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Genome organization in *Oenococcus oeni* strains studied by comparison of physical and genetic maps

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Summary. The genomic organization of nine strains of *Oenococcus oeni* belonging to two previously suggested divergent groups was examined by a top-down approach, including analysis of isolated genes and construction of physical and genetic maps. Genomic sequence data from *Oenococcus oeni* strain PSU-1 were also examined by a bottom-up approach, using sequence data accessible from the U.S. Joint Genome Institute (Walnut Creek, CA, USA), which enabled the confirmation of gene location and the assessment of transcription direction. A comparison of the genomic maps revealed that *O. oeni* is a homogeneous species and supported the existence of two different genomic groups, although in a phase of divergence much too early for the recognition of subspecies. The genomic organization of *O. oeni* is characterized by an unusual conserved distribution of the two *rrn* operons, located at least 500 kb apart from the putative chromosome replication origin. Differential degrees of conservation are observed in *O. oeni* chromosomes, the neighboring region of the replication terminus being the most conserved one. Since most of the structural polymorphisms can be correlated to the presence of transposase genes and sites of prophage integration, the occurrence of macrodiversity events, such as insertions-deletions, duplications, or inversions of larger genomic regions, can most likely be ruled out in *O. oeni* evolution. [**Int Microbiol** 2008; 11(4):237-244]

Key words: *Oenococcus oeni* · physical mapping · genomic organization · phylogeny

Introduction

The genus *Oenococcus* was created in 1995 and consisted of only one species, *Oenococcus oeni* [8], which thrives in wine and related habitats. The bacterium had been previously classified in 1967 as *Leuconostoc oenos*, in the family "Leuconostocaceae" (name without standing in the nomenclature) [1]. Recently, a new non-acidophilic, non-malolactic-fermenting species, *O. kitaharae*, was described [9]. In fact, in the winemaking process, alcoholic fermentation, carried out by yeast, is followed by malolactic fermentation (MLF), which is promoted by lactic acid bacteria, mainly *O. oeni*. MLF is generally considered a beneficial occurrence since it deacidifies the wine and results in the production of compounds that contribute to the final aroma and taste. Additionally, the development of *O. oeni* in wine prevents the multiplication of undesirable microorganisms, thus leading to microbiological stability [15,20,27].

Many microorganisms of potential interest in the food fermentation industry have been widely studied over the last several decades [10]. Although *O. oeni* is a major microorganism in the wine industry, the laborious growth conditions together with the unavailability of gene transfer processes have been a major drawback in genomic and genetic studies of this species. Nevertheless, metabolic and chemotaxonomic criteria point to high intraspecific variability [7,11], while evolutionary analysis, based on 16S rRNA [25] or concatenated ribosomal proteins [22], and the increased levels of mutational input [24] suggest that this species should be tachytelic (fast-evolving) [2]. Several molecular studies have

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revealed the genetic diversity [18,35,37] and high recombinational activity [6] in *O. oeni*. So far, two strains of this species have been sequenced: PSU-1 (GenBank accession no. CP000411, [26]), and BAA-1163 (GenBank accession no. AAUV00000000, sequences deposited by Guzzo and colleagues).

A large amount of genomic sequence information has been made available through genome sequencing projects. Nevertheless, physical methods for the construction of bacterial chromosome maps, by a "top-down" approach (producing a macrorestriction map using pulsed-field gel electrophoresis [PFGE]), remain a powerful tool in the study of genome structure and plasticity, in the framework of phylogenetics and epidemiology [4,19], and in comparative evolutionary studies [14,17,32,33].

Comparative analysis of genome structure at the intraspecific level enables the identification of genetic events (namely, homologous recombination, insertion/deletion, duplication, transposition) and some DNA sequences possibly involved in rearrangements (such as IS elements, prophages, and duplicated regions/genes). Macrodiversity is displayed through the comparative analysis of gene positioning and macrorestriction polymorphisms in the chromosome maps of each strain. A comparison of genomes of strains belonging to divergent groups can also provide insight into the genomic mechanisms driving evolution in a bacterial phylogenetic group.

The physical map of strain PSU-1 has been described previously [38] and the comparison with a divergent strain, GM, described by Zé-Zé et al. [39], suggested that the genome structure of *O. oeni* is highly conserved. In the present study, to further investigate genome plasticity in *O. oeni*, the genomic groups suggested by macrorestriction [35] were analyzed by restriction endonuclease analysis (REA) and ribotyping, and then used for the selection of strains representative of the diversity within this species. For the seven selected strains (CECT 217^T, bOg18, bOg30, bOg34, bOg36, bOg38 e bOg44), physical maps were constructed and then compared with those of PSU-1 and GM, to determine the characteristic chromosomal organization in this bacterial species. Previous to the release of the completed PSU-1 genome, we analyzed available draft sequences of this genome available from the Joint Genome Institute (Walnut Creek, CA, USA) in order to determine the transcription direction of the mapped genes and to evaluate the reliability of the constructed genomic maps. Conservation in the region of replication origin was further analyzed by DNA sequencing and PCR.

Materials and methods

Bacterial strains and growth conditions. To select the *O. oeni* strains for physical map construction, data from a taxonomic study based on macrorestriction profiles and including 30 *O. oeni* strains of different origins [35] were integrated with those from RFLP analysis, using both restriction endonuclease analysis (REA) and ribotyping, as described below. The data comprised those obtained from eight strains derived from several culture collections (namely, the type strain CECT 217^T) and 22 strains isolated from Portuguese red wines: 21 from Dão, northern Portugal, and one from Vila Nova de Ourém, central Portugal. (The strains are listed in Table 1-SI, supplementary information online.) Based on visual inspection of the REA profiles, improved by the strain ordering in gels according to the clusters obtained by macrorestriction analysis [35], strains were grouped as described in Table 1-SI (online). The bacterial isolation procedures from wine and the growth conditions used for all *O. oeni* strains were described previously [34].

