Identification of pOENI-1 and Related Plasmids in *Oenococcus oeni* Strains Performing the Malolactic Fermentation in Wine

Marion Favier¹,2, Eric Bihère¹, Aline Lonvaud-Funel¹, Virginie Moine³, Patrick M. Lucas¹*

¹ University of Bordeaux, ISW, Unit of Enology EA 4577, Villenave d’Ornon, France, ² SARCO, research subsidiary of the Laffort group, BP 40, Bordeaux, France, ³ Laffort, BP 17, Bordeaux, France

**Abstract**

Plasmids in lactic acid bacteria occasionally confer adaptive advantages improving the growth and behaviour of their host cells. They are often associated to starter cultures used in the food industry and could be a signature of their superiority. *Oenococcus oeni* is the main lactic acid bacteria species encountered in wine. It performs the malolactic fermentation that occurs in most wines after alcoholic fermentation and contributes to their quality and stability. Industrial *O. oeni* starters may be used to better control malolactic fermentation. Starters are selected empirically by virtue of their fermentation kinetics and capacity to survive in wine. This study was initiated with the aim to determine whether *O. oeni* contains plasmids of technological interest. Screening of 11 starters and 33 laboratory strains revealed two closely related plasmids, named pOENI-1 (18.3-kb) and pOENI-1v2 (21.9-kb). Sequence analyses indicate that they use the theta mode of replication, carry genes of maintenance and replication and two genes possibly involved in wine adaptation encoding a predicted sulphite exporter (tauE) and a NADH:flavin oxidoreductase of the old yellow enzyme family (oye). Interestingly, pOENI-1 and pOENI-1v2 were detected only in four strains, but this included three industrial starters. PCR screenings also revealed that tauE is present in six of the 11 starters, being probably inserted in the chromosome of some strains. Microvinification assays performed using strains with and without plasmids did not disclose significant differences of survival in wine or fermentation kinetics. However, analyses of 95 wines at different phases of winemaking showed that strains carrying the plasmids or the genes tauE and oye were predominant during spontaneous malolactic fermentation. Taken together, the results revealed a family of related plasmids associated with industrial starters and indigenous strains performing spontaneous malolactic fermentation that possibly contribute to the technological performance of strains in wine.

**Introduction**

Lactic acid bacteria (LAB) contribute to winemaking during the malolactic fermentation (MLF). MLF usually takes place after the yeast-driven alcoholic fermentation (AF) and lasts a few days to several months depending on wine composition, temperature and LAB population [1,2]. MLF mainly consists in the conversion of the strong dicarboxylic L-malate into the softer L-lactate and CO₂. It is beneficial in that it reduces the acidity of wine, improves its taste and aromas and contributes to its microbiological stability [3-5]. MLF is present in six of the 11 starters, being probably inserted in the chromosome of some strains. Microvinification assays performed using strains with and without plasmids did not disclose significant differences of survival in wine or fermentation kinetics. However, analyses of 95 wines at different phases of winemaking showed that strains carrying the plasmids or the genes tauE and oye were predominant during spontaneous malolactic fermentation. Taken together, the results revealed a family of related plasmids associated with industrial starters and indigenous strains performing spontaneous malolactic fermentation that possibly contribute to the technological performance of strains in wine.

**Impact of MLF, winemakers can make use of industrial O. oeni strains. A few dozens of malolactic starters are available to date. They are natural strains selected on the basis of their tolerance to wine stressors, kinetics of MLF, aromas production and safety regarding undesirable metabolisms such as the production of biogenic amines, bitterness or ropiness [10].**

**The molecular mechanisms at the origin of O. oeni survival and growth in wine, and differences existing between strains are still poorly understood. Diverse genes possibly involved in wine adaptation were described in the past decades. This includes genes related to general stress response, membrane composition and fluidity, pH homeostasis, multidrug resistance, or response to oxidative stress and DNA damage [11–18]. Comparative genomic analyses also revealed a number of genes that were statistically more often present in O. oeni strains of technological interest [19,20,21]. However, all the genes identified to date do not satisfactorily explain differences of survival in wine and kinetics of MLF observed amongst O. oeni strains [22].**
Until now, little attention was paid to the plasmids of *O. oeni*. Plasmids are known as a source of phenotypic and genetic diversity in LAB and occasionally confer adaptive advantages to host strains. Besides maintenance and transfer mechanisms, they encode important traits such as secondary metabolisms, resistance to bacteriophages, antibiotics or heavy metals, and production of exopolysaccharides, bacteriocins and immunity proteins [23,24]. Plasmids are often associated to starter cultures used in the food industry and could be a signature of their technological superiority and individuality [24]. In the dairy starter *Lactococcus lactis*, they confer phenotypes that reflect adaptation to the dairy environment, such as lactose catabolism, protease activity, peptide and amino acid uptake and bacteriophage resistance [25]. Six small cryptic plasmids of *O. oeni* were sequenced and described to date: pLo13 [26], pO428 [27], pOG32 [28], pRS1 [29], pRS2 and pRS3 [30]. They encode replication and mobilization proteins but do not carry any gene potentially involved in wine adaptation. Large plasmids were detected in a number of *O. oeni* strains but no sequence was reported to date [31–35]. A functional role has been assigned to the 22.5-kb plasmid pBL34 that seems to confer pesticide resistance to its *O. oeni* host cells [35].

This work was initiated with the aim to investigate whether plasmids may contribute to wine adaptation of *O. oeni* strains. Two large plasmids, named pOENI-1 (18.3 kb) and pOENI-1v2 (21.9 kb), were described for the first time in *O. oeni*. Their contribution to the technological properties of strains was investigated by analyzing their sequences, their distribution in the *O. oeni* species, their associated phenotypes and their frequency in wines at different steps of winemaking.

