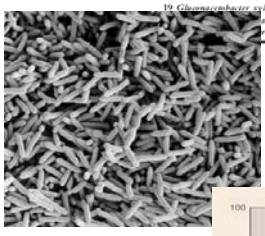
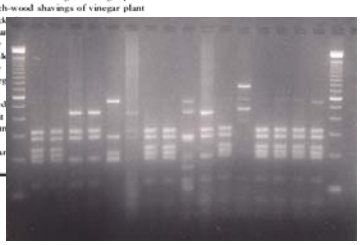
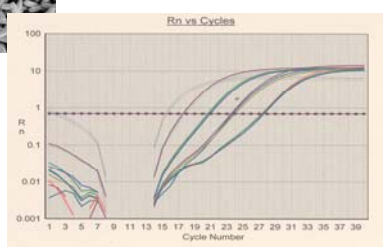
Departament de Bioquímica i Biotecnologia
Facultat d'Enologia

Application of molecular techniques for identification and enumeration of acetic acid bacteria







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Tesis Doctoral
Tarragona, 2005

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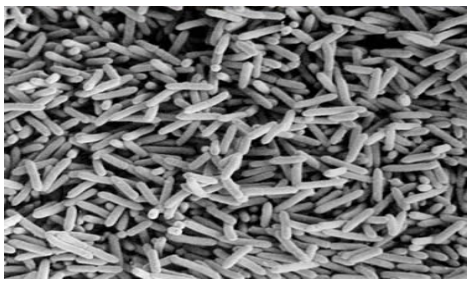
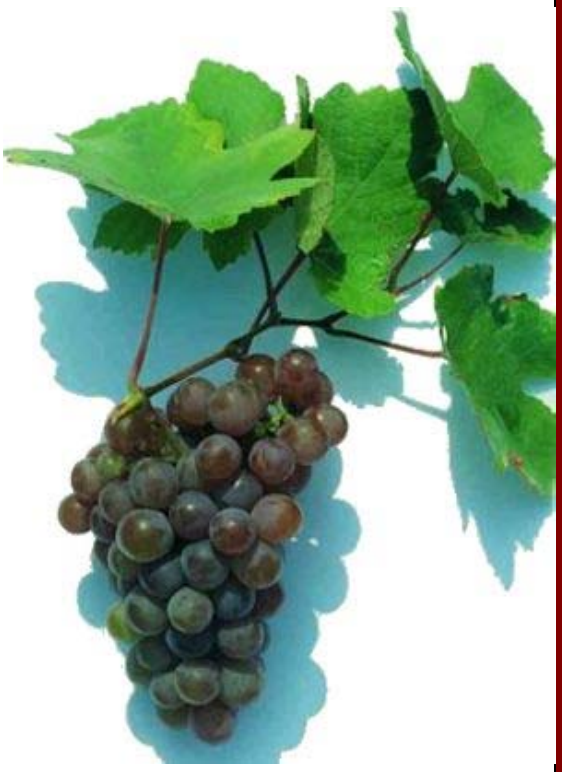


Objectives and Justification.



UNIVERSITAT ROVIRA I VIRGILI

TARRAGONA, 2005

OBJECTIVES AND JUSTIFICATION

I joined the research group "Oenological Biotechnology" of the Department of Biochemistry and Biotechnology at the Faculty of Oenology in Tarragona in the year 2000. Since then, the group has received funds for a number of projects, among which is the present study: grants AGL2001-0467 and AGL2004-07494-C02-02/ALI from the *Comisión Interministerial de Ciencia y Tecnología*, Spain.

The projects in which the group has been involved deal with the development of fast and reliable molecular biology techniques for the characterization of microorganisms involved in alcoholic fermentations. These techniques are basically applied to yeasts, and some work was done on acetic acid bacteria. Our objective was to continue the work on acetic acid bacteria, which are the least studied oenological microorganisms.

The first objective was to develop fast and reliable techniques for characterizing, identifying and enumerating acetic acid bacteria at:

- Strain level: two molecular techniques based on the amplification of repeated regions of the bacterial genome were tested in some bacterial families and adapted to acetic acid bacteria.
- Species level: two techniques based on the amplification of a conserved region of the bacterial chromosome and the restriction of the amplification product were tested in acetic acid bacteria, and new genera and species that have recognised in recent years.
- Enumeration: a technique that allows the enumeration of acetic acid bacteria without the need of culturing them. This technique is the real-time PCR and is based on the determination of the initial template concentration, and, therefore, allows an accurate estimation of the cells numbers.

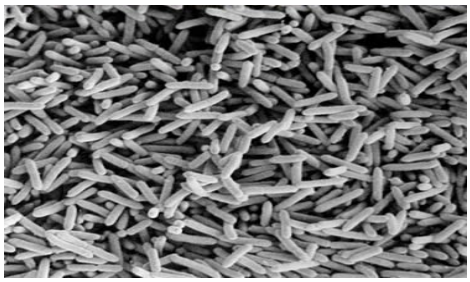
To validate these techniques we made a variety of ecological studies in wine, including grape, must and wine samples in different fermentation conditions.

The objective of the first part of the present study is to develop and apply fast, efficient and reliable methods for identifying acetic acid bacteria species because the methods currently used are complex and slow. The number of known species of acetic acid bacteria has grown quite considerably because of new research done in new microbiological niches such as plants and flowers. Our objective here was to discriminate among those new species using the abovementioned techniques so that they can be distinguished with a routine protocol.

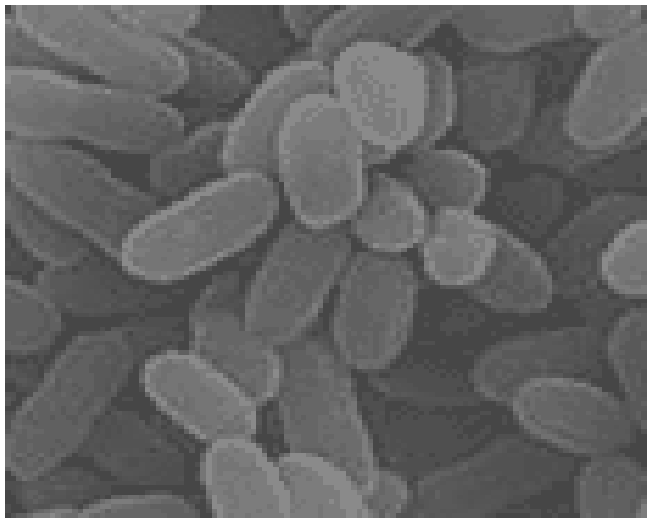
Molecular techniques were also applied to discriminate acetic acid bacteria at strain level and the techniques were used in a survey study during an alcoholic fermentation to test their usefulness in routine assays.

The third objective was to extend our knowledge of how acetic acid bacteria behave during alcohol fermentation. We did this by means of a survey study of acetic acid bacteria including grape, must and wine samples in different fermentation conditions (that is to say, SO_2 addition and yeast inoculation, both commonly used techniques in wine-making processes).

The goal of the fourth part of the study was to quantify total AAB in microbiological samples such as wine or vinegar. Quantitative-PCR made it possible to rapidly enumerate and detect the presence of this bacterial group. For this enumeration, plate culturing was not necessary so viable but non-cultivable cells could also be detected.



Introduction.



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INTRODUCTION

The microorganisms present in wine-making processes are mainly yeasts, lactic acid bacteria and acetic acid bacteria, because of the extreme conditions in grape must such as the low pH (between 3-4) or the high sugar concentration. *Non-Saccharomyces* yeasts are present in the first stages of alcoholic fermentation and *Saccharomyces* species (mainly *Saccharomyces cerevisiae*) are responsible for converting the sugars in grape must into ethanol and CO_2 (Drysdale & Fleet, 1988).

Lactic acid bacteria decrease the acidity of the wine and convert malic acid into lactic acid and CO_2. This is a one-step reaction known as malolactic fermentation, which usually takes place once the alcoholic fermentation is over (Ribéreu-Gayon *et al*., 2000).

Acetic acid bacteria (AAB) play a negative role in the wine-making process because they alter the organoleptic characteristics of the wine and, in some cases, can also lead to stuck and sluggish fermentations. AAB modify wine, mainly because they produce acetic acid, acetaldehyde and ethyl acetate. They are also involved in other industrial processes of considerable interest for biotechnology such as the production of cellulose, sorbose and vinegar (Du Toit & Pretorius, 2002).

Acetic acid bacteria can be found in different stages of the wine-making process: for example, grape ripening, must, alcoholic fermentation, and bottled and stored wine. Although it has been known that wine can be altered by acetic acid bacteria ever since Pasteur, and they have a highly undesirable impact on the alcoholic fermentation processes, relatively little is understood about how they behave. Other microorganisms such as yeasts and lactic acid bacteria are also present during alcoholic fermentations and have been studied in much greater depth.

The aim of the present study is to extend our knowledge of how acetic acid bacteria behave during wine making and other industrial processes of high biotechnological interest, such as vinegar production.

1. General characteristics of acetic acid bacteria

Acetic acid bacteria (AAB) are gram negative, ellipsoidal to rod-shaped, and can occur singly, in pairs or in chains. They are motile due to the presence of flagella, which can be both peritrichous or polar. They do not form endospores as a defensive resistance. They have an obligate aerobic metabolism, with oxygen as the terminal electron acceptor. The optimum pH for the growth of AAB is 5-6.5 (Holt *et al*., 1994). However these bacteria can grow at lower pH values of between 3-4. They vary between 0.4-1 μm wide and 0.8-4.5 μm long. They are catalase positive and oxidase negative. AAB can present pigmentation in solid cultures and can produce different kinds of polysaccharides (De Ley *et al*., 1984).

AAB occur in sugar and alcoholised, slightly acid niches such as flowers, fruits, beer, wine, cider, vinegar, souring fruit juices and honey. On these substrates they oxidise the sugars and alcohols, resulting an accumulation of organic acids as final products. When the substrate is ethanol, acetic acid is produced, and this is where the name of the bacterial group comes from. However, these bacteria also oxidize glucose to gluconic acid, galactose to galactonic acid, arabinose to arabinoic acid. Some of these transformations carried out by AAB are of considerable interest for the biotechnological industry. The best known industrial application of AAB is vinegar production but they are also used to produce sorbose, from sorbitol, and cellulose.

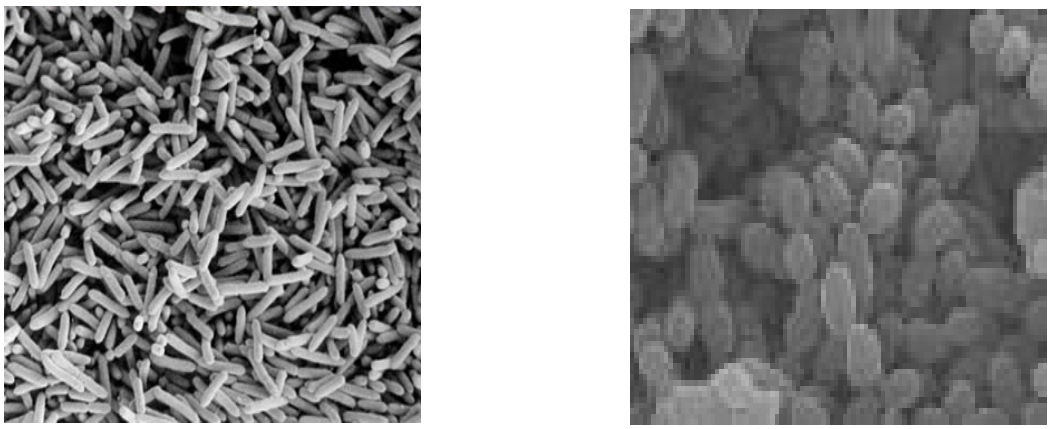

Fig. 1: Electron microscope photography of AAB

2. Metabolism

One of the main characteristics of AAB is their ability to oxidize a wide variety of substrates and to accumulate the products of their metabolism in the media without toxicity for the bacteria. This ability is basically due to the dehydrogenase activity in the cell membrane. These dehydrogenases are closely related to the cytochrome chain (Matsushita *et al*., 1985).

Ethanol metabolism

The oxidation of ethanol to acetic acid is the best known characteristic of acetic acid bacteria. Ethanol oxidation by AAB takes place in two steps. In the first one, ethanol is oxidized to acetaldehyde and in the second step acetaldehyde is oxidized into acetate. In both reactions, electrons are transferred and these are later accepted by oxygen (Fig. 2).

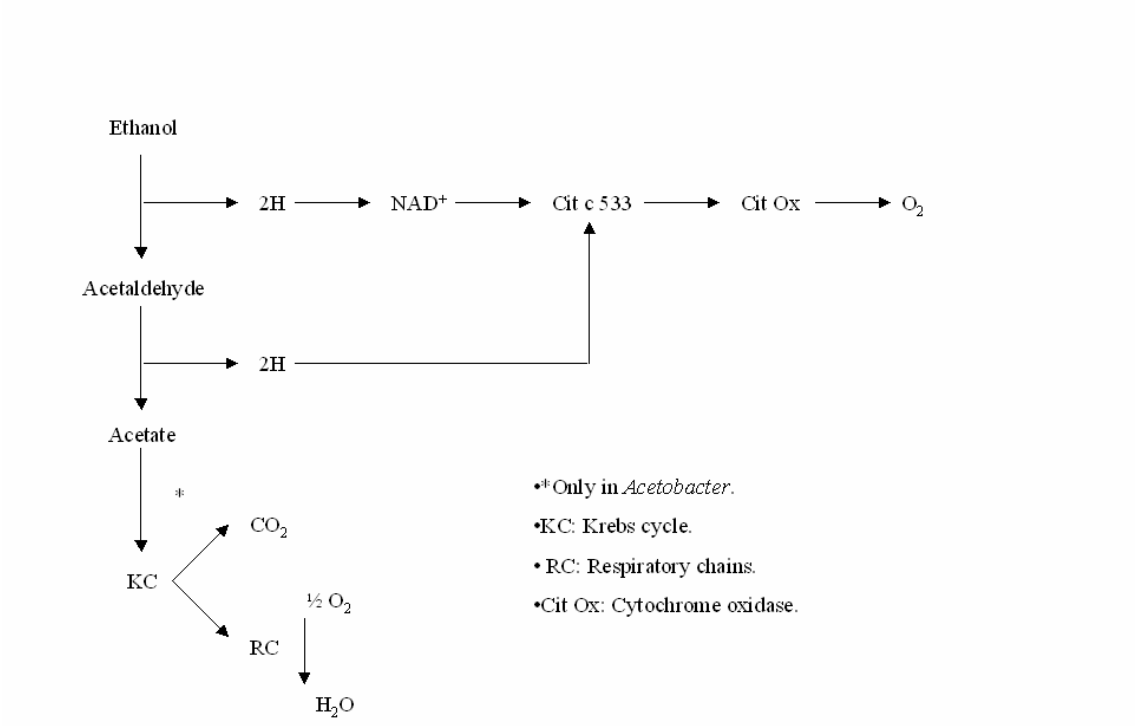

Fig. 2: Ethanol oxidation by AAB

Two enzymes play a critical role in this oxidation process, both of which are bound to the cytoplasmic membrane: they are alcohol dehydrogenase and aldehyde dehydrogenase. Both enzymes have their active sites on the outer surface of the

cytoplasmic membrane (Adachi *et al*., 1978; Saeki *et al*., 1997). The alcohol dehydrogenase enzyme consists of two or three subunits, which include the dehydrogenase (72-78 kDa) and cytochrome c (48 kDa) that are essential for the activity of the enzyme. The third subunit is a protein of 20 kDa, found in *A. aceti* and *A. pasteurianus*. The two larger subunits play a role in the intramolecular transport of electrons from the alcohol dehydrogenase to ubiquinone, and further to the terminal ciytochrome oxidase during the oxidation of ethanol. The smallest one helps the two functional subunits with their association to the membrane (Kondo & Horinouchi, 1997; Saeki *et al*., 1997). This membrane-bound alcohol dehydrogenase has pyrroloquinoline as a cofactor and is independent of $NADP^+$. A cytoplasmatic $NADP^+$-dependent alcohol dehydrogenase has also been identified, although it has a much lower specific activity than the membrane-bound alcohol dehydrogenase and a higher optimal pH (6-8), which limits its contribution to the oxidation process of ethanol (Adachi *et al*., 1978; Takemura *et al*., 1993; Matsushita *et al*., 1994). The $NADP^+$ independent enzyme has an optimal pH of 4, but it is still active at lower pH levels. The alcohol dehydrogenase activity of *Acetobacter* is more stable under acetic conditions than that of *Gluconobacter*, which explains why *Acetobacter* produces more acetic acid (Matsushita *et al*., 1994).

The other enzyme involved in the oxidation of ethanol is aldehyde dehydrogenase. It is also a $NADP^+$ independent enzyme and located in the cytoplasmatic membrane. Its optimum pH is between 4 and 5, although it can catalyse the oxidation of acetaldehyde to acetate at lower pH values (Adachi *et al*., 1980). It is an enzyme that is sensitive to oxygen concentrations, and when these are low its activity decreases, accumulating acetaldehyde. It is also more sensitive to the presence of ethanol than alcohol dehydrogenase (Muraoka, 1983).

These bacteria can produce high concentrations of acetic acid, up to 150 g l^{-1} (Sievers *et al*., 1997; Lu *et al*., 1999), which makes them very important to the vinegar industry. Their resistance is strain dependent (Nanba *et al*., 1984). The enzyme citrate synthase plays a key role in this resistance, because it detoxicates acetic acid by incorporation into the tricarboxylic or glyoxylate cycles, but only when ethanol is not present in the media. Menzel & Gottschalk (1985) reported that Acetobacter strains decrease their

internal pH in response to a lower external pH. However, an adaptation to high acetate concentrations seems to be a prerequisite for high tolerance (Lasko *et al*., 2000).

Metabolism of primary and polyalcohols

A considerable number of AAB can oxidize alcohols into sugars; mannitol into fructose; sorbitol into sorbose or eritritol into eritrulose. An important ability in oenology is to use glycerol as a carbon source (De Ley *et al*., 1984), which is converted into dihydroxyacetone, a small amount of which is used for energy synthesis.

The enzymes that catalyse all these reactions are located in the cell membrane and induce a high accumulation of substrates in the media, which make AAB suitable microorganisms for the biotechnological industry (Deppenmeier et al., 2002).

Carbohydrate metabolism

AAB can metabolise different carbohydrates as carbon sources. *Acetobacter* species can use sugars through the hexose monophosphate pathway (Warburg-Dickens pathway) (De Ley *et al*., 1984; Drysdle & Fleet, 1988) and also through the Embden-Meyerhof-Parnas and Entner-Doudoroff pathways (Attwood *et al*., 1991), although such authors as Drysdale & Fleet (1988) say that this last pathway is not used by AAB to metabolise glucose. From here they are further metabolised to CO_2 and water via the tricarboxylic acid pathway, which is not functional in *Gluconobacter* species, although the complete oxidation is only functional when there is no carbon source in the media.

Sugar is more preferred as a carbon source by *Gluconobacter* than by *Acetobacter* because the species of this genus can obtain energy more efficiently by the metabolisation of the sugars via pentose phosphate pathway (De Ley *et al*., 1984).

