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Intraspecific Genetic Diversity of *Oenococcus oeni* as Derived from DNA Fingerprinting and Sequence Analyses

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The intraspecific genetic diversity of *Oenococcus oeni*, the key organism in the malolactic fermentation of wine, has been evaluated by random amplified polymorphic DNA (RAPD), ribotyping, small-plasmid content, and sequencing of RAPD markers with widespread distribution among the strains. Collection strains representing the diversity of this species have been studied together with some new isolates, many of which were obtained from wines produced by spontaneous malolactic fermentation. The RAPD profiles were strain specific and discerned two main groups of strains coincident with clusters obtained by macrorestriction typing in a previous work. Ribotyping and the conservation of RAPD markers indicates that *O. oeni* is a relatively homogeneous species. Furthermore, identical DNA sequences of some RAPD markers among strains representative of the most divergent RAPD clusters indicates that *O. oeni* is indeed a phylogenetically tight group, probably corresponding to a single clone, or clonal line of descent, specialized to grow in the wine environment and universally spread.

Leuconostoc oenos is the only acidophilic member of the genus *Leuconostoc* and occurs naturally in wine and related habitats (8, 12, 13). This species is the one most frequently associated with malolactic fermentation, and the growing awareness of its contribution to wine flavor and complexity has stimulated the development of cultures to be used as starters in wine making (18). Differentiation of *L. oenos* strains becomes a major concern, and reliable methods are required for certifying the identity of exchanged strains with diverse origins to monitor the survival of inoculated and indigenous bacteria. Results of chromosomal DNA-DNA (3–5, 10, 15, 30, 32) and rRNA-DNA (11, 30) hybridizations and recent 16S and 23S rRNA sequence analyses (20–22) have suggested that *L. oenos* is worthy of a separate generic status. The name *Oenococcus oeni* [corrig.] gen. nov., comb. nov. has been proposed (2) and will be used below. Several studies have generated controversial data related to the intraspecific taxonomic structure of *O. oeni*. Lactate dehydrogenases, carbohydrate fermentation, and cellular fatty acid patterns have shown considerable diversity among strains of *O. oeni* (8, 9, 12, 13, 35, 36). Pulsed-field gel electrophoresis (PFGE) has shown the existence of 20 genomic patterns in *O. oeni*, and the strains used fell in two major groups (34). However, analysis of total soluble cell proteins and DNA-DNA hybridization studies indicated that *O. oeni* is genomically homogeneous (3, 4). Recent sequence analysis of the 16S–23S rDNA intergenic spacer region (ISR) in 37 *O. oeni* strains has demonstrated that this species is phylogenetically homogeneous (42). The ISR sequences may be useful in separating *O. oeni* from other *Leuconostoc* species, but more sensitive methods are required for strain identification.

Arbitrarily primed PCR or random amplified polymorphic DNA (RAPD) is a very useful technique for typing the genomes of bacteria (39, 40), and it has been used to characterize microorganisms at the strain level (7, 23, 37), as well as to generate species-specific oligonucleotide probes with known sequences (24, 38). As identical RAPD patterns (for a considerable number of randomly chosen primers) are expected only from duplicates of the same strain, the use of this rapid method to evaluate strain authenticity has been suggested (25).

In the present study, patterns and sequence analyses of DNA fragments randomly amplified from 70 strains of *O. oeni* were assessed to investigate global genomic similarity at the intraspecific level and to develop a sensitive method for strain identification.

MATERIALS AND METHODS

Bacterial strains and cultivation. The bacterial strains used in this study are listed in Table 1. A total of 37 strains of *O. oeni*, representing isolates from widely different origins that have been used in several previous studies, were obtained from the culture collections (3, 4, 42). An additional set of 33 *O. oeni* strains were isolated in our laboratory (see below) from wine samples obtained from Spain, Peru, and Chile. Two isolates were obtained from each sample (with the exception of the Chilean wine), and the geographic origin is shown in Table 1. The samples from Cigales (Valladolid, Spain) were provided by the Consejo Regulador de la Denominacion de Origen (the institution that guarantees the origin of the wine), and each represents a different winery located in this region. All the Cigales and Peruvian samples were reported to be produced by spontaneous malolactic fermentation (no starter inoculum used). All strains of *O. oeni* were grown at 25°C in MLOS broth (ADSA MICRO, Barcelona, Spain) supplemented with 10% (vol/vol) tomato juice.

Isolation and RFLP-ISR analysis. New strains of *O. oeni* from wine were isolated by the method of Beelman et al. (1) with some modifications. Samples (200 μl) of wine were plated onto MLOS agar with 75 μg of cycloheximide (Sigma) per ml and incubated at 25°C in an anaerobic jar for 8 days. Two colonies of each sample were isolated and grown in tubes with MLOS broth at pH 3.5 and 10% ethanol–MLOS broth at pH 4.8. The 16S-23S rDNA of these isolates was amplified and characterized by digestion with *Taq*I as previously described (42).

