World Journal of Microbiology & Biotechnology (2006) 22: 515–518
DOI 10.1007/s11274-005-9065-5

Species attribution and distinguishing strains of *Oenococcus oeni* isolated from Chinese wines

Hua Li, Chunhui Zhang and Yanlin Liu*
The College of Enology, Northwest A & F University, 712100, Yangling, Shaanxi, P.R. China
**Author for correspondence: Tel.: +86-29-87091346, Fax: +86-29-87092107, E-mail: putj@126.net*

Received 4 February 2005; accepted 21 September 2005

Keywords: Chinese wines, genetic differentiation, genotypic diversity, *Oenococcus oeni*

Summary

Twenty-four strains of *Oenococcus oeni* were isolated from different Chinese wines. Differentiation of isolates was carried out by analysis of total soluble cell protein patterns and random amplified polymorphic DNA (RAPD) patterns. The results indicated that the total soluble cell protein patterns could be used to distinguish different genera but fail to distinguish different strains. It was also found that strain RAPD pattern can successfully distinguish isolates by UPGMA analysis. The RAPD profiles (107 different prints) were strain specific and two main groups of strains were screened.

Introduction

Malolactic bacteria are lactic acid bacteria (LAB) that carry out a secondary fermentation (malolactic fermentation, MLF) of new wine after alcoholic fermentation. *Oenococcus* is the lactic acid bacterium genus most often involved in spontaneous MLF of wine. Strains of the species *Oenococcus oeni* are used increasingly as starter cultures to enhance the organoleptic properties and microbiological stability of wines (Lonvaud-Funel 1999; Li 2000). Several researchers have reported that harshness in wine and organoleptic quality is strain-dependent (Lonvaud-Funel 1999; Beneduce *et al*. 2004). The availability of reliable methods for the differentiation of *O. oeni* strains is essential for monitoring the fate of inoculated and autochthonous bacteria and also to certify the identity of the selected cultures. The classification of LAB into different genera was formerly based on their morphology, metabolism and physiological characteristics. In recent years, soluble protein patterns, DNA–DNA hybridization, 16S rDNA sequencing and 16S–23S rDNA inter-genic spacer regions, and random amplified polymorphic DNA (RAPD)-PCR have led to descriptions of new genera (Dicks *et al*. 1995; Villani *et al*. 1997; Zavaleta *et al*. 1997; Zapparoli *et al*. 1998; Zapparoli *et al*. 2000; Blaiotta *et al*. 2002; Margheri *et al*. 2003; Rodas 2003; Beneduce 2004; Plessis *et al*. 2004). RAPD-PCR is applied for the fingerprinting of large numbers of strains as it offers the advantages of rapidity, reliability, and technical simplicity (Zapparoli *et al*. 2000; Reguant & Bordons 2003). However, this technique has not been used extensively, and, in particular, it has never been used for differentiation of *O. oeni* strains in China. In this study, identification of species and strains of the bacterium, *O. oeni*, indigenous to Chinese wines, originally characterized by conventional phenotypic and biochemical methods (Zhang *et al*. 2005), was carried out by analysis of total soluble cell protein patterns and RAPD patterns.

Materials and methods

Bacterial strains

A collection of 24 malolactic bacteria strains was isolated from Chinese regional dry red wines that underwent spontaneous MLF. Identification of the isolates was performed according to the criteria established in *Bergey's Manual of Determinative Bacteriology* (1994) and the result showed that the 24 isolates were *O. oeni* (Zhang *et al*. 2003, 2005). Seven isolates (*O. oeni* SD-1a, SD-1b, SD-1c, SD-1d, SD-1e, SD-1f, SD-1g) were from Qingdao in Shandong Province; 10 isolates (*O. oeni* SD-2a, SD-2b, SD-2c, SD-2d, SD-2e, SD-2f, SD-2g, SD-2h, SD-2i, SD-2j) were from Yantai, Shandong; two isolates (*O. oeni* SX-1a, SX-1b) were from Yangling in Shaanxi Province; three isolates (*O. oeni* HB-1a, HB-1b, HB-1c) were from Shacheng in Hebei Province; two isolates (*O. oeni* SC-1a, SC-1b) were from Xichang in Sichuan Province. *Oenococcus oeni* 31DH (a type strain, from USA) was provided by the China Center of Industrial Culture Collection (CICC) and two other type strains

(*O. oeni* 1^T and 2^T) were from Denmark. Two type strains of *Leuconostoc* spp. (*Leuconostoc* 1^T, and 2^T) and one type strain of *Lactococcus lactis* subsp. *cremoris* 1.18^T were also provided by CICC. All strains were grown anaerobically at 28 °C in ATB.

