Physical map of the genome of *Oenococcus oeni* PSU-1 and localization of genetic markers

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A physical map of the chromosome of *Oenococcus oeni* PSU-1 was constructed. This represents the first map for a strain of this species. A total of 37 restriction sites for the rare-cutting endonucleases *Ascl*, *FseI*, *NotI* and *SfiI* were mapped on the chromosome, which was found to be circular with an estimated size of 1857 kb. Fragment order was determined using several approaches: analysis of partial and double digestions, two-dimensional pulsed-field gel electrophoresis, isolation of linking clones, and Southern hybridization with labelled restriction fragments both from PSU-1 and from *O. oeni* strain GM. *Oenococcus* genes *alsS/alsD*, *mleA* and *mir*, two phage attachment sites and recurrent sequences such as IS1165-like elements and *rrn* loci were located on the physical map. Specific fragments hybridizing with gene probes from *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Bacillus subtilis* were also identified. The two ribosomal operons have been precisely located and their transcription direction determined.

Keywords: *Oenococcus oeni*, physical map, genetic map, *rrn* organization

INTRODUCTION

Oenococcus oeni, formerly known as *Leuconostoc oenos* (Dicks *et al.*, 1995), is a lactic acid bacterium occurring naturally in wine and related habitats, which is characterized by its peculiar acidophilic nature and growth in media containing high ethanol levels (Garvie, 1986). These properties, associated with the ability to carry out malolactic fermentation and a suggested enhancement of the organoleptic properties of wine, has promoted its use in starter cultures for wine-making (Kunkee, 1991).

Recent research concerning *O. oeni* has been focused in five main fields: (i) bioenergetics of organic acid transport and elucidation of metabolic pathways (Ramos & Santos, 1996; Salema *et al.*, 1996; Veiga da Cunha *et al.*, 1993); (ii) analysis of evolutionary divergence and tachytely (Martinez-Murcia *et al.*, 1993; Morse *et al.*, 1996; Zavaleta *et al.*, 1996); (iii) assessment of intraspecific variability and development of reliable typing methods (Kelly *et al.*, 1993; Tenreiro *et al.*, 1994;

Viti *et al.*, 1996; Zavaleta *et al.*, 1997); (iv) molecular characterization of plasmids and phages (Brito *et al.*, 1996; Fremaux *et al.*, 1993; Santos *et al.*, 1996; Zúñiga *et al.*, 1996); and (v) analysis of genes related to its oenological properties and stress response (Garmyn *et al.*, 1996; Jobin *et al.*, 1997; Labarre *et al.*, 1996).

Despite the multidisciplinary interest in this species, a major drawback remains, in that no effective means of genetic transfer has been reported so far for *O. oeni*. Classical genetic tools are also hampered by the requirement of this species for rich growth media (Garvie, 1986). Therefore, only analysis of isolated genes and construction of physical and genetic maps by the top-down method (Cole & Saint-Girons, 1994) may allow some progress in the understanding of genome expression and structure, dynamics of chromosome rearrangements and intraspecific variability in *O. oeni*.

Pulsed-field gel electrophoresis (PFGE) techniques represent powerful tools for estimation of genome size and construction of physical and genetic maps. In lactic acid bacteria, such maps are only available for strains of *Lactococcus lactis* and *Streptococcus thermophilus* (Davidson *et al.*, 1996; Le Bourgeois *et al.*, 1992; Roussel *et al.*, 1994, 1997; Tulloch *et al.*, 1991).

This paper presents a physical map of *O. oeni* PSU-1, a

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Abbreviations: 2D, two dimensional; CHEF, contour-clamped homogeneous electric field; TAFE, transverse alternating field.

strain commonly used as starter in wine-making and for which the macrorestriction profile with endonucleases that recognize GC-rich sequences was already known (Tenreiro *et al.*, 1994). Oenococcal genetic markers, as well as putative genes inferred from hybridization studies with probes from heterologous Gram-positive bacteria, were located on the map constructed.

METHODS

Bacterial strains and growth conditions. *O. oeni* strains PSU-1 (provided by R. Kunkee, University of California at Davis, USA), GM (Microlife Techniques, Sarasota, FL, USA) and L217^T (Colección Española de Cultivos Tipo, Valencia, Spain) were grown at 28 °C in MTJ broth pH 5.5 (Tenreiro *et al.*, 1993). *Escherichia coli* JM109 was grown at 37 °C in LB broth, supplemented with 100 µg ampicillin ml⁻¹ when required.

Preparation and restriction of genomic DNA. Intact genomic DNA was prepared and digested with restriction endonucleases as previously described (Tenreiro *et al.*, 1994), except that 5–10 U of *AscI* and *FseI* were used for each agarose plug. Partial digestions with *AscI* were similarly performed using 2–10 U enzyme for 0.5–2 h. Double digestions were performed sequentially by washing the agarose plugs with TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and changing the buffer to the appropriate one between incubations. All enzymes were purchased from New England BioLabs.

