Short Communication

Application of culture culture-independent molecular biology based methods to evaluate acetic acid bacteria diversity during vinegar processing

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A B S T R A C T

Acetic acid bacteria (AAB) are considered fastidious microorganisms because they are difficult to isolate and cultivate. Different molecular approaches were taken to detect AAB diversity, independently of their capacity to grow in culture media. Those methods were tested in samples that originated during traditional vinegar production. Bacterial diversity was assessed by analysis of *16S rRNA* gene, obtained by PCR amplifications of DNA extracted directly from the acetification container. Bacterial composition was analyzed by RFLP-PCR of *16S rRNA* gene, Temporal Temperature Gradient Gel Electrophoresis (TTGE) separation of amplicons containing region V3–V5 of *16S rRNA* gene and cloning of those amplicons. TTGE bands and clones were grouped based on their electrophoretic pattern similarity and sequenced to be compared with reference strains. The main microorganism identified in vinegar was *Acetobacter pasteurianus*, which at the end of the acetification process was considered to be the only microorganism present. The diversity was the highest at 2% acetic acid, where indefinite species of *Gluconacetobacter xylinus*/*europaeus*/*intermedius* were also present.

1. Introduction

Acetic acid bacteria (AAB) are the main microorganisms responsible for the elaboration of vinegar through the oxidation of ethanol into acetic acid by an obligatory aerobic metabolism with oxygen as the terminal electron acceptor (De Ley et al., 1984). They are also the main spoilage microorganisms in some food products, especially those that may contain ethanol or sugar. AAB species have traditionally been identified by testing physiological and homotaxonomic abilities (De Ley et al., 1984), but these methods are not completely reliable and are time consuming. These phenotypic properties have now been complemented or replaced by such molecular techniques as DNA and rRNA hybridization methods (Urakami et al., 1989), sequence analysis or various PCR methods (Ruiz et al., 2000; Trcek and Teuber, 2002; Bartowsky et al., 2003; Trcek, 2005; Gonzalez et al., 2005, 2006a,b; Gullo et al., 2006; Prieto et al., 2007). It has to be emphasized that a greater diversity has been observed when some of these molecular techniques have been combined with culture-independent methods and used to study the whole bacterial community in complex natural habitats or ecosystems. Food ecosystems have been shown to be no exception to this diversity, and culture-independent analyses have been applied to wines, sausage, cheese, sourdough, and other foods, as reviewed by Fleet (1999) and Giraffa (2004). In the particular case of acetic acid bacteria, it has been reported that conventional plate counts were considerably lower than the optical counts of viable microbial cells from wines (Millet and Lonvaud-Funel, 2000) or industrial acetators (Mesa et al., 2003), indicating the possibility of viable but not culturable (VBNC) status. Some approaches to identify AAB by culture-independent methods have used systems that include quantitative PCR (Gonzalez et al., 2006b) and Denaturing Gel Gradient Electrophoresis (DGGE) analysis in wines (Lopez et al., 2003), traditional rice vinegar (Haruta et al., 2006), Aceto Balsamico tradizionalle (De Vero et al., 2006; De Vero and Giudici, 2008), or traditional fermented foods (Nielsen et al., 2007).

The aim of the present work was to analyze several systems for identifying AAB using different culture culture-independent methods. These methods were applied to samples obtained from different acetification phases in traditional vinegar production in Chile. It should be emphasized that while Chile has fully consolidated its position in the wine industry, no tradition or relevant production of Chilean vinegar exists and, thus, this marks the first attempt to characterize AAB in Chilean vinegar.

2. Materials and methods

2.1. Samples collection and processing

Mother of vinegar was generated by exposing Chilean wine to air in plastic trays whilst protected by cheese cloth. The vinegar

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mothers were maintained by addition of small quantities of wine. An appropriate mixture of wine and mother was adjusted to 14 g acetic acid l^{-1} and 80 g ethanol l^{-1}. The mixtures were maintained at 32 °C. Samples were taken for microbiological analysis at 20 (acetification start), 40 and 60 g acetic acid l^{-1}, when acetification was considered to have finished. Wine, mother and the mixture were also analyzed.

2.2. DNA extraction and purification

DNA from the acetification samples, the wine and the mixtures were obtained from homogenates by using a PowerSoil™ DNA Isolation Kit from MoBio following the manufacturer's instructions. For reference strains, 1 ml of an overnight bacterial culture was centrifuged and the DNA of the pelleted cells was extracted using the Genomic DNA Purification kit from Promega (Madison, WI, USA).