Plasmid extraction. The alkali lysis procedure was used as described by Sambrook et al. (28) with the following modifications. A 10-ml volume of estimated late-log-phase culture of *O. oeni* was centrifuged at 2,000 × *g* at 4°C for 30 min, and the pellet was washed with 1.5 ml of water. The cells were resuspended in 200 μl of 50 mM glucose–50 mM Tris-HCl (pH 8.0)–10 mM EDTA–10 mg of lysozyme per ml and incubated at 37°C for 2 h. Then 200 μl of 0.2 N NaOH–1% sodium dodecyl sulfate (SDS) was added and gently mixed by inverting the tube several times. After this, 200 μl of 3 N sodium acetate (pH 4.8) was added and mixed, and the mixture was left for 60 min. The chromosomal DNA, RNA, and

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TABLE 1. Strains of *O. oeni* and selected characteristics

Strain(s)[a]	Origin[b]	Ribotype	RAPD group	Plasmid pattern
NCDO1668	France	B	B	—[c]
NCDO1669	France	B	B	I
NCDO1670, NCDO1705	France	B	B	—
NCDO1706	France	B	B	III
NCDO1707	France	B	B	IV
NCDO1708	France	B	B	V
NCDO1709	France	B	B	IV
CECT4028	France	B	B	IV
NCDO1671, NCDO1672, NCDO1673, NCDO1674, NCDO1879	France	A	A	—
NCDO1675	France	A	A	II
NCDO1694, NCDO1695, NCDO1696	Germany	B	B	—
CECT4029	Germany	B	B	—
NCDO1821, NCDO1822	United States	B	B	—
NCDO1823 (ML34)	United States	B	B	—
CECT218	United States	A	A	—
NCDO1883, NCDO1884	Australia	A	A	—
NCDO1890, NCDO1893, NCDO1894	Italy	A	A	VI
NCDO1892, NCDO1895	Italy	A	A	—
NCDO1891	Italy	B	B	VI
NCDO1983	Unknown	A	A	—
NCDO2119, NCDO2122	Japan	B	B	VI
NCDO2120	Japan	B	B	—
NCDO2121	Japan	B	B	IV
NCDO2123	Japan	A	A	—
A5	Spain	A	ND[d]	VII
A7	Spain	A	A	VIII
Ch2	Spain	A	ND	—
Ch6	Spain	A	A	—
D1	Spain	A	A	—
D2	Spain	A	ND	—
P8	Spain	A	ND	—
P9	Spain	A	A	—
R12	Spain	A	A	—
R15	Spain	A	ND	V
F2, M2, A13, A21, A31	Spain	A	A	—
502, 573, 801, 811, 832	Spain	A	A	—
F6, M5, A12, A22, A32	Spain	A	ND	—
501, 571, 802, 812, 831	Spain	A	ND	—
C11, C22	Peru	A	A	—
Co1	Chile	A	A	IX

[a] NCDO, National Collection of Diary Organisms, Reading, United Kingdom; NCFB, National Collection of Food Bacteria, Reading, United Kingdom; CECT, Colección Española de Cultivos Tipo, Valencia, Spain.

[b] Origin of the isolates: A5 and A7, Rivadavia (Orense); Ch2, Ch6, Chinchilla (Albacete); D1, D2, Roa (Burgos); F2, F6, Pesquera de Duero (Valladolid); M2, M5, Quintanilla Onésimo (Valladolid); P8, P9, Pinoso (Alicante); R12, R15, Requena (Valencia); A**, 5**, 8**, Cigales (Valladolid); C11, C22, Contumazá (Peru); Co1, Valle de Maipó (Chile). (information obtained from NCFB and CECT catalogs).

[c] —, no plasmids.

[d] ND, not determined.

cell debris were sedimented at 14,000 rpm for 5 min. Plasmid DNA was extracted twice with 1 volume of phenol-chloroform (1:1, vol/vol), precipitated with isopropanol, incubated at −20°C for 1 h, washed with 70% ethanol, and resuspended in 50 μl of TE (10 mM Tris-HCl [pH 8], 1 mM EDTA). A 2-μl volume of RNase (500 μg/ml) was added, and the mixture was incubated at 37°C for 30 min. A 10-μl volume of DNA solution was analyzed by electrophoresis on a 0.7% (wt/vol) agarose–TAE (40 mM Tris-acetic acid [pH 8], 1 mM EDTA) gel.

