The *Oenococcus oeni* genome: physical and genetic mapping of strain GM and comparison with the genome of a 'divergent' strain, PSU-1

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The physical and genetic maps of the *Oenococcus oeni* strains GM and PSU-1, which represent two genomic divergent groups on the basis of macrorestriction and ribotyping analysis, were compared. To achieve this comparison, the GM maps were constructed and the PSU-1 maps, already established, were improved. All the recognition sites of the restriction enzymes *Ascl*, *I-CeuI*, *FseI*, *NotI* and *SfiI* were located in both chromosomes and the position of 26 genetic markers, including two *rrn* operons and 14 new putative *oenococcal* genes, were allocated to the restriction fragments generated by the five enzymes. The comparative analysis of *O. oeni* GM and PSU-1 genomes revealed extensive conservation of loci order. As for the differences encountered in the locations of restriction sites, they seem to be a reflection of the differences in restriction fragment sizes, explainable by insertion/deletion events and point mutations. No evidence for major genomic rearrangements was found. The genomic conservation between the two strains is in agreement and suggests homogeneity within the species, which was not unexpected in view of the restricted ecological niche of *O. oeni*. Further comparisons of physical maps, both of *O. oeni* strains and related species, will certainly help to assess whether *O. oeni* is really an homogeneous species and physical mapping is suitable for taxonomic purposes, both at the supra- and intraspecific levels.

Keywords: *Oenococcus oeni*, genomic maps, comparative genome analysis

INTRODUCTION

The genus *Oenococcus*, first described by Dicks *et al.* (1995), contains a sole species, *Oenococcus oeni*, formerly known as *Leuconostoc oenos*. *O. oeni* has a restricted ecological niche (wine and related habitats), a peculiar acidophilic nature and viability at high ethanol levels (Garvie, 1986) and, although its dissimilarity to other leuconostocs is fully accepted, there is some controversy regarding both the evolution rate of the species (Martínez-Murcia *et al.*, 1993; Morse *et al.*, 1996) and its diversity (Dicks *et al.*, 1990; Zavaleta *et al.*, 1996). Based on metabolic/physiological criteria, splitting of *O. oeni* into two species was once proposed (Peynaud & Domercq, 1968), but the levels of DNA–DNA homology (73–110%; Dicks *et al.*, 1990) are in agreement with the genomic concept of bacterial species. More recently, the characterization of 30 strains of *O. oeni* of different origins on the basis of macrorestriction profiles and ribotyping, clustered them in two groups

and led to the suggestion that the species might be divided into two subspecies (Tenreiro *et al.*, 1994 and unpublished results).

To determine the validity of this assumption and gain insight into the genomic variability within *O. oeni*, we report here the comparison of the genomes of strains GM and PSU-1. These strains were chosen since, besides being representative of the above-mentioned clusters, they are used as starters for induction of the malolactic fermentation of wines and are therefore industrially important. To achieve this comparison, we constructed a physical and genetic map of the *O. oeni* GM chromosome and improved the existing physical and genetic maps of *O. oeni* PSU-1 (Zé-Zé *et al.*, 1998).

METHODS

Bacterial strains and growth conditions. Growth conditions for *O. oeni* strains GM (Microlife Techniques) and PSU-1 (R. Kunkee, University of California at Davis, USA) were de-

Table 1. PCR conditions used to amplify gene sequences used as probes

Gene	Function	Primer pair (5'-3')*	PCR cycles†	Approx. fragment size (kb)	Reference for primers
<i>ccpA</i>	Catabolite control protein A	<u>CGGAATTC</u> ATHAYGAYGTNGC and <u>CGGGATCC</u> ACNGCNCCDATRTCTA	ID + 35 cycles: 94 °C 1 min, 44 °C 2 min, 72 °C 1 min + FE	0.4	This study‡
<i>gyrB</i>	DNA gyrase subunit B	GAAGTCATCATGACCGTTCTGCAY GCNNGNGGNAARTTYGA and AGCAGGGTACGGATGTGCGAGCCR TCNACRTCNGCRTCNGTCAT	ID + 30 cycles: 94 °C 1 min, 60 °C 2 min, 72 °C 2 min + FE	1.4	Yamamoto & Harayama (1995)
<i>rpoC</i>	β' -subunit DNA polymerase	TGYTCWTGYGGWAARTAYAA and CATWACYTCWACRTGYTTTRTC	ID + 30 cycles: 94 °C 1 min, 48 °C 1 min, 72 °C 2 min + FE	1.1	Morse <i>et al.</i> (1996)
<i>secA</i>	SecA ATPase	<u>CGGAATTC</u> ATHGAYGARGCNMGNAAC and <u>CAGGATCC</u> CKNCCNGCCATRT TNGT	ID + 35 cycles: 94 °C 1 min, 44 °C 2 min, 72 °C 2 min + FE	0.9	This study‡
<i>hexB</i>	Mismatch repair	CTGTCAGTAACTTTGGAG and AAATGGACAATCGTTGGC	ID + 30 cycles: 94 °C 1 min, 56 °C 2 min, 72 °C 1 min + FE	0.6	Duwat <i>et al.</i> (1997)