Pulsed-field gel electrophoresis. PFGE was carried out using the Gene Navigator system (Pharmacia) with contour-clamped homogeneous electric field (CHEF), and Geneline (Beckman) with transverse alternating field (TAFE). The CHEF system was used with 1% (w/v) agarose gels (Seakem GTG, FMC) and 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). Three run-types were applied for resolution of fragments in different ranges (<150 kb, 150–750 kb, >750 kb), as previously described (Tenreiro *et al.*, 1994). The TAFE system was used with 1% (w/v) agarose gels (Seakem LE, FMC) and 1 × TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). In this case, separation of fragments was performed using a two-phase run. In the first phase, at 195 mA, pulse time varied from 1 to 9 s for 5 h, by increments of 1 s; in the second, at 120 mA, pulse time varied by 2 s increments from 10 to 20 s. Electrophoresis was performed at 10 °C in both systems.

The mean size of each fragment was estimated from several gels by linear interpolation with two flanking size standards (Heath *et al.*, 1992). The chromosomes of *Saccharomyces cerevisiae* (Bio-Rad), lambda DNA, and mid-range and low-range PFG ladders (New England BioLabs) were used as size markers.

The two-dimensional PFGE (2D-PFGE) as described by Bautsch (1988) was initially used in this work. Later, owing to ambiguities in assigning the spots arising from this procedure to specific fragments of double digests having similar mobilities, the following version was adopted. Restriction fragments obtained with one enzyme were individually excised from the gel and, after sequential digestion with another enzyme, submitted to the second electrophoresis. The double-digested fragments were inserted in the gel ordered by size, to reconstruct the original restriction profile. Single and double digestions were co-electrophoresed. The designation 2D-PFGE has been adopted throughout this paper, irrespective of the version used.

Isolation of linking clones. Genomic DNA of strain PSU-1 was partially digested with *Sau3AI* and fragments of approximately 6 kb were recovered after centrifugation in a sucrose gradient (Ohman, 1988). After incubation with T4 DNA ligase (New England BioLabs), the self-ligated DNA was digested with *NotI* and cloned into a *NotI*-cleaved pBluescript KS II vector (Stratagene). This mixture was used to transform *E. coli* JM109 by electroporation using the ProGenetor II system (Hoefer Scientific Instruments).

Probes. Plasmids containing fragments used as probes are listed in Table 1. pRT1 was obtained by cloning in pBluescript SK II (Stratagene) a 1342 bp PCR product resulting from amplification of *O. oeni* L217^T genomic DNA, with primers 5'-TTGATCCTGGCTCAGGAT-3' and 5'-ACTTCGTGGA-GTCGAGTT-3', corresponding to positions 13–30 and 1337–1354 of the published 16S RNA sequence (Martinez-Murcia & Collins, 1990). pZZ1 was constructed by inserting in the same vector a 1123 bp *EcoRI* fragment, corresponding to positions 104–1226 of the published 23S RNA sequence (Martinez-Murcia *et al.*, 1993). This fragment was originally present in a lambda clone selected from a genomic bank of *O. oeni* PSU-1 in lambda DASH II (Stratagene) by hybridization with the 1342 bp *rrs* probe. Comparison of the insert restriction map with that expected from published sequences revealed that the clone contained an intact *rrn* operon.

Table 1. Plasmids used in this study

Plasmid	Gene (function) description	Source of relevant marker	Stringency conditions*	Reference
pRT1	<i>rrs</i>	<i>Oenococcus oeni</i> L217 ^T	H	This study
pZZ1	<i>rrl</i>	<i>O. oeni</i> PSU-1	H	This study
pLB3	<i>mir</i> (resistance to mitomycin C)	<i>O. oeni</i> PSU-1	H	Brito (1996)
pJ2C3	<i>mleA</i> (malolactic enzyme)	<i>O. oeni</i> Lo84.13	H	Labarre <i>et al.</i> (1996)
pGID600	<i>alsS</i> , <i>alsD</i> (α-acetolactate synthase and decarboxylase)	<i>O. oeni</i> Lo84.13	H	Garmyn <i>et al.</i> (1996)
pDU36-1	<i>recA</i> (DNA recombination)	<i>Leuconostoc mesenteroides</i>	L	P. Duwat (unpublished)
pAK6.9	IS1165 (insertion sequence)	<i>L. mesenteroides</i>	L	Johansen & Kibenich (1992)
pFS21	<i>citP</i> (citrate permease)	<i>Lactococcus lactis</i>	L	Sesma <i>et al.</i> (1990)
pSNL4	<i>araA</i> (L-arabinose isomerase)	<i>Bacillus subtilis</i>	L	I. Sá-Nogueira (unpublished)

* H, high stringency; L, low stringency.

Table 2. Sizes (kb) of restriction fragments obtained by cleavage of the chromosome of *O. oeni* PSU-1

The observed sizes are mean values of restriction fragments, calculated from 2–19 determinations. The maximal deviation in size was estimated to be less than 4%. The map sizes are theoretical sizes of fragments calculated by placing the restriction sites on the map.