Random primers and nucleotide sequences

Forty-eight random primers were used and their nucleotide sequences are listed in Table 1.

Total soluble cell protein SDS-PAGE

Cultures of strains were prepared as described by Zavaleta *et al.* (1997). Total soluble proteins were extracted and SDS-PAGE was carried out according to Sambrook *et al.* (1989).

DNA manipulations

Genomic DNA was extracted according to the procedure of Zavaleta *et al.* (1997) with some modifications. Concentration of the DNA obtained was estimated by electrophoresis in 1.0% (w/v) agarose gel with Tris–Acetate–EDTA (TAE) buffer.

RAPD-PCR reaction

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% (v/v) Triton X-100, 2.5 mM $MgCl_2$, 200-μM dNTP, 100 pmol of each primer, and 2 U of *Taq* DNA polymerase in a final volume of 25 μl. The reaction mixtures were overlaid with mineral oil and subjected to amplification for 40 cycles; 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. PCR products (5 μl) were electrophoresed in 1.2% (w/v) agarose–TAE gels stained with ethidium bromide for 1 h and photographed.

Random amplified polymorphic DNA (RAPD)-PCR analysis

The RAPD pattern of the genome DNA from *O. oeni* strains was analyzed and a dendrogram constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm.

Table 1. Random primer sequences and codes from RAPD analysis.

Code	Sequence (5′-3′)	Code	Sequence (5′-3′)	Code	Sequence (5′-3′)	Code	Sequence (5′-3′)	Code	Sequence (5′-3′)
OPA1	CAGGCCCTTC	OPA11	CAATCGCCGT	OPG10	AGGGCCGTCT	S5	TGCGCCCTTC	S15	GGAGGGTGTT
OPA2	TGCCGAGCTG	OPA12	TCGGCGATAG	OPI18	TGCCCAGCCT	S6	TGCTCTGCCC	S16	TTTGCCCGGA
OPA3	AGTCAGCCAC	OPA13	CAGCACCCAC	A1	TGCGGCTTAC	S7	GGTGACGCAG	S17	AGGGAACGAG
OPA4	AATCGGGCTG	OPA14	TCTGTGCTGG	A11	CAAACGGCAC	S8	GTCCACACGG	S18	CCACAGCAGT
OPA5	AGGGGTCTTG	OPA15	TTCCGAACCC	A16	AGCCAGCGAA	S9	TGGGGGACTC	S19	ACCCCCGAAG
OPA6	GGTCCCTGAC	OPA16	AGCCAGCGAA	A20	GTTGCGATCC	S10	CTGCTGGGAC	S20	GGACCCTTAC
OPA7	GAAACGGGTG	OPA17	GACCGCTTGT	S1	GTTTCGCTCC	S11	GTAGACCCGT	S333	GACTAAGCCC
OPA8	GTGACGTAGG	OPA18	AGGTGACCGT	S2	TGATCCCTGG	S12	CCTTGACGCA	S412	GGGACGTTGG
OPA9	GGGTAACGCC	OPA19	CAAACGTCGG	S3	CATCCCCCTG	S13	TTCCCCCGCT		
OPA10	GTGATCGCAG	OPA20	GTTGCGATCC	S4	GGACTGGAGT	S14	TCCGCTCTGG		