Restriction fragment length polymorphism (RFLP) analysis. Total DNA was extracted from 50–100 ml bacterial cultures with 10^8–10^9 colony-forming units (CFU)/ml (about 3 days of growth) as described earlier [28]. Genomic DNA (30 mg) of each strain was completely digested with 90 U of *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I, in a final volume of 100 µl at 37°C for ~14 h, following the manufacturer's instructions. Six to 10 µg of digested DNA was electrophoresed in 0.8% agarose gels and 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) at 35–40 V for ~24 h together with a 1-kb DNA ladder (Gibco BRL, Paisley, UK) as size marker. REA profiles obtained with *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I digestion were directly analyzed and used for ribotyping analysis by Southern blotting, with a 1.3-kb cloned fragment of 16S rDNA of *O. oeni* type strain (CECT 217^T) serving as probe. Hybridizations were carried out at 68°C following the procedures described below. The mean size of each fragment (kb) detected after hybridization was estimated from several gels by linear interpolation with two flanking markers [13]. The ribotyping clusters were determined by global analysis, including data from the four restriction enzymes, with the Dice similarity (S_D) coefficient [29] and the unweighted pair group method with arithmetic averages (UPGMA), using NTSYS software [Rohlf J (1987) Numerical taxonomy and multivariate analysis system for the IBM PC microcomputer. Applied Biostatistics Inc., Setauket, NY, USA].

PFGE macrorestriction analysis and fragment nomenclature. Intact genomic DNA was prepared in agarose plugs and then single and double digested with the restriction endonucleases *Asc*I, *Fse*I, and *Not*I and the intron-encoded endonuclease I-*Ceu*I, as described previously [35,38,39]. All enzymes were purchased from New England Biolabs (Beverly, MA, USA). Restriction fragments are indicated by the initial letter of the endonuclease. All fragments are numbered in size order, from the largest to the smallest. Co-migrating fragments were numbered with sequential numbers (Table 2-SI, online). PFGE and 2D-PFGE were performed using the contour-clamped homogeneous electrical field (CHEF) system [Gene Navigator (Pharmacia, Uppsala, Sweden)], as previously described [38]. The mean size of each fragment was estimated from several gels by linear interpolation with two flanking size standards [13] using Kodak 1D 2.0 software (Kodak, Rochester, NY, USA). *Saccharomyces cerevisiae* chromosomes (Bio-Rad, Hercules, CA, USA), lambda DNA, and mid- and low-range PFG ladders (New England Biolabs) were used as size markers.

DNA probes and Southern hybridization. The DNA sequences used as probes are listed in Table 3-SI (online). Manipulation of the plasmids and PCR products, as well as preparation of [α-^{32}P] dCTP-labeled DNA probes and Southern hybridization conditions were as previously described [38,39].

Analysis of chromosomal origin. The partial sequences of the *dnaA* gene of nine *O. oeni* strains were obtained by PCR amplification and sequencing using internal primers dnaA-36F (5′-GAAGGGAATCCAGGG CCGT-3′) and dnaA-666R (5′-AAGCATCTGAATGTCATCGAC-3′). After purification, the amplification products were sequenced using the same PCR primers. DNA sequences were determined in a CEQ 2000-XL automated DNA capillary sequencer (Beckman Coulter, Fullerton, CA, USA) by a dye-labeled dideoxy termination method (DTCS, Dye Terminator Cycle

sequencer start kit, Beckman Coulter). To assess the genetic arrangement in the region of chromosomal origin, PCR amplifications were performed using primers directed to *dnaA*, *dnaN*, and *gyrB* PSU-1 gene sequences (dnaN1001-F, 5′-GTTCAATCGGTGAAAGCAAATTG-3′; dnaN1021-R, 5′-ATTTGCTTTCACCGATTGAACTC-3′; gyrB462-R 5′-GTAATAGATTTTGTTGTCCCG-3′). The numbers assigned to all primers reference their position in the respective *O. oeni* PSU-1 mRNA sequences. PCR reaction mixtures consisted of 100-500 ng of template DNA, 50 pmol of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$ and 1.75 U *Taq* polymerase in the supplied buffer (all from Invitrogen, Paisley, UK); the final volume was 50 μl. PCR amplification consisted of 35 cycles with 1 min denaturation at 94ºC, 1 min annealing at 52°C, and 3 min extension at 72°C, after a previous denaturation step (94°C for 4 min) and followed by a final extension step (72ºC for 5 min). For partial *dnaA* amplification, the extension time at 72°C was 1 min. Partial *dnaA* sequences were aligned using the Clustal W program [36]. Phylogenetic analyses including neighbor-joining (NJ) and maximum-parsimony (MP) as well as trees of DNA sequence alignments were conducted using PAUP* 4.0b10 software [Swofford DL (2003) PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. Sinauer Associates, Sunderland, MA, USA]. Bootstrap analysis consisted of 1000 replicates.

Bottom-up approach. The draft sequences obtained from the PSU-1 genome project and the analyzed data presented by Mills et al. [26] were integrated in the constructed genomic map [38]. This approach enabled the location of several scaffold sequences within the PSU-1 chromosome by sequence blast analysis and a search of restriction sites using the pDRAW32 software. The specific assignment of gene transcription direction was also possible for all markers allocated to specific draft sequences. Data already available regarding the presence of plasmids (L. Brito, PhD thesis, Technical University of Lisbon, 1996; [2]), bacteriophage [30,31] and transposase elements [26,39] were also included.

Results and Discussion

Selection of oenococcal strains. The ability to differentiate the data obtained by REA depends on the enzyme(s) used. Here, *Eco*RI and *Pst*I distinguished 22, *Hin*dIII 23, and *Bam*HI 24 different profiles, thus providing a discrimination power of 73, 77, and 80%, respectively. The distinctive power of the REA profiles is illustrated in Fig. 1A; for instance, the group formed by bOg27, bOg29, bOg35, and bOg39 (macrorestriction group F3) has the same *Bam*HI REA profile (B16), whereas strains bOg38 and bOg44 (macrorestriction group G) have clearly different *Bam*HI profiles (B17 and B18; see bands in the range of 2–3 kb), as do strains ML34 and PSU-1 (macrorestriction group F1; *Bam*HI profiles B13 and B14; see bands in the range of 1.6–2 kb). The profiles obtained for strain pairs CECT 217^T/NC and CECT 218/ML34 were identical with the four enzymes, in accordance with the proposed identity of these strains.

Although a different discriminatory ability was reported by Lamoureux et al. [16] for *Eco*RI (54%) and *Hin*dIII (73%), and the six strains common to our study (CECT 217^T, ML34, PSU-1, GM, CECT 4028 and CECT 4029) were also discriminated by *Hin*dIII, the lower electrophoretic resolution achieved by these authors did not reveal the highest polymorphism, obtained with the *Bam*HI REA profiles, nor did it distinguish GM from CECT 217^T and L4029 from PSU-1 and ML34 using *Eco*RI.