DNA extraction and purification. Genomic DNA from *O. oeni* was extracted by the following procedure. A 5-ml volume of a late-log-phase culture was harvested, washed in 50 mM Tris-HCl (pH 8)–10 mM EDTA, and resuspended in 0.7 ml of the same buffer. Lysozyme was added to a final concentration of 10 mg/ml, and the reaction mixture was kept at 37°C for 1 h. Then 80 μl of 10% (wt/vol) SDS and 5 μl of 500-μg/ml RNase were added, and the mixture was incubated at 37°C for 1 h. The homogenate was digested by the addition of 6 μl of 20-mg/ml proteinase K, and the mixture was incubated at 50°C for 5 h. DNA was extracted twice with 1 volume of phenol-chloroform (1:1), precipitated with isopropanol, and washed with 70% (vol/vol) ethanol. The DNA was then resuspended in 200 μl of TE buffer. The concentration and purity (with respect to the protein content) of the DNA obtained were estimated by using the GeneQuant DNA/RNA Calculator (Pharmacia).

Oligonucleotide primers. The nucleotide sequences and characteristics of the primers used are listed in Table 2. Primers A1, A8, A10, A11, and A22 were synthesized in an Applied Biosystems 396 DNA/RNA synthesizer. The products were incubated at 55°C overnight, vacuum dried, and resuspended in 400 μl of sterile distilled water. The concentration was estimated by using the GeneQuant DNA/RNA Calculator. Primers OPA9, OPA11, OPA12, OPA16, and OPA20 were obtained from Operon-Biotechnologies.

Ribotyping. Total DNA was digested with *Hin*dIII or *Eco*RI, and the products were separated by electrophoresis with 0.8% agarose in 1× TAE buffer at 60 V for 5 h. The gels were stained with ethidium bromide and photographed. Digested DNA was transferred to Hybond-N nylon membrane (Amersham) by the method of Southern (33). Probe 16S rDNA was obtained by PCR from *O. oeni* NCDO1674^T and labelled with ^{32}P as previously described (42). Hybridization was performed overnight at 65°C in 0.1% SDS–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The membranes were washed twice for 10 min each at 65°C in 0.1% SDS–2× SSC, twice in 0.1% SDS–1× SSC, and twice in 0.1% SDS–0.1× SSC under the same conditions. The blots were exposed to Hyperfilm-MP (Amersham) overnight at −70°C.

RAPD. Approximately 50 ng of total DNA was subjected to PCR amplification in a reaction mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 9.0), 0.1% (vol/vol) Triton X-100, 2 mM $MgCl_2$, 200 μM each deoxyribonucleotide (dATP, dCTP, dGTP, and dTTP; Pharmacia LKB Biotechnology), 100 pmol of each primer (A1, A8, A10, A11, and A20) or 15 pmol of each primer (OPA9, OPA11, OPA12, OPA16 and OPA20; Operon Technologies, Inc.), and 2 U of *Taq* DNA polymerase (Promega Corp.) in a final volume of 25 μl. The reaction mixtures were overlaid with mineral oil (light white oil; Sigma) and subjected to amplification for 40 cycles in an OmniGene thermocycler (Hybaid); conditions were 94°C, 1 min; 36°C, 1 min; and 72°C, 2 min, with a final extension step at 72°C for 7 min. Negative controls with no addition of template DNA were included. Aliquots (5 μl) of PCR products were electrophoresed in 1.2% (wt/vol) agarose–TBE (90 mM Tris-borate [pH 8], 2 mM EDTA) gels stained with ethidium bromide and photographed with Polaroid. The size marker was the 1-kb DNA ladder (Gibco BRL).

Purification and cloning of the PCR products. Selected RAPD products were electrophoresed in 1% (wt/vol) agarose, and DNA fragments were recovered from the gel by using the GeneClean II kit (Bio 101), incubated with 2 U of Klenow polymerase at 37°C for 15 min, and purified with MicroSpin S-300 columns (Pharmacia). RAPD fragments were inserted into the *Sma*I site of pUC-18 vector (Pharmacia) by ligation at 16°C overnight. Competent *Escherichia coli* DH5-α was transformed by a standard method (28). Plasmid DNA was extracted with the Magic Miniprep DNA purification system (Promega).

Sequencing of RAPD fragments. The sequence of the cloned RAPD fragments was determined by the dideoxy-chain termination method (29) with the Cy5 AutoRead sequencing kit (Pharmacia). Both strands from two separately cloned RAPD fragments amplified in different PCR experiments were sequenced for each strain. The sequencing products were separated on the ALFexpress DNA sequencer (Pharmacia) as specified by the manufacturer.

Data analysis. The molecular size of the RAPD and ribotyping fragments (referred to as the number of base pairs) was estimated by using the GelBase Windows software for the UVP System 5000 (Ultra Violet Products). RAPD prints were converted to two-dimensional binary matrices. If a band was present, it was scored as positive at that location; if it was absent, it was assigned a negative score. Pairwise comparisons and constructed symmetrical similarity matrices were obtained by using the NTSYS-pc program (version 1.80; Exeter

TABLE 2. Characteristics of the arbitrary primers used in the RAPD analysis of *O. oeni* strains