Glucose metabolism by these species produces a considerable number of industrially important metabolites (Olijve & Kok, 1979; Weenk *et al*., 1984; Qazi *et al*., 1991 and 1993; Velizarov & Beschkov, 1994). Some of these metabolites are 2-ketogluconic, 5-ketogluconic and 2,5-diketogluconic acids. The most characteristic reaction is the direct oxidation of glucose into Glucono-δ-lactone, which is oxidised into gluconic acid. This last reaction is particularly active in *Gluconobacter* species in media with high

concentrations of sugars such as grapes and must. This metabolite can be used as an indicator of the presence of these bacteria.

Acetic acid bacteria can also use other carbohydrates, such as arabinose, fructose, galactose, mannitol, mannose, ribose, sorbitol ando xylose (De Ley *et al*., 1984)

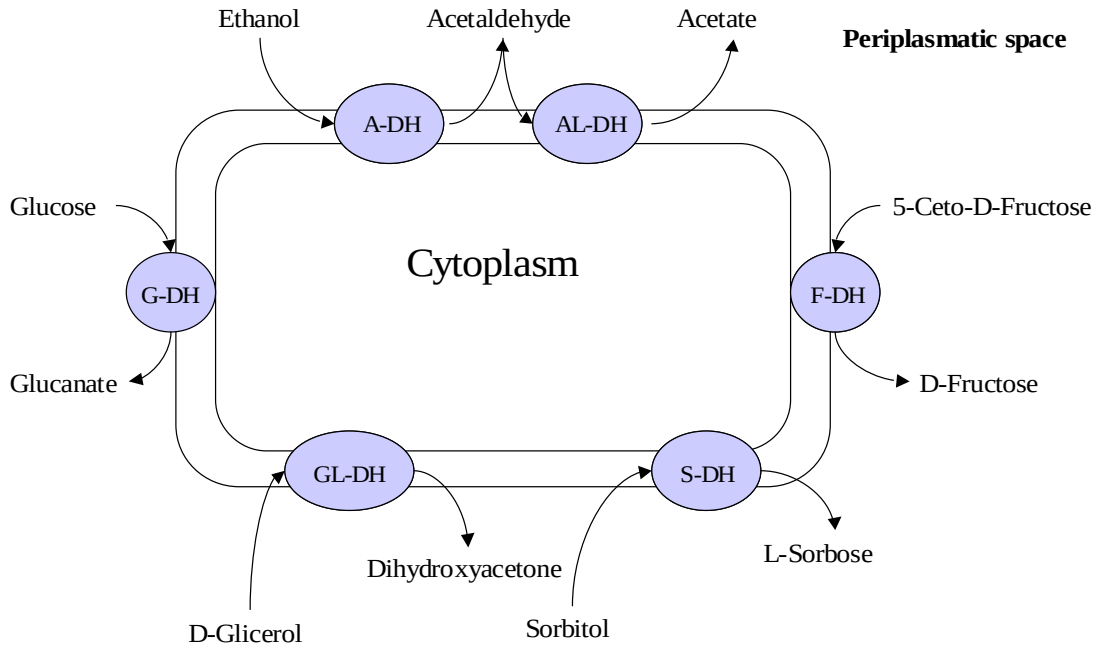

Fig. 3 : Alcohol and sugar oxidation systems in the bacterial membrane. G-DH, Glucose dehydrogenase; A-DH, Alcohol dehydrogenase; AL-DH, Aldehyde dehydrogenase; F-DH, Fructose dehydrogenase; SL-DH, Sorbitol dehydrogenase; GL-DH, Glycerol dehydrogenase.

Organic acid metabolism

AAB are able to metabolise a variety of organic acids. They do so through the tricarboxylic acid cycle which oxidises these acids to CO_2 and water. *Gluconobacter*, which lacks a functional tricarboxylic acid cycle, is unable to oxidise most organic acids (Holt *et al*., 1994). Acetic, citric, fumaric, lactic, malic, pyruvic, and succinic acids are completely oxidised to CO_2 and water. These changes are very important in winemaking, because they mean that the quality of the wines decreases.

Another important metabolic byproduct of lactate metabolism is acetoin (important in the world of oenology) (De Ley, 1959). The buttery aroma of this compound is

considered to be an unwanted flavour in wine, in which its detection limit is 150mg l^{-1} (Romano & Suzzi, 1996; Du Toit & Pretorius, 2000).

Nitrogen metabolism

Although some AAB species (*Ga. diazotrophicus*; Gillis *et al*., 1989) can fix atmospheric nitrogen, most of them use ammonium as a carbon source (De Ley *et al*., 1984). So these bacteria can synthesise all the amino acids and nitrogenated compounds from ammonium. Depending on the amino acid in the media, their growth can be either stimulated or inhibited. So, glutamate, glutamine, proline, and histidine stimulate the growth of AAB, whereas valine for *G. oxydans* and treonine and homoserine for *A. aceti* seem to have an inhibitory effect (Belly & Claus, 1972).

Nevertheless, no studies have been made on the nutritional needs of AAB nitrogen in wine. It has been observed that AAB selectively prefer some aminoacids during vinegar production (Valero *et al*., 2003), and leave significant amounts of ammonium in the media.

3. Taxonomy and identification

3.1. History

The French scientist Pasteur should be considered as the first taxonomist of this bacterial group. Studying the Orléans method of vinegar production, he demonstrated that the acetic acid came from ethanol oxidation and that long-term oxidation of acetic acid converted it into CO_2 and water. His results led him to formulate the involvement of the microorgansism in the process of transforming alcohol into vinegar, and confirmed the existence of *Mycoderma aceti* which Persoon had already described in 1822. Subsequently, in the year 1879, Hansen observed that the microbial flora which converted alcohol into acetic acid was not pure and consisted of various bacterial species. The genera *Acetobacter* was proposed later by Beijerinck (1899).

The first classifications were proposed by Hansen in 1894, based on the occurrence of a film in the liquid media, and its reaction with iodine. The first to propose a classification based on biochemical and physiological criteria was Vissert Hooft (1925). Asai (1934-35) formulated the proposal of classifying AAB into two genera: *Acetobacter* and

Gluconobacter. The main differences between these two genera were both cytological and physiological. The main physiological difference was that *Acetobacter* oxidized ethanol into acetic acid and, subsequently, completed the oxidation of acetic acid into water and CO_2. *Gluconobacter* species, on the other hand, were unable to complete this oxidation of acetic acid.

It was Frateur (1950) who formulated a classification based mainly on five physiological criteria: the presence of catalase, gluconic acid production from glucose, the oxidation of acetic acid into CO_2 and water, the oxidation of lactate into CO_2 and water, and the oxidation of glycerol into hydroxyacetone. On the basis of these criteria he divided *Acetobacter* genera into four groups: *peroxydans*, *oxydans*, *mesoxydans* and *suboxydans*.

Leifson (1954) grouped those AAB that had peritrichous flagella and were able to oxidize ethanol into the geneus *Acetobacter* and those that had polar flagella and unable to perform the complete oxidation into the genera *Gluconobacter*. The taxonomical keys for bacteria taxonomy have been historically collected in Bergey's Manual of Systematic Bacteriology. In its last edition (De Ley *et al*., 1984), some molecular techniques were included as fatty acid composition, soluble protein electrophoresis, % of G + C content, and DNA-DNA hybridisation. *Gluconobacter* and *Acetobacter* genera were included in the family *Acetobacteraceae*. *Acetobacter* geneus was composed by 4 species: *A. aceti*, *A. pasteurianus*, *A. liquefaciens* and *A. hansenii*. The *Gluconobacter* genera only consisted of *G. oxydans*.

3.2. Current situation of the taxonomy of AAB

However, the taxonomy of these microorganisms, initially based on morphological and physiological criteria, has been submitted to continuous variations and reorientations. These variations are due, basically, to the application of molecular techniques to the taxonomic study. The most common techniques used for this purpose are:

DNA-DNA hybridisation: From a taxonomic point of view, this is the most widely used technique for describing new species within bacterial groups. The technique measures the degree of similarity between the genomes of different species. When several species are compared in this way, the similarity values make it possible to

arrange the species in a phylogenetic tree, which shows the degree of intraspecific and interspecific similarity.

% Base ratio determination: This was one of the first molecular tools to be used in bacterial taxonomy. It calculates the percentage of G + C in a bacterial genome. Bergey's Manual of Systematic Bacteriology (De Ley *et al*., 1984) includes these values to differentiate among *Acetobacteraceae* species. Although this percentage must be taken into consideration, by itself it cannot identify a given microorganism. In AAB, the % values of G + C vary between 55.9 and 64.5 %.

16S rDNA sequence analysis: The 16S rDNA gene is a highly preserved region with small changes that can be characteristic of different species. Ribosomal genes are compared in most taxonomical studies of bacteria.

The *Acetobacteraceae* family is no exception in this reorganization of species and genera. Six new AAB genera have been added to both the genera mentioned above: *Acidomonas* (Urakami *et al*., 1989), *Gluconacetobacter* (Yamada *et al*., 1997), *Asaia* (Yamada *et al*., 2000), *Saccharibacter* (Jojima *et al*., 2004), *Swminathania* (Loganathan & Nair, 2004) and *Kozakia* (Lisdiyanti *et al*., 2002). At present, the *Acetobacteraceae* family consists of 8 genera and 38 species (Table 1). It has been proposed that the following species should be added to what was previously established by Bergey's (De Ley *et al*., 1984): *Acetobacter cerevisiae*, *A. malorum* (Ceenwerck *et al*., 2002), *A. tropicalis*, *A. orleaniensis*, *A. lovaniensis* and *A. estuniensis*, *A. syzgii*, *A. cibinongenesis* and *A. orientalis* (Lisdiyanti *et al*., 2001), *A. pomorum* and *A. oboediens* (Sokollek *et al*., 1998), *A. intermedius* (Boesch *et al*., 1998), *Kozakia baliensis* (Lisdiyanti *et al*., 2002), *Gluconacetobacter johannae* and *Ga. azotocaptans* (Fuentes-Ramirez *et al*., 2001), *Ga. swingsii* and *Ga. rhaeticus* (Cleenwerck *et al*., 2005) and *Ga. sacchari* (Franke *et al*., 1999), *Asaia krungthepensis* (Yukuphan *et al*., 2004), *As. siamensis* (Katsura *et al*., 2001), *As. bogorensis* (Yamada *et al*., 2000), *Saccharibacter floricola* (Jojima *et al*., 2004), *Swaminathania salitolerans* (Loganathan & Nair, 2004). *A. oboediens* and *A. intermedius* were subsequently reclassified as *Gluconacetobacter* by Yamada (2000).

Acetobacter	***Gluconacetobacter***	***Gluconobacter***	***Acidomonas***
A. aceti	*Ga. liquefaciens*	*G. oxydans*	*Ac. methanolica*
A. pasteurianus	*Ga. diazotrophicus*	*G. frateurii*	
A. pomorum	*Ga. xylinus*	*G. assaii*	
A. peroxydans	*Ga. hansenii*		
A. indonesiensis	*Ga. europaeus*	***Asaia***	***Kozakia***
A. tropicalis	*Ga. oboediens*	*As. bogorensis*	*K. baliensis*
A. syzygii	*Ga. intermedius*	*As. siamensis*	
A. cibinongenesis	*Ga. sacchari*	*As. indonesiensis*	
A. orientalis	*Ga. entanii*	*As. rugthepensis*	
A. orleaniensis	*Ga. johannae*		
A. lovaniensis	*Ga. azotocaptans*	***Swaminathania***	***Saccharibacter***
A. estuniensis	*Ga. Swingsii*	*S. salitolerans*	*Sa. floricola*
A. malorum			
A. cerevisiae			

Table 1: Species of AAB

3.3. Identification

3.3.1. Classical methods

Classical microbiological taxonomy has traditionally used morphological and physiological differences among the species to discriminate between them. The tests could only discriminate at the species level, although the physiological methods would not be able to distinguish the currently described species. At the genus level, several characteristics can contribute to the differentiation. The *Gluconobacter* genus cannot completely oxidise acetic acid into CO_2 and water. The main characteristic of *Acidomonas* is that it can grow in methanol, and *Asaia* is characterised by its inability to grow in a media with an acetic acid concentration higher than 0.35%. The other two genera, *Gluconcetobacter* and *Acetobacter*, can be differentiated on the basis of their ubiquinone content. Ubiquinone Q9 is present in *Acetobacter*, and ubiquinone Q10 in *Gluconacetobacter* (Trcek & Teuber, 2002). *Kozakia* have low similarity values of the % G + C content among the other genera (7-25% lower than the other species), the major ubiquinone is Q10 and have a weak activity in oxidation of lactate and acetate into carbon dioxide and water. The genus *Saccharibacter* has a negligible or very weak productivity of acetic acid from ethanol and the osmophilic growth properties distinguished this genus from other AAB. *Swaminathania* genus is able to fix nitrogen and solubilized phosphate in the presence of NaCl. Some of the phenotypic characteristics of the former speceis described in Bergey's Manual are shown in table 2.

Characteristics	*A. aceti*	*A. liquefaciens*	*A. pasteurianus*	*A. hansenii*	*G. oxydans*
Ethanol overoxidation	+	+	+	+	-
Growth in:					
Ethanol	+	+	v	-	-
Sodium acetate	+	v	v	-	-
Dulcitol	-	-	-	v	v
Glycerol Cetogenesis	+	+	-	+	+
Lactate oxidation	+	+	+	+	-
Pigment production	-	+	-	-	+

Table 2: Phenotypic characteristics of the species belonging to the *Actobacter* and *Gluconobacter* genera (De Ley *et al*., 1984). (+: positive for more than 90% of the strains, -: negative for more than 90% of the strains. V: positive between 11 and 89 % of the strains).

3.3.2. Isolation

These physiological differences among genera made it possible to develop differential culture media. Various culture media have been reported for isolating AAB whose carbon source is glucose, mannitol, ethanol, etc. Some of these media can also incorporate CaCO3 or bomocresol-green as acid indicators (Swings & De Ley, 1981; De Ley et al., 1984; Drysdale & Fleet, 1988). Culture media are usually supplemented with pimaricin in the agar plates to prevent the yeasts from growing and with penicillin to eliminate lactic acid bacteria.

Some of the most widely used culture media are GYC (5% D-glucose, 1% yeast extract, 0.5% $CaCO_3$ and 2% agar (w/v)), described by Carr and Passmore (1979), and , YPM (2.5% mannitol, 0.5% yeast extract, 0.3% peptone and 2% agar (w/v)). Plates must be incubated for between 2 to 4 days at 28ºC under aerobic conditions. These culture media are suitable for wine samples (Bartowsky *et al*., 2003; Du Toit & Lamberchts, 2002), and no problems have been detected culturing and isolating AAB from wine samples.

Nevertheless, some works indicate the difficulty of culturing this bacterial group from vinegar samples (Sokollek *et al*., 1998). This problem has been partially solved by introducing a double agar layer (0.5% agar in the lower layer and 1% agar in the upper layer (w/v)) into the cultures and media containing ethanol and acetic acid in an attempt

to simulate the atmosphere of the acetification tanks, such as AE medium (Entani *et al*., 1985).

	Culture media			
	GYC	**YPM**	**AE**	**EM**
Components:				
Yeast extract	1%	0,50%	0,30%	1%
Peptone		0,30%	0,40%	
Glucose	5%		0,75%	
Mannitol		2,50%		
$CaCO_3$	0,50%			2%
Acetic acid *			3%	
Ethanol *			3%	2%

Table 3: Different media for AAB. GYC, Glucose; YPM, Mannitol, AE, Entani; EM, ethanol. The values are given in w/v. * means v/v

3.3.3. Molecular techniques

The main objective of microbial classification is to identify an isolated microorganism up to species level. However, discriminating or typing the different strains or genotypes of a species is gaining increasing importance from an industrial point of view. Not all the strains of a species have the same ability to oxidize ethanol into acetic acid. Therefore, it is important to be able to determine how well each technique can discriminate among strains and to know how many species or strains are involved in the various processes.

Depending on the polymorphism degree obtained with the different molecular markers, those are more suitable either for inter-specific or for intra-specific discrimination. In that sense we divided the different molecular techniques into two main groups: those that can discriminate up to species level and those that can discriminate up to strain level.

Species level:

- PCR-RFLP of the rDNA 16S: This technique was used by Ruiz *el al*. (2000) to identify AAB and is appropriate for differentiating and characterising microorganisms on the basis of their phylogenetic relationships (Carlotti & Funke, 1994). In eubacterial DNA, the rRNA loci include 16S, 23S and 5S

rRNA genes, which are separated by internally transcribed spacer (ITS) regions. The technique consists on the amplification of the 16S rDNA region followed by the digestion of the amplified fragment with a restriction enzyme. The DNA fragments obtained are separated by electrophoresis. The resulting patterns are characteristic of every species and make it possible to characterize almost all the AAB species. This technique is explained and used in the present study.

- PCR-RFLP of the 16S-23S rDNA Internally Transcribed Spacer (ITS): This technique was used by Sievers *et al*. (1996), Ruiz *et al*. (2000) and Trcek and Teuber (2002) to characterize AAB species and is also used in the present study. The 16S-23S ITS region have a great variation and length difference among the species (Barry *et al*., 1991; Navarro *et al*., 1992), and conserved sequences with functional roles such as tRNA genes and antitermination sequences (Sievers et al., 1996). This technique consists in the amplification of a region of the ITS (this one spanning between the 16S and 23S rRNA genes) followed by the digestion of the amplified products with one restriction endonuclease. The result is DNA patterns that are characteristic of every species. Intergenic sequences are known to have higher variability than functional sequences, and they make it possible to distinguish below the species level (Navarro *et al*., 1992). The results obtained by Ruiz et al. (2000) and Trcek & Teuber (2002) only differentiated up to species level.

- Denaturing gradient gel electrophoresis (DGGE): DGGE separation of bacterial DNA amplicons is a common method used to characterize microbial communities from specific environmental niches (Muyzer & Smalla, 1998). This technique has been used by Lopez *et al*. (2003) and by De Vero *et al*. (2004) to characterize all of the microorganisms in wine (yeasts, lactic acid bacteria and AAB). It does not require the microorganisms to be isolated. The most commonly used genes for the DGGE method are the 16S and 23S rDNA genes because they present bacterial specificity.

  The technique consists on the amplification of a region of the genome. The amplicons obtained are resolved in a polyacrylamide electrophoresis in denaturing conditions created by using urea and formamide in the running

buffer. The DNA fragments are separated because of their low electrophoretic motility in these denaturing conditions. The 5' primer used in the amplification has a poly GC tale of about 40 bp (Muyzer & Smalla, 1998). During the electrophoretic process, the DNA remains doubled stranded until the gel zone in which the denaturing conditions are the same as its melting temperature (Tm). At that point, the DNA double strand is partially denatured and its motility reduced. Molecules with different base sequences will have different denaturing behaviours and, therefore, different migration rates in the polyacrylamide gel.

DGGE makes it possible to separate DNA fragments that are of equal lengths but which have different base sequences (Myers *et al*., 1987). The band pattern obtained is indicative of the number of different species present in a sample.

- Fluorescence in situ hybridisation (FISH): This technique has been used by Franke *et al.* (1999) to detect *Ga. sacchari* and other wine-related microorganisms such as lactic acid bacteria (Blasco et al., 2003). FISH allows the direct identification and quantification of bacterial species at microscopic level without previous cultivation. It consists of DNA fluorescent labelled probes that will specifically hybridise each of the species or genera.