The results of *Pst*I, *Hin*dIII, *Eco*RI, and *Bam*HI ribotyping in all 30 strains under study were consistent with clustering in two to four profiles, depending on the enzyme, and thus indicated a low discriminative power (7% with *Pst*I and *Hin*dIII; 10% with *Eco*RI, and 13% with *Bam*HI).

Although no published data are yet available for comparative analysis of *Pst*I and *Bam*HI ribotyping, *O. oeni* was previously ribotyped with both *Eco*RI and *Hin*dIII [6,37]. Beyond the general agreement concerning the size of the fragments for common ribotypes, lower discrimination (only two ribotypes for *Eco*RI) and even several arguable results were obtained in those studies. In fact, distinct ribotypes were found for ML34 and

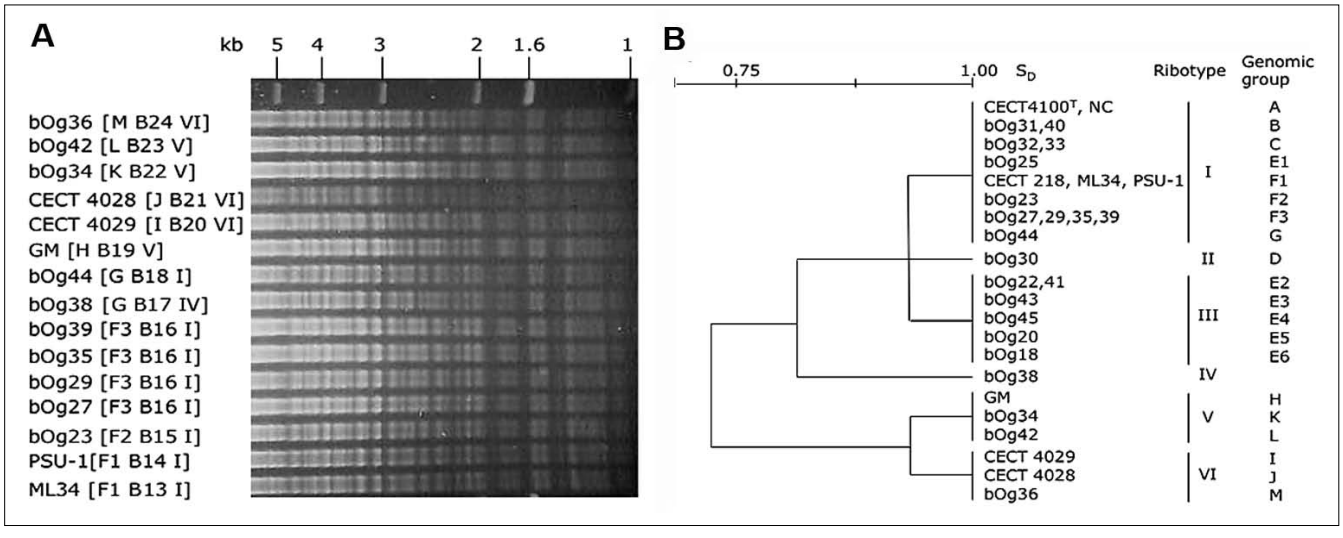

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Fig. 1. RFLP analysis in *Oenococcus oeni* strains. (**A**) Restriction endonuclease analysis profiles observed with *Bam*HI. (**B**) Ribotyping clusters obtained with integrated analysis of *Eco*RI, *Pst*I, *Hin*dIII, and *Bam*HI data, using Dice coefficient (S_D) and UPGMA.

CECT 218 (the same strain) by Zavaleta et al. [37], and the type strain was incorrectly described by De las Rivas et al. [6] as having a ribotype identical to that of CECT 4028 and CECT 4029. From a global analysis of all ribotyping data, we clustered the strains in six ribotypes (I–VI; Fig. 1B), which increased the discriminative ability of this method to 20%.

A previous macrorestriction analysis [35] had sorted the 30 strains under study into 20 genomic groups based on different combinations of *Not*I and *Sfi*I macrorestriction patterns (19 *Not*I and 20 *Sfi*I different profiles were identified). Clustering analysis made it possible to include 16 of the 30 strains (groups D, E and F) in a single cluster (71% significance level), in which the genomic diversity was mainly associated with differences in the second and third largest *Not*I fragments. A global analysis of macrorestriction and ribotyping clustered the strains in two distinctive genomic groups, supporting the previous suggestion that *O. oeni* should be divided in two subspecies [39]. Strain PSU-1 (macrorestriction group F1; ribotype I) belongs to cluster I and strain GM (macrorestriction group H; ribotype V) to the divergent cluster II. Besides including representatives from all major macrorestriction groups and all ribotyping groups, the criteria used for additional strain selection, included the strains: (i) with the largest estimated genome size difference, bOg34 (1.78 Mb) and bOg18 (1.93 Mb); (ii) with unique ribotyping profiles, bOg30 (*Bam*HI ribotyping RB3) and bOg38 (*Eco*RI ribotyping RE2); and (iii) belonging to non-related macrorestriction and ribotyping clusters.

Macrorestriction analysis of selected *Oenococcus oeni* strains. The restriction fragments produced by the endonucleases *Asc*I, I-*Ceu*I, *Fse*I, and *Not*I in all the strains studied here are presented in Table 2-SI (online), except for PSU-1 and GM, for which physical maps were already available. Macrorestriction polymorphisms varied considering the different endonucleases used. In all strains, I-*Ceu*I profiles were highly similar, with only small size differences. The major difference was a 136-kb increase in fragment C1 between strains bOg34 and bOg18 (respectively, the smallest and second largest genome represented in this study). The most variable profiles were obtained using endonucleases *Asc*I (3–5 restriction fragments) and *Not*I (8–14 restriction fragments). The genome size for all strains was determined as the mean size obtained with the four used enzymes and is presented in Table 1-SI (online). For comparative purposes, restriction data for strain PSU-1 and GM [38,39] are also presented.