2.3. PCR amplification and analysis of the products by RFLP and TTGE

The almost complete *16S rRNA* gene was amplified as described by Romero et al. (2002) and amplicons were analyzed by gel electrophoresis as described by Espejo and Escanilla (1993). To obtain profiles of different samples PCR amplification of V3–V5 region of *16S rRNA* gene was carried out as described Magne et al. (2006) using directly extracted DNA. Temporal temperature gradient electrophoresis (TTGE) was performed as described by Romero and Navarrete (2006). Restriction fragment length polymorphism (RFLP) was performed to analysis of amplicons of the *16S rRNA* gene or eluted TTGE bands using AluI and TaqI or HaeIII as described by Romero and Navarrete (2006).

2.4. Cloning

Amplicons were cloned using TOPO TA cloning kit according to the manufacturer's protocol (Invitrogene). Plasmids containing an insert were digested with AluI for separation of the different clones.

2.5. Sequence analysis

The intense bands and some weak bands in each TTGE pattern were excised from the gel and eluted (see Fig. 3). Intense bands could represent the most abundant or dominant bacterial population in the sample, so we called dominant bands. The *16S rRNA* gene amplicons from the reamplified bands or from cloning library were sequenced as described by Romero and Navarrete (2006). Sequences were deposited in GenBank (EU077240–EU077262) and analyzed in Ribosomal Database Project II Web site (Cole et al., 2007). Phylogenetic analysis was performed using the TREECON program with neighbor-joining method (Van de Peer and De Wachter, 1997) as described (Romero and Navarrete, 2006).

2.6. Bacterial counts and cultivation

Total bacterial counts present in vinegar were performed by light microscopy using a Petroff Hausser counting chamber. Triplicate samples of vinegar were plated using an automatic spiral plater in GYC solid medium (De Ley et al., 1984). Colonies were counted after 3 days.

3. Results and discussion

3.1. Bacterial enumeration

The starting of the acetification process was considered when the mixture of wine and vinegar reached 20 g acetic acid l^{-1} and was analyzed to a concentration of 60 g acetic acid l^{-1}. The bacterial enumeration under the microscope gave values ranging from 1.3 to 1.8×10^8 cells ml^{-1} in the main production phase (20–40 g acetic acid l^{-1}) to 8×10^7 cells ml^{-1} at the end. However the number of colonies recovered after plating was much smaller, ranging from 2–4×10^5 ufc ml^{-1}. Thus, a significant reduction of recovery was found in plating, similar to that reported by others (Mesa et al., 2003). The reason could be related to an inappropriate culture medium, VBNC status (Millet and Lonvaud-Funel, 2000), or the agglomeration of cells and the difficulty in isolating or separating them to form single-cell colonies. In fact, a combination of the three factors may explain the final result. The culture medium is a generic, rich medium where normally many bacteria are able to grow and it is a recommended medium for AAB isolation (De Ley et al., 1984). Although several good media have been proposed for AAB cultivation (Entani et al., 1985; Sievers et al., 1992; Sokollek et al., 1998), there are still recovery limitations. The VBNC status might be a real possibility given that observation under the microscope reveals on the one hand the absence of growth, and on the other the survival of AAB in the vinegar medium due to the acetic acid concentration. Surviving cells may not be able to grow as they need to form colonies. Finally, it is evident that AAB form cell groupings of variable numbers which, in the case of forming a colony, may come from a bunch of AAB cells instead of as a single cell. Vigorous shaking or treatments with cellulases or similar glucanases did not improve the recovery in solid medium (results not shown).

3.2. Analysis of RFLP-PCR 16S rRNA gene profiles

Amplicons including almost the entire *16S rRNA* gene were obtained after DNA extraction and PCR amplification from vinegar and wine samples, digested with AluI and TaqI and compared with profiles derived from reference strains including *Acetobacter pasteurianus*, *Acetobacter aceti*, *Gluconobacter oxydans* (Fig. 1). The results of the RFLP analysis of the *16S rRNA* gene AAB type strains were very similar to those reported by Ruiz et al. (2000) and Gonzalez et al. (2006a) also using *16S rRNA* gene amplicons. The RFLP profiles from the vinegar mother and the early vinegar samples showed a mixture of bands that can indicate the coexistence of different microorganisms. However, as the acidity increases in the vinegar process, the number of bands reduces progressively to find a single profile at 60 g acetic acid l^{-1}, which is identical to that of *A. pasteurianus* type strain. These electrophoretic bands were observed in all the vinegar or acetification samples. However, in the wine samples faint and limited bands were found, indicating a very low microbial population. It is evident that this technique is very limited when used with mixed cultures where more than two species are present, yet it might be resolutive when applied to single species cultures.