Fragment	<i>AscI</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
1	990	987	680	692	795	798	330	330
2	513	520	352	353	270	265	295	295
3	214	220	240	233	250	252	280	271
4	130	130	235	230	133	135	160	160
5			163	157	128	128	137	137
6			99	99	102	102	130	130
7			75	75	58	58	110	110
8			18	18	44	44	100	100
9					44	44	72	72
10					15	15	62	62
11					10	10	44	44
12					6	6	44	44
13							35	35
14							35	35
15							26	26
16							6	6
Total	1847	1857	1862	1857	1855	1857	1866	1857

Labelling and hybridization. Gene probes were excised from cloning vectors with suitable enzymes and after agarose gel electrophoresis recovered with the Sephaglas BandPrep kit (Pharmacia). Labelling with [α - 32 P]dCTP was performed using the Multiprime labelling system (Amersham). Some restriction fragments generated by the enzymes *AscI*, *NotI* and *SfiI* from chromosomal DNA of strains PSU-1 and GM were also used as probes for mapping purposes. These fragments were excised, recovered and labelled as above. PFGE gels were treated as described by Wahl *et al.* (1987) and restriction fragments were transferred to a nylon membrane (Hybond-N, Amersham) by blotting overnight with $10 \times$ SSC (1.5 M NaCl, 0.15 M sodium citrate). Hybridizations were performed as previously described (Santos *et al.*, 1996), at 68°C for high stringency (homologous probes) and at 50°C for low stringency (heterologous probes). Filters were washed at the hybridization temperature, twice in $2 \times$ SSC for 15 min, twice in $2 \times$ SSC/ 0.1% (w/v) SDS for 30 min, and once in $0.1 \times$ SSC/ 0.1% (w/v) SDS for 15 min (high stringency) or 5–10 min (low stringency).

DNA fragment nomenclature. Fragments produced by digestion of PSU-1 DNA with a single restriction endonuclease were named by the initial letter of the enzyme (A, *AscI*; F, *FseI*; N, *NotI*; S, *SfiI*). In the case of DNA from strain GM the superscript GM was added. Fragments resulting from partial digestions were named as above, preceded by the letter P. All fragments were numbered in order, from the largest to the smallest. For co-migrating fragments, sequential numbers were assigned. Fragments that could only be obtained by double digestion were designated using the names of the respective single-digestion overlapping fragments joined by a hyphen.

RESULTS

Restriction digestion patterns of genomic DNA

A macrorestriction analysis previously performed on 30 strains of *O. oeni* (Tenreiro *et al.*, 1994) showed that *AscI*, *NotI* and *SfiI*, generating an average number of fragments between 4 and 17, were suitable enzymes for physical mapping. In strain PSU-1 these enzymes cleaved the chromosome into 4, 12 and 16 restriction fragments, respectively (the 12th *NotI* fragment, of 6 kb, had not been previously detected). Two other 8 bp specific enzymes, *FseI* and *SrfI*, generated 8 and 9 fragments when tested on this strain. As *SrfI* profiles were usually interspersed with partial digestion fragments, only *FseI* was further retained. The estimated mean sizes of the fragments produced by each enzyme are listed in Table 2 and the restriction profiles are shown in the PFGE photographs. Fragments ranged from 6 to 990 kb and were resolvable under the PFGE conditions described, except for one *NotI* (N8/N9) and two *SfiI* bands (S11/S12 and S13/S14) that were assumed to be doublets. The genome size of *O. oeni* PSU-1 was determined to be 1857 kb, the mean of the sizes obtained with the four enzymes.

Electrophoretic mobility of macrorestriction fragments was always dependent on run pulse time, indicating that neither of the fragments corresponded to circular molecules (Beverly, 1988; Smith & Condemine, 1990). On the other hand, no signal was obtained in Southern

blots using oenophage DNAs as hybridization probes (Santos, 1995; Santos *et al.*, 1996). Therefore, no plasmids and prophages that could contribute to the cleavage patterns were found in strain PSU-1.

Ascl cleavage map of the *O. oeni* PSU-1 genome

Partial digestions with *Ascl* were performed to determine the order of the four *Ascl* fragments. As shown in Fig. 1, six partial fragments were obtained, PA1 to PA6, with approximate sizes of 1870, 1350, 1125, 860, 730 and 350 kb, respectively. Partial fragments PA3, PA5 and PA6 were interpreted as A1 plus A4, A2 plus A3 and A3 plus A4, respectively, whereas the PA2 and PA4 products should correspond to the linkages A3-A4-A1 and A2-A3-A4. The largest partial fragment, PA1, had an estimated size equivalent to the linearized chromosome. According to these data, the four *Ascl* fragments were ordered as A1-A2-A3-A4 and the circular topology of the chromosome was confirmed.

Alignment of *NotI* fragments

Comparison of double and sequential digests (2D-PFGE) with single *Ascl* and *NotI* profiles allowed the positioning of three *NotI* sites in the *Ascl* physical map and the allocation of all *NotI* fragments to the corresponding *Ascl* fragments, except for N12. Since fragments N1, N2 and N9 disappeared in the *Ascl*-*NotI* double digestion (Fig. 2), the *Ascl* sites were assigned to them (see physical map in Fig. 5). As shown in Table 3 and partially in Fig. 2, fragments N3, N4, N8 and N11 could be allocated to A1 and fragments N5, N6, N7 and N10 to A2. Owing to its small size (6 kb), N12 could only be assigned to fragment A1 by hybridization (Table 4).