Physical mapping strategies. The available physical and genetic maps of PSU-1 and GM chromosomes [38,39], established with the enzymes *Asc*I, I-*Ceu*I, *Fse*I, *Not*I and *Sfi*I, enabled the selection of probes scattered on these genomes and the development of experimental strategies to map polymorphic macrorestriction fragments in other strains. The macrorestriction endonuclease *Sfi*I was not used in this study, as it generates more complex profiles with several co-migrating fragments. The first step in physical map construction consisted of partial digestion analysis of the *Asc*I fragments. The addition of Southern hybridization results (Table 3-SI, online) allowed the independent mapping of several *Asc*I, I-*Ceu*I, and *Fse*I fragments. This combined approach enabled the location of all *Asc*I restriction sites and at least 30% of the *Fse*I cleavage sites in the chromosomes of all strains. I-*Ceu*I mapping is direct, as this enzyme generates only two restriction fragments, thus identifying the two *rrn* operons of *O. oeni*. The relative positioning of I-*Ceu*I and *Fse*I maps in the *O. oeni* genome was readily assessed through the location of restriction sites in the *rrn* operons (23S and 16S rDNA, respectively). Additionally, the presence of an *Fse*I site in the *dnaA* gene sequence (994 nucleotides in PSU-1 mRNA sequence) furthered the assessment of the *Fse*I map (Table 3-SI, online). Sequential analysis of double digestions profiles generated by *Asc*I-I-*Ceu*I and *Asc*I-*Not*I or I-*Ceu*I-*Not*I enabled the global mapping of all restriction sites previously located in the chromosome for each isolated enzyme. The relative positioning of the majority of the restriction sites was achieved by size determination of the *Asc*I-*Not*I double digestion fragments in the region of *rrnB* (Fig. 2). Sequential digests (2D-PFGE) with *Asc*I and I-*Ceu*I confirmed the fragment linkage and the location deduced by previous approaches.

Physical mapping of the seven studied strains was completed by comparative analysis with the known maps of strains PSU-1 and GM. Fragments with differences of < 5 kb (average estimated variation) in the observed size between the macrorestriction profiles of one or both of these strains were considered as co-migrating and were putatively mapped. Shaded restriction sites in the maps indicate those for which no other result confirmed this allocation (Fig. 2).

Analysis of chromosomal origin. The partial sequences of the *dnaA* genes from nine *O. oeni* strains (GenBank accession nos. AY768692-AY768700) showed that these are readily separated in two groups. The recurrent formation of the two clusters, indicated by high bootstrap values in maximum parsimony phylogenetic tree, is in agreement with previous results and supports the existence of two natural groups or genomovars in *O. oeni*. PCR in the neighborhood of the chromosomal origin confirmed the overall similarity of this genetic region. Accordingly, fragments amplified using primers dnaA36-F and dnaN1021-R had a size of 2.5 kb (2512 bp), and those obtained using primers dnaN1001-F and gyrB462-R 2.0 kb (2034 bp), confirming the colinearity and spacement of the *dnaA*, *dnaN*, and *gyrB* genes, as expected from sequence analysis of *O. oeni* PSU-1 [5]. The 2.5-kb fragments that contained partial *dnaA* and *dnaN* genes were all restricted by *Fse*I (data not shown), confirming the results of mapping analysis (Fig. 2).

Bottom-up approach. The PSU-1 chromosome comprises 1,780,517 nucleotides, as determined by sequencing [26], and is

