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Comparison of *Leuconostoc oenos* Strains by Pulsed-Field Gel Electrophoresis

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Pulsed-field gel electrophoresis of chromosomal DNA digested with *Not*I or *Sfi*I was used to differentiate individual strains of *Leuconostoc oenos*. *L. oenos* isolates with 13 different restriction digest patterns were detected in New Zealand wines undergoing malolactic fermentation. The average genome size was estimated to be 1,800 kb.

In cool-climate wine-growing regions, such as New Zealand, malolactic fermentation (MLF) has a significant role in wine quality. In this fermentation, lactic acid bacteria convert L-malic acid from grapes to L-lactic acid and CO_2, thus reducing the acidity of the wine (11). These bacteria also influence wine flavor and aroma (17). *Leuconostoc oenos* is the organism considered preferable for achieving MLF in wine because of its tolerance to low pH and high ethanol levels (11).

Several strains of *L. oenos* may occur in a single fermentation (6); and with increasing use of *L. oenos* starter cultures in wine making, especially if strains with particular flavor characteristics become available (8), a method for accurately identifying *L. oenos* strains will be required to monitor the survival and contribution of inoculated and indigenous bacteria. The aim of this work was to determine whether pulsed-field gel electrophoresis (PFGE) patterns of restriction enzyme-digested chromosomal DNA could be used to differentiate individual strains of *L. oenos*. The *L. oenos* reference strains used in this study were NCFB 1674, NCFB 1694, NCFB 1696, NCFB 1707, NCFB 1709, ML34, OENO, L181, and Lo23. An additional 108 *L. oenos* strains were isolated from 25 red and white wines (see Table 1), undergoing malolactic fermentation (10). Bacteria were grown at 28°C in a medium containing (per liter): glucose (10 g), fructose (5 g), tryptone (10 g; Difco), yeast extract (5 g; Difco), cysteine (0.5 g), 1 M $MgSO_4 \cdot 7H_2O$ (1.0 ml), 0.25 M $MnSO_4 \cdot 4H_2O$ (1.0 ml), apple juice (200 ml; Fresh-up; Apple and Pear Marketing Board, New Zealand), and distilled water to 1 liter. Cultures were stored at −70°C in 40% (vol/vol) glycerol and were also freeze-dried for long-term storage.

Plasmid DNA was isolated by using the method developed by Anderson and McKay (1), and samples were electrophoresed in 0.6% (wt/vol) agarose gels in Tris-acetate buffer (19) for 4 h at 4 V/cm. Plasmids from *Escherichia coli* V517 (14) were used as size standards. To detect lysogenic strains, early-log-phase *L. oenos* cultures were treated with mitomycin (1 μg/ml; Sigma Chemical Company, St. Louis, Mo.) and incubated in the dark overnight. The culture was filtered to remove bacterial cells, and the supernatant was tested for the presence of bacteriophage by using the double-layered agar plate method, with a range of *L. oenos* strains as indicators.

To prepare DNA in agarose blocks for PFGE analysis, cells from a mid- to late-log-phase culture were set in agarose and lysed essentially as described by Tanskanen et al. (20). The agarose blocks were stored in TE 10/100 (10 mM Tris-Cl, 100 mM EDTA [pH 8.0]) at 4°C until required. For restriction enzyme digestion, slices (1 to 2 mm) were cut from the agarose blocks and washed three times with TE 10/0.1 (10 mM Tris-Cl, 0.1 mM EDTA [pH 8.0]) and once with restriction enzyme buffer before incubation for 18 to 24 h with 20 U of *Not*I or *Sfi*I (New England Biolabs, Beverly, Mass.) in a total volume of 100 μl. Following digestion, the slices were washed once with TE 10/1 for 1 h at 4°C and electrophoresed through 1% (wt/vol) PFGE-grade agarose (Sigma) gels in 0.5× Tris-borate buffer (19) for 20 to 24 h at 150 V and 14°C in a CHEF DR II PFGE apparatus and a model 1000 minichiller (Bio-Rad). For strain differentiation a ramped pulse time of 1 to 45 s was used with *Not*I and *Sfi*I digests. To measure the size of the various fragments, other ramped and fixed pulse times were used in accordance with the conditions for optimal separation reported by Birren et al. (2, 3), with *Saccharomyces cerevisiae* chromosomes, multimers of phage lambda, and *Hin*dIII digests of phage lambda (New England Biolabs) as molecular size standards.