**Materials and Methods**

**Bacteria strains and culture conditions**

*O. oeni* strains used in this study are listed in Table 1. They consist in 11 industrial starters, 14 strains of laboratory collections and 18 strains isolated for this study from red and white wines collected during spontaneous MLF of vintages 2008 and 2009. New isolates were deposited in the SARCO collection (SARCO Laboratory, Bordeaux, France). All the strains were stored at −80°C in the presence of 30% (v/v) glycerol and propagated under anaerobic conditions at 25°C in grape juice medium (GJ) containing 25% (v/v) commercial red grape juice, 0.5% (wt/v) yeast extract, 0.1% (v/v) Tween 80, pH 4.3.

**Strain typing**

Strain typing was performed by NotI restriction of bacterial DNA followed by pulse field gel electrophoresis (PFGE) of restriction fragments as previously described [36]. DNA restriction patterns were compared in a dendrogram generated by the unweighted pair group method using arithmetic means (UPGMA) with the Dice coefficient of similarity and a tolerance limit of 2.3% in Bionumerics 3.1 software (Applied Maths, Kortrijk, Belgium). Multilocus sequence typing (MLST) was also performed for several strains according to the procedure described in [36]. MLST data was processed in a neighbor-joining tree constructed using MEGA4 [37].

**Plasmid sequencing and analysis**

Plasmid pOENI-1 was isolated from a 10-ml culture of commercial strain *O. oeni* C9 by the alkaline lysis method [38]. The plasmid DNA preparation was digested by EcoRI and PstI (New Englands Biolabs) to construct a library of inserts in the *E. coli* vector pBluescript II SK+ (Stratagene). Two inserts (1.3 and 2.5 kb) were sequenced. The rest of the plasmid was amplified in two PCR products (4.0 and 10.5 kb) obtained using primers designed in the first sequences and a high-fidelity DNA polymerase (iProof, Bio-Rad). The two PCR products were cloned in pGEM-T Easy (Promega), transferred in *E. coli* and sequenced.

The sequences were assembled using Lasergene (DNA Star) in a single circular DNA molecule of 18,332-bp. The sequence of plasmid pOENI-1v2 was obtained by sequencing the genome of strain S11 by the 454 technology (single reads of 450-bp on average, GenoToul, Toulouse, France). Sequences assembled with Lasergene (DNA Star) formed a circular DNA molecule of 21,926-bp (coverage >100X all along the plasmid sequence). Open reading frames (ORFs) were predicted with GeneMark [39] and Glimmer [40]. Gene annotation was performed manually using Blast and Interproscan analyses [41].

**PCR-based detection of pOENI-1 in *O. oeni* strains**

Bacterial genomic DNAs were extracted using the Wizard genomic DNA purification kit (Promega) according to the manufacturer’s instructions. DNA preparations were used as template in PCR assays to detect the plasmid genes repA, oye and tauE, the chromosomal gene ooeOE_0812 (locus tag of the *O. oeni* PSU-1 genome, NC_008528), and to confirm the integrity of plasmids using a combination of three overlapping PCRs that extend over the whole plasmid sequences. The primers used are listed in Table 2. PCR amplifications were performed in 20-μl mixtures containing 25 ng of template DNA, 0.25 μM of each primer and the Taq-&GO™ PCR mix (MP Biomedicals). The standard PCR program was 95°C for 5 min; followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s and a final step of 10 min at 72°C. PCR products were visualized under UV light exposure after electrophoresis in 1.2% (w/v) agarose gels and staining with ethidium bromide.

**Determination of plasmid/oye gene copy number per cell**

Copy number of plasmids pOENI-1, pOENI-1v2 and gene oye (ORF 11) per cell was determined by quantitative real-time PCR (qPCR) using a GoTaq® qPCR Master Mix (Promega) on a CFX96™ Real-Time Detection System (Bio-Rad). Amplification conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s and a final step of 70°C to 90°C with an increment of 0.5°C each 5 s. Two primer pairs (Table 2) were used to quantify the chromosomal gene *tpdB* and the plasmid gene *oye* in order to calculate their relative proportion.

Serial decimal dilutions of *O. oeni* ATCC BAA 1163 genomic DNA were used to produce the standard curves. In this strain, both *oye* and *tpdB* are present on the chromosome at one copy per cell.

Standard curve equations and coefficients of correlation calculated from three independent experiments were: C_T = −3.33x + 35.11, R² = 0.998 (*tpdB*), C_T = −3.49x + 37.28, R² = 0.993 (*oye*). Genomic DNA of all tested strains was extracted from bacterial colonies suspended in 200-μl sterile H₂O and heating for 10 min at 80°C prior cooling on ice. All determinations were done in triplicates.