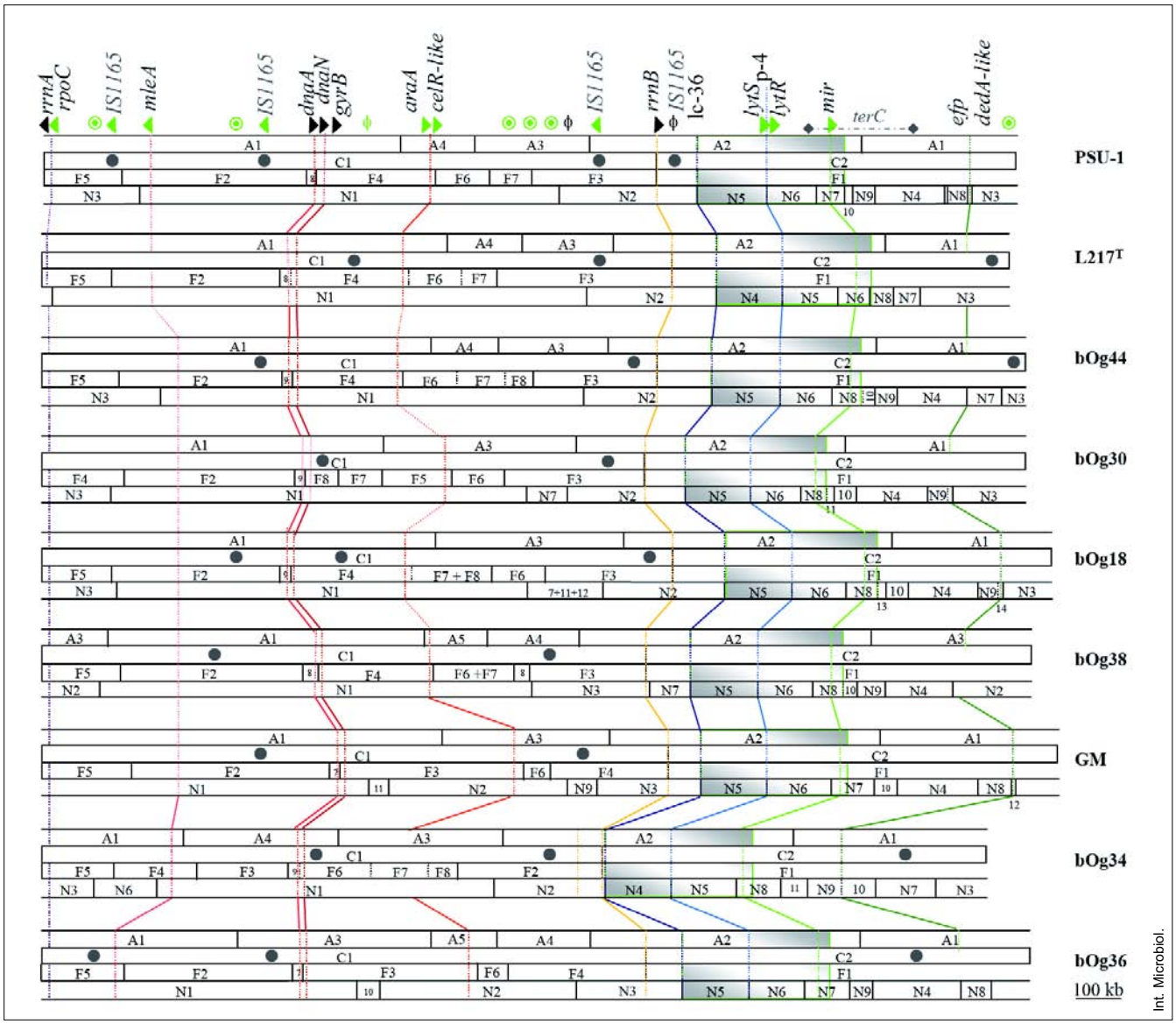

Fig. 2. Physical and genetic maps of nine *Oenococcus oeni* strains. Restriction sites for *Asc*I, I-*Ceu*I, *Fse*I, and *Not*I are indicated. The circular genomes are shown linearized from one of the *rrn* operons (*rrnA*, the closest to replication origin). Genetic markers are presented in the PSU-1 chromosome and their position in the other strains is indicated by transversal lines. Gene transcription direction obtained from PSU-1 genomic sequence analysis is shown in gray arrows; when the data are applicable to all strains, arrows are indicated in black. ● represents the allocation of insertion sequence IS1165, determined by Southern hybridization in each strain; ⦿ represents the location of other transposase genes or gene fragments in the PSU-1 genome [26], and ϕ possible prophage integration sites (in black, *attB1* and *attB2* determined by Zé-Zé et al. [35]; in gray, proposed by São-José et al. [31]). The termination region in the PSU-1 chromosome, as defined by Mills et al. [26], is indicated by a dashed line. The genomic region showing the highest conservation, in all analyzed genomes, is highlighted by a gray box.

thus approximately 76 kb smaller than the size estimated by physical mapping (1857 kb; [38]). In previous work, we estimated the maximal deviation in size to be <4% and that the actual deviation error, considering the sequenced size, was ~4.1%. Other studies of PFGE reproducibility [12] showed that these values are in the normal range, deviating by <2% for fragments in the range of 100–500 kb. Despite this deviation, in a global approach BLAST and restriction analysis confirmed the reliability of the PSU-1 constructed physical and genomic map (Fig. 3), the precise position of the *Sfi*I site being in fragment N9; the position of a *Not*I fragment N11 (sized in an earlier map as 10 kb and by sequencing determined to be 8.458 kb) 44 kb away from the previously presented location (which represents the "other end" of fragment N8); and the relative position of two *Sfi*I fragments, S7–S10 that are linked, but counterclockwise and not clockwise as previously reported [38,39]. These three differ-

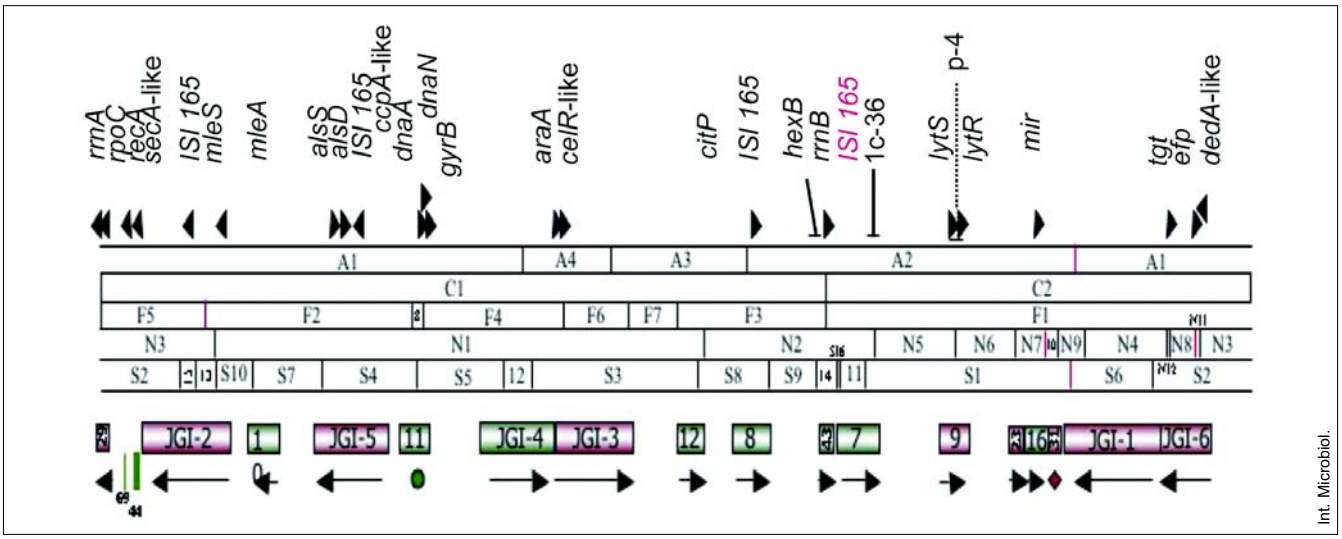

Fig. 3. Physical and genetic maps of *Oenococcus oeni* PSU-1, with allocation of JGI scaffolds. Corrected positions of restriction sites, relative to the previously published map [38,39], are indicated in red. Scaffolds are numbered according to the Joint Genome Institute and the following color code was used: red, sequence and map in the same direction; green, sequence in opposite direction of the map; blue, sequence direction undetermined. The arrows indicate the preferential transcription direction.

ences were the only disagreements we found by analyzing the available data (Fig. 3). The analysis of genomic sequences also enabled the precise allocation of fragment N10 (Fig. 3), the only unresolved restriction site in the PSU-1 physical map, by the following top-down approach [39].

We combined our mapping information with: (i) a thorough analysis of a draft genomic sequence available at JGI; (ii) the reported preliminary genomic analysis of *O. oeni* PSU-1 [26]; (iii) the presence of transposase elements [26,39]; (iv) the presence of temperate bacteriophages ([30] (Table 1); and (v) the identified prophage integration sites in PSU-1 [31]. This approach enhanced comparisons of the constructed *O. oeni* physical and genetic maps and allowed us to assess the transcription direction of several genetic markers (Fig. 3).

In a previous study [38], the locations of two prophage attachment sites (*attB1* and *attB2*; see Fig. 2) in the PSU-1 genome were determined by Southern hybridization with the PSU-1 lysogenic derivatives for phage fOg44 constructed by Santos et al. [30]. As noted by Mills et al. [26], São-José and coworkers [31] identified five prophage integration sites adjacent to tRNA genes in the draft PSU-1 sequence: $tRNA^{Arg\_CCT}$, $tRNA^{Glu\_CTC}$, $tRNA^{Glu\_TTC}$, $tRNA^{Leu\_CAG}$, and $tRNA^{Lys\_TTT}$. The $tRNA^{Glu\_CTC}$ and $tRNA^{Lys\_TTT}$ integration sites represent *attB1* and *attB2* sites, respectively, the first having been ascertained with oenophage fOg44 integration and the second with fOgPSU1. In our study, the putative prophage integration sites $tRNA^{Glu\_TTC}$ and $tRNA^{Leu\_CAG}$ were also assigned in the PSU-1 chromosome (Fig. 2, 4); however, with the available data it was not possible to precisely localize the $tRNA^{Arg\_CCT}$ gene.