Reference and New Zealand strains of *L. oenos* were tested for plasmids and lysogenic phage in initial attempts to differentiate the strains. Strains NCFB 1707 and 1709 both harbored a plasmid with a size of 4.3 kb (Fig. 1), while the remaining reference cultures were plasmid free. Three New Zealand isolates, 1081 (4 kb), 1050 (22 kb), and 1044 (33 kb), also contained plasmids (Fig. 1). Their metabolic role is not known. Four strains (1002, 1095, AM20, and reference culture L181) were found to be lysogenic and released bacteriophage after treatment with mitomycin. ML34, 1050, and 1081 were sensitive to all these induced phage, while other strains varied in their sensitivity. The plasmid in strain 1707 (9) and the bacteriophage from strain L181 (16) have been documented previously. These approaches did not provide sufficient information to adequately distinguish all the strains tested. However, the data obtained was valuable for use in conjunction with PFGE analysis.

The DNA base composition of *L. oenos* is reported to be in the range of 37 to 42 mol% G+C (5, 7), so that restriction enzymes with recognition sequences that contain only G and C nucleotides could be expected to generate a limited number of fragments (15). The enzymes *Apa*I, *Bgl*I, *Bss*HII, *Eag*I, *Ksp*I, *Mlu*I, *Rsr*II, *Sgr*AI, *Sma*I, and *Xho*I were used

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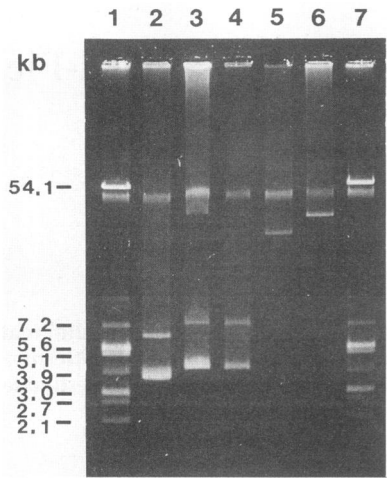

FIG. 1. Plasmid profiles of *L. oenos* strains. Lanes: 1, *E. coli* V517; 2, 1081; 3, 1707; 4, 1709; 5, 1050; 6, 1044; 7, *E. coli* V517.

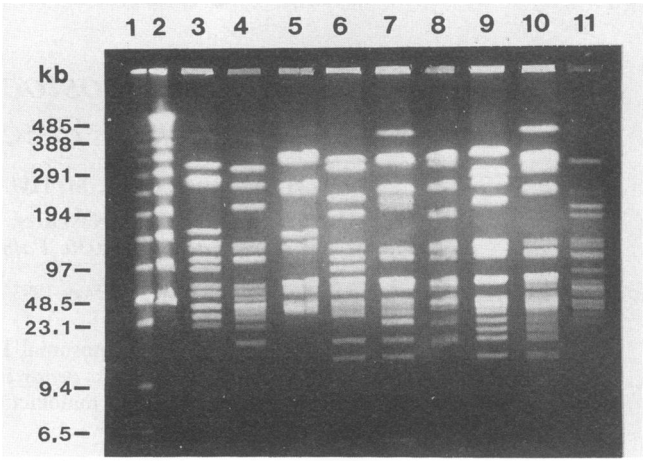

FIG. 3. PFGE patterns of *Sfi*I digests of genomic DNA from *L. oenos* strains. Lanes: 1, low-range PFGE marker; 2, phage λ concatamers; 3, ML34; 4, 1674; 5, 1707; 6, 1694; 7, 1696; 8, 1709; 9, L181; 10, OENO; 11, Lo23. Electrophoresis was done for 22 h with a pulse time ramped from 1 to 45 s.