**Quantification of oye/tauE genes in wine**

Determinations were performed by qPCR as described above, except that template DNAs were extracted from total microorganisms of 10-ml samples of must or wine by the method reported in [31]. Standard curves were produced using DNA extracted from decimal dilutions of *O. oeni* ATCC BAA 1163 or S24 (*tpdB* and *tauE* at one copy per cell) inoculated in sterile wine (10⁶ to 10⁵ cells/ml⁻¹). The corresponding equations indicate cycle threshold values for 1 ml of wine: ATCC BAA 1163, C_T = −3.61x +41.66, R² = 0.906 (*tpdB*), C_T = −3.64x +42.26, R² = 0.961 (*oye*); S24, C_T = −3.98x +39.38, R² = 0.975 (*tpdB*), C_T = −3.86x +39.14,
Plasmids in *O. oeni*

Table 1. *O. oeni* strains used in this study.

| Strain* | Collection | Origin |
|---|---|---|
| C1 | IOEB | Commercial product Lactooenos 350 Preac, Laffort |
| C2 | IOEB | Commercial product Lactooenos 450 Preac, Laffort |
| C3 | IOEB | Commercial product Lactooenos 816, Laffort |
| C4 | IOEB | Commercial product Vitilactic BL01, Martin Vialatte |
| C5 | IOEB | Commercial product Viniflora Ciné, CHR Hansen |
| C6 | IOEB | Commercial product Lalvin 31, Lallemand |
| C7 | IOEB | Commercial product Oeno2, Lamothé-Abiet |
| C8 | IOEB | Commercial product Lactoenos SB3, Laffort |
| C9 | IOEB | Commercial product Vitilactic F, Martin Vialatte |
| C10 | IOEB | Commercial product Lalvin VP41, Lallemand |
| PSU1 | ATCC | Commercial starter, Red wine, California, 1977 |
| IOEB 0026 | IOEB | Red wine, France, 2000 |
| IOEB 0501 | IOEB | Red wine, France, 2005 |
| IOEB 0608 | IOEB | Red wine, France, 2006 |
| IOEB 8419 | IOEB | Red wine, France, 1984 |
| IOEB 9115 | IOEB | Red wine, France, 1991 |
| IOEB 9304 | IOEB | Cider, France, 1993 |
| IOEB 89006 | IOEB | Red wine, France, 1989 |
| IOEB 89127 | IOEB | Red wine, France, 1989 |
| IOEB S268 | IOEB-SARCO | Red wine, France, 2000 |
| IOEB S384 | IOEB-SARCO | White wine, France, 2002 |
| IOEB S422 | IOEB-SARCO | White wine, France, 2002 |
| IOEB S343a | IOEB-SARCO | Red wine, France, 2002 |
| IOEB S455 | IOEB-SARCO | White wine, France, 2003 |
| S4 | SARCO | Red wine, France, 2009 |
| S11 | SARCO | Sparkling white wine, France, 2008 |
| S12 | SARCO | White wine, France, 2009 |
| S13 | SARCO | Red wine, France, 2009 |
| S14 | SARCO | Red wine, France, 2009 |
| S15 | SARCO | Red wine, France, 2009 |
| S17 | SARCO | Red wine, France, 2009 |
| S18 | SARCO | Red wine, France, 2009 |
| S19 | SARCO | Red wine, France, 2009 |
| S20 | SARCO | Red wine, France, 2009 |
| S22 | SARCO | White wine, France, 2009 |
| S23 | SARCO | White wine, England, 2009 |
| S24 | SARCO | Red wine, England, 2009 |
| S25 | SARCO | Red wine, France, 2009 |
| S26 | SARCO | Red wine, France, 2009 |
| S27 | SARCO | Red wine, France, 2009 |
| S28 | SARCO | Red wine, France, 2009 |
| S29 | SARCO | Red wine, France, 2009 |
| ATCC BAA 1163 | ATCC | Red wine, France, 1984 |

*IOEB: Institute of oenology of Bordeaux, S: SARCO, ATCC: American type culture collection.
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$R^2 = 0.979$ ($\tau_E$). The tested samples were 95 red wines and musts collected at different stages of winemaking (must, alcoholic fermentation, MLF) in 86 wineries of Bordeaux’s area. No industrial strain was employed to conduct MLF in these wines.

Plasmid curing

*O. oeni* strains carrying pOENI-1 or pOEni-1v2 were cultivated in GJ medium for about 20 generations. Cultures were plated to analyze 30 colonies and determine the presence or absence of the plasmids. DNA templates were prepared by suspending each
Results

Sequence analysis of pOENI-1

During a survey of O. oeni strains, we have detected a large plasmid in the industrial strain O. oeni C9. This plasmid, named pOENI-1, was analyzed to determine whether it contributes to the technological properties of O. oeni C9. Its complete sequence was obtained by sequencing diverse restriction fragments and PCR products. pOENI-1 is a circular DNA molecule of 18,332-bp in length. Its GC% is 40.8, compared to 38% in the O. oeni chromosome [19]. Sequence annotation revealed 18 complete ORFs and two truncated ORFs (ORFs 4 and 20) ranging from 210 to 1512-bp (Fig. 1A and Table 3). A function was ascribed to 15 of the 20 encoded proteins. The protein encoded by ORF 15 shares more than 70% sequence identity with replication initiator protein A (RepA) encountered in theta type plasmids plca36 of Lactobacillus casei [43,44], pLgLA39 of Lactobacillus gasseri [40] and pSF118-44 of Lactobacillus salivarius UCC118 [44]. The intergenic region located between ORF 14 and ORF 15 shows all the hallmarks of the theta-type replication origin [45,46]. It is located upstream of repA, contains an AT-rich region (positions 12,832–12,915, 71% AT) and an 18-bp repetition present at 7 copies (atatatcttgata, positions 12,862–12,987). Therefore, it is likely that pOENI-1 is the first large theta-type plasmid described in O. oeni.