  This technique has not been widely used with AAB but should be taken into account in further studies because there is no need to cultivate the sample, which makes the analysis faster, and due to the possible presence of viable but non culturable (VBNC) strains in vinegar samples.

- Real Time PCR: This technique identifies and enumerates bacterial species without having to culture. Campbell and Wright (2003) used it to VBNC species of *Vibrio vulnificus*, and also to detect other bacterial species such as the fecal bacteria (Rousselon et al., 2004) *Bacillus cereus* (Fykse *et al*., 2003). It is a fast and reliable method for identification and enumeration. Real-time PCR determines the initial template concentration by continuously measuring the product throughout the reaction and the initial number of cells can be accurately estimated by comparing it to a standard curve (Bleve et al., 2003). The technique

is explained here (chapter 4) although it is only used to enumerate AAB in different samples.

Strain level:

- Random amplified polymorphic DNA-PCR (RAPD-PCR): RAPD fingerprint is based on the amplification of the genomic DNA with a single primer of arbitrary sequence, of 9 or 10 bases of length, which hybridise with sufficient affinity to chromosomal DNA sequences at low annealing temperatures so that they can be used to initiate the amplification of bacterial genome regions. The amplification is followed by agarose gel electrophoresis, which yields a band pattern that should be characteristic of the particular bacterial strain (Caetano-Anolles *et al*., 1991; Meunier & Grimont, 1993).

  The technique has already been used by Nanda *et al*., (2000) to characterize rice vinegar AAB. They managed to discriminate among AAB strains and the patterns yielded between 7 and 8 DNA fragments.

- Amplified fragment length polymorphism (AFLP): AFLP has not been used to characterize AAB, but has been used for bacterial typing. It is a genome fingerprint based on a selective amplification of DNA fragments that are generated by restriction enzyme digestion (Vos *et al*., 1995). The bacterial DNA is extracted and digested with two different restriction enzymes (*Eco*RI and *Mse*I). The restriction fragments are then ligated to linkers containing each restriction site and a sequence homologous to a PCR primer binding site. These primers contain DNA sequences that are homologous to the linker and have one or two selective bases at their 3' ends. These selective nucleotides, then, make it possible to amplify only a subset of the genomic restriction fragments. To visualize the patterns, one of the primers contains either a radioactive or a fluorescent label, and these patterns can be alternatively resolved in agarose gels (Olive & Bean, 1999).

  This technique is laborious and costs more than the others because it uses labelled primers. However, it should be taken into consideration in further studies of AAB characterization.

- Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Repetitive Extragenic Palindromic-PCR (REP-PCR): ERIC and REP elements have been described as consensus sequences derived from highly conserved palindromic inverted repeat regions found in enteric bacteria (Pooler *et al*., 1996). However, these sequences seem to be widely distributed in the genomes of various bacterial groups. The amplification of the sequences between these repetitive elements has generated DNA fingerprints of several gram-negative and gram-positive species (Beyer *et al*. 1998; Guinebretier *et al*. 2001; Pooler *et al*. 1996; Sander *et al*. 1998; Wieser and Busse 2000). ERIC-PCR has already been used by Nanda *et al*. (2001) to identify AAB strains isolated from vinegar.

  These techniques are used in the present study and they are described in detail in chapters 2 and 3, where they are used in survey studies.

4. Ecological studies

4.1. AAB in wine

Alcoholic fermentations

Alcoholic fermentations are carried out by yeasts (mainly *Saccharomyces cerevisiae*), which are responsible for transforming the sugars present in musts (glucose and fructose) into ethanol. The second group of microorganisms involved in wine production is lactic acid bacteria. These bacteria are responsible for the malolactic fermentation, the process by which the malic acid is transformed into lactic acid, thus deacidifying and softening the wine. The third group of wine microorganisms are the AAB. Unlike the other microorganisms involved in fermentation processes, they have received very little attention, and little is known about their behaviour and dynamics in wine making processes or their contribution to the spoilage of musts and wines.

According to Margalith (1981), acetic acid in wine becomes objectionable at concentrations exceeding 0.7-1.2 g l^{-1}. Acetic acid is the main volatile acid in wines and its presence is frequently described as volatile acidity. An excess of acetic acid in wines

is one of the main problems found nowadays in wineries. Another of the contributions of the consequences of high volatile acidity in wines is the presence of ethyl acetate, which also gives the wines a vinegary taint and makes the wine smell like glue.

The wine-making process begins in the vineyard. The grapes acquire and harbour the right sugar and physiological composition of their juice so that, once they have been crushed, it can be transformed into wine by yeasts. The growth of AAB has been reported during various steps of the wine-making process, including some conditions in which they would not be expected to grow.

AAB in grapes and musts

As the grapes become mature, the amount of sugars (glucose and fructose) increases. Those sugars are an optimum growing media for AAB, and in particular for *G. oxydans*, because this species prefers sugars as the carbon source, while *Acetobacter* and *Gluconacetobacter* species clearly prefer ethanol as the carbon source. In these conditions the predominant species in grapes is usually *G. oxydans*, and the most common populations are around 10^2-10^5 cfu g^{-1} (Joyeux *et al*., 1984a; Du Toit & Lamberchts, 2002). Because of *G. oxydans'* low tolerance of ethanol, it disappears in the first stages of alcoholic fermentation. *Acetobacter* and *Gluconacetobacter* species have also been isolated from unspoiled grapes, although in very low amounts (Du Toit & Lamberchts, 2002).

Damaged, rotten or *Botrytis*-infected grapes can be infected by yeasts and AAB. Yeasts can start metabolising the sugars in grapes into ethanol, which are then oxidized into acetic acid by AAB. Damaged grapes contain AAB populations, mainly belonging to *Acetobacter* species (*A. aceti* and *A. pasteurianus*) up to 10^6 cel g^{-1} (Joyeux *et al*., 1984b; Grossman & Becker, 1984). These grapes contain high concentrations of acetic acid, ethanol and glycerol, and small amounts of ethyl acetate (Sponholz & Dietrich, 1985; Drysdale & Fleet, 1989b). Both ethanol and glycerol are the products of yeast metabolism. The glycerol produced can be metabolised by AAB into dihydroxyacetone, which affects the sensory quality of the wine and can bind to SO_2, thus decreasing its antimicrobial properties. Gluconic acid arises from the metabolism of glucose by AAB

(Drysdale & Fleet, 1988), and can be further oxidized to produce 5-keto and 2-ketogluconic acid.

Thus, grape juice composition can be significantly altered if the berries are infected with AAB. The changes not only have an adverse effect on the sensory quality of the wine but also on the growth of yeasts during alcoholic fermentation (Drysdale & Fleet, 1989a) and the possible growth of lactic acid bacteria (Joyeux *et al*., 1984b).

Adding SO_2 to the musts is common practice in cellars, because it inhibits the microorganisms and hinders the development of undesirable organisms such as AAB. So the presence and growth of AAB in must will depend on the concentrations of SO_2 and whether it is present in the free or the bonded form The free form consists of molecular sulphur dioxide, bisulphate and sulphite ions. Only the molecular SO_2 has anti-microbial effects. The proportion of molecular SO_2 represents from 1% to 10% of the free form depending on the pH of the wine, therefore, the lower the pH is, the higher proportion of molecular SO_2 will exist, and the higher anti-bacterial effect (Ribéreau-Gayon *et al*., 2000).

In this process the must may also be contaminated by AAB resident in the cellar because of such processes as grape juice racking and pumping.

AAB during fermentation

During alcoholic fermentation both *Saccharomyces* and *non-Saccharomyces* yeasts develop enormously and can reach populations up to 10^7-10^8 cfu ml^{-1}. During this process, sugars from must are transformed into ethanol by yeasts, which makes this new media more suitable for *Acetobacter* and *Gluconacetobacter* species. In this process, a considerable amount of CO_2 is produced because of the yeast metabolism, and this creates anaerobic conditions that are theoretically unsuitable for AAB growth. Recent studies by Du Toit *et al*. (2005), however, suggest that some AAB strains can survive for a prolonged period under relatively anaerobic conditions in wine. The pH is usually around 3.5, and the optimum pH for AAB development is 5.5-6.3 (Holt et al., 1994), although some AAB have been isolated at pH values of 3.0. The pH is also important for the state in which we can find SO_2 in wine. Low concentrations of SO2 do not affect the culturability of some AAB strains, and sulphur dioxide does not completely

eliminate the presence of AAB (Du Toit et al., 2005). AAB are able to grow in wines containing 20 mg l^{-1} of free SO_2 (Joyeux et al., 1984a), which means that the common levels of SO_2 in wines are not enough to inhibit AAB growth. Watanabe and Iino (1984) found that 100 mg l^{-1} of total SO_2 were needed to inhibit the growth of *Acetobacter* species in grape must.

The temperature at which alcoholic fermentation takes place depends on the type of vinification. Red wine fermentations take place between 25 and 30ºC, which is the same as the optimum temperature for AAB growth (Holt et al., 1994), and therefore does not seem prevent AAB development. The temperature of white and rosé fermentations ranges from 18-20º C and the effect of low-temperature fermentations on the AAB population has not been studied yet.

The growth of these bacteria during alcoholic fermentation may also be linked to the number of bacteria and yeast in the must at the start of the fermentation (Watanabe & Iino, 1984). The predominant species during alcoholic fermentation are commonly *A. aceti*, *A. pasteurianus*, *Ga. liquefaciens* and *Ga. hansenii* (Joyeux *et al*., 1984b; Poblet *et al*., 2000; Du Toit & Lamberchts, 2002), although *G. oxydans* have also been isolated as the only species during the fermentation (Drysdale & Fleet, 1985).

In spite of these adverse conditions during alcoholic fermentation, some authors (Du Toit *et al*., 2005) have detected that AAB can survive and even grow during this process. If the quality of the wines is to be good, it is of vital importance to keep the numbers of AAB low. This can be done by using healthy grapes, inoculating a high quantity of yeast, adding SO_2, clarifying the must and lowering the pH by adding acid (Du Toit &Pretorius, 2002).

If AAB grow a lot in the first stages of alcoholic fermentation, fermentations may become stuck or sluggish and there may be renewed growth of AAB and the reduction in the quality of the wines during their storage (Joyeux *et al*., 1984b).

We have made several survey studies in various fermentation conditions. The results are shown in chapters 2 and 3.

AAB during ageing and wine maturation

During storage, the major species found belong to *Acetobacter* (*A. aceti* and *A. pasteurianus*). These bacteria have been isolated from the top, middle and bottom of the tanks and barrels, suggesting that AAB can actually survive under the semi-anaerobic conditions occurring in wine containers. This can be explained by the ability of AAB to use compounds, such as quinones and reducible dyes, as electron acceptors (Du Toit & Pretorius, 2002).

The main product obtained from the presence of AAB at this point is acetic acid, although considerable amounts of acetaldehyde and ethyl acetate are produced (Dupuy & Maugenet, 1963; Drysdale & Fleet, 1989b) and glycerol metabolises to dihydroxyacetone (Sponholz & Dittricht, 1984).

The pumping over and racking of wine may stimulate the growth of AAB and lead to populations up to 10^8 cel ml^{-1} (Joyeux *et al*., 1984b; Drysdale & Fleet, 1989b), because of the intake of oxygen during these operations. The number of bacteria usually decreases drastically after bottling, because of the relatively anaerobic conditions in a bottle. However, the excessive addition of oxygen during bottling can increase the number of AAB (Millet & Lonvaud-Funel, 2000).

The ethanol concentration of wine is around 10-15% (v/v). As mentioned above, ethanol is a good carbon source for AAB, but it can also inhibit AAB growth at high concentrations. However, it is well known that these bacteria can grow in wine containing between 10-14% (v/v) (Joyeux *et al*. 1984a; Drysdale & Fleet 1989a; and Köselbalan and Özlingen 1992 Du Toit & Pretorius, 2002). It has been reported by Saeki *et al*. (1997) that AAB can overcome the inhibitory effect, and become tolerant to ethanol. In this respect, AAB have been isolated from sake and tequila (beverages with a much higher ethanol concentration than wine) (Swings & De Ley, 1981).

Cellar temperatures in which wine is stored for ageing are 10-15ºC. These temperatures seem to inhibit AAB growth (Joyeux *et al*., 1984a), although Drysdale & Fleet (1989b) observed a weak growth of AAB even at 10ºC.

4.2. AAB in vinegar production

Vinegar has been very important in the human diet since ancient times as a condiment and food preservative. For many centuries, acetic acid from vinegar was the strongest acid, until sulphuric acid was discovered around the year 1300. Although little is known about the role played by microorgansims in vinegar production, vinegar has been produced mainly from wine, alcohol and rice. Nowadays knowledge is much more advanced, above all as far as the analytical and industrial processes are concerned, but the microbiology of the process is still not well understood. At the beginning of the 21st century, the species and strains responsible for vinegar production are still not very clear. Nowadays, there are three different biotechnological processes for producing vinegar (Greenshields, 1978): the Orleans method, the German method and the submerged method.

Few ecological studies have been made of vinegar, the existence of viable but non culturable AAB have made these surveys more difficult (Guidici *et al*., 2003). Guidici *et al*. (2003) identified *Ga. xylinus* as being mainly responsible for acetobalsamic vinegar production. Bermúdez *et al*. (2003) studied the submerged method and identified *A. aceti* and *A. pasteurianus* as the main species, but it should be taken into account that these two studies only analysed viable cells. On the other hand, there were some profiles in both studies that did not match any of the reference strain profiles and, therefore, are unclassified.

4.3. Factors affecting AAB development during wine-making processes

The main factors affecting the development of AAB during the wine-making process are the pH of the must and wine, the temperature, the ethanol concentration and, mainly, the dissolved oxygen in the media (Drysdale & Fleet, 1988). Although few studies have been done about the effect of these parameters on AAB development, some conclusions can be drawn.

Temperature

The optimum growth temperature for AAB is 25-30ºC (Holt *et al*., 1994). No growth has been observed above 37ºC, although some thermotolerant AAB growing at 37-40ºC have been isolated (Saeki *et al*., 1997). AAB can still be active at lower temperatures. Growth has been observed in stored wines at temperatures of 18ºC (Joyeux *et al*.,

1984a), and a weak growth has even been detected at 10ºC. Although the range in which these bacteria are able to grow has not been well determined (Drysdale & Fleet, 1988), the temperatures used during the wine-making process do not seem to prevent these bacteria from growing.

Ethanol concentration

Ethanol is a good carbon source for AAB, but it is also inhibiting at concentrations that are too high. However, the ability of these bacteria to remain viable and even to grow at different ethanol concentrations is dependent on the strain. Vaughn (1995) stated that the maximum alcohol concentration tolerated by these bacteria is between 14 and 15% (v/v).

pH

The optimum pH for AAB growth is 5.5-6.3 (Holt *et al*., 1994). However, they can survive at low pH values of between 3.0 and 4.0. The ethanol sensitivity of these bacteria may also differ at different pH values.

AAB have been isolated from media containing acetate and during vinegar production at pH values of as low as 2.0-2.3, even with low levels of oxygen. pH has a synergic effect with SO_2. As mentioned above, the anti-bacterial effect of SO2 is higher at lower pH values.

SO_2

SO_2 is widely used against AAB in oenology. Nevertheless, its effects have not been studied in detail (Riberéau-Gayon et al., 2000). According to Lafon-Lafourcade and Joyeux (1981), the concentrations of SO_2 used in normal wine-making practice are not sufficient to control the growth of AAB. They reported the growth of *A. aceti* in red wine containing 25 mg l^{-1} of free SO_2. Generally, the effects of SO_2 on the survival and growth of AAB have not been examined in detail (Drysdale & Fleet, 1988).

Oxygen

Oxygen may be the most limiting factor for AAB growth. AAB have an obligate aerobic metabolism because they use oxygen as the terminal electron acceptor during respiration (Matsushita *et al*., 1994). Some results have shown that other compounds,

such as quinones and reducible dyes, can be used as electron acceptors (Aldercreutz, 1986), which suggests that these bacteria can survive and grow in an aerobic to semi-anaerobic environment (Drysdale & Fleet, 1988). Du Toit *et al*. (2005) also suggested that *A. pasteurianus* can survive in a viable but non culturable state when no oxygen is added to a wine.

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Applications of molecular techniques for routine identification of acetic acid bacteria

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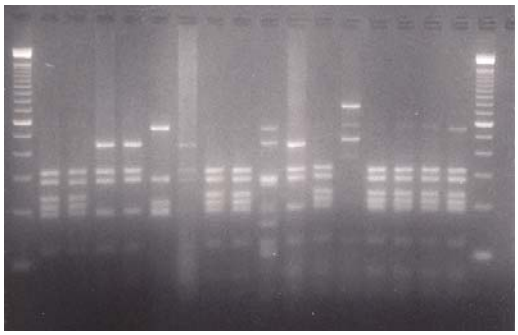

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Abstract:

Recently many new species of Acetic acid Bacteria have been described. The description and identification as new species was based on molecular techniques (sequencing of the 16S rDNA, DNA base ratio (% GC) determinations and DNA-DNA hibridization) and phenotypic characterization. In the present paper we propose a fast and reliable method for the identification of the species currently described based on the RFPLP-PCR of the 16S rDNA. According to the proposed protocol, 1 species can be identified with the use of a single enzyme, 13 with a combination of 2 enzymes, 2 species with a combination of 3 enzymes, 2 with a combination of 4 enzymes. To differentiate 5 more species RFLP-PCR of the ITS was also needed, after using 3 enzymes. Finally, a pair of species (*A. pasteurianus* and *A. pomorum*) could not be distinguished with the proposed method. However, doubts can be raised about their differentiation as separate species.

Keeping these limitations in mind, the method is fast and reliable, allowing the processing of large number of samples in relatively short periods of time (less than 24 hours after the isolation).

Keywords: Acetic acid bacteria, RFLP, 16S, ITS

Introduction:

Acetic acid bacteria (AAB) are Gram negative, ellipsoidal to rod-shaped cells. They have an obligate aerobic metabolism with oxygen as the terminal electron acceptor. Acetic acid is one of the main products of AAB metabolism and it is found in many foods as the result of these bacteria's presence and activity. AAB are classified into the family *Acetobacteraceae* as a branch of the acidophilic bacteria in the α–subdivision of the *Proteobaceria* (De Ley et al., 1984; Sievers et al., 1994). Genotyping relationships among AAB, 16S rDNA sequences and ubiquinone systems revealed the existence of six defined genera (Gillis et al., 1989; Mason & Claus, 1989; Urakami et al., 1989; Yamada and Kondo, 1984; Yamada et al., 1997; Sokollek et al., 1988; Boesch et al., 1998; Franke et al., 1999). Currently, thirty-four species of AAB are recognized, divided into these 6 genera: *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconacetobacter*, *Asaia* and *Kozakia*. Most of these new species have been described since 1998 by studying AAB isolated from fruits and fermented foods collected in tropical countries.

Those new species purposed are *A. cerevisiae* and *A. malorum* (Cleenwerck et al., 2002), *A. tropicalis*, *A. orleaniensis*, *A. lovaniensis*, and *A. estuniensis* (Lisdiyanti et al., 2001), *A. syzygii*, *A. cibinongenesis* and *A. orientalis* (Lisdiyanti et al., 2001), *Kozakia baliensis* (Lisdiyanti et al., 2002), *A. pomorum* and *A. oboediens* (Sokollek et al., 1998) *A. intermedius* (Boesch et al., 1998). *Ga. johannae* and *Ga. azotocaptans* (Fuentes-Ramirez et al., 2001), *Ga. sacchari* (Franke et al., 1999). *A. oboediens* and *A. intermedius* were reclassified to *Gluconacetobacter* by Yamada (2000).