From the results of sequential digestion (Table 3) it was possible to deduce the clockwise linkage N2-N1. The corresponding junction site could be located in the *Ascl* map, since A3 was cleaved by *NotI* into two fragments, A3-N1 and A3-N2, of 153 kb and 67 kb, respectively. As two linking clones, lc-4 and lc-36 (Table 4), identified the linkages N2-N5 and N5-N6, the clockwise linkage N6-N5-N2-N1 was also deduced.

To identify other linkages between *NotI* bands, macro-restriction fragments obtained with this enzyme from a strain with a different profile (Tenreiro *et al.*, 1994), *O. oeni* GM, were used as probes. Hybridization with fragment N1^{GM} (690 kb) indicated that N3 is adjacent to N1, whereas a composite probe containing fragments N8^{GM} and N9^{GM} (both 63 kb) hybridized with five *NotI* (N1, N2, N3, N8 and N11) and three *Ascl* (A1, A2 and A3) PSU-1 fragments (Table 4). As linkages N1-N2 and N1-N3 have been established, targets of this composite probe should be localized far apart and separated by a large portion of N1. Thus, one of them must hybridize with N2-N1 and A2-A3, and the other with A1 and N3, N8 and N11. The hybridization signal observed for PSU-1 fragment N8 (44 kb) was stronger than those observed for N3 and N11, suggesting the N3-N8-N11 linkage.

Fig. 1. *Ascl* partial digestion fragments of the *O. oeni* PSU-1 chromosome separated by PFGE. Lanes 1 and 8, lambda DNA PFG ladder; lanes 2-7, PSU-1 DNA cleaved by *Ascl* using 2 U overnight (lane 2), 5 U for 1 h (lane 3), 5 U for 2 h (lane 4), 10 U for 30 min (lane 5), 10 U for 1 h (lane 6), 10 U overnight (lane 7). running conditions: pulse time ramped from 5 to 120 s over 22 h at 210 V (5-10 s/5 h; 10-30 s/6 h; 30-60 s/4 h; 60-80 s/4 h; 80-120 s/3 h); 1% (w/v) SeaKem GTG agarose (FMC) in 0.5 × TBE; CHEF system (Gene Navigator). Bands of complete and partial digestion are referred to as described in Methods.

Since fragments N7 and N10 were located in A2 (Table 3), they must be positioned between N9 and N6, although their relative order could not be ascertained. Altogether, the clockwise linkage N9-(N7,N10)-N6-N5-N2-N1-N3-N8-N11 was deduced.

Cross-hybridization with fragments N4^{GM} and N10^{GM} revealed that the first probe hybridized with N4, S2 and S6, whereas the second hybridized with N9, S6 and S1 (Table 4). As the linkage S2-S6-S1 was established from *SfiI* physical mapping (see below) and PSU-1 fragment N12 hybridized with S2 (Table 4), the clockwise order of *NotI* fragments N1-N3-N8-N11-N12-N4-N9-(N7,N10)-N6-N5-N2 was established, as shown in Fig. 5.

Fig. 2. Two-dimensional PFGE separation of the DNA fragments generated by *NotI*-*AscI* and *AscI*-*NotI* double digestion of *O. oeni* PSU-1 DNA. Migration parameters were identical to those described in Fig. 1. Lane 1, lambda DNA PFG ladder; lanes 2–8, *NotI* fragments isolated from the first dimension and digested with *AscI*; lane 9, *AscI*-digested genomic DNA; lane 10, *AscI*-*NotI* double-digested genomic DNA; lanes 11–14, *AscI* fragments isolated from the first dimension and digested with *NotI*; lane 15, *NotI*-digested genomic DNA; lane 16, *Saccharomyces cerevisiae* chromosomes. The letters under the lanes refer to the fragments (described in Table 2) produced by the endonuclease used in the first digestion.

Alignment of *SfiI* fragments

The analysis of double and sequential digestions with *AscI*, *NotI* and *SfiI* (Fig. 3, Table 3) made possible the allocation of *SfiI* fragments to the *AscI* and *NotI* physical map, except for S13, S15 and S16. As fragments S3 and S8 were cleaved by *AscI* and partially contained in A3, two *AscI* sites were assigned to these fragments and the linkage S8-S3 was deduced. The position in the *AscI* map of the *SfiI* site between these fragments was obtained from the estimated sizes of double-digestion fragments, A3-S3 (146 kb) and A3-S8 (74 kb). The other two *AscI* sites were located in S12 and S6 by cross-hybridization with fragments N2^{GM} and N10^{GM} (Table 4), which also made evident the linkages S8-S3-S12-S5 and S6-S1. Assignment of fragments S9, S11 and S14 to A2-N2, fragments S4, S7 and S10 to A1-N1, and fragment S2 to N3, was sustained both by *AscI*-*SfiI* and *NotI*-*SfiI* double and sequential digestions.

Using fragments S15 and S16 as probes, their positions were assigned to N3 and A2-N2, respectively (Table 4). As fragment S13 was cleaved by *NotI* and fragment N1^{GM} hybridized with S13, N1 and N3, fragment S13 must contain the *NotI* site between N1 and N3. The clockwise linkage S6-S1-(S9, S11, S14, S16)-S8-S3-S12-S5-(S4, S7, S10)-S13-S15-S2 was thus deduced.