Code	Sequence (5′-3′)	Size (bp)	% G+C content	RAPD print no.
A1	TGCGGCTTAC	10	60	10
A8	GTCGCCGAC	9	77.7	5
A10	GTAGACGAGC	10	60	5
A11	CAAACGGCAC	10	60	6
A22	ATGGACACCA	10	50	19
OPA9	GGGTAACGCC	10	70	10
OPA11	CAATCGCCGT	10	60	9
OPA12	TCGGCGATAG	10	60	8
OPA16	AGCCAGCGAA	10	60	10
OPA20	GTTGCGATCC	10	60	12

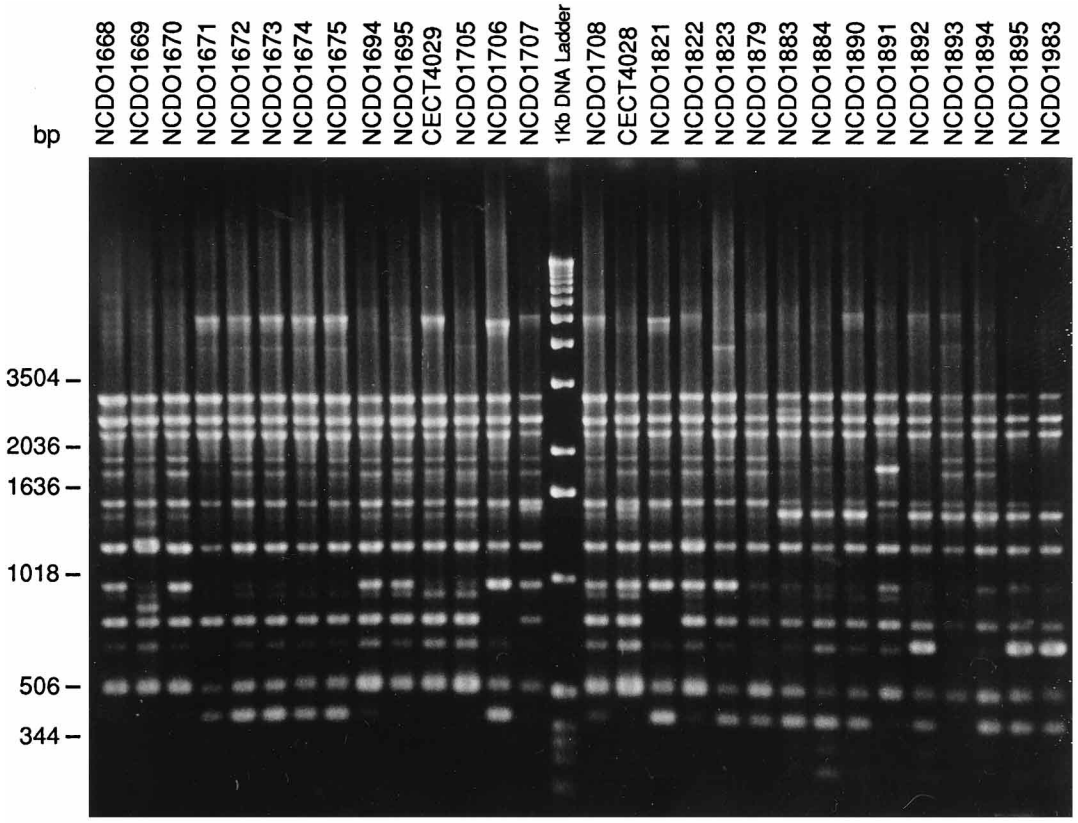

FIG. 1. RAPD patterns obtained with primer A22 and DNA template from *O. oeni* strains.

Software). Finally, a dendrogram from the RAPD similarity matrix was made by the unweighted pair group method with arithmetic near (UPGMA) in the NTSYS-pc program. Similarities between determined RAPD sequences and those from the EMBL data library were calculated with the FASTA program (27) of the Genetics Computer Group sequence analysis package.

Nucleotide sequence accession numbers. The sequences reported in this paper were deposited with the EMBL under the following accession numbers: X99959 for the OPA09-500 DNA fragment, X99958 for the OPA16-700 DNA fragment, X99955 for the OPA11-640A DNA fragment, X99957 for the OPA11-640B DNA fragment, X99954 for the OPA20-650 DNA fragment, and X99956 for the OPA20-560 DNA fragment.

RESULTS

Isolation and RFLP-ISR analyses. The isolates obtained from wine were tested for growth at low pH (3.5) and a relatively high concentration of ethanol (10%, vol/vol). Strains were characterized by restriction of the PCR-amplified 16S-23S rDNA with the endonuclease *Taq*I (42). The resulting pattern of the 33 isolates was identical to that obtained from the 37 *O. oeni* culture collection strains tested (results not shown). The isolates were considered to be *O. oeni* and were subjected to further studies.