* Abbreviations: B = C, G or T; D = A, G or T; H = A, C or T; M = A or C; N = A, C, G or T; R = A or G; S = C or G; Y = C or T. Underlined sequences represent non-complementary restriction sites for cloning strategies.

† ID, Initial denaturation: 94 °C 5 min; FE, final extension 72 °C 5 min.

‡ Supplied by M. A. Santos (DBV, FCUL, Lisbon).

scribed by Tenreiro *et al.* (1993). *Escherichia coli* JM109 and XL-1 Blue (Stratagene) were grown at 37 °C in LB broth, supplemented with 100 µg ampicillin ml⁻¹ when required.

Isolation and restriction of chromosomal DNA, and DNA fragment nomenclature. Intact genomic DNA was prepared in agarose plugs and single or double digested with the restriction enzymes *AscI*, *NotI*, *FseI* and *SfiI* as described previously (Tenreiro *et al.*, 1994; Zé-Zé *et al.*, 1998). I-CeuI digestion proceeded for 10–12 h with 0.1–0.2 U enzyme per agarose plug. All the enzymes were purchased from New England Biolabs.

Restriction fragments produced by digestion with a single enzyme are indicated by the initial letter of the endonuclease. Those obtained by double digestion were designated using the letters of the first and second digestions joined by a hyphen. All fragments were numbered in size order, from the largest to the smallest. Co-migrating fragments were numbered with sequential numbers.

PFGE. PFGE and two-dimensional PFGE were carried out in the Gene Navigator system (Pharmacia) with contour-clamped homogeneous field (CHEF) or Geneline (Beckman) with transverse alternating field (TAFE), as previously described (Zé-Zé *et al.*, 1998). The mean size of each fragment was estimated from several gels by linear interpolation with two flanking size standards (Heath *et al.*, 1992) using KODAK 1D 2.0 software (Kodak). *Saccharomyces cerevisiae* chromosomes (Bio-Rad), lambda DNA, mid-range and low-range PFG ladders (New England Biolabs) were used as size markers.

DNA probes. Preparation of [α -³²P]dCTP-labelled DNA probes and Southern hybridization conditions were as previously described (Zé-Zé *et al.*, 1998).

The DNA sequences used as probes in this study are reported below and listed in Table 3. The restriction fragments generated from *O. oeni* PSU-1 and GM chromosomes by the endonucleases *AscI*, *FseI*, *NotI* and *SfiI* were described previously (Zé-Zé *et al.*, 1998).

Gene sequences obtained by PCR, and primers and amplification conditions are listed in Table 1. PCR was performed in a Robocycler (Stratagene) and the reaction mixtures contained 50 pmol each primer (purchased from Gibco-BRL), 250 µM each dNTP, 1.5 mM MgCl₂ and 2.0 U *Taq* DNA polymerase, in the supplied buffer (Gibco-BRL). Reactions were performed with approximately 200 ng *O. oeni* PSU-1 genomic DNA, except for *hexB* from *Lactococcus lactis* (cloned in plasmid pDU190-1; Duwat *et al.*, 1997), where 100 ng plasmid was used as template. RAPD 1.2 is a randomly amplified polymorphic DNA fragment of ~1.2 kb from strain PSU-1.

Most of the plasmids harbouring sequences from PSU-1 have already been described (Zé-Zé *et al.*, 1998). The plasmid pZAR4.7 was constructed by subcloning, in pBluescript KS II (pKS II; Stratagene), a 4.7 kb *EcoRI* fragment from a clone of a genomic library of *O. oeni* PSU-1 in lambda DASH II (Stratagene) which hybridized with *araA* from *Bacillus subtilis* (pSNL4, Sá-Nogueira *et al.*, 1997). pMIR0.5 was constructed by subcloning in pKS II a 0.5 kb *PstI* fragment from pLB3 (pMK4 with an insert of a 5.1 kb *Sau3AI* fragment from strain

Fig. 1. Physical map of the chromosome of *O. oeni* GM using the enzymes *Ascl*, *I-CeuI*, *FseI*, *NotI* and *SfiI*. Radiating out from the centre, the five annuli show the restriction sites for the five enzymes, respectively. The locations of the genetic markers are also shown, although the order of markers in a single fragment is arbitrary. Direction of transcription of *rrn* operons is indicated by arrows. Scale is in kb.