However, these molecular methods are not appropriate for routine analysis of large

amounts of samples that can be isolated from nature. Some quick and reliable techniques such as RFLP analysis of PCR-amplified 16S rDNA has been considered as an appropriate technique for the differentiation and characterization of microorganisms (Carlotti & Funke, 1994). In eubacterial DNA, the rRNA genetic loci include 16S, 23S and 5S rRNA genes, which are separated by internally transcribed spacer (ITS) regions. It is known that DNA sequences in the 16S-23S ITS exhibit a great deal of sequence and length variation (Barry et al., 1991; Navarro et al., 1992) and conserved sequences with functional roles (tRNA genes, antitermination sequences) are also found within the ITS (Sievers et al., 1996). The goal of the present study is to apply the previously mentioned set of techniques for a fast and efficient classification of the whole described family of new species. These techniques will include the RFLP of PCR-amplified 16S rDNA and RFLP of PCR-amplified ITS region. With these techniques we should be able to discriminate among the species within 24 hours, having also a large capacity for sample processing, needed for routine analysis of large amount of samples.

Methods:

Strains, media and growth conditions. Strains used in this study were those available in reference collections and are listed in table 1. All genera were represented except *Asaia*.

They were grown in different media according to providers specifications (BCCM/LMG, Gent, Belgium). The media used were (% means w/v):

Medium 13: 2.5% D-mannitol, 0.5% yeast extract, 0.3% peptone, 1.5% agar.

Medium 17: 5% glucose, 1% yeast extract, 3% calcium carbonate, 1.5% agar.

Medium 239 (Reinforced AE medium): 4% glucose, 1% yeast extract, 1% peptone,

0.338% $Na_2HPO_4 \cdot H_2O$, 0.15% citric acid, 2% (v/v) ethanol, 1% (v/v) acetic acid.

Table 1: Origin and source of the AAB tested in the present work.

	Strain	Growing medium	Source
A. tropicalis	LMG 1663	13	Fermenting putified meat sample
A. estuniensis	LMG 1626	13	Cider
A.orientalis	DSMZ 15550	17	Canna flower
A. cibiononGenesis	DSMZ 15549	17	Fruit of mountain, *Annona montanae*
Kozakia baliensis	DSMZ 14400	17	Palm brown sugar
A. syzygii	DSMZ 15548	17	Fruit of Malai rose apple
A. malorum	DSMZ 14337	17	Rotting apple
A. cerevisiae	DSMZ 14362	17	Beer (ale) in storage
G. cerinus	LMG 1366	13 or 17	/
A. lovaniensis	LMG 1579	13	Sewage on soil
A. tropicalis	LMG 1754	13	*Ficus capensis*, fruit
Ga. intermedius	LMG18909	239	Tea fungus beverage (Kombucha)
A. indonesiensis	LMG 1571	13	/
A. orleanensis	LMG 1545	13	Film in fermentor of rice vinegar
A. orleanensis	LMG 1592	13	Manufacture of vinegar
A. indonesiensis	LMG 1588	13	/
Ga. Johannae	LMG 21312	13	Rhizosphere of coffee plants
Ga. sacchari	LMG 19747	13	Sugar cane, leaf sheath
Ga. oboediens	LMG 18849	239	Red wine vinegar fermentation
Ga. azotocaptans	DSMZ 13594	13	Rhizosphere of coffee plants
G. cerinus	LMG 1390	13 or 17	*Rheum rhabarbarum*, flower
A. pomorum	LMG 18848	239	Cider vinegar fermentation
Ga. oboediens	LMG 18849	239	Red wine vinegar fermentation

DNA extraction. Extraction was carried out using the method described by Rademaker & De Bruijn (http://www.msu.edu/user/debruijn). A volume of twenty-five ml of growth medium was centrifuged, the supernatant discarded and the pellet resuspended in RB buffer (NaCl, 0.15M; EDTA,0.01M pH 8). It was centrifuged again and the supernatant discarded. The pellet was resuspended in 100 µl of TE buffer (Tris, 10mM; EDTA,1mM pH 8) and 500 µl of GES solution (60% guanidine thiocyanate; 20% EDTA 0.5M; 20% sterile water). A 250 µl aliquot of ammonium acetate and 500 µl of chloroform/isoamilic alcohol was added to the solution and the mixture centrifuged for 10 min at 10,000 rpm. The upper phase was collected and 380 µl of iso-propanol added. It was centrifuged again for 5 min at 10,000 rpm and the supernatant discarded. The

pellet was washed with ethanol 70% and the DNA resuspended in 200 µl of TE buffer. The DNA was treated with 25 µl of DNAase-free RNAase (250 µg μl^{-1}) (Sigma-Aldrich, Steinheim; Germany) for 1 hour at 37ºC. The concentration and purity of DNA were determined using a GenQuant spectrophotometer (Pharmacia, Cambridge, UK).

Oligonucleotide primers. Primers used to amplify the 16S rDNA and 16S-23S ITS region were designed by Ruiz et al. (2000) on the basis of conserved regions of the 5'-end (16Sd, 5'-GCTGGCGGCATGCTTAACACAT) and the 3'-end (16Sr, 5'GGAGGTGATCCAGCCGCAGGT) of 16S rDNA gene and for 16S-23S ITS; 16S (its 1, 5'-ACCTGCGGCTGGATCACCTCC-3') and 23S (its 2, 5'-CCGAATGCCCTTATCGCGCTC-3') rRNA genes conserved among AAB.

PCR amplification. PCR amplification was carried out in a final volume of 50 µl containing 3 µl of DNA solution (ranging from 0.5 to 1.5 µg μl^{-1}), 5µl 10X amplification buffer (ARK Scientific, Darmstadt, Germany), 15 pmol of each primer (Ruiz *et al*., 2000), 200 µM of each of the four dNTPs (Roche Diagnostics GmBh, Manheim, Germany), 100 mM $MgCl_2$, 0.4 µl of BSA (20mg ml^{-1}), 5 µl of DMSO and 2.5U of *Taq* Polymerase (ARK Scientific). The reactions were carried out in Gene Amp PCR System 2700 (Applied Biosystems, Foster city, USA). Samples were incubated for 5 min at 94ºC and then cycled 35 times at 94ºC for 1min, 58ºC for 1min (16S rDNA) and 72ºC for 1 min (16S-23S ITS) and 72ºC for 2 min. The samples were incubated for 10 min at 72ºC for final extension and kept at 4ºC until tested.

Restriction of amplified DNA. An aliquot of five microlitres of each 16S rDNA amplified product was digested with all restriction endonucleases, as recommended by

the manufacturer (Roche Diagnostics GmBh). 16S rDNA amplified product was detected by electrophoresis gel on a 1% (w/v) agarose in 1X TBE buffer.

Agarose gel electrophoresis and microchip electrophoresis. Restriction fragments generated by restriction endonucleases were detected by 3.5% (w/v) agarose electrophoresis gel. Gels were stained with ethidium bromide and photographed. Lengths of both amplification products and restriction fragments were detected by comparison with 100bp DNA ladder (Gibco-BRL, Eggenstein, Germany).

Sizing by electrophoresis was compared with automated capillary electrophoresis using Agilent 2100 bioanlyzer (Agilent Technologies, Böblingen, Germany). A 7500 Labchip was used for sizing both amplified products and restriction fragments. DNA assay separates nucleic acid fragments by capillary electrophoresis in a microchip with micro-fabricated channels and automates both detection and on-line data evaluation. The instrument is equipped with a fluorescence detection system that leads to greater detection sensitivity, and the DNA sample size is estimated by comparison with external standards (DNA sizing ladder) and internal standards (DNA markers), thus providing accurate and reproducible size estimation (Panaro *et al*., 2000; Nachakin *et al*., 2001).

Sequences of 16S rDNA were searched in www.ncbinlm.nih.gov and then aligned by clustalw in http://www.ebi.ac.uk/services/. Fragments of 16S rDNA were also restricted *in silico* in www.yeastgenome.org to compare the experimentally obtained sizes and the theoretical ones.

Results:

Amplified products of the 16S rDNA contained approximately 1450bp and all strains indicated identical size. The expected size was 1458bp. Eight restriction endonuclesases (*Taq*I, *Cfo*I, *Alu*I, *Hae*III, *Tru*9I, *Hin*fI, *Msp*I and *Rsa*I) were tested and digested the amplified 16S rDNA,. These enzymes were chosen randomly between 4-base restriction endonucleases, as Ruiz et al. (2000), because these enzymes yielded enough polymorphism to distinguish among the species. Furthermore, test of two-base cutters *in silico*, using the 16S rDNA genes of each one of the species yielded such a high amount of bands which would not allow any discrimination, and in an agarose electrophoresis this would led to a smear.

Digestion of the 16S rDNA amplicon of all the AAB species with the enzyme *Alu*I produced 9 different DNA patterns. The strains were clustered depending on their genera. With this restriction enzyme *Kozakia baliensis* yielded a unique pattern which allowed straight identification. (Table 2).

Table 2: Classification of the AAB according to the electrophoretic analysis of the PCR RFLP of 16S rDNA and RFLP-PCR of ITS 16-23S rDNA

		*Alu*I	*Cfo*I	*Tru*9I	*Hin*fI	*Taq*I	*Hae*III	*Rsa*I	ITS+ *Alu*I*	ITS+ *Cfo*I	ITS+*Pvu*II
1	*A. tropicalis*	A1	C1	Tr1		T1					
2	*A. indonesiensis*		C1	Tr1		T3					
3	*A. cerevisiae*		C1	Tr2					ITSA1	ITSC2	
4	*A. orleanensis*		C1	Tr2					ITSA2		
5	*A. malorum*		C1	Tr2					ITSA1	ITSC1	
6	*A. estunensis*		C2		Hi1						
7	*A. aceti*		C2		Hi2						
8	*A. orientalis*	A2				T1					
9	*A.cibinongensis*					T3					
10	*A. syzgii*	A3				T4					
11	*A. lovaniensis*					T5					
12	*A. pasteurianus*					T2					
13	*A. pomorum*					T2					
14	*K. baliensis*	A4									
15	*G. cerinus*	A5	C3								
16	*G. oxydans*		C4								
17	*Ga.intermedius*	A6					H1				
18	*Ga. xylinus*						H4				ITSP1
19	*Ga. europaeus*						H4				ITSP2
20	*Ga. johannae*	A7						R2			
21	*Ga. azotocaptans*							R1			
22	*Ga. sacchari*	A8					H3				
23	*Ga. liquefaciens*						H2				
24	*Ga. oboediens*	A9			Hi2						
25	*Ga. hansenii*				Hi3						

* ITS + restriction endonuclease means the use of PCR-RFLP of the 16-23S ITS rDNA.

Treatment with *Taq*I yielded 8 different patterns. With this enzyme *Kozakia baliensis*, and *A. syzgii* had different patterns from the others and, thus, could be identified directly. Similarly, the *Cfo*I digestion generated 8 patterns. In this case, 3 especies, *Kozakia baliensis*, *Ga. sacchari* and *G. oxydans* could be identified by this treatment. Despite these restriction patterns and the ability of these two enzymes to discriminate directly a major number of species, the other species were grouped in more complex

and numerous clusters. Thus, these groups of species were more difficult to differentiate, and more enzymes needed.

*Tru*9I yielded only 3 different patterns and none of the species could be identified directly. All the *Gluconacetobacter* species yielded the same patterns, together with *Kozakia baliensis* and some *Acetobacter* species. In the case of *Hae*III 5 different patterns were obtained. As occurred with *Tru*9I none of the species could be identified only with this enzyme.

*Hin*fI treatment yielded 5 different patterns. *Ga. hansenii* and *G. oxydans* could be identified directly with this enzyme. *Rsa*I gave 6 different patterns. *Gluconacetobacter* species shared the same pattern (except for *Ga. azotocaptans*). With this enzyme *Kozakia baliensis*, *A. syzgii* and *G oxydans* could be identified. Finally, the less resolutive enzyme was *Msp*I with only 2 different patterns generated. No identification was observed with this enzyme.

After the use of all these enzymes, we propose a simple classification system for all the species described in the *Acetobacteriaceae* family. As mentioned, the most discriminating endonuclease is *Alu*I (Table 2). Thus, the species are grouped considering the pattern obtained with this first restriction enzyme (A1 to A9). Fig. 1 shows the typical patterns obtained by 16 different species after treatment with *Alu* I. The rest of the treatments are chronologically arranged. Each of the groups (A1 to A9) has to be considered individually. Except those species grouped showing the pattern A1 and A6, all the other species can be classified by using an additional endonuclease. In table 3 are shown the patterns obtained with each of the restriction endonucleases for

both PCR-RFLP of the 16S rDNA and PCR-RFLP of the 16S-23S ITS rDNA needed to identify the studied species.

Table 3: Restriction fragment sizes of the amplified 16S DNA used to classify the AAB.

*Alu*I		*Cfo*I	
A1	330-280-250-210-200-120-50	C1	500-350-210-150-150
A2	450-320-290-200-120-50	C2	500-420-210-150-150
A3	450-320-290-200-110-50	C3	400-350-180-150-140-110-100
A4	550-290-210-190-190	C4	430-340-180-175-130-110-90
A5	550-290-200-180-120-70-50		
A6	790-490-120-50	*Hin*fI	
A7	750-290-210-180-90	Hi1	950-275-200
A8	750-280-210-120-70	Hi2	950-210-150-80
A9	740-470-120-70-50	Hi3	980-200-180-120
*Tru*9I		*Rsa*I	
Tr1	530-350-350-150-110	R1	500-400-250-150-110
Tr2	530-350-340-150-110	R2	500-400-400-150
*Taq*I		*Hae*III	
T1	650-375-210-180	H1	550-290-190-180-170-100
T2	500-375-370-210	H2	550-280-210-180-170
T3	850-375-210	H3	550-290-280-180-170
T4	500-375-210-175-160	H4	550-220-200-180-170-100
T5	500-375-200-190-150		
ITS			
ITSA1	480-210-120-50	ITSC1	400-210-160-70
ITSA2	480-280-110	ITSC2	500-190-160
ITSP1	250-550*		
ITSP2	300-500*		

* Data obtained from Sievers *et al*. (1996)

Group A1 is the most complex, needing up to 4 different enzymes to differentiate most of the species. The seven species that share pattern A1 can be differentiated in two different groups after using *Cfo* I. Those species showing pattern C2 can be separated by an additional enzyme, *Hin*f I, which differentiates *A. estuniensis* from *A. aceti*.

Instead, pattern C1 species have to be also treated with *Tru* 9I, clustering them again in two groups. One of them (Tr1) allows classification after the use of *Taq* I, which yields two different patterns that allow the separation between *A. tropicalis* and *A. indonesiensis*. In the case of *A. cerevisiae, A. orleaniensis* and *A. malorum* we are not able to distinguish them with any of the tested enzymes. In fact, the 16S rDNA gene sequence of those species have only 6 non consecutive base pairs differences (bp numbers 45, 93, 559, 564, 565 and 666), and the only restriction enzymes with recognition site for those bases found were 2-base cutters and yielded fragments far too small to distinguish among them. Thus, in order to be able to differentiate between those species we have chosen the PCR-RFLP of 16S-23S ITS rDNA technique, already used by Ruiz *et al*. (2000) and Treck & Teuber (2002) with AAB species. The length of the 16S-23S ITS rDNA amplified product was 835bp for all these species. We are able to differentiate between *A. cerevisie, A. orleaniensis* and *A. malorum* using *Alu*I and *Cfo*I as restriction enzymes.

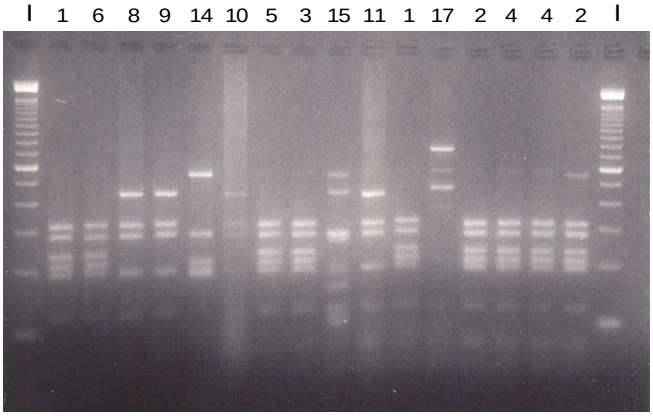

Fig. 1: Typical PCR-RFLP of 16S rDNA profile obtained with *Alu*I. Lane (I) is molecular size marker: 100bp DNA ladder. Lane assignments correspond to the numbers given in table 2.

For the rest of the groups only a second restriction endonuclease is needed. Group A2 and Group A3 can be differentiated by treatment with *Taq* I, which is the second more

discriminating enzyme. In A2 group, we can differentiate *A. orientalis* from *A. cibinongensis.* In A3 group, *A. syzgii and A. lovaniensis* can be differentiated with characteristic profiles. *A.pasteurianus* and *A. pomorum* yield the same profiles and no other endonuclease can help us to differentiate them. In fact , the 16S rDNA gene differs in 3 non consecutive base pairs (bp 576, 757 and 765). Using PCR-RFLP of the 16S-23S ITS rDNA no differences were found between these species, so we are not able to differentiate them.

As mentioned, A4 profile corresponds to a single species, *K. baliensis*. The group A5 is formed by *G. cerinus* and *G. oxydans,* which are effectively separated by the additional use of *Cfo*I. The group A6 is formed by three species of *Gluconoactebacter*, *Ga. intermedius, Ga. xylinus* and *Ga. europaeus. Ga. intermedius* can be differentiated from the other two species by the use of *Hae*III. However, we are not able to distinguish *Ga. xylinus* from *Ga. europaeus* with any of the restriction enzymes tested. The 16S rDNA gene of *Ga. europaeus* and *Ga. xylinus* have only 5 base pairs difference (bp 6, 288, 565, 581 and 1070), and one of them (bp 6) is excluded with the set of primers used. To differentiate these two species we can use PCR-RFLP of the 16S-23S ITS rDNA as in Sievers *et al*. (1996) who obtained different patterns using *Pvu*II as a restriction endonuclease. In fact, this technique was also tested with the rest of the species but the degree of polymorphism yielded was not as high as it was with PCR-RFLP of the 16S rDNA.

Groups with electrophoretic patterns A7, A8 and A9 are composed by two species each, which are easily resolved by the use of one more restriction enzyme, that is *Rsa* I for group A7, *Hae* III for A8 and *Hin*fI for the last group, A9.

Discussion

Identification has traditionally been performed by studying physiological and chemotaxonomic properties (Buchanan & Gibbons, 1984) but these methods are not completely reliable and are time-consuming. The proposal of novel species are based in studies that include DNA-DNA hybridisations, DNA base ratio determinations, sequence analysis, which are also time-consuming and expensive.