Cross-hybridization with fragment N4^{GM} confirmed the linkage S2-S6, whereas probes S6^{GM} and S17^{GM} confirmed the location of fragments S7 and S10 in N1.

Taking into account that the linkages S1-S11 and S5-S4 were obtained from composite probe S1^{GM}/S2^{GM} (Table 4), a further alignment of *SfiI* fragments was performed, leading to the linkage S8-S3-S12-S5-S4-(S7, S10)-S13-S15-S2-S6-S1-S11-(S9, S14, S16).

As shown in Fig. 3 and Table 4, the *rrs* probe hybridized with fragments S2 and S14 and *FseI* fragments F1, F3 and F5. Associated with the *FseI* map described below, these results led to the *SfiI* physical map presented in Fig. 5. In this map, the relative order of fragments S7 and S10 remains unknown, as does the exact position of fragment S16. Nevertheless, this fragment must be located in the region between S11 and S8 (Fig. 5) because it hybridizes with N2 (Table 4).

Alignment of *FseI* fragments and physical map of *O. oeni* PSU-1

Analysis of the *FseI*-*NotI* double digestion (see Fig. 4) showed that fragments F1, F2 and F3 disappeared as well as N1, N2 and N3, allocating all the restriction sites of both enzymes to these fragments. From results of cross-hybridization with fragments A4 (Fig. 4b, Table 4) and S11/S12 (Table 4), the clockwise linkage F6-F4 was established and fragments S11 and S12 were assigned to F1 and F4, respectively. Using fragment N1^{GM} as probe (Table 4), the linkage F6-F4-(F2, F5, F8)-F1 was deduced. When *rrs* was used as probe, hybridization signals were obtained in F1, F3 and F5 (Table 4), revealing that F1

Table 3. *AscI*, *NotI* and *SfiI* double restriction fragments of *O. oeni* PSU-1 chromosome

First enzyme	Fragment*	Products after digestion with the second enzyme†
<i>AscI</i>	A1	<i>NotI</i> : A1-N1 (515), N3, N4, N8, A1-N9 (25), N11, N12‡ <i>SfiI</i> : S2, S4, S5, A1-S6 (123), S7, S10, A1-S12 (39), S13‡, S15‡
	A2	<i>NotI</i> : A2-N2 (198), N5, N6, N7, A2-N9 (19), N10 <i>SfiI</i> : S1, S9, S11, S14, A2-S8 (26), A2-S6 (7)‡, S16‡
	A3	<i>NotI</i> : A3-N1 (153), A3-N2 (67) <i>SfiI</i> : A3-S3 (146), A3-S8 (74)
	A4	<i>NotI</i> : A4 <i>SfiI</i> : A4-S3 (125), A4-S12 (5)‡
<i>NotI</i>	N1	<i>AscI</i> : N1-A1 (515), N1-A3 (153), A4 <i>SfiI</i> : S3, S4, S5, S7, S10, S12, N1-S8 (8)‡, N1-S13 (6)‡
	N2	<i>AscI</i> : N2-A2 (198), N2-A3 (67) <i>SfiI</i> : N2-S8 (92), S9, S11, S14, N2-S1 (16)‡, S16‡
	N3	<i>AscI</i> : N3 <i>SfiI</i> : N3-S2 (198), N3-S13 (28)‡, S15‡
	N4	<i>AscI</i> : N4 <i>SfiI</i> : N4-S6 (98)§, N4-S2 (37)‡
	N5	<i>AscI</i> : N5 <i>SfiI</i> : N5
	N6	<i>AscI</i> : N6 <i>SfiI</i> : N6
	N7	<i>AscI</i> : N7 <i>SfiI</i> : N7
	N8	<i>AscI</i> : N8 <i>SfiI</i> : N8
	N9	<i>AscI</i> : N9-A1 (25), N9-A2 (19) <i>SfiI</i> : N9-S6 (33)§, N9-S1 (11)‡
	(N10)	<i>AscI</i> : N10 <i>SfiI</i> : N10
	(N11)	<i>AscI</i> : N11 <i>SfiI</i> : N11
	(N12)	<i>AscI</i> : N12 <i>SfiI</i> : N12
<i>SfiI</i>	S1	<i>AscI</i> : S1‡ <i>NotI</i> : N5, N6, N7, S1-N2 (16)‡, N10‡, S1-N9 (11)
	S2	<i>AscI</i> : S2 <i>NotI</i> : S2-N3 (198), N8, S2-N4 (37)‡, N11‡, N12‡
	S3	<i>AscI</i> : S3-A3 (146), S3-A4 (125) <i>NotI</i> : S3
	S4	<i>AscI</i> : S4 <i>NotI</i> : S4
	S5	<i>AscI</i> : S5 <i>NotI</i> : S5
	S6	<i>AscI</i> : S6-A1 (123), S6-A2 (7)‡ <i>NotI</i> : S6-N4 (98)§, S6-N9 (33)§
	S7	<i>AscI</i> : S7 <i>NotI</i> : S7
	S8	<i>AscI</i> : S8-A3 (74), S8-A2 (26) <i>NotI</i> : S8-N2 (92), S8-N1 (8)‡
	S9	<i>AscI</i> : S9 <i>NotI</i> : S9
	S10	<i>AscI</i> : S10 <i>NotI</i> : S10
	(S11)	<i>AscI</i> : S11 <i>NotI</i> : S11

Table 3. (cont.)