Different size DNA plasmids were also detected in some of the selected oenoccocal strains, namely, bOg18 and bOg38, with a plasmid of ~40 kb, and bOg34 and bOg36, with a smaller plasmid of ~4.5 kb [2]. Southern hybridization using the oenococcal plasmids as probes against PFGE profiles ruled out the presence of similar integrated plasmids or remnants of plasmid DNA in the chromosomes of the selected strains.

Featuring chromosomal dynamics in *Oenococcus oeni*. As previously discussed in the comparison of *O. oeni* PSU-1 and GM genomic structure [39], and although some macrorestriction polymorphisms (regarding the number of fragments and their mean sizes) were identified with the endonucleases used, there is a high degree of conservation in oenococcal chromosomes. Also, the location of genetic markers besides the restriction sites is homogeneous, which suggests that the observed differences are due to point mutations and small insertion-excision or duplication events. However, distinctive features, specifically, the presence of a highly conserved region nearby the chromosomal replication terminus (highlighted in a gray box; Fig. 2), surrounded by a variable region, were detected. Upstream of this region (in the *oriC* direction) and limited by the *rrnB* operon, we identified a highly polymorphic region showing insertion/deletion events over most of the ~100 kb (comparing bOg44 or bOg18 with bOg34). This insertion-deletion hotspot could be related to prophage integration events (*attB2* location and a putative integration site related to a $tRNA^{Glu\_TTC}$ sequence) and/or to transposase genes (*IS1165* probe) found in the *O. oeni* PSU-1 genome. Correlating these data with bacteriophage presence and phagotyping studies ([35]; data not shown), we found that strain bOg34 was infected by only one phage and that CECT 217^T, bOg38, GM and bOg36 were resistant to all phage collections. Even if the other poten-

Table 1.* Presence of temperate bacteriophage in *Oenococcus oeni* strains

Strain	Bacteriophages[b]	
	Lysogenic	Hibridization with phage DNA
PSU-1[a]	(fOgPSU-1 [36.4 kb])	Negative
217^T	–	Negative
bOg44	fOg44 (36.5 kb)	Negative
bOg30	fOg30 (40.9 kb)	Positive
bOg18	fOg18 (39.8 kb)	Positive
bOg38	fOg38 (39.8 kb)	Positive
GM	–	Negative
bOg34	–	Negative
bOg36	–	Negative

[a]Although strain PSU-1 is prophage-free, the lysogenic derivatives constructed (PSU-1C; [30]) proved the possible integration of phages in two locations of the chromosome, *attB1* and *attB2* [38].

[b]In a study of mitomycin induction and host capacity of the 30 *O. oeni* strains (Table 1-SI), 19 phages were isolated (R. Santos, PhD thesis, Univ. of Lisbon, 1995). The phagotyping study of these strains [35] and unpublished results showed that strains PSU-1 and bOg44 are sensitive to 19 and 17 phages, respectively; bOg30 and bOg18 to 3 and 4 phages, respectively; bOg34 is only infected by 1 phage and CECT 217^T, bOg38, GM and bOg36 are resistant to all *O. oeni* phage collection.

*Previous tables (T1-SI to T3-SI) are online.

tial prophage integration sites were underestimated, these strains, and mainly *O. oeni* GM, bOg34 and bOg38, presented the highest ranges of "deletion" events in the considered region. As shown in Table 4, the only strains that hybridized with phage DNA were bOg30, bOg18, and bOg38. However, considering bOg44 as lysogenic, we ascertained the presence of bacteriophage DNA in the *attB2* region (or a putative phage integration site in RNA^{Glu_TTC}) can be, by phage similar size increments (about 40 kb), to bOg44 and bOg18 strains. Although the fragment size in bOg30 was identical to the corresponding site in the PSU-1 chromosome, additional hybridization data of phage DNA in bOg18 and bOg30 *Not*I profiles (R. Santos, PhD thesis, 1995), allocated the temperate oenophages to fragments N2 in both strains, confirming deductions based on genomic mapping analysis. In *O. oeni* bOg38, the temperate phage integration site was probably in fragment A2 because it showed an increase of ~38 kb compared to all other strains (Table 2-SI [online]), Fig. 2). The putative integration site adjacent to tRNA^{Leu_CAG} was located in a region with no observed difference in all strains, and thus considered to be the most conservative one (see above). Despite the downstream presence of an ORF with similarity to fOg44 genome (by draft PSU-1 genome analysis), chromosome stability in this specific putative oenococcal phage integration site suggests that a site-specific recombination capacity was lost long ago.

Regarding transposase elements relevant to PSU-1, no *IS1165* Southern hybridization signals were obtained for the same highly polymorphic region in any of the remaining strains. Downstream, the replication terminus region, which is limited by the other *rrn* operon (*rrnA*), also showed considerable size variability, especially when the largest and the smallest genomes (strains GM and bOg34) were compared. The possibility of site-specific recombination could be related to the presence of transposase genes or gene fragments detected in PSU-1, CECT 217^T, bOg44, bOg34, and bOg36 (Fig. 2).

A difference of ~30 kb was observed in the *Not*I fragments, where the genetic marker *mir* hybridizes, between strains of PSU-1 and GM genomovars. The gene denominated *mir* [38] represents a cloned fragment selected by mitomycin C resistance that was used as a probe. According to similarity search by BLAST analysis, this sequence targets a possible DNA-binding protein in the PSU-1 genome. PSU-1 genome draft analysis showed that this marker was located between two possible recombinational spots, ORFs with similarity to integrases (upstream to a *Bacillus anthracis* phage integrase and downstream to a *Listeria monocytogenes* integrase/recombinase gene). As the *mir* upstream region appears to be highly conserved, the downstream putative integrase/recombinase gene is the most probable candidate to represent, in the PSU-1 genome, the remnants of an insertion/deletion event that occurred prior to the divergence of the two defined genomic groups.

Comparison with other studies. Our results of an overall genomic conservation apparently contradict the high genetic (allelic) diversity suggested by multilocus sequence typing (MLST) analysis performed by De las Rivas et al. [6]. However, genetic conversion events might explain such differences; the lack of some important mutator genes, such as *recQ*, *mutS*, and *mutL*, in *O. oeni* [24] should be a major drawback to the occurrence of recombinational events dependent on mismatch repair systems.