to digest chromosomal DNA from *L. oenos* ML34, and all gave multiple bands mainly in the 25- to 100-kb range. None of these were ideal for PFGE analysis. Two other enzymes with 8-bp recognition sequences (*Not*I and *Sfi*I) proved useful for PFGE separation. Chromosomal DNA preparations from nine reference strains of *L. oenos* could be distinguished, as the patterns obtained were distinctly different (Fig. 2 and 3). A third enzyme (*Asc*I) gave three to five bands for each strain.

When genomic DNA from *L. oenos* strains isolated from wines undergoing MLF was digested with *Sfi*I (Fig. 4) or *Not*I and examined by PFGE, 13 different restriction digestion patterns resulted. Some wines provided two or three distinct isolates (Table 1), indicating that more than one strain may be involved in carrying out the MLF. One pattern could be correlated with that of strain OENO and proved to be from a winery at which that strain had been used as a starter culture, although it was not the sole *L. oenos* strain present.

Of the indigenous strains, some (1078, 1081, and AM21) were found at only one winery, but others were more widely distributed. A group of strains with very similar PFGE restriction patterns (1002, 1004, 1041, 1044, 1050, and 1095) were found at wineries in all regions and in both red and white wines. The six strains in this group varied slightly in their restriction patterns and in some of their properties. Strains 1002 and 1095 released bacteriophage particles at low titers when treated with mitomycin, while strains 1044 and 1050 contained plasmids not found in other *L. oenos* strains. The predominance of these strains, and their variability, suggests that they are well adapted to New Zealand wine-making conditions and that further adaptation to the winery environment is occurring. The genome size of each strain was determined as the sum of the individual restriction fragments in each digest, and the results for NCFB 1674 and

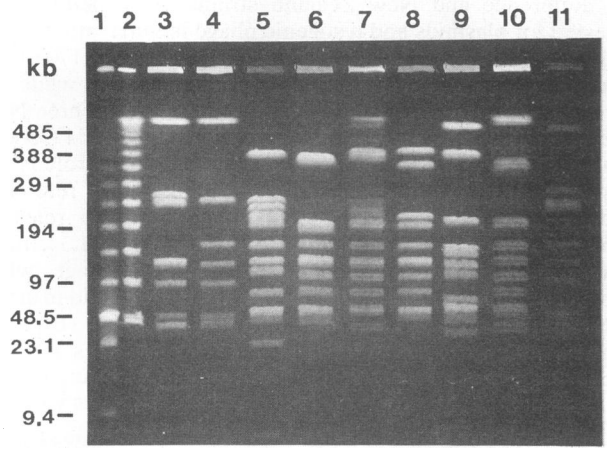

FIG. 2. PFGE patterns of *Not*I digests of genomic DNA from *L. oenos* strains. Lanes: 1, low-range PFGE marker (phage λ concatamers and *Hin*dIII fragments); 2, phage λ concatamers; 3, ML34; 4, 1674; 5, 1707; 6, 1694; 7, 1696; 8, 1709; 9, L181; 10, OENO; 11, Lo23. Electrophoresis was done for 22 h with a pulse time ramped from 1 to 45 s.