First enzyme	Fragment*	Products after digestion with the second enzyme†
	(S12)	<i>AscI</i> : S12-A1 (39), S12-A4 (5) <i>NotI</i> : S12
	(S13)	<i>AscI</i> : S13 <i>NotI</i> : S13-N3 (28), S13-N1 (6)
	(S14)	<i>AscI</i> : S14 <i>NotI</i> : S14
	(S15)	<i>AscI</i> : S15 <i>NotI</i> : S15
	(S16)	<i>AscI</i> : S16 <i>NotI</i> : S16

* Fragment sizes are presented in Table 2. Cleavage products from fragments in parentheses were deduced from double digestions in one-dimensional PFGE. Otherwise, separation of products from the second digestion was performed by two-dimensional PFGE.

† Numbers in parentheses refer to theoretical sizes in kb obtained after location of restriction sites on the physical map.

‡ Fragments not observed in sequential digests (2D PFGE).

§ Fragments observed in double and/or sequential digests that were assigned to the second enzyme only after hybridization.

linked F3 with F5. A hybridization signal with this gene probe was also observed in a partial digestion fragment smaller than F2 (approximately 320 kb; data not shown), suggesting the linkage of F7 (75 kb) to F3 (240 kb). This probe further hybridized to the double-digestion fragment A2-F3 (120 kb; data not shown) providing the necessary information to locate the F1-F3 joining site in the *AscI* physical map.

The last piece of the puzzle came from the double digestion *FseI*-*SfiI*. In fact, S4 and S5 were both cleaved with *FseI* (Fig. 4), allocating F8 to the linkage S4-S5 and indicating the clockwise order F4-F8-F2-F5-F1-F3-F7-F6.

The physical map of the PSU-1 chromosome obtained with the four enzymes referred to above is shown in Fig. 5. The observed sizes of the restriction fragments were corrected to map sizes (Table 2), to agree with all the data from hybridization and double and sequential digestions.

Analysis of macrorestriction sites on the PSU-1 genome showed that map intervals, defined by two consecutive sites, varied between 5 kb (F5-S13) and 151 kb (F2-S4).

Mapping of genetic markers in the PSU-1 chromosome

The probes described in Table 1 were used to locate six homologous and four heterologous genes on the physical map of the *O. oeni* PSU-1 genome (Fig. 5). The probes have been assigned to the restriction fragments to which they hybridized (Table 4), although their exact positioning on the fragment is unknown. For probes that hybridized with the same fragment the relative posi-

tioning was arbitrary, not reflecting their real order in the chromosome. Only *rrn* gene probes were hybridized with *FseI*-digested DNA.

(i) ***rrn* operons.** *O. oeni* strains contain two *rrn* operons on their chromosome, with the typical 5'-*rrs*-*rrl*-*rrf*-3' organization found in the majority of bacteria (Zavaleta *et al.*, 1996). According to the physical map (Fig. 5) and the hybridization results obtained with *rrs* and *rrl* probes on PSU-1 DNA digested with *AscI*, *NotI* or *SfiI* (Table 4), the two operons must be located in fragments S2-N3 and S14, which are at least 600 kb apart. When the *rrs* published sequence (Martinez-Murcia & Collins, 1990) was screened for the presence of restriction sites, one *FseI* cutting site was found at position 324, allowing a more precise location of *rrn* genes. In addition, hybridization results obtained on *FseI* digests revealed three positive fragments with the *rrs* probe (F1, F3 and F5) and only one with the *rrl* probe (F1), showing that *rrs* genes are 692 kb apart and that the operons have opposite orientation in the PSU-1 chromosome, with *rrnA* transcribed clockwise and *rrnB* counterclockwise (Fig. 5).

(ii) **Oenococcal genes.** The *mleA* gene, which encodes the malolactic enzyme and belongs to the *mle* locus (Labarre *et al.*, 1996), and the *alsS/alsD* genes, encoding α -acetolactate synthase and decarboxylase (Garmyn *et al.*, 1996), were located in fragments S7 and S4 respectively.

A fragment of PSU-1 DNA cloned in pMK4 (Sullivan *et al.*, 1984) and conferring mitomycin C resistance upon a *recA* *E. coli* strain was obtained in our laboratory (Brito, 1996). The locus responsible for this phenotype (*mir*, for mitomycin resistance) was located in fragment N7 (Fig. 5).