The explanation for a high allelic diversity as a result of the absence of *mutS* and *mutL* (and the consistently higher mutation rate) and the acquisition of functional alleles by horizontal transfer, proposed by Marcobal et al. [24], are in agreement with our findings. In fact, the major diversity at the chromosomal level seems to be related to prophage integration/movements and transposition events, and a genomic island of at least 17 kb, including genes coding for transposases and phage proteins, has been described in *O. oeni* [23].

The small genome size, the product of progressive and substantial gene loss [21], together with the adaptations to the restrictive environment where this species has subsequently thrived, could have driven *O. oeni* to an evolutionary dead end. Even though a high mutation capacity is functional whenever positive selection is imposed, most random mutations should be lethal in the natural habitats of *O. oeni*. Therefore, the driving forces for oenoccocal branch divergence from other lactic acid bacteria may paradoxically be a restriction to a diverse intraspecific genomic structure. In conclusion, the occurrence of macro-

diversity events, such as insertions-deletions, duplications, or inversions of larger genomic regions, can thus far be ruled out in *O. oeni* evolution.

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Table 1-SI. Origin, genomic size and distribution by the genomic macrorestriction, REA and ribotyping groups of the *Oenococcus oeni* strains

Strain[a]	Origin/Source[b]	MGG[c]	REA profiles[d]				Ribotyping profiles[d]					Genomic size¶
			*Eco*RI	*Pst*I	*Hin*dIII	*Bam*HI	*Eco*RI	*Pst*I	*Hin*dIII	*Bam*HI	Global	
CECT 217^T	France/CECT	A	1	1	1	1	RE1	RP1	RH1	RB1	I	**1848**
NC (=CECT 217^T)	France/NCIMB											1846
bOg31, bOg40	Portugal/CGBM	B	2	2	2	2	RE1	RP1	RH1	RB1	I	1835
bOg32	Portugal/CGBM	C	3	3	3	3	RE1	RP1	RH1	RB1	I	1866
bOg33			3	3	3	4	RE1	RP1	RH1	RB1	I	1866
bOg30	Portugal/CGBM	D	4	4	4	5	RE1	RP1	RH1	RB3	II	**1879**
bOg25	Portugal/CGBM	E1	5	5	5	6	RE1	RP1	RH1	RB1	I	1915
bOg22	Portugal/CGBM	E2	6	6	6	7	RE1	RP1	RH1	RB4	III	1850
bOg41			7	7	7	8	RE1	RP1	RH1	RB4	III	1850
bOg43	Portugal/CGBM	E3	8	8	8	9	RE1	RP1	RH1	RB4	III	1874
bOg45	Portugal/CGBM	E4	9	9	9	10	RE1	RP1	RH1	RB4	III	1847
bOg20	Portugal/CGBM	E5	10	10	10	11	RE1	RP1	RH1	RB4	III	1817
bOg18	Portugal/CGBM	E6	11	11	11	12	RE1	RP1	RH1	RB4	III	**1923**
ML34	California, USA	F1	12	12	12	13	RE1	RP1	RH1	RB1	I	1858
CECT 218 (=ML34)	California,		12	12	12	13	RE1	RP1	RH1	RB1	I	1858
PSU-1	USA/CECT		12	12	13	14	RE1	RP1	RH1	RB1	I	**1857**#
	Pennsylvania, USA											
bOg23	Portugal/CGBM	F2	13	13	14	15	RE1	RP1	RH1	RB1	I	1802
bOg27, bOg29, bOg35, bOg39	Portugal/CGBM	F3	14	14	15	16	RE1	RP1	RH1	RB1	I	1843
bOg38	Portugal/CGBM	G	15	15	16	17	RE2	RP1	RH1	RB4	IV	**1891**
bOg44			16	16	17	18	RE1	RP1	RH1	RB1	I	**1885**
GM	France/MT	H	17	17	18	19	RE3	RP2	RH2	RB1	V	**1932**¥
CECT 4029	Germany/CECT	I	18	18	19	20	RE3	RP2	RH2	RB2	VI	1894
CECT 4028	France/CECT	J	19	19	20	21	RE3	RP2	RH2	RB2	VI	1898

bOg34	Portugal/CGBM	K	20	20	21	22	RE3	RP2	RH2	RB1	V	**1807**
bOg42	Portugal/CGBM	L	21	21	22	23	RE3	RP2	RH2	RB1	V	1893
bOg36	Portugal/CGBM	M	22	22	23	24	RE3	RP2	RH2	RB2	VI	**1889**

[a]Strains in bold were selected for genomic comparison by physical mapping construction; [T] Type strain.

[b]CECT, Colección Española de Cultivos Tipo, Valencia, Spain; CGBM, Centro de Genética e Biologia Molecular, Lisbon, Portugal; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK; MT, Microlife Techniques, Sarasota, Florida, USA; Ml34 and PSU-1 were provided by R. Kunkee, University of California at Davis, USA.

[c]MGG: macrorestriction genomic group as defined by Tenreiro et al. [35].

[d]For each enzyme, Arabic numbers refer to different REA profiles and alphanumeric codes to different ribotypes. For *Pst*I, it was possible to define two patterns (RP1 and RP2) with three common fragments (10, 8.1, and 7.6 kb) and one distinct fragment, with 4.5 kb in RP1 and 4.7 kb in RP2. For *Hin*dIII ribotyping, clustering results were identical to those obtained with *Pst*I, with the definition of two patterns (RH1 and RH2), with three common fragments (two co-migrating fragments of 1.1 kb and a 4.4 kb fragment, and a different fragment of 10.3 kb in RH1 and 5.6 kb in RH2. *Eco*RI and *Bam*HI ribotyping had a higher discriminative ability, yielding three and four distinct profiles, respectively. The observed *Eco*RI patterns (RE1, RE2 and RE3) shared a 2.8-kb fragment; in addition, we identified a 4.2-kb fragment common to RE1 and RE2, as well as two 1.7-kb co-migrating fragments present both in RE1 and RE3. Patterns RE2 and RE3 presented, as specific fragments, two 1.6-kb co-migrating fragments for RE2 and a 7.1-kb fragment in RE3. In *Bam*HI ribotyping, the four recognized profiles (RB1, RB2, RB3, and RB4) had a common 3.2-kb fragment and were distinguished by a variable second fragment with 15.7 kb in RB1, 15 kb in RB2, 12.8 kb in RB3, and 9.6 kb in RB4. Ribotype I includes strains belonging to RP1, RH1, RE1, and RB1; ribotype II, strains with RP1, RH1, RE1, and RB3 profiles; ribotype III, strains with RP1, RH1, RE1, and RB4 profiles; ribotype IV, strains with RP1, RH1, RE2, and RB4 profiles; ribotype V, strains with RP2, RH2, RE3 and RB1 profiles; and group VI, strains with RP2, RH2, RE3, and RB2 profiles.