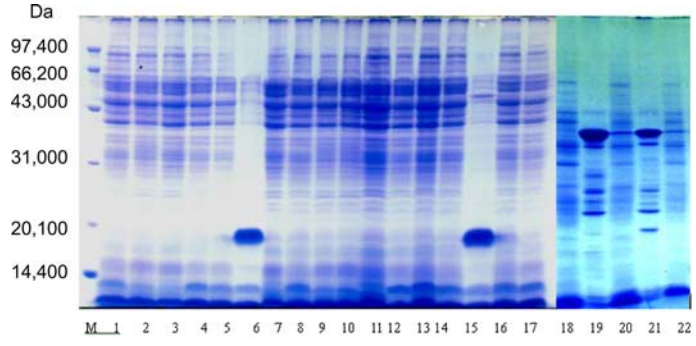

Figure 1. SDS-PAGE pattern of whole-cell proteins of partly strains M, protein marker; Lane 1, *O. oeni*-SD-1g; Lane 2, *O. oeni*-SD-2a; Lane 3, *O. oeni*-SD-2b; Lane 4, *O. oeni*-SD-2c; Lane 5, *O. oeni*-SD-2d; Lane 6, *O. oeni*-SD-2e; Lane 7, *O. oeni*-SD-2g; Lane 8, *O. oeni*-SD-2h; Lane 9, *O. oeni*-SX-1a; Lane 10, *O. oeni*-SD-2i; Lane 11, *O. oeni*-SD-2j; Lane 12, *O. oeni*-SX-1b; Lane 13, *O. oeni*-HB-1a; Lane 14, *O. oeni*-HB-1b; Lane 15, *O. oeni* 31DH; Lane 16, *O. oeni*-HB-1c; Lane 17, *O. oeni* no. 1; Lane 18, *L. lactis* no. 1; Lane 19, *Leuconostoc* spp. no. 1; Lane 20, *L. lactis* no. 2; Lane 21, *Leuconostoc* spp. no. 2; Lane 22, *L. lactis* no. 3.

Results and Discussion

Soluble whole-cell protein SDS-PAGE patterns and inter-specific diversity

Different pure cultures of malolactic bacteria were carefully prepared and total soluble proteins were extracted for performing SDS-PAGE. SDS-PAGE patterns of soluble whole-cell proteins of *O. oeni*, *Leuconostoc* spp. and *Lactococcus lactis* are shown in Figure 1. Four groups can be classed by analyzing soluble whole-cell protein SDS-PAGE patterns of the 32 strains used in this study.

Twenty-five strains of *O. oeni* are in group one. Protein patterns of *O. oeni* SD-2b and *O. oeni* SD-2h, *O. oeni* SD-2j and *O. oeni* SD-1a, *O. oeni* SD-1c and *O. oeni* SD-1f resemble in this group.

Two strains (lane 6, lane 15) of *O. oeni* are in group two. There was a high content of soluble protein with molecular weight of 18,000 Da in this group, with distinctively different features to the pattern of group one. So it is thought that this protein stripe is a key characteristic for differentiation and it is the main difference between the two sub-groups of *O. oeni*, as found by other researchers (Dicks *et al.* 1995).

Two strains (lane 19, lane 21) of *Leuconostoc* spp. constitute group three. They have stripes with a high content of protein with molecular weights: 37,000, 35,000, 25,000 and 22,000 Da. This is also a key characteristic and differentiates them from the patterns of the other two genera. The result indicates that *Leuconostoc* spp. have less resemblance to *Oenococcus* and *L. lactis*.

Two strains (lane 18, lane 20, lane 22) of *L. lactis* and *L. lactis* subsp. *cremoris*1.18T comprise group four. Their protein patterns bear less resemblance to that of *O. oeni*.

All the results indicated that total soluble cell protein patterns could distinguish diversity among inter-specific genera of LAB but failed to distinguish different strains of the same genus.

RAPD patterns of O. oeni *and intra-specific diversity*

From analysis of RAPD patterns of the genome DNA from 25 *O. oeni* strains, polymorphism of DNA (DNA fingerprinting) can be used successfully to distinguish strains. Their correlation can also be estimated by the similitude in the extent of RAPD patterns. The patterns were analyzed by UPGMA. Profile strips of bacteria in RAPD were quantified (codes: 1 for strip, 0 without strip). The *r*-value (resemblance coefficient) refers to the comparability of strains. Computer cluster analysis of *r*-values of strains is shown in Figure 2.