3.3. Analysis of TTGE profiles

The amplicons, including V3–V5 regions of *16S rRNA* gene from vinegar and wine samples, were also separated using TTGE (Fig. 2). The observed profiles showed in general one dominant band as well as the presence of some other minor bands. The main dominant band showed the same migration both in mother vinegar and the rest of the vinegar samples. This band was only absent in the starting wine. After elution and sequencing, the main band of all the vinegar samples could be grouped within *A. pasteurianus* with an identity range from 99.2 to 100%. Other weak bands migrating near to this dominant band were identified as *Acetobacter* sp., and could correspond to heteroduplexes formed in the last cycle of the PCR amplification (Espejo et al., 1998). A faint band containing greater electrophoretic migration (higher %GC) was observed only in the 20 g acetic acid l^{-1} sample. After sequencing this band was associated with a group which had, along with strains from *Gluconacetobacter xylinus*/*europaeus*/

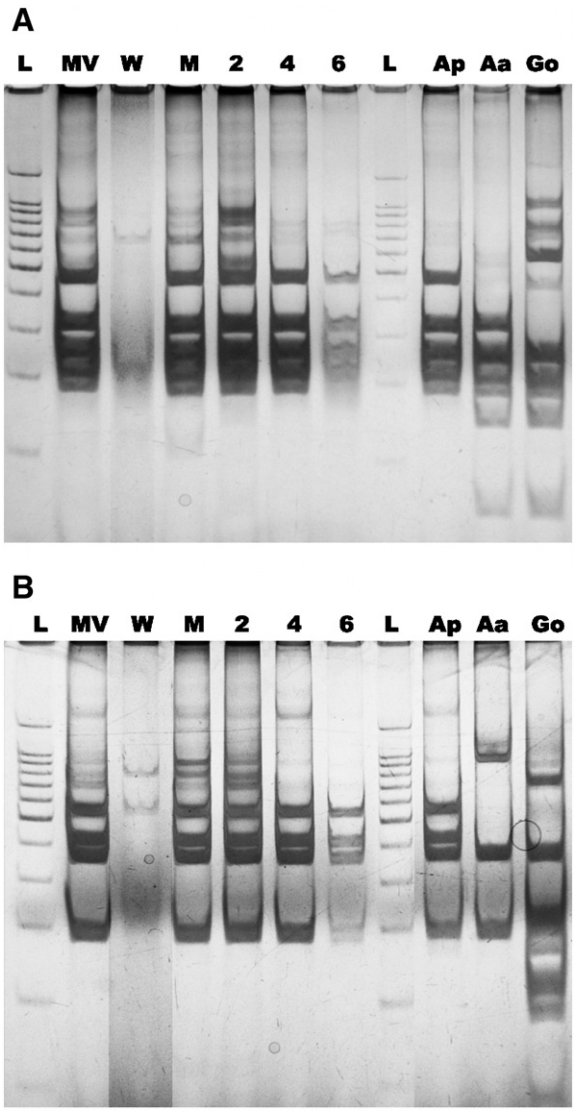

Fig. 1. Electrophoretic profiles of the RFLP-PCR of 16S rDNA of the different samples. Lanes: MV vinegar mother, W wine, M mix of wine and vinegar mother, 2, 4, 6 acetification samples containing 20, 40 and 60 g acetic acid l-1, L Ladder 100 bp. Invitrogen, Ap *A. pasteurianus*, Aa *A. aceti*, Go *G. oxydans*. A: Digested with AluI, B: Digested with TaqI.

intermedius species, 99.5% homology. In the wine sample, a unique and dominant band was also observed and was identified as *Pediococcus* spp. with 100% identity. The *Pediococcus* spp. band was also detected in the mixture of wine and vinegar mother. The presence of *A. pasteurianus* and *G. xylinus* in vinegar produced by traditional methods has been previously reported by Gullo et al. (2006). These authors, however, found more *G. xylinus* colonies growing on a solid medium. On similar samples and using DGGE, De Vero et al. (2006) identified a main microorganism that was identified as belonging to the *A. pasteurianus*/*A. aceti* group. On the other hand, *Pediococcus* spp. is regularly present in wine, being one of the most frequent Lactic Acid Bacteria in wines (Ribereau-Gayon et al., 2000).