ORFs 13 and 14 encode partitioning proteins ParB and ParA respectively, involved in plasmid segregation during cell division. A putative toxin/antitoxin system (PemK-like and RelB-like proteins) contributing to plasmid stability is encoded by ORFs 16 and 17. pOENI-1 does not encode the full set of proteins required for plasmid conjugation, but only two (ORFs 1 and 3). ORFs 7 and 8 code for a TetR transcriptional regulator and a putative permease of the TauE family. Permeases of this family are known to act as sulfite transporters [47,48]. It is likely that ORF 7 and ORF 8 encode proteins that are functionally related since pairs of similar genes were detected in Lactobacillus mali (Table 3) and in the O. oeni phages iOg30, iOgPSU1 and iOg44 [49], ORFs 10 and 11 encode a LysR transcriptional regulator and a NADH:flavin oxidoreductase of the old yellow enzyme (OYE) family, group 4.

Table 2. Primers list.

| Primer name | Forward sequence (5’-3’) | Reverse sequence (5’-3’) | Target | Product (bp) |
|-------------|-------------------------|-------------------------|--------|--------------|
| Detection of plasmid genes | | | | |
| B/a/b | TAAAGCAAGGGGGTCAACTTC | TCAGGCGGGAGGATCAAATAC | ORF 8 | 142 |
| oye1/oye2 | TAAAGATGGCTCCTTAGCATA | ATGAATGGGCTTCCTTAGCATA | ORF 11 | 602 |
| oye1/oye2b | TAAAGATGGCTCCTTAGCATA | ATGAATGGGCTTCCTTAGCATA | ORF 11 | 106 |
| repA1/repA2 | ATCACCTAGTAGACGAAGAG | GGTAGGGAGGTTCTAATC | ORF 15 | 911 |
| orf20a/orf10 | ATCACCTAGTAGACGAAGAG | GGTAGGGAGGTTCTAATC | ORF 15 | 6464 |
| orf20a/orf20b | ATCACCTAGTAGACGAAGAG | GGTAGGGAGGTTCTAATC | ORF 15 | 821 |
| Detection of chromosomal genes | | | | |
| OO1/OO2 | GTGCGCCAGTTTTATGATATTA | AGGCGATTTTATCTTTATAGCT | mleA | 431 |
| 0812a/0812b | GTAGTATTTACCATTGCGCTT | AGCCGGAATAATGTAATAG | mleA | 540 |
| rpoB1/rpoB2 | ATGGAAAGTATCTGGAATGCTC | AACAGGATCATAGTACATCAC | rpoB | 148 |

*Primer sequences used in qPCR assays.

aProduct sizes obtained for pOENI-1 and pOENI-1v2.

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Population dynamics during growth in wine and MLF kinetics

O. oeni strains carrying pOENI-1 or pOENI-1v2 and isogenic plasmid-less derivatives were used in two types of experiments: direct inoculation in wine for monitoring MLF kinetics and inoculation in grape juice for monitoring cell growth in wine and MLF kinetics. For the first experiment, cells were produced as freeze-dried industrial preparations (SARCO) and inoculated to 107 cell ml−1 according to manufacturer’s instructions (Laffort, France). AF was monitored daily by weight-loss determinations. MLF and bacterial populations were determined three times per week as described above. Plasmids stability was investigated by PCR-analysis (see the paragraph “plasmid curing”) of 30 to 50 colonies picked up on plates produced at the inoculation time, at the end of AF and the end of MLF.

Sequencing accession numbers

The nucleotide sequences of pOENI-1 and pOENI-1v2 were submitted to GenBank and are available under the accession numbers JX416328 and JX416329, respectively.

colony in 200-μl sterile H2O and heating for 10 min at 80°C prior cooling on ice. Multiplex PCR were performed in order to detect simultaneously a chromosomal gene (PCR positive control, mleA) and a plasmid gene (ORF 20). PCRs were carried out in 20-μl reaction mixtures containing 1-μl of cell suspension, 0.25 μl of each primer and the Taq&Go® PCR mix (MP Biomedicals). Clones without plasmid were controlled by NotI-PFGE typing as described above and compared with parental strains.

Table 2. Primers list.
The biological role of OYEs is still poorly understood, but they can contribute to the oxidative or general stress response [50,51,52]. The rest of pOENI-1 includes a resolvase (ORF 9), a transposase (ORF 12) and five hypothetical proteins of 69 to 128 amino acids in length.

Investigations in public databases revealed that several ORFs of pOENI-1 involved in plasmid maintenance or replication are conserved in other LAB plasmids. The most similar are plasmids pH10 of Lactobacillus helveticus H10 (ORFs 1 to 6, ORF 9, ORF 15 and ORFs 18 to 20) and p1 of Lactobacillus casei ATCC 1163 by an 10 genes insert comprising four genes conserved in pOENI-1 (red arrows) and six genes unrelated to pOENI-1 (pink arrows). The insert is bordered by an 8-bp repeated sequence (dark triangles). D. Genetic organization of pOENI-1v2. ORFs numbered from 1 to 20 share more than 99% nucleotide sequence similarity with corresponding ORFs in pOENI-1. ORFs shaded in purple are not detected in pOENI-1 and code for transposases (a, e, f), hypothetical proteins (b, c) and a recombinase (d). Pseudogenes are symbolized by arrowheads containing the symbol y. Regions of sequence similarity are indicated in percentages and shaded in blue. ori: putative origin of replication.