Table 2. Sizes (kb) of restriction fragments obtained by cleavage of the *O. oeni* GM chromosome

Fragment	<i>Ascl</i>		<i>I-CeuI</i>		<i>FseI</i>		<i>NotI</i>		<i>SfiI</i>	
	Observed size*	Map size†	Observed size*	Map size†	Observed size*	Map size†	Observed size*	Map size†	Observed size*	Map size†
1	1088	1089	1195	1200	746	733	701	696	345	340
2	521	520	736	732	376	382	344	350	336	330
3	318	323			325	330	200	202	294	295
4					233	233	154	154	242	242
5					174	175	128	128	130	137
6					61	61	105	102	111	110
7					16	18	85	83	73	73
8							63	63	73	72
9							62	62	65	62
10							47	46	53	53
11							38	38	39	39
12							6	7	39	39
13									35	35
14									33	35
15									25	26
16									21	21
17									16	16
18									7	7
Total	1927	1932	1931	1932	1930	1932	1933	1932	1934	1932

* The observed sizes are mean values, calculated from 3–24 determinations. The maximal deviation in size was estimated to be less than 4.9%.

† The map sizes are theoretical sizes of fragments calculated by placing restriction sites on the map.

Table 3. DNA sequences used as probes in this study and their location on the *O. oeni* GM chromosome

T, type strain; ND, not determined.

Probe	Gene (function/description)	Origin	Hybridizing fragment(s)					Reference
			<i>AscI</i>	<i>I-CeuI</i>	<i>FseI</i>	<i>NotI</i>	<i>SfiI</i>	
Plasmids								
pRT1	<i>rrs</i> (16S rRNA gene)	<i>O. oeni</i> L217 ^T	A1, A2	C1	F1, F4, F5	N1, N3	S3, S12	Zé-Zé <i>et al.</i> (1998)
pSNL4	<i>araA</i> (L-arabinose isomerase)	<i>B. subtilis</i>	A3	C1	F3	N2	ND	Sá-Nogueira <i>et al.</i> (1997)
pZAR4.7	<i>araA</i> (L-arabinose isomerase)	<i>O. oeni</i> PSU-1	A3	C1	F3	N2	ND	This study
pZN11	<i>celR</i> -like (regulator gene)	<i>O. oeni</i> PSU-1	A1	ND	F1	N8, N12	S3	This study
pZN12	<i>dedA</i> -like (unknown)	<i>O. oeni</i> PSU-1	A1	ND	F1	N8, N12	S3	This study
p-4	<i>efp</i> (elongation factor)	<i>O. oeni</i> PSU-1	A1	ND	ND	N12	S3	This study
p-4	<i>tgt</i> (tRNA ribosyltransferase)	<i>O. oeni</i> PSU-1	A1	ND	ND	N12	S3	This study
p-4	<i>lytS/lytR</i> (<i>NotI</i> linking clone)	<i>O. oeni</i> PSU-1	A2	C2	ND	N5, N6	ND	Zé-Zé <i>et al.</i> (1998)
pLB3	<i>mir</i> (mitC resistance)	<i>O. oeni</i> PSU-1	A2	C2	ND	N7	ND	Brito (1996)
pMIR0.5	<i>gcg</i> (GTP binding protein)	<i>O. oeni</i> PSU-1	A2	C2	ND	N7	ND	This study
pJ2C3	<i>mleA</i> (malolactic enzyme)	<i>O. oeni</i> Lo84.13	A1	C1	F2	N1	S6	Labarre <i>et al.</i> (1996)
pGID600	<i>alsS, alsD</i> (α -acetolactate synthetase and decarboxylase)	<i>O. oeni</i> Lo84.13	A1	C1	ND	N1	S2	Garmyn <i>et al.</i> (1996)
pDU36-1	<i>recA</i> (DNA recombination)	<i>Leuconostoc mesenteroides</i>	A1	ND	ND	N1	S3	Duwat <i>et al.</i> (1992)
pAK6.9	IS1165 (insertion sequence)	<i>Leu. mesenteroides</i>	A1, A3	C1	F2, F4	N1, N9	S2, S7	Johansen & Kibenich (1992)
pFS21	<i>citP</i> (citrate permease)	<i>Lac. lactis</i>	A3	ND	ND	N2	S4	Sesma <i>et al.</i> (1990)
lc-36	<i>NotI</i> linking clone	<i>O. oeni</i> PSU-1	A2	C2	ND	N3, N5	S1	Zé-Zé <i>et al.</i> (1998)
p-20	<i>NotI</i> linking clone	<i>O. oeni</i> PSU-1	A3	ND	ND	N9	S7	This study
PCR products								
<i>ccpA</i>	Catabolite control protein A	<i>O. oeni</i> PSU-1	ND	ND	ND	N1	S2	This study
<i>gyrB</i>	DNA gyrase subunit B	<i>O. oeni</i> PSU-1	A1	ND	F3	N1	S2	This study
<i>rpoC</i>	DNA polymerase β' -subunit	<i>O. oeni</i> PSU-1	A1	C1	F5	N1	S3, S16	This study
<i>secA</i>	SecA ATPase	<i>O. oeni</i> PSU-1	A2	ND	ND	N5	ND	This study
<i>hexB</i>	Mismatch repair	<i>Lac. lactis</i>	A2	C1	ND	N3	S8, S12	This study
RAPD 1.2	RAPD fragment	<i>O. oeni</i> PSU-1	A3	C1	F4	N2	S4	This study
PSU-1 DNA fragments								
3' <i>rrl</i>	<i>rrl</i> (23S rRNA fragment)	<i>O. oeni</i> PSU-1	A1, A2	C2	F1	N1, N3	S3, S12	This study
A3		<i>O. oeni</i> PSU-1	A3	ND	ND	N3, N9	S4, S7	This study
A4		<i>O. oeni</i> PSU-1	ND	ND	ND	N2	ND	This study
F1		<i>O. oeni</i> PSU-1	A1, A2	C2	ND	N1, N3, N4, N5, N6, N7, N8, N10	S1, S3, S5, S11, S12	This study
F3		<i>O. oeni</i> PSU-1	A2, A3	ND	F4, F6	N2, N3, N9	S4, S7, S8, S12, S15	This study