3.4. Analysis of 16S rRNA gene cloning

One way to avoid culturing, whilst being able to enumerate the individuals of the different species, is to clone the sample DNA into competent microorganisms by means of incorporating it into a plasmid and cloning it in a microorganism which is easier to cultivate. The cloning was done using the microbiologically more complex vinegar sample (20 g acetic acid l^{-1}) and thus more than 100 clones were obtained. Of these, 60 clones with the insert were analyzed by RFLP with AluI. Three different groups were set according to those patterns; the most abundant group was represented by 53 clones and showed the same restriction pattern as the main TTGE band. The sequencing of eleven of these clones allowed us to identify them as *A. pasteurianus*, which agreed with the results seen in both RFLP-PCR *16S rRNA* gene and TTGE. The second group was represented by 6 clones and clustered with *Gluconacetobacter xylinus*/*europaeus*/*intermedius*. It is well known that these species have very limited variation in their *16S rRNA* gene and the grouping methods based on the variability of this sequence are not able to discriminate them properly (Ruiz et al., 2000; Gonzalez et al., 2006a; De Vero and Giudici, 2008). Finally, one clone was grouped with a *Gluconacetobacter*-like microorganism.

3.5. Phylogenetic analysis of bacterial populations obtained by TTGE and cloning

We compared partial *16S rRNA* gene sequences of approximately 370 bases obtained from bands detected in TTGE (bands V2,V4,V6 Fig. 2) and *16S rRNA* gene clones with sequences available in the RDP II database (Table 1, Fig. 3). Our results indicated that all the microorganisms represented by the main bands in all the vinegar samples were AAB. The main species found were *A. pasteurianus*, as seen in the three methods used. Using the sequences available in the RDP II database, the strains considered as *A. pasteurianus* showed differences of up to 1.4% in their 355 bp *16S rRNA* gene sequences with respect to other *A. pasteurianus* strains. The other cluster observed was *Gluconacetobacter* genus. Due to the sequence similarity, several related species described in vinegars were included, such as *G. xylinus*,

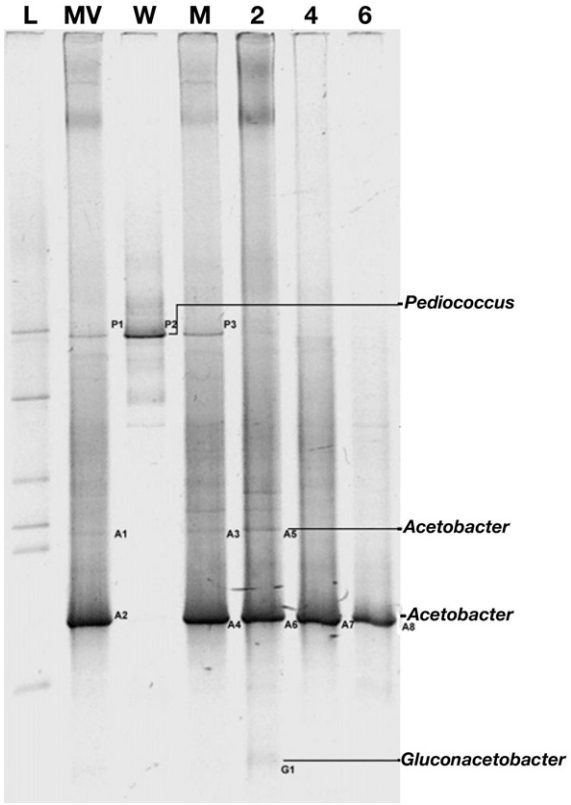

Fig. 2. TTGE electrophoretic profile of the different samples. Lanes: MV vinegar mother, W wine, M mix of wine and vinegar mother, 2, 4, 6 acetification samples containing 20, 40 and 60 g acetic acid l-1, L ladder with different %GC. Bands eluted and sequenced were marked.

Table 1
Sequence variability in the sequenced bands and clones

	Sequence variability % 16S fragment 355	No of sequences compared
Acetobacter pasteurianus		
Clones	0–1.4	11
Bands	0–0.8	8
Reference sequences RDP II *A. pasteurianus* (LMD 22.1, IFO 13755, CICCHLJ Q40, CICCHLJ Q61, LMG 1633, LMG 1629, MHM 10-1, OR56-1, A74, CWBI\B-419, NCI 1193).	0–1.7	11
Gluconacetobacter cluster		
Clones	0.9	2
Bands	NA	1
Reference sequences RDP II *G. xylinus* (BPR2001, LMG1515, JCM 10150, JCM 7644, JCM 9730, NBRC 15237).	0–0.6	6
Gluconacetobacter sp.		
Clones	NA	1

NA: Not applicable.