(iii) **Heterologous genes.** Since very few genes of *O. oeni*

Table 4. Assignment of hybridization probes to restriction fragments of *O. oeni* PSU-1 chromosome

Fragment or marker used as probe	Hybridizing fragment(s)			
	<i>AscI</i>	<i>FseI</i>	<i>NotI</i>	<i>SfiI</i>
Genetic markers				
<i>rrs</i>	A1, A2	F1, F3, F5	N2, N3	S2, S14
<i>rrl</i>	A1, A2	F1	N2, N3	S2, S14
<i>mir</i>	A1	NT	N7	S1
<i>mleA</i>	A1	NT	N1	S7
<i>alsS/alsD</i>	A1	NT	N1	S4
<i>recA</i>	A1	NT	N3	S2
<i>IS1165</i>	A1, A2	NT	N1, N2, N3	S1, S2, S4, S8
<i>citP</i>	A3	NT	N1	S8
<i>araA</i>	A4	NT	N1	S3
Other markers*				
<i>attB1</i>	A3	F3	N2	S8
<i>attB2</i>	A2	F1	N2	S11
lc-4	A2	NT	N5, N6	S1
lc-36	A2	NT	N2, N5	S1
PSU-1 DNA fragments				
A3	A3	NT	N1, N2	S3, S8
A4	A4	F4, F6	N1	S3
N4/5	A1, A2	NT	N4, N5	S1, S2, S6
N11	A1	F1	N11	S2
N12	A1	NT	N12	S2
S11/S12	A1, A2	F1, F4	N1, N2	S11, S12
S15	A1	NT	N3	S15
S16	A2	NT	N2	S16
GM DNA fragments				
N1	A1	F1, F2, F4, F5, F8	N1, N3	S2, S4, S5, S7, S10, S13, S15
N2	A1, A3, A4	NT	N1	S3, S5, S12
N4	A1	NT	N4	S2, S6
N5	A2	NT	N5	S1
N8/N9	A1, A2, A3	NT	N1, N2, N3, N8, N11	S2, S3, S8
N10	A1, A2	NT	N9	S1, S6
N11	A1	NT	N1	S5
S1/S2	A1, A2	NT	N1, N2, N5, N6, N7, N9, N10	S1, S4, S5, S11, S12
S6	A1	F2	N1	S7
S15	A2	NT	N2	S14
S17	A1	F2	N1	S10
S18	A1, A4	NT	N1	S12

NT, Not tested.

* *attB1* and *attB2* refer to attachment sites of phage fOg44 and were localized on *AscI*, *NotI* and *SfiI* fragments by hybridization of phage DNA to lysogenic derivatives of strain PSU-1 (Santos, 1995; Santos *et al.*, 1996). Assignment to *FseI* fragments resulted from size shifting analysis as described. lc-4 and lc-36 represent *NotI* linking clones.

have been described, gene probes from other Gram-positive bacteria with low G+C content were used under low-stringency hybridization conditions (Table 1). The map location of these genes is also presented in Fig. 5.

(iv) **Phage attachment sites.** Although PSU-1 is a phage-free strain, lysogenic derivatives have been constructed (Santos *et al.*, 1996). Two attachment sites for phage fOg44 (*attB1* and *attB2*) were previously assigned to *AscI*, *NotI* and *SfiI* macrorestriction fragments (Santos,

Fig. 3. (a) Single and double digestion profiles of *O. oeni* PSU-1 chromosomal DNA obtained with *Ascl*, *NotI* and *SfiI*. Lane 1, lambda DNA PFG ladder; lane 2, *Ascl*; lane 3, *Ascl-NotI*; lane 4, *NotI*; lane 5, *NotI-SfiI*; lane 6, *SfiI*; lane 7, *SfiI-Ascl*. Running conditions: pulse time from 1 to 9 s at 180 mA for 5 h, followed by 10–30 s at 90 mA for 15 h; 1% (w/v) SeaKem LE agarose (FMC) in 1× TAE; TAFE system (Geneline). compr., compression zone for DNA. (b) Southern blot of gel in (a) hybridized with *rrs* probe from *O. oeni* L217^T.

Fig. 4. (a) *FseI* single and double restriction profiles of *O. oeni* PSU-1 chromosomal DNA. Migration parameters were identical to those described in Fig. 1. Lane 1, lambda DNA PFG ladder; lane 2, *Ascl*; lane 3, *FseI-Ascl*; lane 4, *FseI* (partial fragment F3-F7 is shown by an arrow); lane 5, *FseI-NotI*; lane 6, *NotI*; lane 7, *SfiI*; lane 8, *FseI-SfiI*; lane 9, *FseI*. (b) Southern hybridization of gel in panel (a) using fragment A4 from *O. oeni* PSU-1 as probe.

1995; Santos *et al.*, 1996). These results have been confirmed in the present study (Table 4), which extended the localization of *attB1* and *attB2* to the *FseI* map. In fact, a 40 kb size increase (corresponding to the phage genome size; Santos *et al.*, 1996) was observed in

fragment F3 for a single lysogen, whereas both F3 and F1 increased by this amount in a double lysogen (data not shown). The position of the phage attachment sites in these *FseI* fragments is in agreement with mapping results described above.

Fig. 5. Physical map of the chromosome of *O. oeni* PSU-1 using the enzymes *Ascl*, *FseI*, *NotI* and *SfiI*. Radiating out from the centre, the four annuli show the restriction sites for the four respective enzymes. Restriction fragments are identified as shown in Table 2; the scale is in kb. Fragments N7 and N10 could not be oriented, nor could S7 and S10. Fragment S16 is not shown on the map as its position was not assessed with precision. The locations of several 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 an arrow.