Table 3 (cont.)

Probe	Gene (function/description)	Origin	Hybridizing fragment(s)					Reference
			<i>AscI</i>	<i>I-CeuI</i>	<i>FseI</i>	<i>NotI</i>	<i>SfiI</i>	
N1		<i>O. oeni</i> PSU-1	A1, A3	ND	ND	N1, N2, N11	S2, S4, S6, S7/8, S9, S13/14	This study
N2		<i>O. oeni</i> PSU-1	A2, A3	ND	ND	N3, N9	S1, S7/8, S11/12, S15	This study
N2/3		<i>O. oeni</i> PSU-1	ND	ND	ND	N1, N2, N3, N9	S1, S3, S4, S7/8, S13/14, S15, S16	This study
N4/5		<i>O. oeni</i> PSU-1	ND	ND	ND	N4, N5	S1, S3, S5	This study
N6		<i>O. oeni</i> PSU-1	A2	ND	ND	ND	S1	This study
N11		<i>O. oeni</i> PSU-1	A1	ND	F1	N8, N12	S3	This study
S1		<i>O. oeni</i> PSU-1	A2	ND	ND	N3, N5, N6, N7, N10	S1	This study
S10		<i>O. oeni</i> PSU-1	ND	ND	ND	N1	S9	This study
S11/12		<i>O. oeni</i> PSU-1	A1, A2, A3	ND	F1, F3	ND	S2, S11, S18	This study
S15		<i>O. oeni</i> PSU-1	A1	ND	ND	N1	S14, S16	This study
S16		<i>O. oeni</i> PSU-1	A2	ND	ND	N3	S11/12	This study
GM DNA fragments								
N1		<i>O. oeni</i> GM	A1	ND	ND	ND	S2, S3, S6, S9, S10, S13 S14, S16	This study
N2		<i>O. oeni</i> GM	A1, A3	ND	ND	N2	S2, S4, S18	This study
N4		<i>O. oeni</i> GM	A1	C2	ND	N4	S3, S5	This study
N8/9		<i>O. oeni</i> GM	A1, A3	C1, C2	ND	N8/9	ND	This study
N10		<i>O. oeni</i> GM	A1, A2	ND	ND	N10	S1, S5	This study
N11		<i>O. oeni</i> GM	A1	ND	ND	N11	S2	This study
S1/2		<i>O. oeni</i> GM	A1, A2	ND	ND	N1, N2, N3, N5, N6, N7, N10, N11	ND	This study
S6		<i>O. oeni</i> GM	A1	ND	ND	N1	S6	This study
S9		<i>O. oeni</i> GM	ND	ND	ND	N1	S9	This study
S15		<i>O. oeni</i> GM	ND	ND	ND	N3	S15	This study
S17		<i>O. oeni</i> GM	A1	ND	F2	N1	S17	This study
S18		<i>O. oeni</i> GM	A1, A3	ND	ND	N2	S18	This study