G. europaeus and *G. intermedius*. After comparison the differences among these sequences were less than 1% (Table 1). The exact species could not be defined because of the limited variation in the sequenced region within the genus *Gluconacetobacter*. However, it can be seen that the sequence variability is higher than in the database strains and, thus, the possibility of different *Gluconacetobacter* species cannot be ruled out. Overall the results suggest the presence of a diversity of strains in the vinegar samples, with *A. pasteurianus* probably being the better adapted species as it is able to survive in a more acidic environment.

This study shows that using rapid molecular methods for identifying AAB species still yields inconclusive identification. Thus, some more work has to be done in order to have a reliable, quick and easy-to-use method. However, a clear advantage of these molecular quick methods is that they allow groupings of different microorganisms into definite clusters where representative individuals can be chosen for further analysis using more reliable and complete methods (*16S rRNA* gene sequencing), and thus they can be identified definitely. Several culture-independent methods can be used yielding very similar results, as seen in the present study. TTGE allows a quick insight into overall and rough diversity, while cloning allows enumeration of different species and observation of fine molecular diversity. Thus, both methods complement each other in offering a view of quantitative microbial diversity. Finally, the present paper analyzes for first time the presence of AAB in Chilean vinegars, and it is evident that the main species producing vinegar are the same as those previously described in other countries, that is *A. pasteurianus* and members of the *Gluconacetobacter xylinus*/*europaeus*/*intermedius* cluster.

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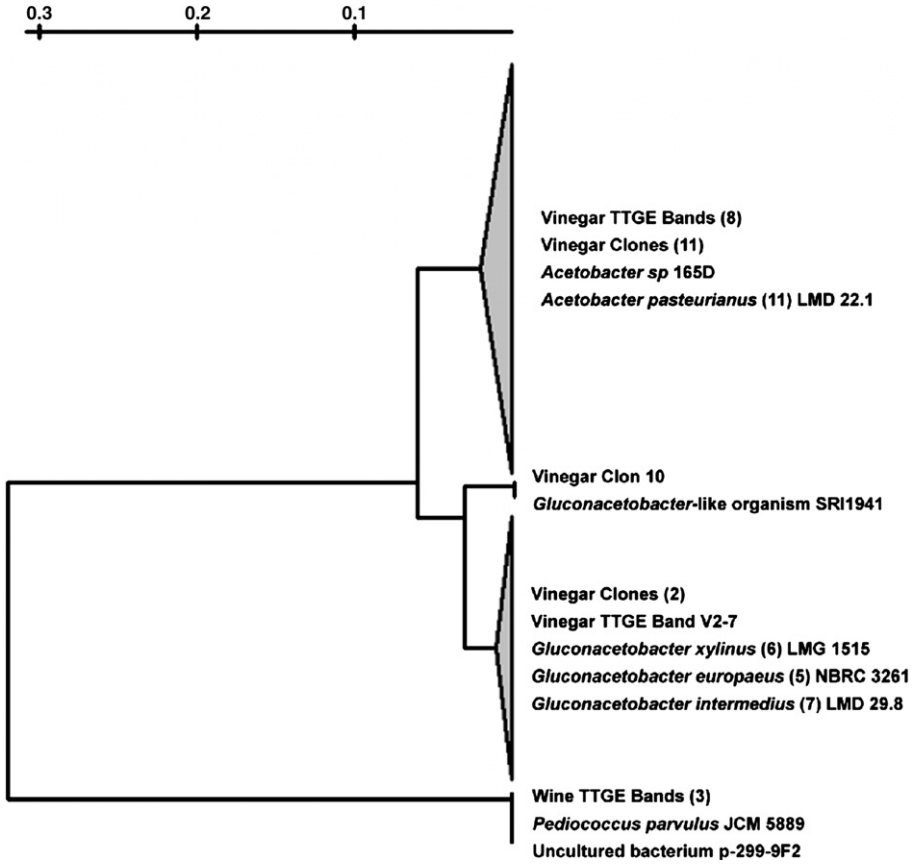

Fig. 3. Neighbor-joining phylogenetic tree showing the relationship between sequences retrieved from the TTGE profiles, clones and their closest relative sequences deposited in the RDP II. The tree was based on the 341–788 region of the *16S rDNA* genes. Distance matrices were constructed from the aligned sequences and corrected for multiple base changes at single nucleotide positions by the method of Jukes and Cantor using Treecon.

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