Twelve strains of *O. oeni* were in cluster A ($r \geq 0.722$). In this cluster, RAPD prints of *O. oeni*-SD-1a and *O. oeni*-SD-1d, *O. oeni*-SD-1c and *O. oeni*-SD-1d, *O. oeni*-HB-1a and *O. oeni*-HB-1b were very similar ($r \geq 0.98$).

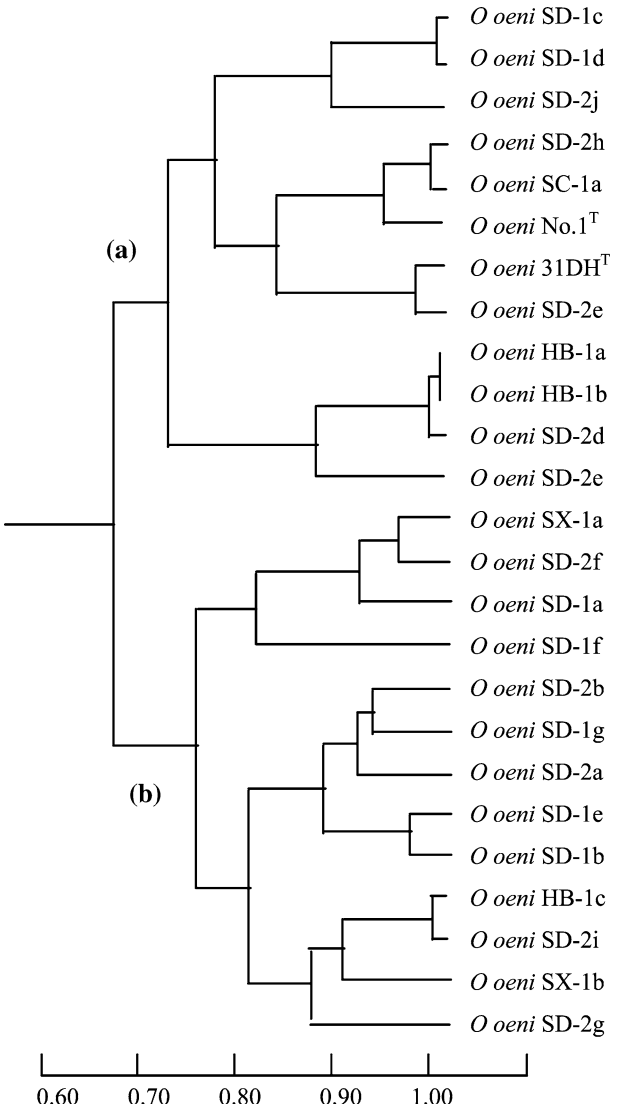

Figure 2. UPGMA Dendrogram of *O. oeni* strains deduced by RAPD prints obtained with random primers A and B refer to the groups A and B from the main RAPD grouping.

Thirteen strains of *O. oeni* were in cluster B ($r \geq 0.684$). RAPD prints of *O. oeni*-HB-1c and *O. oeni*-SD-2i were very similar ($r \geq 0.98$).

The RAPD profiles were strain specific and discerned two main groups of strains, coincident with the clusters obtained in other researchers' results (Zavaleta *et al.* 1997; Zapparoli *et al.* 2000) and also confirmed the results of total soluble cell protein patterns and characterization by morphology, metabolism and physiology. The results seem to indicate that the RAPD patterns could successfully differentiate malolactic bacteria isolates at strain level, thus representing a potential tool for the control of inoculated malolactic fermentation.

References

Beneduce, L., Spano, G., Vernile, A., Tarantino, D. & Massa, S. 2004 Molecular characterization of lactic acid populations associated with wine spoilage. *Journal of Basic Microbiology* **44**, 10–16.

Bergey, Holt, J.G. & Krieg, N.R. 1994 *Bergey's Manual of Determinative Bacteriology*, Lippincott: Williams & Wilkins. ISBN 0-683-00603-7.