PCR-RFLP of the 16S rDNA has already been used to identify AAB isolates and to characterize reference strains (Poblet et al., 2000; Ruiz et al., 2000). In this work we apply this technique to identify the new reference strains. In the cases where we are not able to identify them we have used PCR-RFLP of the 16S-23S ITS rDNA. In 16S-23S ITS rDNA 2 to 11 copies of the rRNA loci are present on the chromosomes of most bacterial species and, in some bacterial groups, the different copies of the intergenic spacer regions show extensive length variation (Dolzani et al., 1995; Lagatolla et al., 1996). In our case all the amplified products yielded the same length, and the sum of the length of the restriction fragments obtained with all the restriction endonucleases equalled the length of the corresponding PCR-amplified ITS sequence, as occurred with Ruiz et al. (2000). PCR-RFLP of the 16S rDNA and 16S-23S rDNA, combining the different restriction endonucleases allows the identification of the AAB in a shorter period of time, as was demonstrated by Ruiz et al. (2000).

The groups obtained with *Alu*I (table 2) generated clusters dividing the species depending on their genera, being separated *Acetobacter*, *Gluconobacter*, *Kozakia* and

Gluconacetobacter. As it is done with yeasts (Querol et al., 1992) and lactic acid bacteria (Rodas et al., 2003) this clustering does not respond to phylogenetic grouping, nor taxonomic classification of the species. If the initial endonucleases was different (*i.e*. *Taq*I) almost the same clusters were obtained (data not shown) except for *A. tropicalis*, *A. estuniensis* and *A. lovaniensis*, which appeared in two clusters together with *Gluconacetobacter* species. We could also discriminate some of the species with other restriction endonucleases but it is much longer because we needed to use a higher number of enzymes for most of the species.

We were able to differentiate all the strains using RFLP-PCR of the 16S rDNA except for *A. malorum, A. cerevisiae* and *A. orleaniensis*, which were discriminated using 16S-23S rDNA, as occurred with the pair *Ga. xylinus* and *Ga. europaeus*. Also, we are unable to differentiate *A. pasteurianus* from *A. pomorum*. In the case of the first group of species (*A. malorum*, *A. cerevisiae* and *A. orleaniensis*) the identification of these strains at species level, genotypic characterization was required, because phenotypic characteristics are very similar (Cleenwerk et al., 2002). The % G+C content showed similar values, and 16S rDNA sequences showed more than 99.9% of similarity. DNA-DNA hibridization showed relatedness at low levels among these species (Cleenwerk et al., 2002). This similarity between them is what makes it more difficult to discriminate and justifies the use of the other technique.

In the case of the pair *Ga. xylinus/Ga europaeus* they were differentiated by Sievers et al. (1996) using RFLP-PCR of the 16-23S rDNA. They share 99.6% of the 16S rDNA sequence and they were separated as different species on the basis of DNA-DNA hybridisation and phenotypic differences such as the absolute requirement of acetic acid

for the growth of *A. europaeus* (Sievers & Teuber, 1995).

The separate species status of *A. pasteurianus* and *A. pomorum* was proposed by Sokollek et al. (1998) based on DNA-DNA hibridization, 16S rDNA sequencing and phenotypic characterization. The results obtained by Sokollek et al. (1998) differ from the ones obtained by Cleenwerck et al. (2002) who disagree in almost all the results obtained previously and also with some methods used for the calculation of the DNA relatedness. In our case we are unable to differentiate these two species neither with RFLP-PCR of 16S rDNA nor RFLP-PCR of ITS 16-23S rDNA, which, together with Cleenwerck et al. (2002) suggests that the classification of these two species as separate species should be revised.

This is a methodology already used for AAB (Ruiz et al., 2000) and now extended to all the AAB strains which allows the routine identification of AAB samples in a short period of time, compared with the time-consuming techniques (such as DNA-DNA hybridisation) which are also not appropriated for laboratory routine investigation, or population studies where a high amount of samples must be analysed. The use of these techniques for AAB differentiation is proposed for routine laboratory analysis because of its easiness, the general use of a single PCR and limited restriction analysis, and for the low cost as compared to the techniques used to identify the novel AAB species.

Acknowledgements

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Applications of molecular methods for the differentiation of acetic acid bacteria in a red wine fermentation

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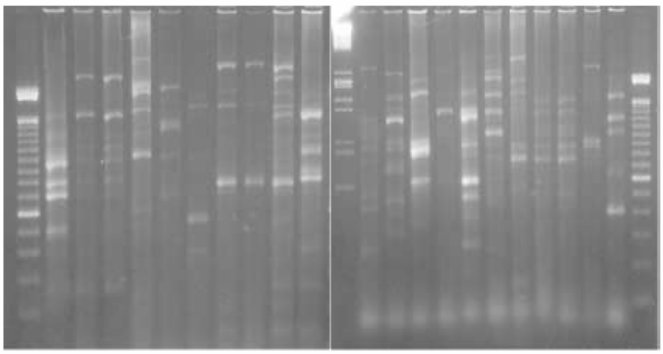
Species	Strain	Origin
1 *Gluconobacter oxydans*	LMG 1408^{T}	Beer
2 *Gluconobacter oxydans*	CECT 360*	Beer
3 *Gluconobacter oxydans*	LMG 1484*	Beer
4 *Gluconobacter oxydans*	LMG 1414	Grap
5 *Gluconobacter frateurii*	LMG 1365^{T}	*Frag*
6 *Gluconobacter asaii*	LMG 1390^{T}	*Rheu*
7 *Acetobacter aceti*	LMG1261^{T}†	Beec
8 *Acetobacter aceti*	CECT 298^{T}†	Beec
9 *Acetobacter aceti*	LMG 1505	Quic
10 *Acetobacter aceti*	LMG1372	Naka
11 *Acetobacter pasteurianus*	LMG 1262^{T}	Beer
12 *Acetobacter pasteurianus*	LMG 1553	Spoil
13 *Acetobacter pasteurianus*	LMG1282	Beer
14 *Gluconacetobacter hansenii*	LMG 1527^{T}	Vine
15 *Gluconacetobacter hansenii*	LMG 1511	–
16 *Gluconacetobacter liquefaciens*	LMG 1381^{T}	Drie
17 *Gluconacetobacter liquefaciens*	LMG 1347	Fruit
18 *Gluconacetobacter xylinus*	LMG 1515^{T}	Mou
19 *Gluconacetobacter xylinus*	LMG 1518	–
20 *Gluconacetobacter diazotrophicus*	DSMZ 5601^{T}	Suga
21 *Gluconacetobacter europaeus*	LMG 6160^{T}	–



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ABSTRACT

Aims: to develop rapid and reliable molecular techniques for studying the population dynamics of acetic acid bacteria during wine-making processes.

Methods and Results: We tested the usefulness of the Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Repetitive Extragenic Palindromic-PCR (REP-PCR) techniques with reference strains of most of the species of acetic acid bacteria and obtained exclusive patterns for each strain. One hundred twenty isolates from a commercial red wine fermentation were fingerprinted using both techniques. We detected a high degree of strain diversity in the first stage of fermentation that decreased throughout the process. However, several strains and species were dominant in the alcoholic fermentation phases. *Gluconobacter oxydans* dominated the fresh must, while *Acetobacter aceti* was the only isolated species at the end of the process. *Gluconoacetobacter hansenii* and *Gluconoacetobacter liquefaciens* were also isolated in significant numbers at the beginning of fermentation.

Conclusions: Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Repetitive Extragenic Palindromic-PCR (REP-PCR) techniques proved useful for characterising strains of acetic acid bacteria.

Significance and Impact of the study: The availability of molecular techniques for a fast and reliable genotypic characterisation should increase our knowledge of the ecology of acetic acid bacteria and determine their growth behaviour during the various stages of vinification more accurately

Key words: ERIC-PCR- REP-PCR- *G. oxydans*- *A. aceti*- Population dynamics

INTRODUCTION

Acetic acid bacteria are Gram-negative, ellipsoidal to rod-shaped cells that have an obligatory aerobic metabolism with oxygen as the terminal electron acceptor. These bacteria can use different substrates such as glucose, ethanol, lactate or glycerol as energy sources. However, most of these compounds are not completely oxidised into CO_2 and water and several metabolites are accumulated in the growth medium, especially acetic acid. Acetic acid is the major volatile acid in wine and is considered objectionable at levels above 1.2 to 1.4 g l^{-1}, being one of the main problems in the world of oenology (Drysdale and Fleet 1988). Although other microorganisms of the wine, such as yeast and lactic acid bacteria, can produce a cetic acid, acetic acid bacteria are mainly responsible for producing this acid in wine.

Acetic acid bacteria can develop in two stages during the wine-making process: before alcoholic fermentation and during storage or ageing of the wines. Spoiled grapes and those infected with *Botrytis cinerea* have a high population of acetic acid bacteria that can grow in grape must and accumulate gluconic and acetic acids. It has also been reported that excessive growth of acetic acid bacteria on grape berries or in musts can lead to an incomplete alcoholic fermentation (Drysdale and Fleet 1989). The anaerobic conditions set up during alcoholic fermentation seem to make conditions unsuitable for the growth of these bacteria. However, after alcoholic fermentation, there may be enough agitation and aeration when the wine is transferred from fermentation tanks to other storage vessels to encourage the growth of the surviving acetic acid bacteria populations. At this stage, the oxidation of ethanol mainly accumulates acetic acid, but also acetaldehyde and ethyl acetate. All of these products are detrimental to the quality of the wine (Drysdale and Fleet 1988; Drysdale and Fleet 1989)

Taxonomically, acetic acid bacteria are classified in the family *Acetobaceraceae* (De Ley *et al*. 1984). The most recent techniques for classifying within the family *Acetobaceraceae* based on genotypic relationships such as DNA-DNA and DNA-RNA hybridization or 16S rDNA sequence analysis (Gillis and De Ley 1980; Mason and Claus 1989; Urakami *et al*. 1989; Yamada *et al*. 1994; Yamada and Kondo 1984) revealed the existence of four well-defined genera: *Acetobacter* (*A. aceti* and *A. pasteurianus*), *Gluconobacter* (*G. oxydans*, *G. asaii* and *G. frateurii*), *Gluconoacetobacter* (*Ga. liquefaciens*, *Ga. hansenii*, *Ga. xylinus*, *Ga. europaeus* and *Ga. diazotrophicus*) and *Acidomonas* (*Ac. methanolica*). In addition, the new species *A. oboediens*, *A. pomorum*, *A. intermedius* and *Ga. sacchari* have more recently been reported (Boesch *et al*. 1998; Franke *et al*. 1999; Sokollek *et al*. 1998).

Most of these molecular techniques, however, are not completely useful for routinely identifying bacterial isolates because they are time-consuming and difficult to use with a large number of strains. Recently, Ruiz *et al*. (2000) used the RFLP analysis of PCR-amplified 16S rDNA to differentiate acetic acid bacteria at the level of genus and species. This quick and easy method identified reference strains and indigenous acetic acid bacteria isolated from wine fermentations. Other PCR techniques have also been proposed for typing strains. Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Repetitive Extragenic Palindromic-PCR (REP-PCR) have, because of their high degree of polymorphism, recently been proposed as suitably accurate techniques for identifying bacteria strains and for determining taxonomic relationships between bacterial species (Beyer *et al*. 1998; Matheson *et al*. 1997; Nanda *et al*. 2001; Pooler *et al*. 1996; Sander *et al*. 1998; Versalovic *et al*. 1991).

The aim of this study was to evaluate the usefulness of these techniques for characterising acetic acid bacteria and to study the population dynamics of these bacteria during alcoholic fermentation. We also used PCR-RFLP rDNA 16S to identify the different species of acetic acid bacteria involved during the process and to validate the data obtained by ERIC- and REP-PCR.

MATERIALS AND METHODS

Strains, media and growth conditions.

Reference strains used in this study are shown in Table 1. The strains were grown in liquid medium manitol (0.5% yeast extract; 0.3% Peptone; 2.5% Manitol w/v) for DNA extraction. Sampling for the acetic acid bacteria isolation was done during an industrial red wine fermentation during the 2001 vintage. Fresh must was obtained from the Grenache grape variety. This must had a sugar concentration of 215g l^{-1}, a pH of 3,4 and 40 mg SO_2 l^{-1} was added. These colonies were selectively isolated by plating them on glucose solid medium (1% yeast extract; 3% $CaCO_3$; 7.5% Glucose; 2.2% agar w/v) supplemented with Pimaricin (100 mg l^{-1}) to inhibit the growth of yeasts and moulds. This antibiotic was added to the culture medium from stock solution after sterilising the medium. The isolates were then grown for 48h in the previously described liquid medium.

Table 1. Reference strains used in this study

Species	Strain	Origin
1 *Gluconobacter oxydans*	LMG 1408^T	Beer
2 *Gluconobacter oxydans*	CECT 360*	Beer
3 *Gluconobacter oxydans*	LMG 1484*	Beer
4 *Gluconobacter oxydans*	LMG 1414	Grapes
5 *Gluconobacter frateurii*	LMG 1365^T	*Fragaria ananassa.*
6 *Gluconobacter asaii*	LMG 1390^T	*Rheum rhabarbarum*, flower
7 *Acetobacter aceti*	LMG1261^T†	Beech-wood shavings of vinegar plant
8 *Acetobacter aceti*	CECT 298^T†	Beech-wood shavings of vinegar plant
9 *Acetobacter aceti*	LMG 1505	Quick vinegar
10 *Acetobacter aceti*	LMG1372	Nakano Rice-Vinegar Company
11 *Acetobacter pasteurianus*	LMG 1262^T	Beer
12 *Acetobacter pasteurianus*	LMG 1553	Spoiled beer
13 *Acetobacter pasteurianus*	LMG1282	Beer
14 *Gluconacetobacter hansenii*	LMG 1527^T	Vinegar
15 *Gluconacetobacter hansenii*	LMG 1511	–
16 *Gluconacetobacter liquefaciens*	LMG 1381^T	Dried fruit
17 *Gluconacetobacter liquefaciens*	LMG 1347	Fruit
18 *Gluconacetobacter xylinus*	LMG 1515^T	Mountains ash berries
19 *Gluconacetobacter xylinus*	LMG 1518	–
20 *Gluconacetobacter diazotrophicus*	DSMZ 5601^T	Sugarcane roots
21 *Gluconacetobacter europaeus*	LMG 6160^T	–

LMG, Laboratorium voor Microbiologie Universiteit Gent, Belgium. CECT, Colección Española de Cultivos Tipo, Valencia, Spain. DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.
*Synonymous strains.
†Synonymous strains.

DNA extraction.

Extraction was carried out using the method described by Rademaker and De Bruijn (online: http://www.msu.edu/user/debruijn). Twenty-five ml of growth medium were centrifuged, the supernatant was discarded and the pellet was resuspended in RB buffer (0.15 NaCl; 0.01M EDTA, pH 8). It was centrifuged again and supernatant was discarded. Pellet was resuspended in 100 µl of TE buffer (10mM Tris; 1mM EDTA, pH 8) and 500 µl of GES solution (60% guanidine thiocyanate; 20% EDTA 0.5M; 20% sterile water). 250 µl of NH_4 Acetate and 500 µl of chloroform/isoamilic alcohol were added to the solution and the mixture was centrifuged for 10 min at 10,000 rpm. The upper phase was collected and 380 µl of iso-propanol was added. It was centrifuged again for 5 min at 10,000 rpm and supernatant was discarded. Pellet was washed with

ethanol 70% and DNA was resuspended in 200 μl of TE buffer. DNA was treated with 25 μl of DNAase-free RNAase (250 μg μl^{-1}) (SIGMA-ALDRICH; Steinheim, Germany) for 1 hour at 37ºC.

Oligonucleotide primers.

Primers used to amplify the 16S rDNA were designed by Ruiz *et al*. (2000) on the basis of conserved regions of the 5'-end (16Sd, 5'-GCTGGCGGCATGCTTAACACAT.) and the 3'-end (16Sr, 5'GGAGGTGATCCAGCCGCAGGT) of this gene. Primers used for the amplification of ERIC (ERIC1R: ATGTAAGCTCCTGGGGATTCAC ERIC2: AAGTAAGTGACTGGGGTGAGCG) and REP (REPIR-I: IIIICGICGICATCIGGC REP2-I: ICGICTTATCIGGCCTAC) elements were those described by Versalovic *et al* (1991). 'I' represents inosine.

PCR conditions.

PCR amplification of 16S rDNA was carried out in a final volume of 50 μl comprising 3 μl of DNA solution (ranging from 0.5 to 1.5 μg μl^{-1}), 5μl 10X amplification buffer (ARK Scientific; Darmstad, Germany), 15 pmol of each primer (Ruiz *et al*. 2000), 200 μM of each of the four dNTPs, 3 mM $MgCl_2$, 0.4 μl of BSA (20mg ml^{-1}), 5 μl of DMSO and 2.5U of *Taq* Polymerase (ARK Scientific).The reactions were carried out in Gene Amp PCR System 2700 (Applied Biosystems). Samples were incubated for 5 min at 94ºC and then cycled 35 times at 94ºC for 1min, 58ºC for 1min and 72ºC for 2 min. The samples were incubated for 10 min at 72ºC for final extension and kept at 4ºC until tested.

ERIC and REP elements were amplified in 25 μl samples comprising 3 μl of DNA solution (ranging from 0.5 to 1.5 μg μl^{-1}), 5 μl 5X GB Buffer ($(NH_4)_2SO_4$ 1M, Tris-HCl 1M pH 8.8, $MgCl_2$ 1M, EDTA 0.5M pH 8.8, β-mercapto-ethanol 14.4M) (Rademaker and De Bruijn, http://www.msu.edu/user/debruijn), 0.2 μl of BSA (20mg ml^{-1}), 2.5 μl of DMSO, 100 μM of each of the four dNTPs, 15 pmol of each respective primer (Versalovic *et al*. 1991) and 2.5U of *Taq* Polymerase (ARK Scientific). Reactions were carried out in Gene Amp PCR System 2700 (Applied Biosystems). Samples were incubated for 5 min at 94ºC, and then cycled 30 times at 94ºC for 30s, 57ºC (ERIC-PCR) and 47ºC (REP-PCR) for 30s and 65ºC for 4 min The samples were incubated for 8 min at 65ºC for final extension and kept at 4ºC until tested.

Restriction analysis and gel electrophoresis.

Five microlitres of each 16S rDNA amplified product was digested with *Taq*I and *Rsa*I restriction endonucleases, as recommended by the manufacturer (Boehringer Mannheim, Mannheim, Germany). 16S rDNA amplified product was detected by electrophoresis gel on a 1% (w/v) agarose in 1X TBE buffer. Restriction fragments generated by *Taq*I and *Rsa*I were detected by 3.5% agarose electrophoresis gel. Gels were stained with ethidium bromide and photographed. Lengths of both amplification products and restriction fragments were detected by comparing against 100bp DNA ladder (Gibco-BRL, Eggenstein, Germany).

ERIC and REP amplification products were detected by electrophoresis gels on a 1.5% agarose (w/v). Pattern bands lengths were determined by comparison against a 100bp DNA ladder (Gibco-BRL) for the smallest bands and by the mixture of λ phage DNA digested with *Hind*III-*Eco*RI and *Hind*III (Boehringer Mannheim) for the largest bands.

RESULTS

Characterisation of reference strains by ERIC-PCR and REP-PCR.

