*: dextransucrase genes; *levO*: levansucrase gene.
B) Distribution of *eps* genes and EPS phenotypes in 50 *O. oeni* strains. The genome sequences were used for MLST typing in order to construct a consensus dendrogram, using the neighbor-joining method with bootstrap values (cut-off>70%). The two phylogroups A and B are indicated. Legend: *eps1* model: A: light blue, B: medium blue; C: dark blue; *eps2*: each of the 14 complex models displays its own color, while the absence of *eps2* is indicated by a white box bearing the sign and the presence of a truncated inactive *eps2* model is indicated by T. dsrO size: dark box: 4428 nt, medium color box: 3303 nt, light color box: 806 nt and white (-) box: no *dsrO*; *levO* is present when the box is parm and the symbol T indicates a truncated gene; *dsrV* is present when the box is gray and the symbol T indicates a truncated gene; *gtf* is present when the box is pink and *it4* is present when the box is garnet colored.
For EPS production from glucose: 1: [EPS]<20 mg/l: 2: [EPS]<50 mg/l and 3: [EPS]>80 mg/l. The ropy phenotype is indicated by +. For EPS production from sucrose: +++: [EPS]>1000 mg/l: ++: [EPS]>250 mg/l and + [EPS]>100 mg/l. A white box (-) indicates an [EPS]<100 mg/l in the conditions of the assays. The incapacity to produce EPS from sucrose cannot be proved by this method. The presence of a capsule around the cells (negative staining) is indicated by + and its absence by -; ND: Not Determined.

Nevertheless, a recent study suggests that complementation phenomena between the *eps1* and *eps2* clusters contribute to maintain the capsule biosynthesis [55]. Furthermore, the very large diversity of *O. oeni eps2* gene clusters recalls what is described in pathogenic bacteria such as *S. pneumoniae*, in which the *eps* operons direct the capsule synthesis, a proven pathogenicity factor [59]. All these findings reinforces the hypothesis that EPS play a role in the adaptation of the bacterium.

To explore this, we examined the possible link between the original isolation matrix of the strains and their *eps* genes. No special link could be drawn between the *eps1* or *eps2* gene clusters or the dextransucrase gene and the ecological niche of the strains (fruit, cider, red or white wines, or even specific winemaking regions). On the contrary, all the *gtf*+ *O. oeni* strains studied came from white wines or champagnes, which are notably more acidic than red wines. A study was therefore conducted specifically on these particular wines: 56% of *O. oeni* Champagne isolates studied were *gtf*+, while the prevalence of *gtf* in the *O. oeni* overall population is generally described between 8 and 23% [43,50,52,60]. This high prevalence could indicate a better adaptation to the acidity and the specific medium of white wine, as demonstrated in the case of ropy pediococci (see previous paragraph). Nevertheless, these *O. oeni* strains are never responsible for wine spoilage, because (i) the amount of beta-glucan produced is much lower than that observed with pediococci, and (ii) their stage of development is early enough for the defect to be corrected by the winemaker (mixing, filtration, sulfuring) before bottling the wine [43,60,61].

We also looked at whether EPS could protect bacteria during the preparation of malolactic starters. These are generally concentrated by centrifugation, added with cryoprotectors, frozen and optionally lyophilized (Figure 7A). We first looked at whether the presence or absence of a devoid of sucrose and then concentrated and lyophilized. In