DISCUSSION

A physical map of the genome of *O. oeni* PSU-1 has been constructed using rare-cutting enzymes, PFGE and hybridization techniques. To our knowledge this is the first genome restriction map of a strain of this species. The chromosome of *O. oeni* PSU-1 was shown to be circular, as described for the majority of known bacterial genomes (Cole & Saint-Girons, 1994; Krawieck & Riley, 1990), with an estimated size of 1857 kb. This value is in close agreement with previous determinations (Tenreiro *et al.*, 1994), although size corrections smaller than 4% were applied to some restriction fragments over 130 kb to integrate all the data in a coherent map.

This genome size is at the lower extreme of the range described for lactic acid bacteria (3.4–1.75 Mb, as reviewed by Davidson *et al.*, 1996). Such a small genome could reflect the huge requirement for growth factors and the limited biochemical complexity of these Gram-

positive bacteria (Le Bourgeois *et al.*, 1993; Tulloch *et al.*, 1991), as well as the specific adaptation to an almost non-fluctuating ecosystem (Roussel *et al.*, 1994).

Four restriction endonucleases, *Ascl*, *FseI*, *NotI* and *SfiI*, recognizing eight GC-pair sequences, were employed and 37 restriction sites were mapped using several experimental approaches. Analysis of partial digestion products led to the complete *Ascl* physical map, whereas the majority of *NotI* and *SfiI* fragments have been assigned to the overlapping *Ascl* fragments by two-dimensional PFGE. To improve the visualization and assignment of fragments resulting from the secondary cleavage, which are sometimes hindered by the punctiform appearance of these fragments with the 2D techniques commonly used (Bautsch, 1988; Davidson *et al.*, 1992; Tulloch *et al.*, 1991), bands from the first dimension were individually excised and digested, rather than being collectively processed in a single agarose slice (see Methods). Mapping of *NotI*, *SfiI* and *FseI* sites was

achieved mostly by hybridization, by using either linking clones or fragments generated by a different enzyme. The use of random-labelled macrorestriction fragments from a different *O. oeni* strain proved to be a useful additional hybridization strategy owing to the different positioning of restriction sites in the two strains. The occurrence of rearrangements such as inversions of large genomic segments, which could lead in these experiments to wrong assumptions concerning fragment linkage, has been ruled out by the observed internal consistency with data obtained from other approaches. A globally similar genetic organization in *O. oeni* strains exhibiting fragment length polymorphisms is not unexpected, as several lines of evidence have highlighted the homogeneous nature of this species (Zavaleta *et al.*, 1996, 1997).

Although similar-sized fragments were observed with both *NotI* and *SfiI*, their allocation was not difficult since at least one enzyme cleaved only one fragment of the doublet. Three restriction sites could not be precisely positioned on the physical map. Nevertheless, one leads to an *SfiI* fragment of 6 kb with three possible positions in an approximately 160 kb region of the PSU-1 chromosome, whereas alternative positions less than 50 kb apart were assigned for the remaining two.

The localization of 10 genetic markers on the physical map represents a first step towards the construction of a genetic map of the *O. oeni* chromosome. Homologous markers included the genes for malolactic enzyme (*mleA*), α -acetolactate synthase (*alsS*) and α -acetolactate decarboxylase (*alsD*), formerly described in another strain of this species (Garmyn *et al.*, 1996; Labarre *et al.*, 1996), and a cloned fragment of PSU-1 DNA conferring resistance to mitomycin C in *E. coli* (*mir*). The two *rrn* operons reported in the *O. oeni* genome (Zavaleta *et al.*, 1996) were also mapped by using homologous *rrs* and *rrl* probes. As gene organization follows the typical *rrs-rrl-rrf* pattern and *FseI* cleaves the *rrs* sequence of *O. oeni* at a unique site, an accurate positioning and orientation of *rrn* operons was obtained, with *rrnA* transcribed clockwise and *rrnB* counterclockwise. Assuming that chromosome replication and *rrn* transcription are processed in the same direction as generally found (Davidson *et al.*, 1996; Krawieck & Riley, 1990), the origin of replication in *O. oeni* is probably located in the larger region separating the two operons.

Since very few genes of *O. oeni* have been studied, probes from other low-G+C Gram-positive bacteria were used under low-stringency conditions, to minimize the effects of DNA sequence divergence. These heterologous genes (*recA*, *citP* and *araA*) allowed the identification of PSU-1 DNA sequences which may have a similar coding function. Although no definite assumption can be made in this regard, it is worth noting that the relatively conserved nature of *recA* from different species has been established (Eisen, 1995), and the PSU-1 strain in our collection is able to transport citrate and grow on arabinose (unpublished data).

According to a previous report of IS1165 or related

elements in strains of *O. oeni* (Johansen & Kibench, 1992), we found that four segments in the PSU-1 genome hybridized with the IS1165 probe. The phage attachment sites *attB1* and *attB2* previously identified in strain PSU-1 (Santos *et al.*, 1996) were mapped in this work in a 265 kb region which also contains *rrnB* and two putative IS1165 elements.

The physical map of strain PSU-1 reported here has a good resolution for positioning genes, since the majority of the map intervals (62.5%) are smaller than 50 kb and only six fall within 100–200 kb, the minimum acceptable range (Cole & Saint-Girons, 1994). This distribution of restriction sites clearly represents an advantage for the construction of ordered libraries that will be extremely useful for the genomic characterization of this organism by gene sequencing.

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