Plasmid profiles. Low-molecular-weight plasmid DNA was examined in all strains of *O. oeni* available in this study; for some of the patterns obtained, see Fig. 4. Only 26% of the strains showed extrachromosomal DNA (Table 1). Previous reports have shown plasmids in 19% (16) and 8% (6) of analyzed strains. The analysis revealed nine different profiles, but this characteristic did not correlate with the RAPD and ribotype groupings. For instance, strains NCDO1890, NCDO1893, and NCDO1894, and strains NCDO1891, NCDO2119, and NCDO2122, which belonged to RAPD groups A and B, respectively, showed an identical plasmid pattern. However, isolates A5 and A7 (with more than 90% RAPD pattern similarity) showed a different content of plasmids. The results indicated that plasmid transfer between strains of group A and those of group B may occur.

Ribotyping. Ribopattern analysis with *Eco*RI or *Hin*dIII revealed three bands for all *O. oeni* strains. Two different groups of strains were defined on this basis. Ribotype A, containing *O. oeni* NCDO1674^T, showed bands of ca. 1.6, 2.5, and 4 kb for *Eco*RI and ca. 1, 5, and 12 kb for *Hin*dIII; and ribotype B, including *O. oeni* CECT4029, showed bands of ca. 1.6, 2.5, and 8 kb for *Eco*RI and ca. 1, 5, and 6 kb for *Hin*dIII. The assignment of ribotype group A or B to each strain is listed in Table 1. Eighteen strains isolated in this study and identified by the 16S-23S rDNA-RFLP method (42) belonged to ribotype group A.

RAPD analysis. PCR with DNA from *O. oeni* NCDO1674^T and CECT4029 and 47 different oligonucleotides of arbitrary sequence were performed to test the suitability of these sequences for the RAPD study. Ten primers generated at least five sharp RAPD prints (ranging from ca. 350 to 2,900 bp) and were selected for further experiments. The reproducibility of RAPD patterns was tested with the selected primers, and DNA was extracted from separate duplicates of three strains from two different collections. RAPD patterns obtained with primers OPA20 and A22 are shown in Fig. 1 and 2. A total of 94 RAPD prints obtained with the 10 selected primers were used as markers for pattern comparisons, and UPGMA-calculated relationships are presented in a dendrogram (Fig. 3). Although often very similar, most strains showed unique RAPD patterns. In fact, even isolates originating from the same wine samples showed different patterns, with the single exception of strains 831 and 832. This seems to reflect that there is more than one strain growing

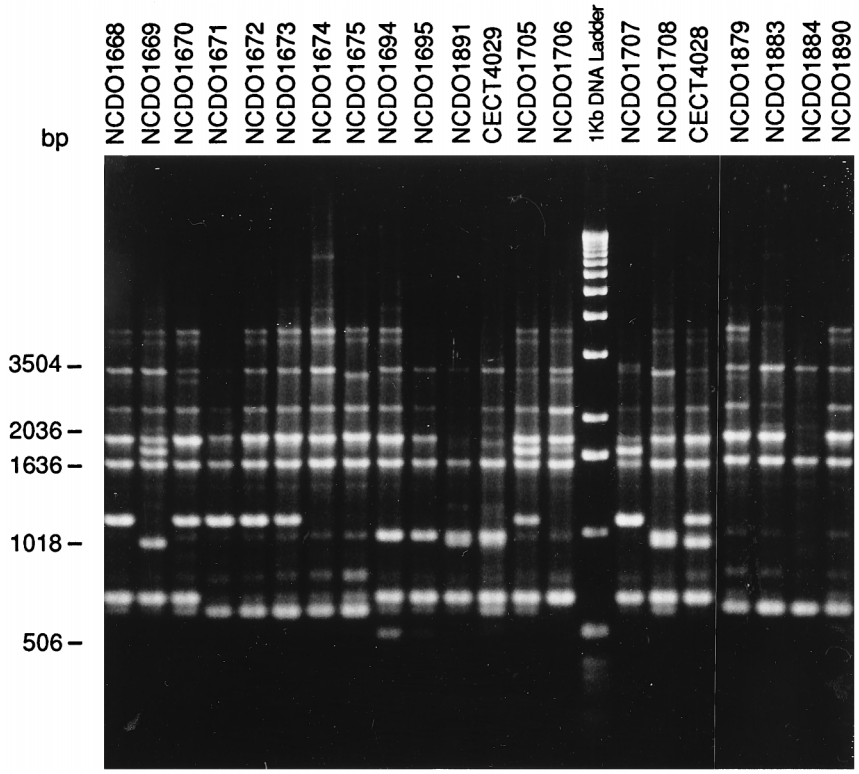

FIG. 2. RAPD patterns obtained with primer OPA20 and DNA template from *O. oeni* strains.

in wine, at least in wines produced by spontaneous malolactic fermentation. On the other hand, identical RAPD patterns were found in the duplicates (same strain from different culture collections; NCDO1709/CECT4028, NCDO1696/CECT 4029, and NCDO1823/CECT218) and in two isolates from the same sample (831 and 832). Also, most strains showed >90% similarity in their RAPD patterns when they were isolated from the same sample, and those from the same region often clustered together, at least in the spontaneous malolactic fermentation samples.