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Figure 1. Genetic organization of pOENI-1 and comparison with related sequences. A. Genetic organization of plasmid pOENI-1. ORFs are represented by numbered arrows and identified by corresponding protein tags (see also Table 3). B. Sequence comparison of pOENI-1 and related plasmids p1 (CP000424) and pH10 (CP002430). ORFs “c, d” (purple arrows) share 99% similarity with ORFs of pOENI-1v2. C. Portions of chromosomes in O. oeni ATCC BAA 1163 and O. oeni PSU1. The gene OEOE_0812 in O. oeni PSU1 (green arrow) is disrupted in O. oeni ATCC BAA 1163 by an 10 genes insert. D. Genetic organization of pOENI-1v2. ORFs numbered from 1 to 20 share more than 99% nucleotide sequence similarity with corresponding ORFs in pOENI-1. ORFs shaded in purple are not detected in pOENI-1 and code for transposases (a, e, f), hypothetical proteins (b, c) and a recombinase (d). Pseudogenes are symbolized by arrowheads containing the symbol y. Regions of sequence similarity are indicated in percentages and shaded in blue. ori: putative origin of replication.

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Distribution of pOENI-1, pOENI-1v2 and plasmid genes amongst O. oeni strains

Forty-four O. oeni strains from diverse origins were analyzed to examine the frequency of pOENI-1 in the species: 11 industrial starters from seven companies, 15 laboratory strains collected between 1983 and 2009, and 18 strains isolated from red and white wines during this study. The phylogenetic relationships of the strains were determined by REA-PFGE and MLST analyses. The dendrogram depicted in Figure 2 shows that all strains belong to two major phylogenetic lineages as suggested in previous studies [36,54]. The presence of pOENI-1 was investigated by a PCR-based strategy targeting the plasmid ORFs 15 (repA), 8 (tauE) and 11 (oye). The three ORFs were detected only in four strains, including three industrial starters (C9, C10, C6) and a new isolate (S11) (Figure 2). A second series of three PCRs targeting large regions of pOENI-1 confirmed that these strains contain complete plasmids (see primers list in Table 2). However, the region extending from ORFs 11 to 13 was 2376-bp long in pOENI-1 of strain C9, whereas it extended over 5942-bp in the other strains (Figure 2). The sequence of this larger fragment was determined by analyzing the genome of O. oeni S11 by the 454 technology. Genome sequence analysis revealed that O. oeni S11 holds a 21,926-bp plasmid that was named pOENI-1v2. It carries the same 20 ORFs as pOENI-1 and six additional ORFs located between ORFs 12 and 13, which accounts for the larger PCR products described above. These ORFs encode a recombinase,
transposases and hypothetical proteins (Figure 1D). All other parts of pOENI-1 and pOENI-1v2 are very similar (>99% sequence identity), except that two mutations disrupting ORFs 4 and 20 in pOENI-1 are not detected in pOENI-1v2 in which these ORFs encode full-length proteins. It is possible that strains C10 and C6 are not detected in pOENI-1v2 in which these ORFs were also transmitted via horizontal transfer events.

PCR screening did not disclose any other strain containing a full plasmid sequence since repA was present only in the four strains above-mentioned. However, tauE and oye were detected together or separately in 7 and 10 additional strains, respectively (Figure 2). Of the 11 industrial starters analyzed in this work, six contained tauE (C9, C10, C6, C4, C3 and C2), while the gene oye was found only in the three starters carrying a plasmid. The tauE and oye genes are randomly distributed among strains, suggesting that they were mostly acquired through horizontal gene transfer events.

The number of plasmids per cell was determined by qPCR (Fig. 1C). Plasmids were also transmitted via horizontal transfer events. In contrast, strains C9 and S11 are distantly related, suggesting that they were acquired via different routes.

**Table 3. ORFs and predicted proteins of pOENI-1.**

| ORF | Position | %GC | Protein | Size (aa) | Predicted function | Best blast (organism, GenBank accession) | % identity |
|-----|----------|-----|---------|----------|-------------------|------------------------------------------|------------|
| 1   | 108-1619 | 44.0| Tral    | 503      | DNA topoisomerase IA, Tral | L. pentosus, CC884017                      | 95         |
| 2   | 1742-1957| 38.9| HP      | 71       | Hypothetical protein    | L. brevis, ZP_03940833                     | 90         |
| 3   | 1961-3082| 42.7| LtrC    | 373      | LtrC-like protein       | L. helveticus, ADX71206                     | 96         |
| 4*  | 3096-4053| 36.9| Gdh     | 319      | Glycerate dehydrogenase  | Lc. kimchii, YP_003621246                  | 70         |
| 5   | 4558-4172| 42.1| HP      | 128      | Hypothetical protein    | P. clauseni, AEV96201                      | 94         |
| 6   | 4871-4551| 40.5| HP      | 106      | Hypothetical protein    | L. brevis, ZP_03940921                     | 99         |
| 7   | 5865-5281| 52.7| TetR    | 194      | Transcriptional regulator, TetR | Lmali, ZP_09449471                      | 99         |
| 8   | 6041-6757| 53.3| TauE    | 248      | Putative permease TauE   | L. mali, ZP_09449472                      | 99         |
| 9   | 7365-7952| 41.8| Res     | 195      | Resolvase               | L. crispatus, YP_003601011                 | 99         |
| 10  | 9124-8192| 35.3| LysR    | 310      | Transcriptional regulator, LysR | O. oeni, ZP_01543901                     | 100        |
| 11  | 9267-10448|42.1| Oye     | 393      | NADH: flavin oxidoreductase | O. oeni, ZP_01543900                     | 100        |
| 12  | 11258-10575|45.5| Tnp     | 227      | Transposase             | O. oeni, ZP_01543898                      | 100        |
| 13  | 11927-11649|41.6| ParB    | 92       | Partition protein, ParB | L. casei, YP_794449                       | 100        |
| 14  | 12831-11908|40.8| ParA    | 307      | Partition protein, ParA | L. casei, YP_794448                      | 99         |
| 15  | 13334-14449|39.5| RepA    | 371      | Replication protein, RepA | P. clauseni, AEV96162                    | 76         |
| 16  | 14709-14990|42.9| RelB    | 93       | Addiction module antitoxin, RelB | L. hilgardii, ZP_03954201                 | 100        |
| 17  | 14980-15486|35.9| PemK    | 168      | Addiction module toxin, PemK | L. paracasei, ABA12818                 | 99         |
| 18  | 15754-15476|34.8| HP      | 92       | Hypothetical protein    | L. brevis, YP_796419                      | 97         |
| 19  | 15985-15776|30.0| HP      | 69       | Hypothetical protein    | L. mali, ZP_0944951                      | 90         |
| 20* | 16256-18317|38.7| TraA    | 687      | Putative nickase, TraA  | L. pentosus, CCC15328                     | 95         |