PSU-1 that confers resistance to mitomycin C; Brito, 1996). Plasmids pZN11 and pZN12 were obtained by shotgun cloning of PSU-1 DNA in pKS II, both digested with *NotI*. Plasmids p-4 and lc-36 contain *NotI* linking clones of *O. oeni* PSU-1 in pGEM5Z (Promega) and pKS II, respectively (Zé-Zé *et al.*, 1998). Plasmid p-20 was isolated when screening for PSU-1 *NotI* linking clones in pGEM5Z vector.

DNA sequencing and analysis. Non-automated DNA sequencing was performed by the dideoxynucleotide triphosphate chain termination method with [α - 35 S]dATP and Sequenase version 2.0 kit (USB, Amersham) or with [γ - 32 P]dATP by the dsDNA Cycle Sequencing system (Gibco-BRL). Automated sequencing was purchased from the '4 base

lab' (Reutlingen) which uses the ALF Sequencer (Pharmacia).

DNA sequences were analysed by DNASIS (Hitachi) and, for database searches, the BLAST programs (Altschul *et al.*, 1997) were used on the NCBI Sequence Database (<http://www.ncbi.nlm.nih.gov>).

RESULTS

Physical mapping of the *O. oeni* GM chromosome

In a previous study, a physical map of the *O. oeni* PSU-1 chromosome was constructed using the enzymes *AscI*,

Fig. 2. Comparison of the physical and genetic maps of *O. oeni* strains GM and PSU-1. Restriction sites for *AscI*, *I-CeuI*, *FseI*, *NotI* and *SfiI* are indicated. The circular genomes are shown linearized from a common *NotI* site, identified by the linking clone p-4. $\triangleright$ represents an insertion and $\triangleleft$ a deletion event in the GM chromosome. $\vdash$ and $\dashv$ indicate genetic markers assigned to endonuclease recognition sites.

FseI, *NotI* and *SfiI* (Zé-Zé *et al.*, 1998). The same rare cutting endonucleases were employed to construct the GM physical map (Fig. 1) and the intron-encoded endonuclease *I-CeuI* sites were added to both maps.

I-CeuI cuts the genome of both strains twice, identifying two *rrn* operons in *O. oeni* located at the limits of the generated fragments (GM C1, 1200 kb; C2, 732 kb; PSU-1 C1, 1166 kb; C2, 691 kb). Under different digestion conditions, i.e. more than 0.2 U enzyme per agarose plug or longer incubation times, restriction with *I-CeuI* gave rise either to four reproducible fragments or to degradation of the DNA. As the presence of extra *rrl* gene sequences could be discarded, taking in account the two precisely located *rrn* operons on the *O. oeni* PSU-1 physical map (Zé-Zé *et al.*, 1998) and the hybridization analysis with *rrl* and *rrs* probes (data not shown), the four-fragment profiles must be artefacts. Although *I-CeuI* is able to cleave the *rrl* sequence in most bacteria and lower eukaryotes, these results suggest that the presence of an *rrn* operon based solely on the cleavage

by *I-CeuI* (Liu *et al.*, 1993, 1999; Marshall & Lemieux, 1992) cannot be taken for granted and must be confirmed by hybridization of *I-CeuI* profiles with *rrl* and *rrs* probes. To our knowledge, reproducible extra cutting sites for *I-CeuI* were never reported. Nevertheless, the absence of *I-CeuI* cleavage of the *rrl* gene due to a single base deletion in the recognition sequence has been described in *Mycobacterium tuberculosis* (Philipp *et al.*, 1996).

Restriction of the *O. oeni* GM circular chromosome with *AscI*, *FseI*, *NotI* and *SfiI* produced 3, 7, 12 and 18 fragments, respectively; their estimated mean sizes are presented in Table 2. All fragments were resolvable under the PFGE conditions used, except for three *SfiI* bands (S7/S8, S11/S12 and S13/S14) which were assumed to be doublets. The size of the *O. oeni* GM chromosome was estimated to be 1932 kb.