The degree of polymorphism found with RAPD is relatively low compared to those often reported for other species (7, 19, 26), indicating that *O. oeni* is, relatively, an exceptionally homogeneous species. The dendrogram in Fig. 3 shows that most strains clustered within the range of 80 to 100% similarity. In spite of this low diversity, two very well defined groups, group A (50 strains) and group B (20 strains), with a similarity level around 56%, were discerned. RAPD clustering was congruent with the two ribotyping groups A and B. All strains showing ribotype A fell in the same RAPD group, separated from the other group comprising all strains with ribotype B. The calculated correlation coefficient for the RAPD dendrogram was $r = 0.852$, indicating a good level of reliability.

Sequencing of RAPD fragments. The fragments called OPA9-500 (ca. 500 bp, generated with primer OPA9), OPA16-700 (ca. 700 bp, primer OPA16), and OPA11-640 (ca. 640 bp, primer OPA11) were obtained in the RAPD analysis for all strains of *O. oeni* and were selected for sequencing. Primer OPA20 generated the OPA20-560 fragment when all the DNA template was from strains belonging only to group A and the OPA20-650 fragment when all the DNA template was from strains of group B. They represented group-specific markers and were also selected for sequencing. The OPA9-500 fragment comprised 524 bp, and sequences from strains NCDO 1674^T and CECT4029 were identical. The sequence showed an open reading frame of 369 bp (positions 69 to 437) that may code for a protein of 123 amino acids with similarity levels of 73% with respect to ribosomal protein S13 of *Bacillus subtilis*, 61% with respect to that of *Haemophilus influenzae*, 56% with respect to that of *E. coli*, and 50% with respect to that of *Chlamydia trachomatis*. The OPA16-700 fragment comprised 708 bp, and sequences from strains NCDO1674 and CECT4029 were identical; the 3′- ends may encode a peptide of 106 amino acids. This stretch showed sequence similarities of 41 to 47% to the enzyme acetoacetyl coenzyme thiolase of *Clostridium acetobutylicum*, *B. subtilis*, *H. influenzae*, and *Alcaligenes eutrophus*. The OPA11-640 fragment, preliminarily considered the same for all strains in the agarose gel, was sequenced; it comprised 642 bp in strain NCDO1674 but 631 bp in strain CECT4029. The sequence comparison revealed no significant homology, considering that they are two strains of the same species. This result demonstrated that the so-called OPA11-640 contained nonhomologous DNA fragments in the strains analyzed. The 642-bp sequence of NCDO1674 may code for a peptide (positions 393 to 641) which showed 51% similarity to the xanthine permease of *B. subtilis*. However, the 631-bp sequence of strain CECT4029 revealed 73.6% nucleotide sequence similarity to the P-type ATPase of *Listeria mocytogenes*. Further digestions of OPA1-640 with *Hin*dIII demonstrated that strain CECT4029 generated fragments of both 631 and 642 bp (results not shown). The OPA20-560 fragment (specific for group A strains), from strains NCDO1674 and NCDO1823, revealed an identical sequence of 577 bp that showed no significant homology to the actual EMBL database. Identical sequences were found from OPA20-650 fragment of strains CECT4029 and NCDO1821. The 646-bp sequence of CECT

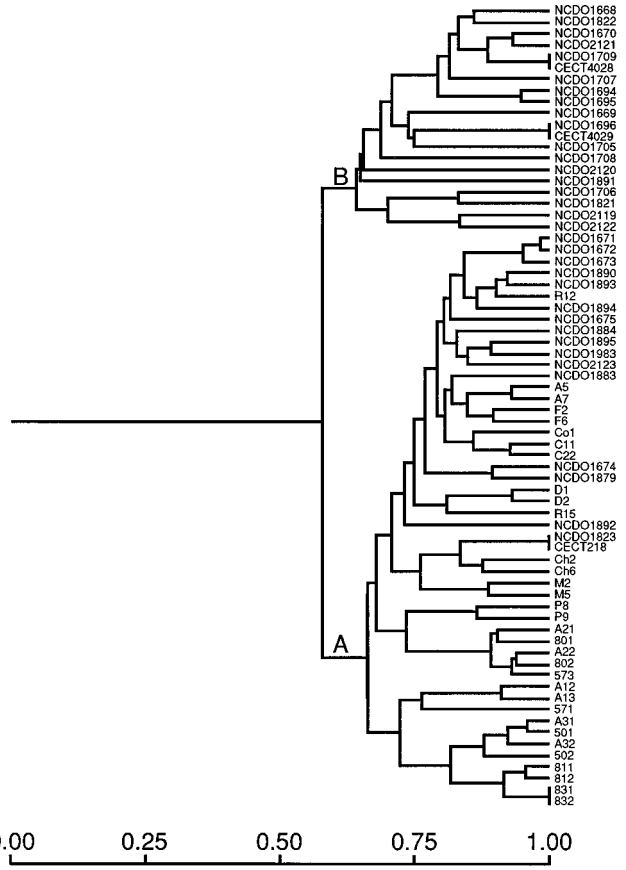

FIG. 3. UPGMA dendrogram showing the relationships between *O. oeni* strains deduced by analysis of 94 RAPD prints obtained with 10 primers of arbitrary sequence. A and B refer to groups A and B from the main RAPD grouping.