*pseudogenes, the characteristics of hypothetical full-length genes and proteins are provided.

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Comparison of plasmid-containing and plasmid-free cells during wine fermentations

Detection of plasmids pOENI-1 and pOENI-1v2 in three industrial starters prompted us to examine whether they contribute to the technological properties of their hosts. In order to compare strains sharing the same genetic background, we have generated plasmid-less derivatives of strains C9 and C10 by growing cells in liquid GJ medium during approximately 20 generations prior to plate samples and to test colonies using a plasmid-specific PCR test. Analysis of 68 and 30 colonies of O. oeni C9 and C10 allowed for the identification of two and one plasmid-less mutants, respectively. Controls performed by REA-PFGE and PCR assays confirmed that the mutants share the same genetic background as parental strains and have lost the plasmids (Figure 3).

To determine if plasmids confer an advantage during MLF, strains with (C9+, C10+) and without (C9−, C10−) plasmids were produced under industrial conditions, freeze-dried and tested in micro-vinification assays. They were inoculated to 10^7 ml^-1 in a red wine and consumption of L-malate and bacterial populations were monitored until completion of MLF. Bacterial populations evolved similarly whichever the strain. They declined rapidly after inoculation in wine, started to grow after 5 to 10 days and showed similar growth curves during all the rest of the experiments. Bacteria started to consume significantly L-malate after a lag phase of about 20 days and completed MLF in 37 to 43 days following inoculation in wine, started to grow after 5 to 10 days and showed similar growth curves during all the rest of the experiments.
A second series of tests was performed to determine if plasmids confer a growth advantage during the phases that precede MLF. Bacteria were inoculated in a sterile grape must to 10⁷ cells ml⁻¹ at the same time as yeasts to perform alcoholic fermentation (Figure 5). The growth of strains with or without plasmids was similar during AF and the ensuing MLF. Additional trials consisting in mixtures of C9+/C9− or C10+/C10− cells inoculated as above showed the same growth curves. The kinetics of MLF were also very similar in all cases, except that strain C9+ achieved MLF two days prior to C9−. To determine if the plasmids were stable during cell growth in wine, samples were collected at the inoculation time, at the end of AF and at the end of MLF and they were plated to isolate colonies that were tested in PCR assays specific for the plasmids (Table 5). At inoculation, C9+ and C10+ contained only 90% of plasmid-containing cells, which denotes the instability of the plasmids during precultures in laboratory. During AF and MLF (approx. 20 generations), plasmids were stable since they were detected in 90 to 100% of the cells. In samples inoculated with equal amounts of plasmid-carrying and plasmid-free cells, the C9+/C9− ratio increased from 48/52 at inoculation time to 57/43 at the end of MLF. However an opposite tendency was noticed for the mixture C10+/C10−.

Figure 2. Distribution of pOENI-1 genes in 44 O. oeni strains. The dendrogram was constructed from DNA banding patterns obtained by NotI-PFGE analysis of 44 O. oeni strains. Oenococcus kitaharae was used as outgroup. Strain S11 was positioned on the basis of MLST data since no NotI-PFGE pattern was obtained for this strain. The presence (filled square) or absence (empty squares) of plasmid genes repA, tauE, oye and of the chromosomal gene OEOE_0812 were determined by PCR. The presence/absence of a region encompassing the oye and parB genes was also investigated. IOEB: Institute of oenology of Bordeaux, S: SARCO, ATCC: American type culture collection. Industrial strains are marked with asterisks. Letters A and B in the dendrogram represent two phylogenetic groups of strains [36].

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C10—(Table 5). We concluded that plasmids did not confer a clear advantage to their host cells in laboratory trials, although they were stably maintained during growth in wine.

Detection of plasmids and plasmid genes in wines
To determine if the plasmids have a technological significance during real winemaking, we have investigated their presence in bacteria of 95 samples collected in 86 wineries at different phases of wine fermentations (must, AF, MLF). Microbial DNAs were purified from each sample and used as template in quantitative PCR assays to determine the \( \text{tauE} \) and \( \text{oye} \) copy numbers. The chromosomal gene \( \text{rpoB} \) was also quantified in order to assess the total \( \text{O. oeni} \) population. As anticipated, the \( \text{O. oeni} \) population ranged from 10 to \( 10^5 \) cells ml\(^{-1} \) in samples collected in must and AF, while it reached up to \( 10^9 \) cells ml\(^{-1} \) during MLF (Figure 6). The genes \( \text{tauE} \) and \( \text{oye} \) were detected in all samples. They were present at high copy numbers in the vast majority of samples collected during MLF: above \( 10^6 \) copies ml\(^{-1} \) in 55.8% (\( \text{tauE} \)) and 78.9% (\( \text{oye} \)) of samples. The average ratios of \( \text{tauE/rpoB} \) and \( \text{oye/rpoB} \) were calculated in samples collected before and during MLF (Figure 6C). The ratios were below 0.6 in must/AF samples and above 0.9 during MLF. This suggests that bacteria carrying \( \text{tauE} \) and \( \text{oye} \) were underrepresented before MLF but proliferated during AF and became predominant in MLF.