A strain-specific DNA fingerprint with 2 to 10 DNA fragments was obtained after gel electrophoresis of the ERIC-PCR product for each of the reference strains (Figure 1). These patterns, made up of bands between 300 and 3000 bp, were used to characterise the strains. The pairs of strains CECT 360/LMG 1484 and LMG 1261/CECT 298 are synonymous and each couple had the same ERIC pattern, which proved the usefulness of the technique for differentiating between strains of acetic acid bacteria.

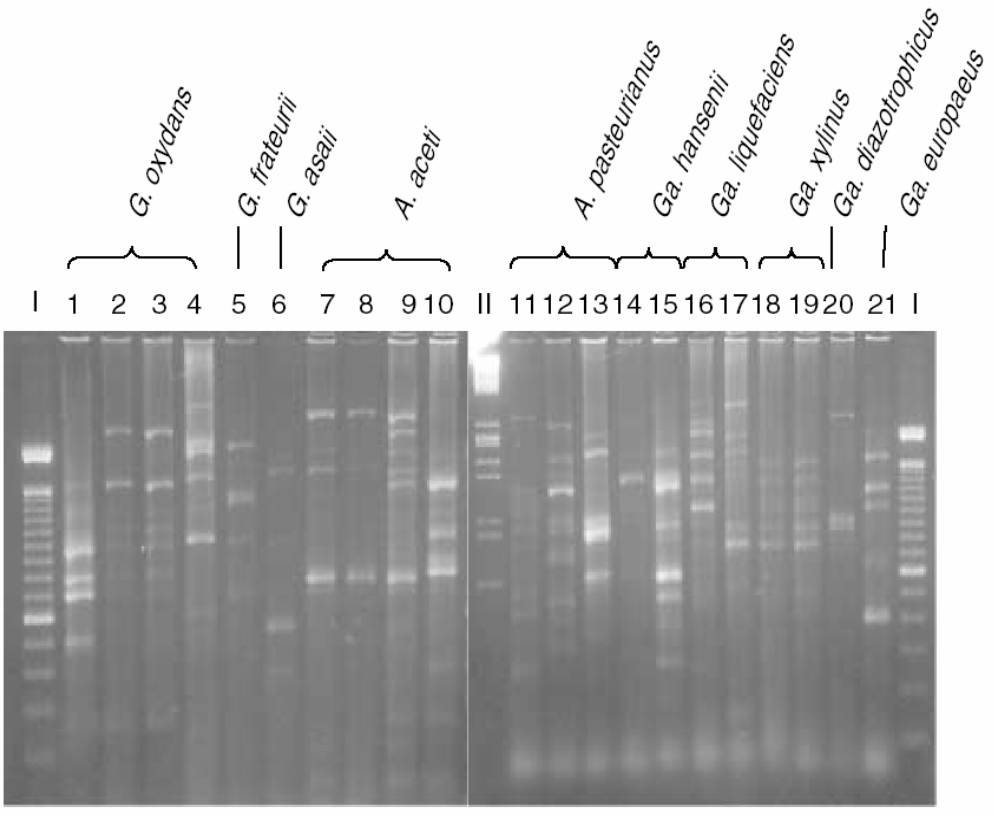

Figure 1. ERIC-PCR patterns of the reference strains. Lane assignments correspond to the numbers given in table 1. Lanes **I** and **II** are molecular size markers: 100bp DNA ladder (**I**) and the mixture of λ phage DNA digested with *Hind*III-*Eco*RI and *Hind*III (**II**).

REP-PCR products also generated strain-specific patterns for most of the same reference strains. Similar sizes of bands were obtained with this technique (between 300 and 3000 bp). REP-PCR patterns were more complex and contained larger number of bands than the ERIC-PCR technique (Figure 2) but the degree of polymorphism was lower. As expected, synonymous strains yielded the same REP patterns, but some different strains could not be distinguished on the basis of the REP patterns. The pairs of strains *A. pasteurianus* LMG 1282/ *Ga. hansenii* LMG 1511; *Ga. liquefaciens* LMG 1347/ *Ga. xylinus* LMG 1515 and *Ga. liquefaciens* LMG 1381/ *Ga. xylinus* LMG 1518 each had identical REP patterns. Previously, with PCR-RFLP of the 16S rDNA the reference strains of the species *Ga. liquefaciens*, *Ga. xylinus* and *Ga. europaeus* (Ruiz *et al*. 2000) could not be distinguished. In this study, these species had very similar patterns and a large number of common bands.

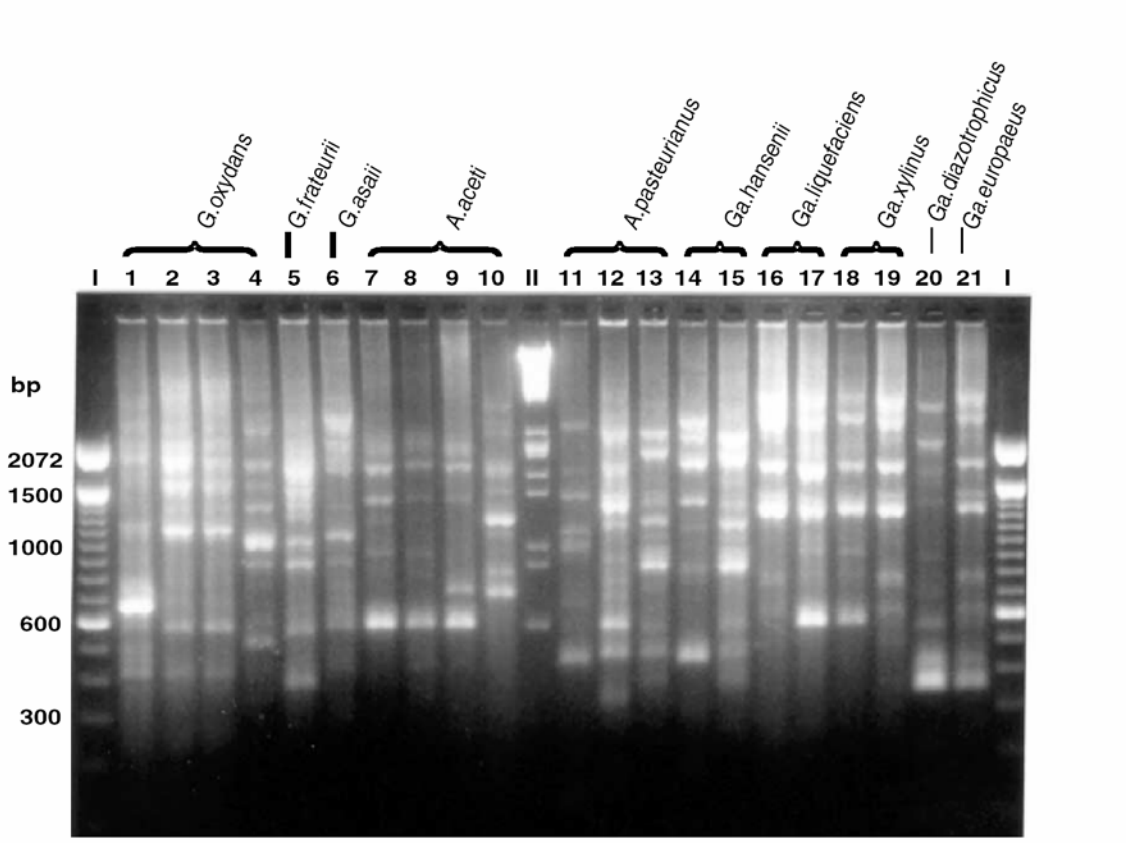

Figure 2. REP-PCR patterns of the reference strains. Lane assignments and molecular size markers are the same as in Fig. 1.

The reproducibility of the two techniques was demonstrated by amplifying the same strains several times, though each amplification was made from different DNA extraction. The patterns obtained were identical for the same strain.

Identification and typing of wine isolates

We studied the diversity of acetic acid bacteria throughout the spontaneous alcoholic fermentation of a red wine. One hundred and twenty isolates of acetic acid bacteria, corresponding to 6 points (20 isolates per day) of the alcoholic fermentation were identified by RFLPs of rDNA 16S, ERIC-PCR and REP-PCR. Population kinetics were also determined throughout fermentation. The initial population of acetic acid bacteria in the must was $3x10^3$ cfu ml^{-1}. This grew slightly to $5x10^3$ cfu ml^{-1} in the first stage of fermentation (the maximum population was reached on day four). The viable population then fell to 50 cfu ml^{-1} at the end of the process.

Isolates of acetic acid bacteria were typed at strain level by ERIC-PCR and REP-PCR. The results from both techniques were identical. The 120 colonies of acetic acid bacteria represented 33 different ERIC patterns and the same number of REP patterns, i.e. 33 different strains. Table 2 shows the percentage of all of the patterns obtained in each sampling of the fermentation. A predominance of different strains was detected in the different stages of fermentation. Strain AAB01 represented 50% of the isolated colonies in the must but this was not detected during the fermentation process (except for day 2 when frequency was only 5%). Strains AAB17 and AAB22 were predominant in the middle of fermentation while strains AAB29, AAB30 and AAB32 were the only ones isolated in the final fermentation. Minor strains (percentages of 5-10%), therefore, were

not detected in the last stage of fermentation. Figure 3 shows the ERIC and REP patterns for the most common strains isolated during the alcoholic fermentation.

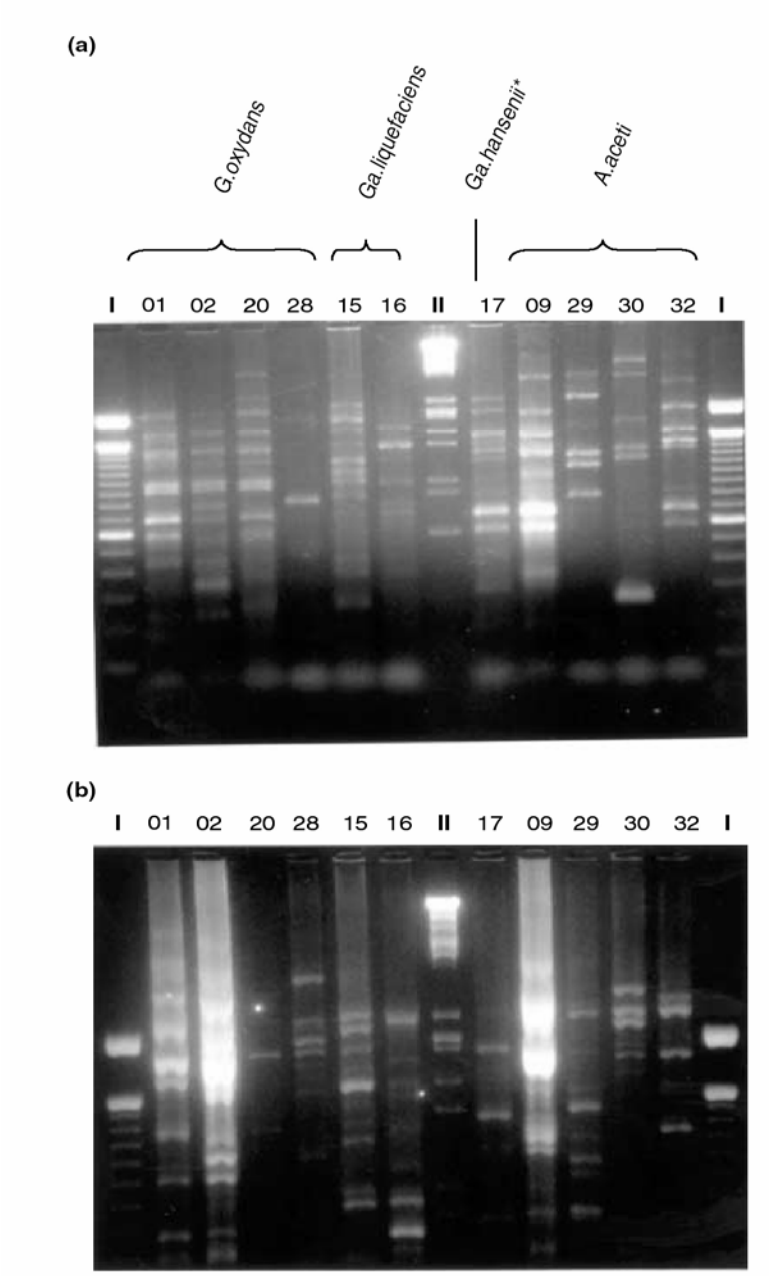

Figure 3. ERIC-PCR patterns (A) and REP-PCR patterns (B) of acetic acid bacteria strains isolated throughout the alcoholic fermentation. Lane assignments correspond to the numbers given in Table 2. Molecular size markers are the same as in previous figures.

**Ga. liquefaciens* means the group of species *Ga. liquefaciens*/ *Ga. xylinus*/ *Ga. europaeus*

The 33 strains were identified at species level by RFLP of 16S rDNA (Ruiz *et al*. 2000). Four different patterns were obtained and these corresponded to four species or groups

of species (Table 2): *G. oxydans, Ga. hansenii, Ga. liquefaciens*/*Ga. xylinus/Ga. europaeus* and *A. aceti*. As the results for diversity of strains, the predominant species was different in each stage of fermentation. Strains of *G. oxydans* were predominant in the must and at beginning of fermentation. *Ga. hansenii* also appeared in a higher proportion during the first days of fermentation, while *A. aceti* was predominant at the end. In fact, the only three strains isolated between day 8 and day 10 of fermentation were *A. aceti*.

Table 2 Prevalence (%) of genetically different acetic acid bacteria during a red wine fermentation as assessed by ERIC-PCR and REP-PCR. The isolates were identified to species level by PCR-RFLP of 16S rDNA

	Days of fermentation						
Strain	1†	2	4	5	8	10‡	Species
AAB 01	50	5	–	–	–	–	*G. oxydans*
AAB 02	10	–	–	–	–	–	*G. oxydans*
AAB 03	10	–	–	–	–	–	*G. oxydans*
AAB 04	5	–	–	–	–	–	*G. oxydans*
AAB 05	5	–	–	–	–	–	*G. oxydans*
AAB 06	5	–	–	–	–	–	*G. oxydans*
AAB 07	5	–	–	–	–	–	*Ga. liquefaciens**
AAB 08	5	–	–	–	–	–	*Ga. liquefaciens*
AAB 09	5	–	–	–	–	–	*A. aceti*
AAB 10	–	5	–	–	–	–	*G. oxydans*
AAB 11	–	5	–	–	–	–	*G. oxydans*
AAB 12	–	5	–	–	–	–	*G. oxydans*
AAB 13	–	5	–	–	–	–	*G. oxydans*
AAB 14	–	5	–	–	–	–	*Ga. liquefaciens*
AAB 15	–	5	–	–	–	–	*Ga. liquefaciens*
AAB 16	–	5	–	–	–	–	*Ga. liquefaciens*
AAB 17	–	45	25	–	–	–	*Ga. hansenii*
AAB 18	–	10	–	–	–	–	*A. aceti*
AAB 19	–	5	–	–	–	–	*A. aceti*
AAB 20	–	–	5	–	–	–	*G. oxydans*
AAB 21	–	–	10	–	–	–	*A. aceti*
AAB 22	–	–	20	–	–	–	*A. aceti*
AAB 23	–	–	10	–	–	–	*A. aceti*
AAB 24	–	–	10	–	–	–	*A. aceti*
AAB 25	–	–	10	–	–	–	*A. aceti*
AAB 26	–	–	5	–	–	–	*A. aceti*
AAB 27	–	–	5	–	–	–	*A. aceti*
AAB 28	–	–	–	10	–	–	*G. oxydans*
AAB 29	–	–	–	20	40	50	*A. aceti*
AAB 30	–	–	–	50	30	30	*A. aceti*
AAB 31	–	–	–	5	–	–	*A. aceti*
AAB 32	–	–	–	10	30	20	*A. aceti*
AAB 33	–	–	–	5	–	–	*A. aceti*

**Ga. liquefaciens* means the group of species *Ga. liquefaciens/Ga. xylinus/Ga. europaeus*.
†Day 1 is the fresh grape must.
‡Day 10 is the final fermentation.

DISCUSSION

In oenology, acetic acid bacteria have received less attention than other wine microorganisms such as yeasts or lactic acid bacteria. However, the increase in acetic acid and other detrimental metabolites in wines as a consequence of the growth of these bacteria is a common problem for wineries. Our objective was to develop rapid and reliable molecular techniques for studying the ecology of acetic acid bacteria population during wine-making processes. Ruiz *et al*. (2000) proved the usefulness of the RFLP of 16S rDNA for identifying acetic acid bacteria at the level of genus and species. In this study, we proposed two simple PCR techniques for identifying acetic acid bacteria isolates.

ERIC and REP elements were described as consensus sequences derived from highly conserved palindromic inverted repeat regions found in enteric bacteria (Pooler *et al*. 1996). However, these sequences seem to be widely distributed in the genomes of various bacterial groups. The amplification of the sequences between these repetitive elements has generated DNA fingerprints of several gram-negative and gram-positive species (Beyer *et al*. 1998; Guinebretier *et al*. 2001; Pooler *et al*. 1996; Sander *et al*. 1998; Wieser and Busse 2000). Recently, Nanda *et al*. (2001) used the ERIC-PCR method to identify acetic acid bacteria isolated from vinegar. We tested the usefulness of this technique with reference strains of most of the species of acetic acid bacteria and obtained exclusive patterns for each strain. REP-PCR has not yet been used with this bacterial group. Although some reference strains could not be differentiated, this technique also produced strain-specific DNA fingerprints. Ruiz *et al*. (2000) could not distinguish the reference strains of the species *Ga. liquefaciens*, *Ga. xylinus* and *Ga. europaeus* with any of the molecular markers they used. Strangely, these same strains

had very similar REP patterns and some of them could not even be distinguished from each other. Further studies should be done with more strains to assure the entity of these species. A common problem with PCR techniques that amplify unspecific sequences (e.g. RAPD-PCR) is the reproducibility of the patterns obtained. In this study, the ERIC and REP patterns were reproducible in the different amplification processes.

The population of acetic acid bacteria in must was relatively small because the grapes were exceptionally healthy. Damaged or spoiled grapes and grapes infected with *Botrytis cinerea* have much higher populations that are reflected in the fresh must (*ca* 10^6 cells ml^{-1}) (Drysdale and Fleet, 1988). The growth of acetic acid bacteria during alcoholic fermentation has not been studied in detail. It appears that the vigorous production of carbon dioxide due to the activity of yeast restricts their growth (Drysdale and Fleet 1988). In the wine fermentation studied, we detected a slight growth of the population during the first few days of fermentation when the yeast had not reached maximal fermentation activity. However, during tumultuous fermentation (maximal fermentation activity), the populations of acetic acid bacteria fell dramatically. At the end of the process, the population was less than 10^2 cfu ml^{-1}.

When we identified the isolated colonies we found a predominance of *G. oxydans* strains in must and during the first few days of fermentation. Joyeux *et al*. (1984) already mentioned the predominance of this species in unspoiled grapes and the predominance of *A. aceti* and *A. pasteurianus* in damaged or rotten grapes. We did not detect this latter species, *A. pasteurinaus*, at any stage of fermentation even though previous studies have reported it to be one of the main species in these environments (Lafon-Lafourcade, 1983; Swings and De Ley, 1981). *A. aceti* was clearly a minority in

must but became progressively more frequent during the process and was the only species isolated at the end of fermentation. These results show that this species must be the one that is most resistant to the anaerobic conditions set up during alcoholic fermentation, to the antimicrobial sulphur dioxide added to the must and to the presence of ethanol. Recently, Du Toit and Lamberchts (2002) identified acetic acid bacteria from South African red wine fermentations and concluded that *Ga. liquefaciens* and *Ga. hansenii* were present in significant numbers although these species had not previously been reported in connection with wine-making processes. Our results also confirm the presence of these species, especially at the beginning of fermentation.

