Short Communication

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

Carolina Ilabaca a, Paola Navarrete b, Pamela Mardones b, Jaime Romero b, Albert Mas c,*

a Laboratorio de Microbiología Enológica, Facultad de Ciencias Agronómicas, Universidad de Chile, Santiago de Chile, Chile
b Laboratorio de Biotecnología, INTA, Universidad de Chile, Santiago de Chile, Chile
c Grup Biotecnologia Enològica. Dept. Bioquímica i Biotecnologia. Facultat d’Enologia de Tarragona. Universitat Rovira i Virgili. Marcel ll Domínguez, sn. 43007 Tarragona, Spain

ABSTRACT

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 aceticification 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 aceticification 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 quantative 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 tradizionale (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 aceticification 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...
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 (aceticification start), 40 and 60 g acetic acid l$^{-1}$, when aceticification was considered to have finished. Wine, mother and the mixture were also analyzed.

2.2. DNA extraction and purification

DNA from the aceticification 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 pellet 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). The bacterial population. It is evident that this technique is very limited 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 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 aceticification 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/
intermedius species, 99.5% homology. In the wine sample, a unique and dominant band was also observed and was identified as *Pedio-
coccus* 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

v
de

intermedius species, 99.5% homology. In the wine sample, a unique and dominant band was also observed and was identified as *Pedio-
coccus* 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

v

intermedius species, 99.5% homology. In the wine sample, a unique and dominant band was also observed and was identified as *Pedio-
coccus* 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).

3.5. 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. 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 aceticification 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.](image)

![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 aceticification samples containing 20, 40 and 60 g acetic acid l−1, L ladder with different %GC. Bands eluted and sequenced were marked.](image)
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.

Acknowledgements

This work is the result of joint collaboration agreements funded by AECI travel bursaries and grant number AGL2004-07494-C02-02/ALI from the government of Spain and partially by FONDECYT 1061121 from Chile. The authors thank the Language Service of the Rovira i Virgili University for revising the manuscript. P. Navarrete (INTA) and P. Mardones (USACH) were supported by a Doctoral scholarship from CONICYT–Chile. C. Ilabaca was supported by Tecnovid Research Consortium scholarship.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Sequence variability in the sequenced bands and clones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sequence variability</td>
</tr>
<tr>
<td></td>
<td>% 16S fragment 355</td>
</tr>
<tr>
<td>Acetobacter pasteurianus</td>
<td></td>
</tr>
<tr>
<td>Clones</td>
<td>0–1.4</td>
</tr>
<tr>
<td>Bands</td>
<td>0–0.8</td>
</tr>
<tr>
<td>Reference sequences RDP II</td>
<td>A. pasteurianus (LMD 22.1, IFO 13755, CICHLJ Q40, CICHLJ Q81, LMG 1633, LMG 1629, MHM 10-1, OR56-1, A74, CWB/B-419, NCI 1193).</td>
</tr>
<tr>
<td>Gluconacetobacter cluster</td>
<td></td>
</tr>
<tr>
<td>Clones</td>
<td>0.9</td>
</tr>
<tr>
<td>Bands</td>
<td>NA</td>
</tr>
<tr>
<td>Reference sequences RDP II</td>
<td>G. xylinus (BPR2001, LMG1515, JCM 10150, JCM 7644, JCM 9730, NBRC 15237).</td>
</tr>
<tr>
<td>Gluconacetobacter sp.</td>
<td></td>
</tr>
<tr>
<td>Clones</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA: Not applicable.

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.
References