Thirty samples of wines were further tested by PCR to determine if the genes \( \text{tauE} \) and \( \text{oye} \) were located on plasmids resembling pOENI-1 or pOENI-1v2. PCR assays were performed using primers bordering a plasmid region that extends from ORF 11 to ORF 13 and extends over 2376-bp in pOENI-1 and 5942-bp in pOENI-1v2. PCR products were obtained for 13 samples (Figure 7). Three samples contained a mixture of products of two different sizes, which denote that cells carrying different plasmids were present together in some samples. Only two and one samples had PCR products of molecular sizes expected for pOENI-1 and pOENI-1-v2, respectively. The other PCR products had intermediate sizes of around 4.0 kb, suggesting that additional forms of

### Table 4. Plasmid/\( \text{oye} \) copy number.

| Strain | Plasmid type | \( \text{oye} \) (copies.ml\(^{-1} \)) | \( \text{rpoB} \) (copies.ml\(^{-1} \)) | ratio \( \text{oye/rpoB} \) |
|--------|--------------|-----------------|-----------------|-----------------|
| C9     | pOENI-1      | \( 7.7 \times 10^3 \pm 2.8 \times 10^2 \) | \( 2.1 \times 10^3 \pm 0.7 \times 10^3 \) | 3.7 \pm 0.6 |
| C10    | pOENI-1v2    | \( 6.6 \times 10^4 \pm 4.9 \times 10^3 \) | \( 1.4 \times 10^4 \pm 1.0 \times 10^4 \) | 4.7 \pm 0.2 |
| C6     | pOENI-1v2    | \( 2.3 \times 10^4 \pm 0.5 \times 10^3 \) | \( 6.7 \times 10^3 \pm 2.4 \times 10^3 \) | 3.7 \pm 1.2 |
| S11    | pOENI-1v2    | \( 6.8 \times 10^5 \pm 1.8 \times 10^4 \) | \( 2.1 \times 10^5 \pm 0.4 \times 10^5 \) | 3.3 \pm 0.4 |
| Type S14* | no plasmid | - | - | 1.1 \pm 0.2 |

*aaverage plasmid copy number in stains carrying a pOENI-1 like plasmid: S14, S19, S20, S24, S25, S27, S29, IOEB S433a, IOEB S455.

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Plasmids of the “pOENI-1 family”

pOENI-1 and pOENI-1v2 are the first large plasmids described in O. oeni. The presence of large plasmids in this species was known from previous works [31–35], but no sequence was available. However, during the preparation of this manuscript, Borneman and coworkers have reported the sequences of 11 O. oeni genomes and found plasmids in four of them (discussed below) [55]. The plasmids pOENI-1 and pOENI-1v2 carry a majority of ORFs involved in maintenance and replication but also a few ones encoding proteins that can benefit to their host cells, such as TauE and Oye. They share a limited sequence similarity with plasmids found in other LAB. The most similar is plasmid p1 of L. casei [56] that shares similarities over a 6-kb region comprising the origin of replication and proteins ParA/ParB (partitioning), RepA (replication), RelB/PemK (toxin/antitoxin system of maintenance) and TraA (transfer). pOENI-1 and pOENI-1v2 most likely use a theta-mode of replication since they encode a RepA protein that is conserved in theta-type plasmids described in other LAB [44]. Their origin of replication also is typical of such plasmids [45,46]. It is noteworthy that they were detected at a low copy number (3 to 5 copies per cell), which is consistent with plasmids using this mode of replication. There is no doubt that pOENI-1 and pOENI-1v2 derive from each other since they share extensive sequence identity (>99% nucleotide sequence identity over the whole pOENI-1 sequence). Their main difference is a 3.5-kb insert that is present between ORFs 12 and 13 in pOENI-1v2 and absent in pOENI-1. This insert encodes recombinase, transposases and hypothetical proteins without apparent functional role. The plasmids also differ at several nucleotide positions. pOENI-1v2 contains full-length ORFs 4 and 20 coding for a glyceraldehyde 3-phosphate dehydrogenase and a DNA nickase, respectively, whereas these ORFs are interrupted by early stop codons in pOENI-1. For example, pOENI-1v2 encodes a nickase (ORF 20, disrupted in pOENI-1) that is typical of mobilizable LAB plasmids [44].

Discussion

Plasmids in O. oeni strains analyzed in this work support well the hypothesis that they were horizontally exchanged. Of the four strains that carry a copy of pOENI-1 (C9) or pOENI-1v2 (C10, C6, S11), only two are closely related in dendrograms and phylogenetic trees constructed from PFGE or MLST analyses (C10, C6). The third strain carrying pOENI-1v2 (S11) and strain C9 with pOENI-1 are positioned on distant branches. This distribution most likely results from a dissemination of plasmids via horizontal transfer events.