The establishment of relationships between restriction fragments and the mapping of all 42 restriction sites was

accomplished by a reasoning similar to the one described for the *O. oeni* PSU-1 physical map (Zé-Zé *et al.*, 1998).

The experimental strategy involved a combined approach using the analysis of double digestions, two-dimensional PFGE and Southern hybridization. Besides the assessment of fragment linkage, cross-hybridization with PSU-1 linking clones and restriction fragments both from GM and PSU-1, as well as the use of probes for genetic markers (plasmid cloned genes and PCR products), were essential to the confirmation of the relative order of restriction sites (see Table 3).

The integration of these data and comparison to the *O. oeni* PSU-1 physical map (Fig. 2) led to the physical map of strain GM presented in Fig. 1.

Assignment of genetic markers to the *O. oeni* GM physical map

The chromosomal locations of 26 genetic markers, namely 23 gene markers, two linking clones and a PSU-1 RAPD fragment, were determined (Fig. 1). The probes used, listed in Table 3, assigned the corresponding sequences to the restriction fragments they hybridized with, although their exact positions remain unknown and, when in the same fragment, the order is arbitrary. Only the markers containing restriction site(s) were precisely located (Fig. 1).

The exact location of the two *rrn* operons in the *O. oeni* GM chromosome was determined by hybridization of *rrs* and *rrl* probes to the *I-CeuI* and *FseI* restriction profiles (Table 3). As in strain PSU-1 (Zé-Zé *et al.*, 1998), these operons have opposite orientations in the genome, *rrnA* being transcribed clockwise and *rrnB* anticlockwise (Figs 1 and 2).

Since very few genes of *O. oeni* have been described, gene-like probes obtained by PCR using degenerate primers (Table 1) and some shotgun clones (Karlyshev *et al.*, 1998) of *O. oeni* PSU-1 were further analysed by DNA sequencing, aiming to find homologies by database searches, a strategy that is suitable for locating essential genes in organisms with low G + C contents when using cloned fragments with G + C-rich restriction sites (Ladefoged & Christiansen, 1992). Therefore, the *NotI* linking clone p-4, plasmid pZAR4.7, as well as small cloned fragments produced by *NotI* digestion of *O. oeni* PSU-1 genomic DNA (pZN11, 10 kb; pZN12, 6 kb), were sequenced ~ 700 nucleotides in each direction using universal vector primers. The insert cloned in pMIR0.5 was sequenced on both strands and its precise size is 557 bp. The putative genes found are presented in Table 4.

Improvement of *O. oeni* PSU-1 physical and genetic maps

The construction of the GM physical map, and the comparative analysis of cross-hybridization results, enabled the mapping of two previously unlocalized *SfiI* sites in the PSU-1 map (Zé-Zé *et al.*, 1998 and Fig. 2). The use of fragments S10^{PSU-1} and S17^{GM} as probes

allowed the assignment of the linkage S7–S10 (clockwise) in the PSU-1 chromosome. As fragment S16^{PSU-1} hybridized to the contiguous GM fragments S11/S12, and S16^{PSU-1} is located in the region S8–S11 (Zé-Zé *et al.*, 1998), its position must lie between S11 and S14 (see Fig. 2).

All the genetic markers were also located on the *O. oeni* PSU-1 physical map, as shown in Fig. 2, except for the RAPD 1.2 fragment (hybridization experiment not performed).

Comparison of *O. oeni* GM and PSU-1 genomic maps

Comparison of the *O. oeni* GM and PSU-1 physical maps showed that, although there are some polymorphisms in restriction profiles with all enzymes used, in fragment length and number (Table 2, Fig. 2 and Zé-Zé *et al.*, 1998), there is a high degree of conservation in cleavage sites (~ 40 %) and in the order of loci. The fact that *NotI*-cloned linking sequences of PSU-1 are maintained in the GM physical map and the unambiguous results of cross-hybridization analysis (Table 3) support the assumption of global genetic linkage preservation.

The smaller region between the *rrn* operons, defined by fragments C2 or F1 in both GM and PSU-1, has an overall conservation of restriction sites, whereas the other region, defined by fragment C1, shows more variation. In terms of size, the C2/F1 region is 41 kb longer in strain GM. As the size difference between the *O. oeni* GM (1932 kb) and PSU-1 (1857 kb) chromosomes is 75 kb, a similar level of insertion/deletion events can be expected in both genomic regions.