4029 may code for a peptide (positions 188 to 646) which showed similarities of 41 to 48% to the enzyme lipoamide dehydrogenase, a component of the pyruvate dehydrogenase complex of *Pseudomonas putida*, *B. subtilis*, and *B. stearothermophilus.*

DISCUSSION

The analysis of ribotyping and RAPD patterns has revealed a high level of genetic homology in *O. oeni* (Table 1). This homogeneity is consistent with the results of DNA-DNA hybridization, total soluble cell proteins (3, 4), and 16S-23S ISR sequences (42). Irrespective of the enzyme used, *Eco*RI or *Hin*dIII, all the strains analyzed in the present study showed only two different ribotypes. However, the RAPD method was strain specific and appears to be potentially useful for the control of strains in wine-making processes. Identical RAPD patterns were found in a few pairs of strains (NCDO1709/CECT4028, CECT4029/NCDO1696, and NCDO1823/CECT 218), supporting the notions that they are duplicates of the same organism or strain and that the RAPD patterns are reproducible (7, 25). The grouping obtained by RAPD (groups A and B), in agreement with the two ribotyping groups, correlated well with previous PFGE results (34). Macrorestriction patterns by PFGE in *O. oeni* isolates from Portuguese wine have shown a major cluster (80% of isolates analyzed) that includes the type strain NCDO1674^T and strain ML34 (NCDO 1823); strains CECT4028 and CECT4029 appeared in a different cluster. Our study included 37 culture collection strains, 17 of which fell in group A (which includes NCDO1674^T and ML 34) and 20 fell in group B (represented by CECT4028 and CECT4029). Nevertheless, the 33 strains isolated from red wine and molecularly characterized by 16S-23S ISR-RFLP were categorized in group A by RAPD; 18 of them were categorized in group A by ribotyping also. The two well-defined and consistent groups of strains shown by all these methods are indicative of two distinct main patterns of DNA arrangement in the genome (14) of the species *O. oeni*. Isolates from red wine (at least Spanish and Portuguese) typically show a group A genomic pattern type, favoring the hypothesis that a particular strain predominates in the wine from Spain and Portugal (34), perhaps due to the transfer of viticulture. Group A could also be better adjusted to relatively warmer climates, while B could be favored by the colder latitudes of France or Germany. The clustering of strains obtained from spontaneous malolactic fermentation samples originating from the same region (i.e., Valladolid) indicates a geographic distribution in the *O. oeni* strains that grow spontaneously in wine; i.e., some clones, in the broad sense of the word (31), have limited geographic distribution.

Two RAPD fragments common to all *O. oeni* strains were selected to assess sequence similarity. The determined sequences of fragments generated with primers OPA9 and OPA16 were identical for strains NCDO1674^T and CECT4029. Although part of the OPA9-500 sequence may code for ribosomal proteins (it is known that such sequences are conserved), it is remarkable that amplified noncoding DNA showed identical sequences in strains with different genomic DNA organization. The finding of these sequences, together with the recently reported 16S-23S ISR in *O. oeni*, clearly indicate that this species is phylogenetically homogeneous.

In the RAPD analysis, it is generally assumed that DNA fragments of the same size belong to a homologous sequence of the genome. In fact, this is true for most RAPD prints: in a random process, the finding of two antiparallel targets (each in a different strand) in nonhomologous sites of the genome located at the same distance in two different organisms is not likely. This makes fragment size determination an important task. For example, primer OPA11 yielded a DNA fragment of ca. 640 bp for all strains of *O. oeni*, as seen in the agarose gel. The sequences obtained from strains NCDO1674^T (642 bp) and CECT4029 (631 bp) showed negligible similarity to each other, indicating that these fragments belong to nonhomologous sites. Possible misinterpreted markers do not alter the RAPD dendrogram, as it was constructed from 94 RAPD prints. Nevertheless, such differences in DNA could be detected by using high-resolution gels. In the present case, we further demonstrated that strain CECT4029 also yielded the homologous sequence of 642 bp.

Primer OPA-20 gave fragments specific for each RAPD group. A ca. 560-bp fragment was group A specific, and a 650-bp fragment was group B specific. Sequences from strains NCDO1674^T and CECT4029 are of potential value for developing oligonucleotide probes of known sequence to specifically identify groups A and B. Oligonucleotide probes with known sequence from RAPD fragments have been reported only in the development of species-specific tests (24, 38). The present article offers a variety of methods for characterizing *O. oeni* unequivocally at the species (16S-23S ISR-RFLP) and the genomic group A and B (OPA20 probes) and strain (RAPD) levels. All these tools are valuable for the purpose of monitoring the process of wine making. They may help to improve quality and avoid spoilage microorganisms.