As far as we know, this is the first survey in which the population dynamics of acetic acid bacteria at strain level have been studied during the fermentation of wine. At the beginning of fermentation, a high degree of strain diversity was detected. On average, one new strain was detected for every two colonies analysed. However, this diversity decreased as the process continued. As mentioned above, the anaerobic conditions and the increasing concentrations of ethanol dramatically reduced the population and selected in favour of the most resistant strains. Regardless of the degree of strain diversity, there were clear dominant strains in all stages. This agrees with the predominance of different species mentioned above. The AAB01 *G. oxydans* strain represented 50% of the colonies analysed in the must. A similar percentage was shown by the *Ga. hansenii* strain AAB17 on the second day of fermentation, while the *A. aceti* strains AAB29, AAB30 and AAB32 clearly dominated in the last days of fermentation.

Different molecular techniques for a fast and reliable genotypic characterisation should increase our knowledge of the ecology of acetic acid bacteria and help us to determine their growth behaviour during the various stages of vinification more accurately.

Drysdale and Fleet (1988) have already indicated the need for these studies, which should examine the effects of the different conditions of vinification, the yeast-to-bacteria ratio in the must and the variations in the behaviour of different species and strains of acetic acid bacteria.

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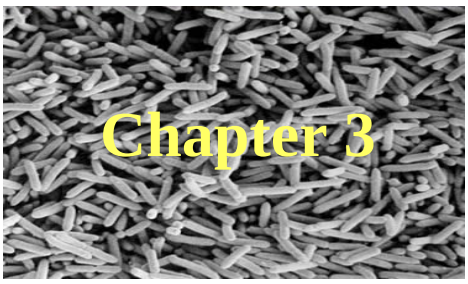

Applications of molecular methods to demonstrate species and strains evolution of acetic acid bacteria population during wine production

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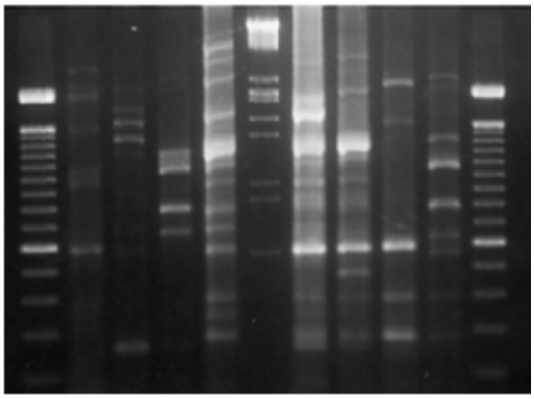

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TARRAGONA, 2005

Abstract

The growth of acetic acid bacteria on grapes or throughout the winemaking process influences the quality of wine, mainly because it increases the volatile acidity. The objective of this study was to analyse how the acetic acid bacteria population evolves in the changing environment of the grape surface and during wine fermentation. We have analysed the influence of yeast inoculation and SO_2 addition on acetic acid bacteria populations. These bacteria were analysed at both the species and the strain level by molecular methods such as Restriction Fragment Length Polimorfism (RFLP) of amplified 16S rDNA, and amplification by Polimerase chain reaction of Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) and Repetitive Extragenic Palindromic (REP-PCR). Our results show that the increases in population size are normally accompanied by a proliferation of *Acetobacter aceti*, which is the main species during fermentation. The diversity of strains is considerable in natural environments such as the grape surface. Changes in the environment during alcoholic fermentation substantially reduce the survival and the diversity of acetic acid bacteria. Few strains are able to survive these conditions and they seem to originate from both the grapes and the winery. To the best of our knowledge this is the first time that acetic acid bacteria are analysed at the strain level in grape surfaces and during winemaking.

Key words: *Acetobacter, Gluconobacter*, REP-PCR, ERIC-PCR, Sulphur dioxide.

Introduction

Acetic acid bacteria (AAB) are Gram-negative, ellipsoidal to rod-shaped cells that have an obligatory aerobic metabolism with oxygen as the terminal electron acceptor. They can use such substrates as glucose, ethanol, lactate or glycerol as energy sources. However, most of these compounds are not completely oxidised into CO_2 and water and several metabolites are accumulated in the growth medium, especially acetic acid. AAB are commonly found in nature, yet because of their high resistance to acidity and the variety of substrates that they can use, they are one of the main food spoilage microorganisms and their presence is mostly related to food modification and human activities for food preservation (De Ley et al., 1984).

Acetic acid is one of the main products of AAB metabolism and it is found in many foods as the result of these bacteria's presence and activity. Acetic acid is the major volatile acid in wine. It is considered objectionable at levels above 1.2 to 1.4 g l^{-1}, and is one of the main reasons for wine spoilage (Drysdale and Fleet, 1988). Although other microorganisms of the wine, such as yeast and lactic acid bacteria, can produce acetic acid, significant populations of AAB are mostly responsible for producing this acid in wine.

Winemaking provides a rather unique series of environments for studying AAB ecology. Spoiled grapes and grapes infected with *Botrytis cinerea* have a high population of AAB, which can grow in grape must and accumulate gluconic and acetic acids. It has also been reported that excessive growth of AAB on grape berries or in musts can lead to incomplete alcoholic fermentation, which is caused by an AAB/yeast interaction that is not fully understood (Drysdale and Fleet, 1989). Subsequently, the

anaerobic conditions of the alcoholic fermentation seem to make conditions unsuitable for the growth of AAB and most of them disappear, although some should be able to survive. However, after alcoholic fermentation, there may be enough agitation and aeration when the wine is transferred from fermentation tanks to other storage vessels to encourage the growth of the surviving AAB populations. At this stage, the oxidation of ethanol accumulates mainly acetic acid, but also acetaldehyde and ethyl acetate. All of these products are detrimental to the quality of the wine (Drysdale and Fleet, 1988; Drysdale and Fleet, 1989). AAB are the main oxidative microorganisms able to survive in high acidic and high ethanol conditions, such as in wine.

AAB species have traditionally been identified by testing physiological and chemotaxonomic 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 (Yamada and Kondo, 1984) or different PCR methods (Bartowsky et al., 2003; Ruiz et al., 2000). Some of these molecular techniques, however, are not suitable for routine identification of bacterial isolates because they are also time-consuming and difficult to use with a large number of isolates. Recently, Ruiz et al. (2000) used the RFLP analysis of PCR-amplified 16S rDNA to differentiate AAB at the level of genus and species. González et al. (2004) have also proposed two PCR techniques for typing strains: Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Repetitive Extragenic Palindromic-PCR (REP-PCR) (Versalovic et al., 1991). These three quick and easy methods proved to be useful for identifying and typing reference strains and indigenous AAB isolated from wine fermentations.

There is an obvious need for further studies into the ecology and development of AAB during the winemaking process (Drysdale and Fleet, 1988). The availability of these molecular methods means that this objective can be achieved and we have used them to study the evolution of the AAB population in a changing environment on the grape surface and during wine fermentations. We have also evaluated two of the most common oenological practices (yeast inoculation and SO_2 addition) and further analysed the interaction at the population level between AAB and yeast.

Methods

Fermentation conditions

This study was done in the experimental cellar belonging to the Faculty of Oenology in Tarragona (Spain) during the 2002 vintage. Red Grenache was the grape variety chosen for the vinifications. The grape must obtained was separated into four 100-litre tanks after destemming and crushing. When SO_2 was added, the concentration was 60 mg l^{-1} and the inoculum used was a commercial strain of Saccharomyces cerevisiae (Lallemand, Canada) added as dry yeast at a concentration of 150 mg l^{-1}. The fermentation conditions in the four tanks studied were (A) inoculum and SO_2, (B) inoculum and no SO_2, (C) no inoculum and SO_2 and (D) no inoculum and no SO_2. All the vinifications were allowed to develop at room temperature (the maximum variation in fermentation temperature was set at 22-28ºC).

Sampling

Samples were taken from grape and fresh must, and also during the fermentation process. Grape samples were taken 8 and 3 days before the harvest and on the day of the

harvest (day 0). One hundred berries were randomly (at least from 50 different vines, the two more exposed berries from the same bunch, without taking into consideration the sanitary status of the berry) and aseptically collected on each sampling day. Grapes were crushed by Stomacher (Stomacher 400, Seward, Norfolk, UK) and different dilutions of the must were spread on plates of glucose solid medium (1% yeast extract; 3% $CaCO_3$; 7.5% glucose; 2.2% agar w/v) supplemented with Pimaricin (100 mg l^{-1}) (Sigma-Aldrich; Steinheim; Germany) to inhibit the growth of yeasts and moulds. This antibiotic was added to the culture medium from the stock solution after the medium had been sterilised. Plates were incubated at 28ºC for 2-4 days under aerobic conditions. Twenty colonies were randomly isolated and purified from each sample. The isolates were then grown for 48h in mannitol liquid medium (0.5% yeast extract; 0.3% peptone; 2.5% mannitol w/v) for DNA extraction.

Must samples (210 g sugar l^{-1}, pH 3.6, density of 1090 mg l^{-1}) were taken after the grapes were crushed and before the addition of sulphur dioxide and inoculation. Samples were also taken at three different stages of the fermentation: at the beginning (density of 1065 mg l^{-1}), in the middle (density of 1030 mg l^{-1}) and at the end (ethanol concentration of wine, 12.5 % v/v). These samples were treated exactly the same as the grape samples for the AAB isolation. They were also plated on media containing 20g l^{-1} glucose; 20g l^{-1} peptone; 10g l^{-1} yeast extract and 15g l^{-1} agar (YEPD media) and on the selective Agar-Lysine medium (ADSA micro, Pharmafaster, Barcelona, Spain) so that the total yeast and non-Saccharomyces yeast populations, respectively, could be enumerated. Saccharomyces species were unable to grow on the Agar-Lysine medium (Angelo and Siebert, 1987).

DNA extraction

Extraction was carried out using the method described by Rademaker and De Bruijn (2003). Twenty-five ml of growth medium was centrifuged, the supernatant discarded and the pellet resuspended in RB buffer (NaCl, 0.15M; EDTA,0.01M pH 8). It was centrifuged again and the supernatant discarded. The pellet was resuspended in 100 μl of TE buffer (Tris, 10mM; EDTA,1mM pH 8) and 500 μl of GES solution (60% guanidine thiocyanate; 20% EDTA 0.5M; 20% sterile water). A 250 μl aliquot of ammonium acetate and 500 μl of chloroform/isoamilic alcohol was added to the solution and the mixture centrifuged for 10 min at 10,000 rpm. The upper phase was collected and 380 μl of iso-propanol added. It was centrifuged again for 5 min at 10,000 rpm and the supernatant discarded. The pellet was washed with ethanol 70% and the DNA resuspended in 200 μl of TE buffer. The DNA was treated with 25 μl of DNAase-free RNAase (250 $\mu g\ \mu l^{-1}$) (Sigma-Aldrich) for 1 hour at 37ºC. The concentration and purity of DNA was determined using a GenQuant spectrophotometer (Pharmacia, Cambridge, UK).

Oligonucleotide primers

For species identification, the primers used to amplify the 16S rDNA were designed by Ruiz et al. (2000) on the basis of conserved regions of the 5'-end (16Sd, 5'-GCTGGCGGCATGCTTAACACAT.) and the 3'-end (16Sr, 5'GGAGGTGATCCAGCCGCAGGT) of this gene.

For strain identification, the primers used for the amplification of ERIC (ERIC1R: ATGTAAGCTCCTGGGGATTCAC ERIC2: AAGTAAGTGACTGGGGTGAGCG) and REP (REPIR-I: IIIICGICGICATCIGGC REP2-I: ICGICTTATCIGGCCTAC)

elements were those described by Versalovic et al. (1991). 'I' represents inosine. All the primers were synthesised by Sigma-ARK (Darmstadt, Germany).

PCR conditions

PCR amplification of 16S rDNA was carried out in a final volume of 50 μl comprising 3 μl of DNA solution (ranging from 0.5 to 1.5 μg μl^{-1}), 5μl 10x amplification buffer (ARK Scientific), 15 pmol of each primer (Ruiz et al., 2000), 200 μM of each of the four dNTPs (Roche Diagnostics GmBh, Manheim, Germany), 3 mM $MgCl_2$, 0.4 μl of BSA (20mg ml^{-1}), 5 μl of DMSO and 2.5U of Taq Polymerase (Sigma-ARK). The reactions were carried out in Gene Amp PCR System 2700 (Applied Biosystems, Foster city, USA). Samples were incubated for 5 min at 94ºC and then cycled 35 times at 94ºC for 1min, 58ºC for 1min and 72ºC for 2 min. The samples were incubated for 10 min at 72ºC for final extension and kept at 4ºC until tested.

ERIC and REP elements were amplified in 25 μl samples comprising 3 μl of DNA solution (ranging from 0.5 to 1.5 μg μl^{-1}), 5 μl 5x GB Buffer ($(NH_4)_2SO_4$ 1M, Tris-HCl 1M pH 8.8, $MgCl_2$ 1M, EDTA 0.5M pH 8.8, β-mercapto-ethanol 14.4M (Sigma-Aldrich) (Rademaker and De Bruijn, 2003), 0.2 μl of BSA (20mg ml^{-1}), 2.5 μl of DMSO, 100 μM of each of the four dNTPs, 15 pmol of each respective primer (Versalovic et al., 1991) and 2.5U of *Taq* Polymerase (Ecogen RSL, Barcelona, Spain). Reactions were carried out in Gene Amp PCR System 2700 (Applied Biosystems). Samples were incubated for 5 min at 94ºC, and then cycled 30 times at 94ºC for 30s, at 57ºC (ERIC-PCR) and 47ºC (REP-PCR) for 30s and at 65ºC for 4 min The samples were incubated for 8 min at 65ºC for final extension and kept at 4ºC until tested.

Restriction analysis and gel electrophoresis

Five microlitres of each 16S rDNA amplified product were digested with *Taq*I and *Rsa*I restriction endonucleases, as recommended by the manufacturer (Roche Diagnostics GmBh). 16S rDNA amplified product was detected by electrophoresis gel on a 1% (w/v) agarose in 1x TBE buffer. Restriction fragments generated by *Taq*I and *Rsa*I were detected by 3.5% agarose electrophoresis gel. Gels were stained with ethidium bromide and photographed. Lengths of both amplification products and restriction fragments were detected by comparison with 100bp DNA ladder (Gibco-BRL, Eggenstein, Germany).

ERIC and REP amplification products were detected by electrophoresis gels on a 1.5% agarose (w/v). Pattern band lengths were determined by comparison with a 100bp DNA ladder (Gibco-BRL) for the smallest bands and by the mixture of λ phage DNA digested with *Hind*III-*Eco*RI and *Hind*III (Roche Diagnostics GmBh) for the largest bands.

3. Results

3.1 Evolution of AAB populations during grape ripening

We analysed the AAB population on grape surfaces eight and three days before harvest and on the harvest day itself in the 2002 vintage (Table 1). The sizer of the population increased during the last week of ripening, but on the day of the harvest was lower. This decrease was due to the berries being washed by rain (day before harvest it rained 61 l m^{-2}). Identification of the isolated AAB showed *Gluconobacter oxydans* and A*cetobacter aceti* as the main species on the grape surface (*Gluconacetobacter hansenii* was also isolated at harvest, but at a low percentage).

Table 1
AAB population dynamics in grapes and must

	Days before harvest			Must
	8	3	0	
Total population size (10^6 cfu/ml)	0.076	1.150	0.013	0.220
Diversity (%)	50	45	70	50
A. aceti (%)	35	90	15	80
Number of strains	1	8	1	8
Repeated strains	1	3	1	3
G. oxydans (%)	65	10	80	5
Number of strains	10	1	12	1
Ga. hansenii (%)	0	0	5	15
Number of strains	0	0	1	1

Number of strains means the different strains of each species found in a given day. Repeated strains are those that were detected in different days. Diversity is calculated as the percentage of number of different strains (no matter the species) on the total colonies analysed. No repeated strains were observed for *G. oxydans* and *Ga. hansenii*.

All the colonies identified at the species level were also typed at strain level by two PCR techniques: ERIC-PCR and REP-PCR. The most common patterns are shown in Figure 1. In grapes and must, a total of 25 different ERIC patterns were obtained from the 80 colonies analysed, i.e. at least 25 different strains, which represent a diversity of 31.5% (calculated as the percentage of different strains of the total colonies analysed). The same number of patterns was obtained with the REP-PCR technique. This strain diversity was considerable and accounted for a variation between 45 and 70% when taken every day (Table 1). Although some of these strains showed higher percentages for several days (i.e. 01Ac was 35% of the total number of analysed colonies), the percentages of most of them were lower than 10%. Interestingly the highest variation was in the *G. oxydans* strain population (with no repeated strains during this pre-harvest period and in the must). However, *A. aceti* did not show such a great variation, yet the number of repeated strains was much higher (Table 1). Overall, two strains of *A. aceti* had the highest presence in grapes (strains 01Ac and 11Ac). The entry of the grapes into

the cellar and exposure to cellar machinery significantly increased (10-fold) the total AAB population and particularly the *A. aceti* population. In this case the two strains mentioned above were also found along with *A. aceti* strain 33Ac, which was not found previously. Strikingly, *G. oxydans*, which was the predominant species on the grape surface on the day of the harvest, was only a small percentage of the must.

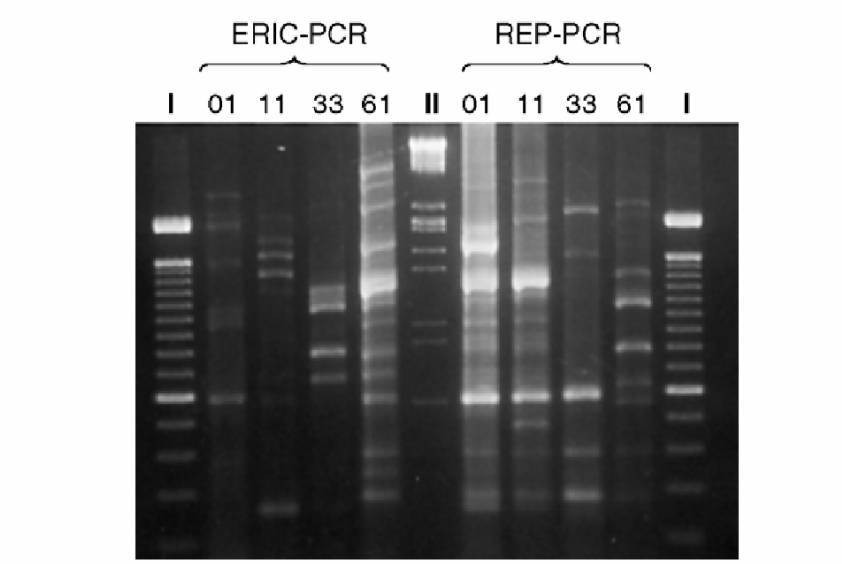

Fig. 1. ERIC- and REP-PCR patterns of the major strains found in fermentations (01Ac, 11Ac, 33Ac and 61Ac). Lanes I and II are molecular size markers: 100 bp DNA ladder (I) and the mixture of λ phage DNA digested with *Hin*dIII–*Eco*RI and *Hin*dIII (II).