Blaiotta, G., Pepe, O., Mauriello, G., Villani, F., Andolfi, R. & Moschetti, G. 2002 16S–23S rDNA intergenic spacer region polymorphism of *Lactococcus garvieae*, *Lactococcus raffinolactis* and *Lactococcus lactis* as revealed by PCR and nucleotide sequence analysis. *Systematic and Applied Microbiology* **25**, 520–527.

Dicks, L.M.T., Loubser, P.A. & Augustyn, O.P.H. 1995 Identification of *Leuconostoc oenos* from South African fortified wines by numerical analysis of total soluble cell protein patterns and DNA–DNA hybridizations. *Journal of Applied Bacteriology* **79**, 43–48.

Li, H. 2000 *Modern Enology*, 2nd edn. Xi'an: Shaanxi People's Publishing House, ISBN 7-224-04005-3.

Lonvaud-Funel, A. 1999 Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie van Leeuwenhoek* **76**, 317–331.

Margheri, M.C., Piccardi, R., Ventura, S., Viti, C. & Giovannetti, L. 2003 Genotypic diversity of oscillatoriacean strains belonging to the genera *Geitlerinema* and *Spirulina* determined by 16S rDNA restriction analysis. *Current Microbiology* **46**, 359–364.

Pinzani, P., Bonciani, L., Pazzagli, M., Orlando, C., Guerrini, S. & Granchi, L. 2004 Rapid detection of *Oenococcus oeni* in wine by real-time quantitative PCR. *Letters in Applied Microbiology* **38**, 118–124.

Plessis, H.W., Dicks, L.M., Pretorius, I.S., Lambrechts, M.G. & Toit, M. 2004 Identification of lactic acid bacteria isolated from South African brandy base wines. *International Journal of Food Microbiology* **15**(91), 19–29.

Reguant, C. & Bordons, A. 2003 Typification of *Oenococcus oeni* strains by multiplex RAPD-PCR and study of population dynamics during malolactic fermentation. *Journal of Applied Microbiology* **95**, 344–353.

Rodas, A.M., Ferrer, S. & Pardo, I. 2003 16S-ARDRA, a tool for identification of lactic acid bacteria isolated from grape must and wine. *Systematic and Applied Microbiology* **26**, 412–422.

Sambrook, J., Fritsch, E.F. & Maniatis T. 1989 *Molecular Cloning: A Laboratory Manual*, New York: Cold Spring Harbor. Cold Spring Harbor Press. ISBN 0-87969-577-3.

Villani, F., Moschetti, G. & Blaiotta, G. 1997 Characterization of strains of *Leuconostoc mesenteroides* by analysis of soluble whole-cell protein pattern, DNA fingerprinting and restriction of ribosomal DNA. *Journal of Applied Microbiology* **82**, 578–588.

Zavaleta, A.J., Martinez-Murcia, A.J. & Rodriguez-Valera, F. 1997 Intraspecific genetic diversity of *Oenococcus oeni* as derived from DNA fingerprinting and sequence analyses. *Applied and Environmental Microbiology* **634**, 1261–1267.

Zapparoli, G., Torriani, S. & Pesente, P. 1998 Design and evaluation of malolactic enzyme gene targeted primers for rapid identification and detection of *Oenococcus oeni* in wine. *Letters in Applied Microbiology* **27**, 243–246.

Zapparoli, G., Reguant, C., Bordons, A., Torriani, S. & Dellaglio, F. 2000 Genomic DNA fingerprinting of *Oenococcus oeni* strains by pulsed-field gel electrophoresis and randomly amplified polymorphic DNA-PCR. *Current Microbiology* **40**, 351–355.

Zhang,Ch., Xia Sh. & Li, H.2003Design and evaluation of culture media to isolate *Oenococcus oeni*Food Science24 2830.

Zhang, Ch. & Li, H. 2005 Study on the identification and enological adaptability of *Oenococcus oeni*. In: *Proceedings of the Fourth International Symposium on Viticulture and Enology*, eds. Li, H. pp. 70–74. Xi'an: Shaanxi People's Publishing House. ISBN 7-224-07030-0.