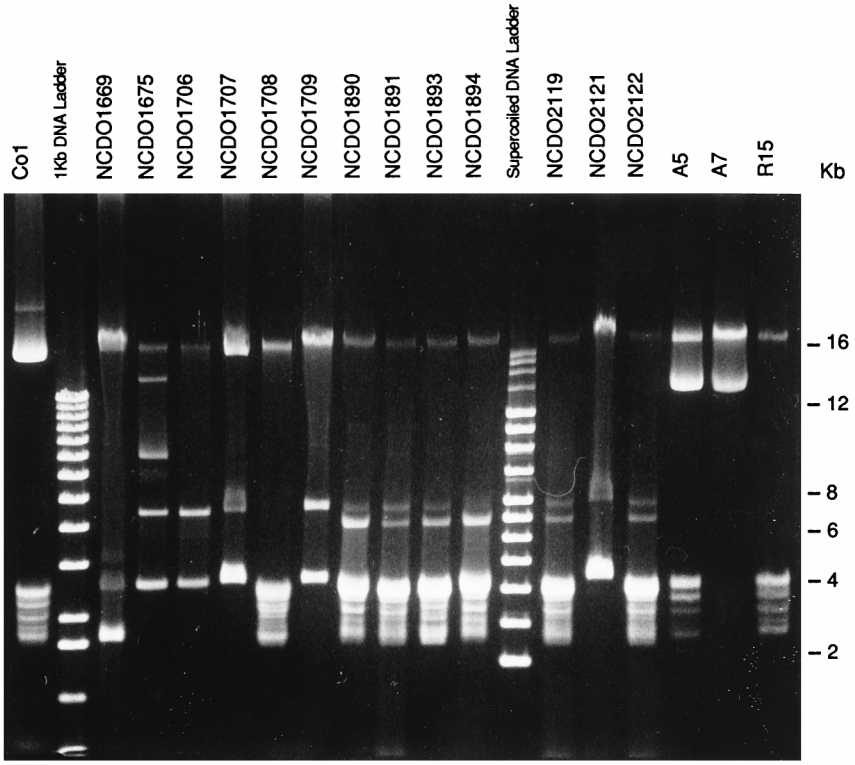

FIG. 4. Plasmid DNA from *O. oeni* strains.

The species *O. oeni* is phylogenetically very homogeneous, as revealed by the homologous DNA fragments analyzed in this study, according to the ISR sequences (42) and the global DNA homology reported (3, 4). The main difference in the genome appears to arise from the occurrence of two forms of DNA arrangement during the relatively short history of this species, as demonstrated by several analyses at the genomic organization level. Although data indicate that *O. oeni* follows a closely clonal model of evolution (31), genetic transfer may occur, as suggested by results of plasmid analysis (Fig. 4). It has been proposed that *O. oeni* (formerly *Leuconostoc oenos*) represents a fast-evolving organism since, compared to all other members of the genus *Leuconostoc* and other lactic acid bacteria, considerable numbers of nucleotide positions in the 16S rRNA show an unusually high rate of substitutions accompanied by an atypical phenotype and no significant DNA-DNA similarity (41). Results from our study showed that homologous sequences, including noncoding DNA apparently not subjected to selection, are, unexpectedly, identical among strains that revealed considerable divergence of the genomic organization and a different plasmid content. It is possible that selective pressure on *O. oeni* was strong over the period when adaptation to the wine-making environment was achieved. Nevertheless, the hypothesis of rapid evolution can be sustained only if we consider this microorganism, at the present stage, to be a true *Leuconostoc*. For example, a single microorganism belonging to a different genus at a relatively long phylogenetic distance from *Leuconostoc* (such as *Lactococcus* or *Streptococcus*) also shows a relatively unusual 16S rRNA sequence and different phenotype. Since this organism has been considered a member of a different genus, *Oenococcus*, the same considerations apply.

In many ways, the intraspecific situation we found is reminiscent of the case of some pathogenic bacterial clones such as those of *Vibrio cholerae*, which were responsible for the cholera pandemics. In this case, an environmental clone that has acquired pathogenic properties is greatly amplified by transmission of the disease, so that virtually identical organisms can be isolated from widely separated regions of the world (17). In the case of *O. oeni*, wine making would be equivalent to an infectious disease that allows the few clones capable of growing in this specialized environment to reach a global distribution. The absence of representatives of "environmental" *O. oeni* in strain collections could reflect the lack of proper isolation techniques or culture media. It is our intention to exploit the molecular information gained here and in other similar works to isolate and identify new members of *Oenococcus* in the natural environment and perhaps to find the actual diversity reservoir of this peculiar organism.

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