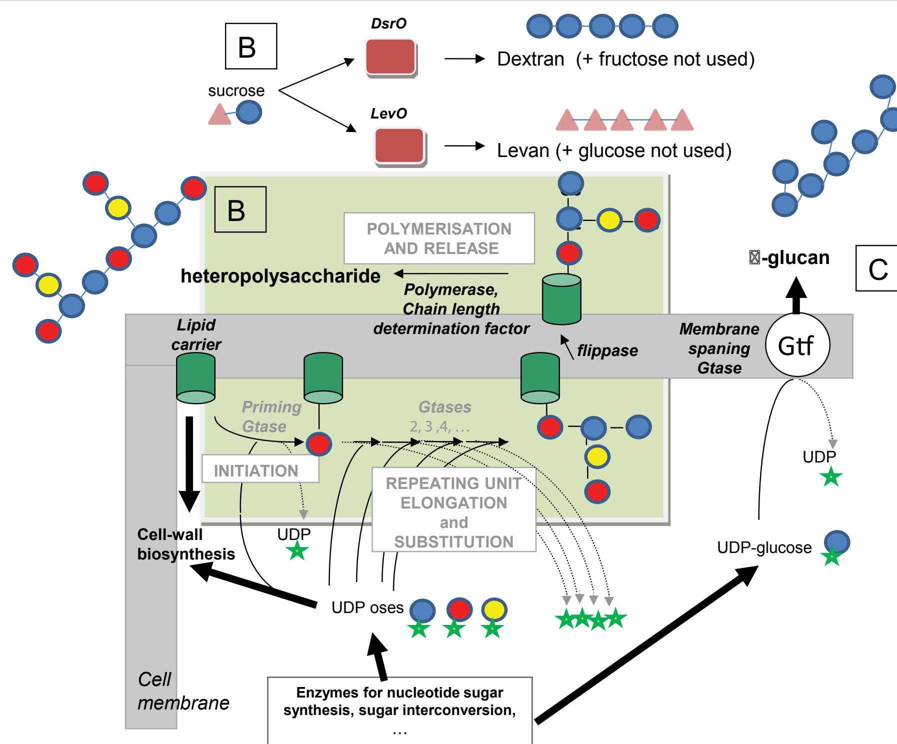

Figure 6: Schematic representation and cellular localization of the different EPS biosynthetic pathways identified in *O. oeni*. The osyls (e.g. glucosyl, rhamnosyl, galactosyl…) are each represented by a distinct colored disc and UDP by a green star), Gtase: Glycosyltransferase; DexO: Dextransucxrase; LevO: Levansucrase. Dextran is an α-glucan and levan a β-fructan.

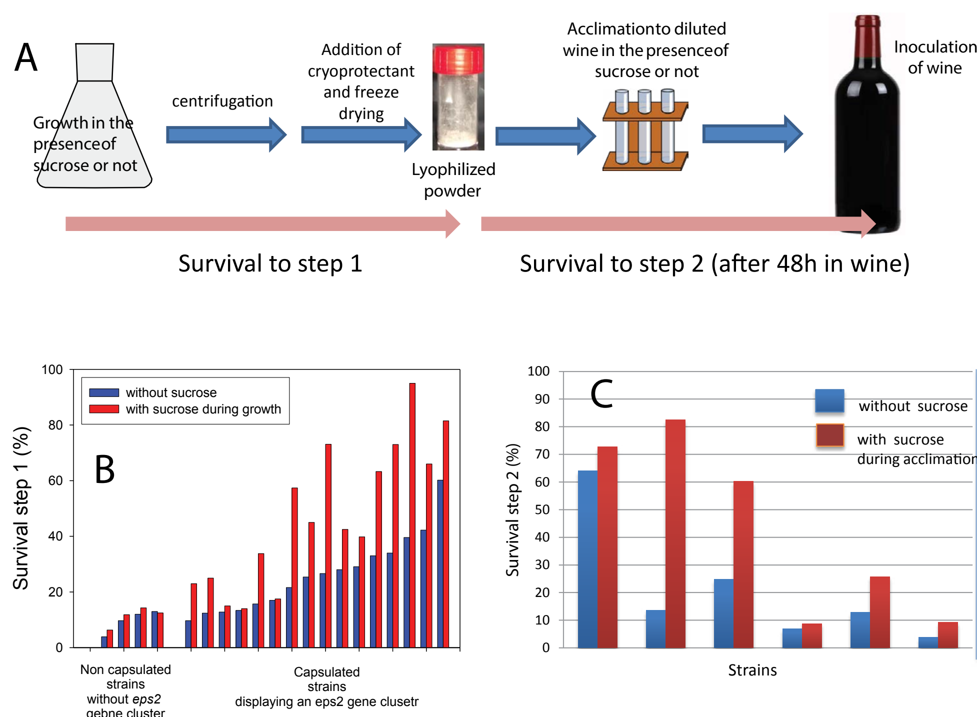

Figure 7: A) Schematic representation of the protocol leading to the production of lyophilized *O. oeni* strains and their inoculation in wine. Definition of the steps examined for survival: step 1 includes centrifugation, cryoprotectant addition and freeze drying; step 2 includes dilution into diluted wine (± sucrose), inoculation in wine and 48 h stay in wine. **B)** Survival rates to step 1, depending on the strain and on the medium used for cell growth (blue: grape juice based medium, red: grape-juice+sucrose based medium). Each group of bars (a blue and a red) represents the results for a strain
C) Survival rates to step 2 of strains produced from grape-juice+sucrose based growth medium depending on the strain considered and on the presence or not of sucrose in the acclimation medium (diluted wine).

this type of growth medium, the only EPS biosynthetic pathway active polysaccharide capsule could protect the bacteria during the entire process. For this, different strains were grown in a medium containing grape juice (and are the Gtf and the Wzy ones, and strains displaying a complete *eps2* gene cluster (Wzy pathway) are encapsulated. Overall, these encapsulated strains seem to better survive to the complete process than un-encapsulated ones, even if disparities exist. If sucrose is added to the culture medium to stimulate the synthesis of dextran and levan, the encapsulated strains resist even better (Figure 7B). Dextran is known for its cryoprotector properties and the endogenous dextran of *O. oeni* also displays this property. Protection with dextran is also putatively effective during inoculation in wine (step 2), (Figure 7C), when the cells are acclimated in the presence of sucrose before inoculation into the wine [62].

Conclusion

The biosynthesis of polysaccharides is a widespread characteristic in lactic acid bacteria, and it is also observed in wine bacteria, and especially in *O. oeni*. Contrary to what was currently described in winemaking handbooks, this phenotypic trait is not always visible and does not necessarily constitute a defect. On the contrary, a better knowledge of this property could make it possible to develop more efficient tools (i) to eliminate the spoilage strains and (ii) to produce more resistant starters.

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