Putting together the cross-hybridization results, relative dimension of homologous fragments and alignment of GM and PSU-1 physical maps, eight insertion events (in N1, N4, N7, N8, N10, S2, S3 and S16) and a deletion (in S4) were deduced in strain GM. For instance, the insertion of a 19 kb sequence in N4 was determined by cross-hybridization of N4^{PSU-1} and N4^{GM} fragments. As N4^{GM} (154 kb) fragment only hybridized with N4^{PSU-1} (135 kb), that region is expected either to contain a duplication or a sequence absent in PSU-1. Although the deletion event in S4 and the insertion in N1 have the same size (29 kb), there is no indication of an unequal crossing-over event involving the flanked region taking into account the maintenance of loci order relative to PSU-1. The conservation of several restriction fragments between strains GM and PSU-1 through the whole genome (e.g. N5 and N5, F7 and F8, S6 and S7, S9 and S10, N6 and N6) is also indicative of the absence of major recombination events, leaving point mutations and localized duplications or transpositions (involving regions smaller than the resolution of the presented map) as the most probable explanation for the observed restriction polymorphisms.

In terms of physical maps, and except for the presence of an extra *SfiI* site in the PSU-1 chromosome, the region defined by *rrnB*–N5–N6 seems to be largely conserved. Nevertheless, minor differences were detected in the genetic maps, namely the presence of an extra IS1165

Table 4. Orthologous genes found after sequencing selected plasmid inserts and PCR fragments

Name*	Size (kb)†	Sequence length (bp)	Homology	EMBL/SWISS-PROT accession no.	Gene product	Percentage identity (aa overlap)‡	G + C content (mol %)
pZAR 4.7/T3	4.7	880	<i>araA</i> (<i>B. subtilis</i>)	emb CAA61585	L-Arabinose isomerase	51 (291)	37.9
pZAR 4.7/T7	4.7	993	<i>celR</i> (<i>B. subtilis</i>)	sp P46321	Putative Cel operon regulator	23 (154)	35.4
			<i>bglG</i> (<i>B. subtilis</i>)	emb CAB13057	Transcriptional antiterminator	25 (155)	
<i>gyrB</i> /UP-1	1.4	222	<i>gyrB</i> (<i>Streptococcus pneumoniae</i>)	emb CAA91553	DNA gyrase subunit B	50 (54)	41.4
<i>gyrB</i> /UP-2R	1.4	372	<i>gyrB</i> (<i>S. pneumoniae</i>)	sp P48373	DNA gyrase subunit B	73 (111)	44.1
pMIR0.5/T3 and T7	0.557	557	<i>ulf-250</i> (<i>Rattus norvegicus</i>)	gi 3978495	ULF-250 protein	70 (100)	35.9
			(<i>B. subtilis</i>)	sp P50743	GTP-binding protein	62 (93)	
pZN11/T3	10	566	<i>dedA</i> (<i>E. coli</i>)	sp P09548	Unknown	43 (89)	38.3
pZN11/T7	10	771	<i>efp</i> (<i>B. subtilis</i>)	sp P49778	Elongation factor P (EF-P)	58 (89)	41.5
pZN12/T3	6	646	<i>tgt</i> (<i>B. subtilis</i>)	sp O32053	Queuine tRNA-ribosyltransferase	56 (193)	38.9
pZN12/T7	6	702	<i>clpB</i> (<i>Mycoplasma pneumoniae</i>)	sp P75247	CLPB ATP-binding protein	58 (29)	45.3
p-4/SP6 and T7	14	1335	<i>lytS</i> (<i>Staphylococcus aureus</i>)	gi 862312	Autolysis control	53 (308)	39.5
			<i>lytR</i> (<i>B. subtilis</i>)	emb CAA99611.1	Autolysin response regulator	50 (118)	
			<i>lytR</i> (<i>S. aureus</i>)	gi 1854577		46 (128)	

* Plasmid and primer(s) used for sequencing reactions.

† Size of the cloned insert or PCR fragment.

‡ Percentages of aa identity (and overlap) between *O. oeni* predicted protein sequences and the similar protein sequences of other organisms.

sequence in strain PSU-1, and the different location of *secA*-like gene in the two chromosomes.