Potential role of plasmids

Previous studies have demonstrated the importance of plasmids in conferring valuable properties to industrial LAB strains [24,43]. Plasmids of the pOENI-1 family encode the proteins TauE and OYE, which could be useful for wine bacteria. TauE belongs to a family of membrane transporters involved in the import/export of
sulfites or sulfur-containing compounds. Enzymes of this family were characterized as exporters in *Cupriavidus necator* and *Neptuniibacter caesariensis*, in which they contribute to the metabolism of taurine (2-aminoethanesulfonate) [48,57]. Sulfites can be added at different phases of winemaking for their antioxidant and antimicrobial properties. They are also naturally produced by yeasts during alcoholic fermentation. High concentrations of sulfites in wine may prevent the development of bacteria and avoid MLF to occur [58]. The protein OYE could be also advantageous for wine bacteria. It has the functional domains conserved in NADH:flavin oxidoreductases of the large “old yellow enzymes” family. These enzymes are involved in diverse biological functions including stress response in a number of living cells [52,59–64]. They were not characterized in LAB so far, but identified in *Bacillus subtilis* in which they are expressed in response to oxidative stress and acidification of the cytosol [50,65]. However, our comparison of strains carrying or not the plasmids has not revealed clear phenotypic differences during MLF or during growth in wine. Strain C9 carrying pOENI-1 has repeatedly completed MLF a few days before its plasmid-less derivative, but this was not confirmed by comparing strains C10 carrying or not pOENI-1v2. Therefore it is yet unclear whether the plasmids confer a significant advantage during growth in wine and what could be this advantage. Further analyses of plasmid genes expression should help to solve this issue. It is noteworthy that besides TauE and OYE, the plasmids encode several hypothetical proteins which could be important for bacteria.

### Predominance of plasmids in starter strains and indigenous strains performing MLF

Despite the absence of clear evidence for a role of the plasmids, their distribution among *O. oeni* strains and bacteria present in wine suggests that they could contribute to the fitness of bacteria performing MLF. Of the 44 strains of our study collection, which included 11 industrial starters and 33 non-starter strains, the plasmids were present in only four strains: three starters (C9, C10, C6) and a new isolate (S11) that performs well MLF (data not shown). This represents a frequency of 27% in starters (3/11) and 3% in other strains (1/33). In their recent study, Borneman and coworkers have also detected plasmids in the genomes of four of

**Figure 6. Frequency of tauE and oye genes during wine fermentations.** A. B. The oye and tauE gene were quantified by qPCR analysis of 95 samples of must or wine collected at different stages of winemaking. Data obtained from rpoB quantifications were plotted on the x-axis to appraise the *O. oeni* population and on the y-axis to make easier the comparison between the *O. oeni* population (rpoB, filled squares) and the tauE or oye copy number (empty squares). Data are means of two independent determinations. C. The average ratios of tauE/rpoB or oye/rpoB were calculated from samples collected in must or AF (10–10⁶ cells.ml⁻¹) and during MLF (10⁵–10⁹ cells.ml⁻¹). The boxes and lines represent the means (small squares), standard errors (large squares) and standard deviations (lines). doi:10.1371/journal.pone.0049082.g006

**Figure 7. PCR detection of pOENI-1 and related plasmids in wines.** PCR assays were performed using DNA templates from *O. oeni* C9 (pOENI-1), *O. oeni* S11 (pOENI-1v2) and 30 samples of wine collected during MLF (A–E). The number of samples sharing the same PCR product is indicated in parentheses. The primers allowed detection of pOENI-1 repA (panel A), tauE (panel B) and a region extending from ORF11 (oye) to ORF 13 (parB) (panel C). M: DNA size markers. doi:10.1371/journal.pone.0049082.g007
the 11 strains that they have analyzed: two starters (AWRIB419, AWRIB422) and two strains (AWRIB565, AWRIB576) sharing close genetic relationships with the starter AWRIB429 [53]. By examining these new genomes, we have found that they contain pOENI-1 (AWRIB419), pOENI-Iv2 (AWRIB565, AWRIB576) and a divergent pOENI-like plasmid containing a different RepA protein and apparently lacking the partitioning proteins (AWRIB422). In addition, although this was not mentioned by the authors, the starter AWRIB429 also contains a plasmid. This starter was investigated in our study under the name “C10” and our results prove that it contains the plasmid pOENI-Iv2 (Figures 2 and 3). This plasmid sequence is split into four contigs of the published sequence of AWRIB429 (ACSE00000000). These new findings confirm the high frequency of plasmids of the pOENI-1 family in starter strains. They are detected to date in eight strains, among which there are five starters (C6, C9, C10 = AWRIB429, AWRIB419, AWRIB422), two strains closely related to one of these starters (AWRIB565, AWRIB576) and one strain performing well MLF (S11). It is noteworthy that we have also detected a large fragment of the plasmid which includes the ORF encoding TauE in the genome sequence of another starter (AWRIB548, named C4 in our study). In this strain, a fragment of the plasmid has possibly been integrated in the chromosome (sequence acc. number ALAH00000000). The same situation was detected in the genome sequence of starter C3 (unpublished data) that is genetically closely related to C4 (Figure 2).

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Our results showed also that the plasmids or the plasmid-encoded genes are frequent in indigenous bacteria performing MLF. The genes taurE and oxy were detected in all of the 95 samples of wine analyzed in this work. They were particularly abundant in samples collected during MLF, reaching the same level as the total cell population. It is very unlikely that the plasmid-encoded genes confer the capacity to survive in wine and to perform MLF. Wine is a complex and harsh environment for most microorganisms [1]. The ability of bacteria to survive in wine and to conduct efficiently MLF involves many genes, many of which have already been described [11–16]. However, the predominance of pOENI-1-like plasmids and plasmid-encoded genes in starter strains and indigenous bacteria performing spontaneous MLF indicates that they contribute positively to the fitness of these bacteria during winemaking.

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Author Contributions

Conceived and designed the experiments: MF EB PL Performed the experiments: MF EB AL VM Contributed reagents/materials/analysis tools: AL VM PL. Wrote the paper: MF PL.
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