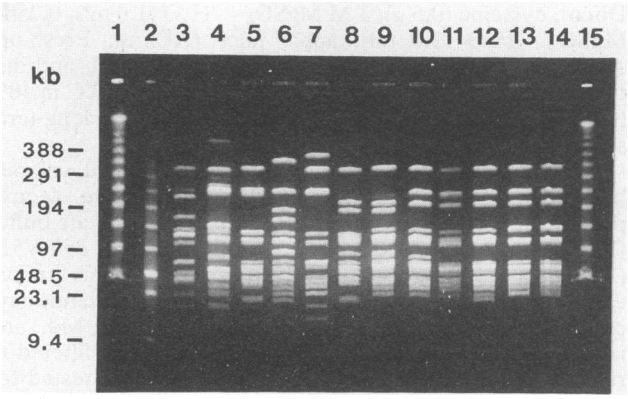

FIG. 4. PFGE patterns of *Sfi*I digests of genomic DNA from New Zealand *L. oenos* strains. Lanes: 1, phage λ concatamers; 2, low-range PFGE marker; 3, 1078; 4, 1081; 5, AM19; 6, AM20; 7, AM21; 8, AM22; 9, 1002; 10, 1095; 11, 1004; 12, 1050; 13, 1041; 14, 1044; 15, phage λ concatamers. Electrophoresis was done for 22 h with a pulse time ramped from 1 to 45 s.

TABLE 1. Presence of *L. oenos* strains in New Zealand wines

Sample no.[a]	Type of wine[b]	Presence of *L. oenos* strain												
		1078	1081	AM19	AM20	AM21	AM22	OENO	1002	1095	1004	1050	1041	1044
A1	CH								+		+			
B1	CH									+				
C1	CH										+			
C2	CH										+			
C3	CS										+			
C4	CH	+												
D1	ME									+				
D2	CH									+				
E1	PN									+	+			
E2	CH									+	+			
F1	SB											+	+	
F2	CH											+		+
F3	CH											+		+
F4												+		
F5	CH												+	+
G1	CH						+	+			+			
G2	CH		+					+			+			
G3	CH		+											
G4	PN		+											
G5	CH			+	+					+				
H1	CH										+			
I1	PN								+		+			
I2	ME			+						+				
J1	CS				+	+	+							
J2	CH			+							+			

[a] The samples are identified by number and region: Auckland (A, C, F, and H), Hawkes Bay (B, D, and J), Martinborough (E), and Marlborough (G and I).
[b] CH, Chardonnay; CS, cabernet sauvignon; ME, merlot; PN, pinot noir; SB, sauvignon blanc.

TABLE 2. Genome sizes of *L. oenos* NCFB 1674 and ML34

Genome no.	Genome size (kb) in strain digested with enzyme indicated					
	1674			ML34		
	*Asc*I	*Not*I	*Sfi*I	*Asc*I	*Not*I	*Sfi*I
1	975	1,020	304	975	800	316
2	505	250	258	505	260	274
3	170	158	208	190	240	268
4	115	124	135	115	128	156
5		94	135		124	135
6		50	124		94	124
7		44	106		50	106
8		40	106		40	90
9			68		40	68
10			60		14	60
11			51		9	42
12			42			42
13			42			30
14			35			30
15			29			23
16			22			6
17			19			
18			6			
19			6			
Total size	1,765	1,780	1,756	1,785	1,799	1,770

ML34 are shown in Table 2. Other strains showed similar genome sizes, with an average of approximately 1,800 kb. The genome size of *L. oenos* has previously been estimated to be 1,320 kb (4), but the multiple bands given with *Apa*I or *Sma*I digestion of *L. oenos* chromosomal DNA in that study may have resulted in an underestimate of the genome size. The larger genome size of 1,800 kb is more consistent with the genome sizes reported for other lactic acid bacteria (12, 13, 18, 20).

This work shows the suitability of PFGE for the differentiation of *L. oenos* strains, with each strain having a characteristic restriction pattern following digestion of chromosomal DNA with *Not*I or *Sfi*I. In a similar study (4), transverse alternating field electrophoresis has been successfully used to differentiate strains of *L. oenos*. With the availability of these techniques, it is now possible to monitor individual strains and determine whether cultures inoculated into wine to perform MLF actually survive and carry out the fermentation.

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REFERENCES

1. **Anderson, D. G., and L. L. McKay.** 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46**:549–552.