Concerning the restriction sites, an uneven distribution can be observed both for specific enzymes and the overall G + C-rich sequences. In spite of the identical G + C content of their recognition sequences, *FseI* and *SfiI* cutting sites predominate in the large fragment defined by the two *rrn* operons (C1), whereas *NotI* sites are more frequent in the smaller region (C2), both for the GM and PSU-1 genomes. Taking into account the G + C content of the restriction sites, and normalizing their distribution according to the size difference of fragments C1 and C2, there is no doubt that GC-rich sequences seem to prevail in fragment C1 for strain GM and in fragment C2 for strain PSU-1. Nevertheless, the general agreement in the order of the genetic markers does not support the hypothesis of differential gene spreading in both genomes.

DISCUSSION

The physical map of *O. oeni* GM was constructed by a top-down approach (Cole & Saint-Girons, 1994) using the rare-cutting enzymes *AscI*, *I-CeuI*, *FseI*, *NotI* and

SfiI, and the chromosome size was estimated as 1932 kb. The map has an average resolution of 46 kb with the majority of map intervals (83.3 %) smaller than 100 kb and, therefore, is in the acceptable range for genetic mapping (Cole & Saint Girons, 1994). The cross-hybridization experiments were invaluable for the GM map construction. The success of this approach and the previous knowledge of the PSU-1 physical map (Zé-Zé *et al.*, 1998), not only made GM mapping relatively easy but also enabled the completion of *SfiI* mapping in PSU-1.

Hybridization experiments allowed the localization of 26 genetic markers in both maps, including 14 new *O. oeni* putative genes: (i) four PCR products – *ccpA*-like, *hexB*, *rpoC*, *secA*-like; (ii) 10 ORFs identified by database searches of DNA sequences – *araA*, *celR*-like, *clpB*, *dedA*-like, *efp*, *gcg*, *gyrB*, *lytS*, *lytR* and *tgt*. The known *O. oeni* genes related to the oenological properties of this species, *mleA* (Labarre *et al.*, 1996) and *alsS/alsD* (Garmyn *et al.*, 1996), as well as genes essential for replication and transcription (*gyrB*, *rpoC*), or involved in mismatch repair (*hexB*) and *NotI* linking clones were also mapped. The heterologous probes for

citP and *recA* also enabled the localization of the putative orthologous sequences in *O. oeni*.

The precise location and opposite transcription direction of the two *rrn* operons (Zavaleta *et al.*, 1996) was determined by Southern hybridization with *rrs* and *rml* homologous probes, taking advantage of the fact that the sequence of these genes is cleaved by *FseI* and *I-CeuI*, respectively. The origin and terminus of replication of the *O. oeni* chromosome remains unknown. However, considering that in most known eubacteria *oriC* is located near *gyrB* and *rpoC*, and that the transcription of *rrn* operons usually occurs in the opposite direction to *oriC* (Cole & Saint Girons, 1994), the suggestion of its location in fragment C1, as proposed by Zé-Zé *et al.* (1998), is reinforced.

In a study involving 30 *O. oeni* strains, Tenreiro *et al.* (1994), chose strains GM and PSU-1 as representatives of two divergent genomic groups. The comparative analysis of the genetic maps of these strains presented here (Fig. 2), showed global similarity, in spite of the restriction fragment polymorphisms (62.5% of the fragments produced by the five enzymes have different sizes). Although we can speculate that some of the differences in the locations of *NotI* and *SfiI* restriction sites in the vicinity of *rrnB* operon (Figs 1 and 2) could be due to the IS1165 element, the cause for the majority of the variation remains unknown. As strain GM has no lysogenic derivatives and considering that the chromosomal regions corresponding to both attachment sites in PSU-1 genome (*attB1*, S8 and *attB2*, S11; Zé-Zé *et al.*, 1998) presents no considerable difference in the two strains, the in and out movement of bacteriophage DNA cannot explain the overall diversity. As, except for *secA*-like and IS1165, the order of genetic markers is identical in the two *O. oeni* chromosomes and the small differences observed are apparently due to insertion/deletion events that do not affect the global genomic similarity, other differences on the restriction maps probably result from point mutations.

The extensive degree of conservation of the order of loci presented here supports the previously suggested homogeneous nature of *O. oeni* (Morse *et al.*, 1996; Zavaleta *et al.*, 1996, 1997). As suggested by St. Jean & Charlebois (1996), there is no objective measure to determine the degree of similarity between genomes and the maps can be preserved despite the potential for rearrangement. Nevertheless, the value of genomic maps *per se*, both at specific and subspecific levels, can only be ascertained by comparisons involving strains from different taxonomic ranks, in order to assess taxonomic divergence and homogeneity.

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