[¶]Genomic sizes in bold were estimated by physical mapping and the remaining by the mean size of the sum of *Not*I and *Sfi*I macrorestriction fragments determined by Tenreiro et al. [35], # Zé-Zé et al. [38], and ¥ Zé-Zé et al. [39].

Table 2-SI. Sizes of macrorestriction fragments obtained by *Asc*I, I-*Ceu*I, *Fse*I and *Not*I cleavage of *Oenococcus oeni* strains chromosome

Macrorescrtion fragments (kb)	Fragment size in strain[a]								
	PSU-1	217^T	bOg44	bOg30	bOg18	bog38	GM	bOg34	bOg36
*Asc*I fragments									
A1	990	1029	1022	987	1051	617	1088	631	677
A2	513	516	518	517	521	558	521	559	521
A3	214	178	215	370	352	415	318	318	373
A4	130	130	130			176		296	183
A5						123			130
I-*Ceu*I fragments									
C1	1170	1127	1178	1155	1219	1157	1195	1083	1157
C2	688	717	705	712	704	724	736	722	719
*Fse*I fragments									
F1	680	713	686	709	699	718	746	718	716
F2	352	337	337	341	335	351	376	282	338
F3	240	251	235	263	257	228	325	174	330
F4	235	228	227	165	229	228	233	164	267
F5	163	133	145	135	134	152	174	143	166
F6	99	99	99	99	92	99	61	143	62
F7	75	63	80	85	92	62	16	99	16
F8	18	20	63	61	62	36		62	
F9			18	18	20	22		18	
*Not*I fragments									
N1	795	1050	811	816	787	821	701	638	678
N2	270	251	268	243	237	251	344	201	379
N3	250	162	246	219	222	237	200	195	203
N4	133	128	132	136	133	135	154	128	167
N5	128	102	128	130	128	128	128	128	129
N6	102	58	101	102	100	102	105	126	105
N7	58	53	70	79	79	80	85	120	86
N8	44	45	58	59	58	59	63	89	58
N9	44		44	45	45	53	62	68	46
N10	15		23	45	45	30	47	66	46
N11	10			15	35		38	55	
N12	6			11	30		6		
N13					15				
N14					10				

[a]Fragment sizes were calculated from 2-19 determinations; PSU-1 and GM strains data are according to previous reports [38,39].

Table 3-SI. DNA sequences used as probes and their location in *Oenococcus oeni* macrorestriction fragments

Genetic marker used as probe[a]	Strains/ Hybridising fragment(s)[b]						
	217[T]	bOg44	bOg30	bOg18	bOg38	bOg34	bOg36
Genetic markers							
rrs CECT 217T	A1, A2	A1, A2	A1, A2	A1, A2	A2, A3	A1, A2	A1, A2
	C1	C1	C1	C1	C1	C1	C1
	F1, F3, F5	F1, F3, F5	F1, F3, F4	F1, F3, F5	F1, F3, F5	F1, F2, F5	F1, F4, F5
	N2, N3	N2, N3	N2, N3	N2, N3	N2, N3	N2, N3	N1, N3
rrl PSU-1	A1, A2	A1, A2	A1, A2	A1, A2	A2, A3	A1, A2	A1, A2
	C2	C2	C2	C2	C2	C2	C2
	F1	F1	F1	F1	F1	F1	F1
araA/celR-like PSU-1	nd	A4	A3	nd	A5	A3	A5
	F4	F4	F5	F4	F4	F6	F3
	N1	N1	N1	N1	N1	N1	N2
pZN11 PSU-1	A1	A1	A1	A1	A3	A1	A1
dedA-like/EF-P	nd	nd	nd	N14	nd	N9/N10	nd
p-4	A2	A2	A2	A2	A2	A2	A2
lytS/lytR PSU-1	C2	C2	C2	nd	C2	nd	C2
	N4, N5	N5, N6	N5, N6	N5, N6	N5, N6	N4, N5	N5, N6
mir PSU-1	A2	A2	A2	A2	A2	A2	A2
	C2	C2	C2	C2	C2	C2	C2
	N6	N8	N8	N8	N8	N8	N7
dnaA PSU-1	F8-F4	F9-F4	F9-F8	F9-F4	F8-F4	F9-F6	F7-F3
	N1	N1	N1	N1	N1	N1	N1
gyrB PSU-1	nd	A1	A1	nd	A1	A4	A3
	F4	F4	F8	F4	F4	F6	F3
	N1	N1	N1	N1	N1	N1	N1
rpoC PSU-1	C1	C1	C1	C1	C1	C1	C1

	F5	F5	F4	F5	F5	F5	F5
mleA [Lo84.13]	A1	A1	A1	A1	A1	A1	A1
	F2	F2	F2	nd	F2	F4	F5
	nd	N1	N1	nd	N1	N3	N1
IS1165[§]	A1, A3	A1, A2	A1, A2	A1, A2	A2, A4	A1, A2, A4	A1, A3
	C1, C2	C1, C2	C1, C2	C1, C2	nd	C1, C2	C1, C2
	F1, F2, F3,	F1, F2	F2, F3, F4,	F2, F3, F4	F3	F1, F2, F4	F1, F2, F5
	F4	N1, N2, N3	F7	N1, N2	N3	N1, N2, N6,	N1, N4
	N1, N2, N3		N1, N2, N3			N9	
Other markers							
lc-36 [PSU-1]	nd	A2	A2	nd	A2	A2	A2
	C2	C2	C2	C2	C2	nd	C2
	nd	N2, N5	N2, N5	N2, N5	N5, N7	N2, N4	N3, N5

nd, Not determined.

[a]The origin of the probe (*O. oeni* strain) is described in superscript.

[b]Macrorestriction fragments are identified as in Table 2, with A corresponding to *Asc*I, C to I-*Ceu*I, F to *Fse*I and N to *Not*I.

[§]Insertion sequence from *Leuconostoc mesenteroides.*