2. **Birren, B. W., E. Lai, S. M. Clark, L. Hood, and M. I. Simon.** 1988. Optimized conditions for pulsed field gel electrophoretic separations of DNA. Nucleic Acids Res. **16:**7563–7582.
3. **Birren, B. W., E. Lai, L. Hood, and M. I. Simon.** 1989. Pulsed field gel electrophoresis techniques for separating 1- to 50-kilobase DNA fragments. Anal. Biochem. **177:**282–286.
4. **Daniel, P., E. de Waele, and J.-N. Hallet.** 1993. Optimisation of transverse alternating field electrophoresis for strain identification of *Leuconostoc oenos*. Appl. Microbiol. Biotechnol. **38:** 638–641.
5. **Dicks, L. M. T., H. J. J. van Vuuren, and F. Dellaglio.** 1990. Taxonomy of *Leuconostoc* species, particularly *Leuconostoc oenos*, as revealed by numerical analysis of total soluble cell protein patterns, DNA base compositions, and DNA-DNA hybridizations. Int. J. Syst. Bacteriol. **40:**83–91.
6. **Fleet, G. H., S. Lafon-Lafourcade, and P. Ribéreau-Gayon.** 1984. Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. Appl. Environ. Microbiol. **48:**1034–1038.
7. **Garvie, E. I.** 1986. Genus *Leuconostoc*, p. 1071–1075. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. Williams & Wilkins, Baltimore.
8. **Gockowiak, H., P. J. Costello, and P. A. Henschke.** 1991. Options for the induction of malolactic fermentation. Aust. N.Z. Wine Ind. J. **6:**133–137.
9. **Janse, B. J. H., B. D. Wingfield, I. S. Pretorius, and H. J. J. van Vuuren.** 1987. Plasmids in *Leuconostoc oenos*. Plasmid **17:**173–175.
10. **Kelly, W. J., R. V. Asmundson, and D. H. Hopcroft.** 1989. Growth of *Leuconostoc oenos* under anaerobic conditions. Am. J. Enol. Vitic. **40:**277–282.
11. **Kunkee, R. E.** 1991. Some roles of malic acid in the malolactic fermentation in wine making. FEMS Microbiol. Rev. **88:**55–72.
12. **Le Bourgeois, P., M. Mata, and P. Ritzenthaler.** 1989. Genome comparison of *Lactococcus* strains by pulsed-field gel electrophoresis. FEMS Microbiol. Lett. **59:**65–70.
13. **Luchansky, J. B., K. A. Glass, K. D. Harsono, A. J. Degnan, N. G. Faith, B. Cauvin, G. Baccus-Taylor, K. Arihara, B. Bater, A. J. Maurer, and R. G. Cassens.** 1992. Genomic analysis of *Pediococcus* starter cultures used to control *Listeria monocytogenes* in turkey summer sausage. Appl. Environ. Microbiol. **58:**3053–3059.
14. **Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCowen.** 1978. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid **1:**417–420.
15. **McClelland, M., R. Jones, Y. Patel, and M. Nelson.** 1987. Restriction endonucleases for pulsed field mapping of bacterial genomes. Nucleic Acids Res. **15:**5985–6005.
16. **Patel, J.** 1990. Lysogenic *Leuconostoc oenos* strain L181, and implications of lysogenic strains in MLF, p. 258. *In* P. J. Williams, D. M. Davidson, and T. H. Lee (ed.), Proceedings of the Seventh Australian Wine Industry Technical Conference. Wine Titles, Adelaide, Australia.
17. **Rodriguez, S. B., E. Amberg, R. J. Thornton, and M. R. McLellan.** 1990. Malolactic fermentation in Chardonnay: growth and sensory effects of commercial strains of *Leuconostoc oenos*. J. Appl. Bacteriol. **68:**139–144.
18. **Roussel, Y., C. Colmin, J. M. Simonet, and B. Decaris.** 1993. Strain characterization, genome size and plasmid content in the *Lactobacillus acidophilus* group (Hansen and Mocquot). J. Appl. Bacteriol. **74:**549–556.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Tanskanen, E. I., D. L. Tulloch, A. J. Hillier, and B. E. Davidson.** 1990. Pulsed-field gel electrophoresis of *Sma*I digests of lactococcal genomic DNA, a novel method of strain identification. Appl. Environ. Microbiol. **56:**3105–3111.