3.2 Fermentation kinetics. Enumeration of yeast populations

Four semi-industrial fermentations were carried out in different conditions of SO_2 addition and yeast inoculation (see Methods, where each condition is designated as A, B, C or D). As expected, yeast inoculation speeds up the beginning of fermentations, especially when SO_2 is not added (Figure 2a). The lag phase of fermentation C was longer than that of the inoculated fermentations (A and B) but finished at the same time. On the other hand, the lack of SO_2 and yeast inoculation in fermentation D delayed the beginning and the end.

It is interesting to compare these fermentation kinetics with the total yeast and non-*Saccharomyces* populations in these fermentations (Figure 2b and 2c). The yeast population size was 8 x 10^6 cfu ml^{-1} in the must (which was the same for all the fermentations). Inoculation increased these populations to a maximum of approximately 50 x 10^6 cfu ml^{-1}, but SO_2 addition (without inoculation; fermentation C) also increased the yeast population. Of these total yeast populations, the non-*Saccharomyces* yeasts were more than 10% of the total number in the must (1 x 10^6 cfu ml^{-1}). This percentage decreased to less than 1% in most of the samples taken from fermentations A, B and C. On the other hand, fermentation D had a higher proportion of non-*Saccharomyces* yeasts during most of the process. (Only at the end of fermentation D was the proportion of *Saccharomyces* vs. non-*Saccharomyces* similar to that of the other fermentations).

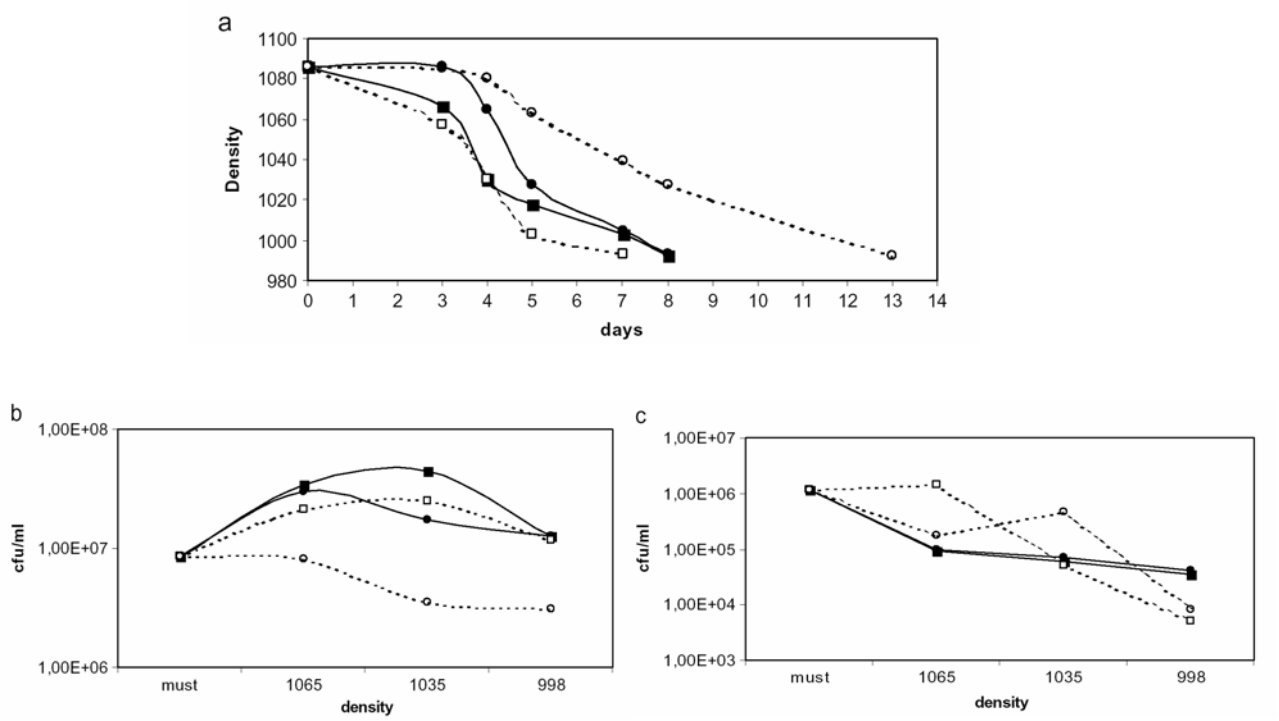

Fig. 2. Fermentation kinetics (as density mg l^{-1}, a) and yeast population (b, total yeast; c, non-*Saccharomyces* yeast) of the 4 experimental fermentations. Fermentations A (inoculated and SO2 added, –■–), B (inoculated no SO2 added, · · · □ · · ·), C (non-inoculated and SO2 added, –●–) and D (non-inoculated and no SO_2 added, · · · ○ · · ·) are represented.

3.3 Identification and typing of the isolated AAB

The number of AAB in the must was 2.2 x 10^5 cfu ml^{-1}. This number decreased sharply at the beginning of the fermentation to reach levels of 10^2-10^3 cfu ml^{-1} at the end (Table 2). Although the differences were very limited, the AAB population sizes of the inoculated fermentations were generally lower than the spontaneous ones. These differences were more evident at the end of the fermentation.

Regardless of the fermentation conditions, *A. aceti* was the predominant species throughout the fermentations (Table 2). This predominance was practically 100% in the middle and at the end of the fermentations. Other species identified at lower percentages were *G. oxydans*, *Ga. hansenii*, *Ga. liquefaciens* and *A. pasteurianus*. Interestingly, *A. pasteurianus* was only found at the end of the fermentation and in the absence of both inoculation and SO_2.

Table 2
AAB population dynamics during alcoholic fermentations

Density	1065 (beginning)				1035 (mid)				998 (end)			
Fermentation condition	A	B	C	D	A	B	C	D	A	B	C	D
Total population size (10^3 cfu/ml)	3.50	2.80	3.25	9.10	2.00	2.50	14	3.10	0.27	0.74	0.28	1.70
Diversity (%)	45	60	45	80	35	35	20	35	25	20	15	40
A. aceti (%)	70	55	85	85	90	95	100	100	100	100	100	90
Number of strains	6	4	8	14	5	6	4	7	5	4	3	7
Repeated strains	2	1	5	7	3	3	4	4	3	2	3	1
Strains from must/grape	1	1	2	3	1	2	2	2	1	2	2	1
G. oxydans (%)	15	15	15	0	10	5	0	0	0	0	0	0
Number of strains	2	3	1	0	2	1	0	0	0	0	0	0
Repeated strains	1	2	1	0	1	0	0	0	0	0	0	0
Strains from must/grape	1	1	1	0	0	0	0	0	0	0	0	0
Ga. hansenii (%)	15	5	0	15	0	0	0	0	0	0	0	0
Number of strains	1	1	0	2	0	0	0	0	0	0	0	0
Repeated strains	0	0	0	1	0	0	0	0	0	0	0	0
Strains from must/grape	0	0	0	1	0	0	0	0	0	0	0	0
Others (%)	0	25l	0	0	0	0	0	0	0	0	0	10p

Number of strains and diversity were as in Table 1. Repeated strains are those that were detected in different stages of fermentation. Other strains: l: *Ga. liquefaciens*, p: *A. pasteurianus*.

The AAB strain diversity was also high during the fermentations. In fact, a new strain was detected about every two or three colonies analysed at the beginning of fermentation. However, this diversity was clearly different in the various stages of the fermentations. The decrease in the population as the fermentation progresses led to a decrease in the strain diversity. Of all the different fermentation conditions, fermentation D had the highest strain diversity at the end of fermentation (40%). Inoculation and the presence of SO_2 also had a role in decreasing diversity, although the differences were not dramatic. In fact, in all the fermentations one or two major strains accounted for more than 50% of the total population in the middle and at the end of fermentation (Figure 3). All of these strains belonged to the *A. aceti* species, which was clearly the most resistant and the most adapted to the fermenting medium. A total of four *A. aceti* strains survived the adverse conditions and were found at some stage of the fermentations. Of these, 01Ac was also significantly present in grapes and must, and in at least two fermentations (B and C) present at the end. The other major strain, 33Ac, was not detected in grape, yet appeared in must for the first time. Its massive presence at the end of the fermentations could easily be related to the cellar. Of the other two repetitive strains, 11Ac was also present in grapes and appeared at the beginning of fermentation in low numbers. The other strain, 61Ac, was absent in the grapes or must yet present at the beginning of some fermentations.

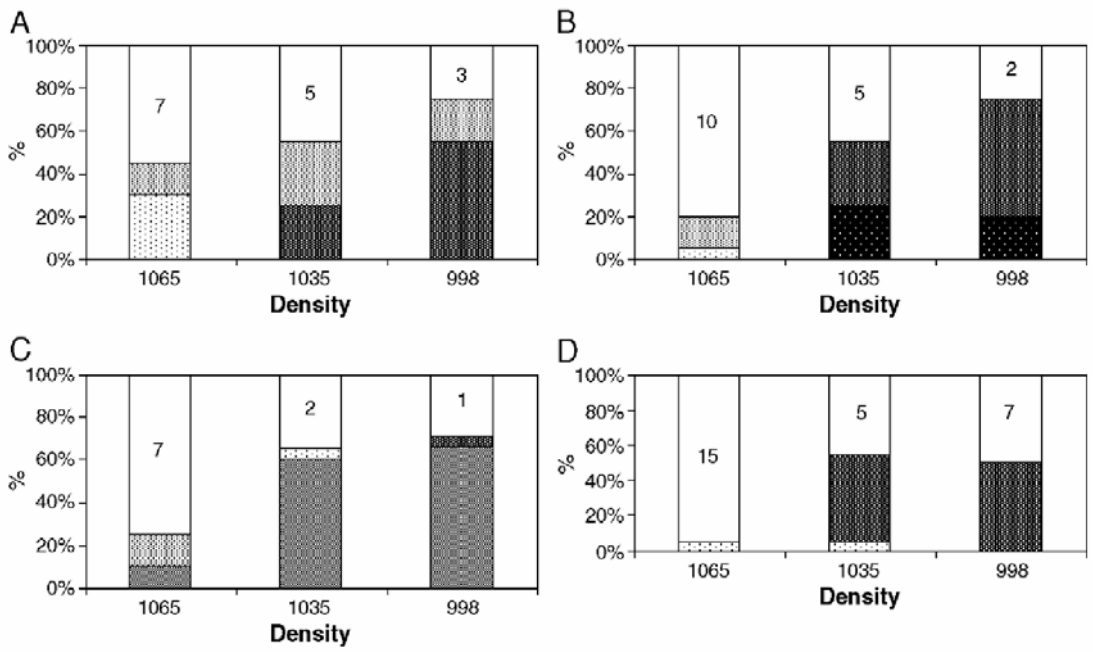

Fig. 3. AAB strain variation during fermentations, expressed as % of the total strains analysed. Major strains are represented (01Ac, ■; 11Ac, □; 33Ac, ▩; 61Ac ▨ and others, □). Numbers means the amount of other strains. A, B, C and D mean the fermentation conditions.

4. DISCUSSION

In oenology, AAB have received less attention than other wine microorganisms such as yeasts or lactic acid bacteria. However, the increase in acetic acid and other detrimental metabolites in wines as a consequence of the growth of these bacteria is a common problem for wineries (Du Toit and Lamberchts, 2002). Drysdale and Fleet (1988) recommended the need for studies into the origin of AAB, the growth behaviour of AAB during the different stages of vinification, the influence of different oenological practices, the significance of the yeast to bacteria ratio in the must, and variations in the behaviour of different species and strains of AAB. Our objective in the present study completely fits this need. In particular our aim was to examine the origin of AAB during vinification and the influence of two widespread oenological practices on the AAB population: yeast inoculation and SO_2 addition. For this purpose, we are currently applying rapid and reliable molecular techniques for the identification and typing of AAB (González et al., 2004; Poblet el al., 2000; Ruiz et al., 2000).

4.1 Evolution during grape ripening

It should be noted that the grape maturation during the 2002 vintage was characterised by unusual rainfall for this period. These climatic conditions caused higher grape spoilage and damage than other vintages. Although we detected more AAB on the grape surface than expected from healthy grapes, the numbers were within the limits reported (Joyeux et al., 1984; Du Toit and Lamberchts, 2002). Other studies have determined the predominance of *G. oxydans* in healthy grapes and of *A. aceti* and *A. pasteurianus* in spoiled grapes (Joyeux et al., 1984). However, we detected a major presence of both *G. oxydans* and *A. aceti* in spoiled grapes. Likewise, Du Toit and Lambrechts (2002) isolated mainly *G. oxydans* on spoiled grapes and Barbe et al. (2001) also found *G. oxydans* to dominate *Botrytis*-infected grapes. Therefore, the health state of the grape may not be the only parameter that determines the dominant species of AAB on the grape surface. As in the case of yeast microbiota, such parameters as the climatic conditions, the geographical location of the vineyard, the grape variety, antifungal treatments, etc. may influence these populations. However, it is rather evident that the increase in AAB correlates well with high counts of *A. aceti* (Table 1), which could be an indication that spoiled grapes facilitate the growth of these species (Barbe et al., 2001).

Furthermore, it is noteworthy that the AAB population in the must is ten times higher just after it comes into contact with cellar equipment than in crushed grapes in sterile conditions. This increase can mostly be accounted for by the population of *A. aceti*.

4.2. Fermentation kinetics. Enumeration of AAB and yeasts

As previously reported (Constantí et al., 1998) yeast inoculation increased the *Saccharomyces* strains *vs*. non-*Saccharomyces* strain ratio. These differences in population led to shorter lag phases and a quicker fermentation start. The lag phase when SO_2 was added to spontaneous fermentations was longer than when they were inoculated. However, SO_2 also selected *Saccharomyces* over non-*Saccharomyces*, and the fermentation rate was similar to that of inoculated fermentations. On the other hand, when there was no inoculation or SO_2 addition the non-*Saccharomyces* species had an excessive influence, which delayed the beginning of the fermentation and slowed down the fermentation rate. Constantí et al. (1998) showed that the concentration of acetic acid in spontaneous fermentations was higher without SO_2 addition than in the inoculated and/or sulphited fermentations. This high volatile acidity could be due to the considerable development of the non-*Saccharomyces* species but also to high numbers of AAB. In all the fermentative conditions, the production of carbon dioxide by yeasts during alcoholic fermentation substantially reduced the population of AAB.

4.3. Identification and typing of the isolated AAB

Other studies on the evolution of AAB species throughout wine fermentations have established certain general trends (Drysdale and Fleet, 1988; Du Toit and Pretorius, 2002; Joyeux et al., 1984). *G. oxydans* is usually the dominant species in fresh must and the first stages of fermentation. It is rarely isolated from wines. On the other hand, *A. aceti* is the major strain in the last phases of fermentation, which we have also noted, regardless of the yeast inoculation or SO_2 addition. Du Toit and Lambrechts (2002) suggested that *A. pasteurianus* is more resistant to SO_2 than *A. aceti*. Our results did not confirm this hypothesis. In the present study, *A. aceti* mostly dominated in the fermentations where SO_2 had been added but we only found *A. pasteurianus* in the

absence of SO_2. This considerable predominance of *A. aceti* could be due to the unhealthy state of the grapes because, in the same cellar during the previous vintage (González et al., 2004) we also found *G. oxydans*, *Ga. liquefaciens* and *Ga. hansenii* in higher percentages as well as *A. aceti.*

The study at the strain level makes it possible to follow or presume the origin of the different species. Apart from our previous paper (González et al., 2004) this is the first time that the population dynamics of AAB during a wine fermentation has been monitored at the strain level. In the present study, we have applied the same PCR techniques to study fermentations with different environments (effect of SO_2 addition and microbial competition through yeast inoculation). Few strains survived the transfer from grapes to must and the ones that did were mostly *A. aceti* species. However, the survival of *A. aceti* was not due only to those that can be considered "cellar-resident" strains (present in cellar equipment and environment), and thus adapted to wine making conditions. In fact, a strain that was already present in grapes (01Ac) was able to survive until the end of fermentation and be one of the most relevant strains. However, the other most important strain during fermentation (33Ac) was only present in must after "contamination" with winery equipment, which could raise doubts about its origin, although its presence in the middle and at the end of all the fermentations could indicate that it adapts very well to the harsh conditions of winemaking. Of the other two strains, 11Ac is clearly a grape-associated strain which hardly survives the limiting fermentation conditions, while 61Ac does survive those conditions. Furthermore, the clear reduction in strain diversity and AAB population size at the end of fermentation indicates that this medium is extremely deleterious for AAB and very few of them are able to survive.

Further knowledge about the strains that are most adapted to the changing conditions of wine fermentations would include the analysis of their physiological and genetic characteristics. The genetic and physiological characteristics will explain their resistance and growth behaviour during the winemaking processes. Other information needed concerns the main origin of these bacteria is. It is clear that both the grape itself and the cellar equipment could be the point at which wine contamination by AAB starts. However, further studies should be done on the origin and prevalence of AAB during wine fermentations. Better knowledge on this subject will make it possible for winemakers to control the growth and deleterious effects of these bacteria.

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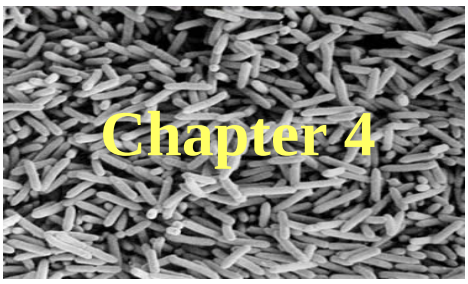

Enumeration and detection of acetic acid bacteria by real-time PCR and nested-PCR.

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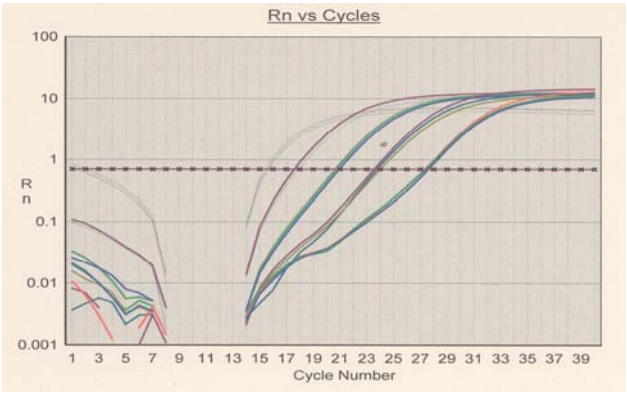

UNIVERSITAT ROVIRA I VIRGILI

TARRAGONA, 2005

Abstract:

Acetic acid bacteria play a negative role in wine-making by increasing the volatile acidity of the wines. They are able to survive and grow in the different phases of the alcoholic fermentation and it is very important to control their development. The most commonly used techniques used for enumeration can not detect viable but not cultivable cells, state which have been described in this bacterial group. The main objective of the present work is to test fast, sensitive and reliable techniques to enumerate and detect the presence of this bacterial group without the need of cultivation such as real time-PCR and nested-PCR. Primers were designed based on the available 16S rDNA gene sequences and tested successfully with reference acetic acid bacteria strains. Usefulness of rt-PCR was demonstrated by comparing the results with traditional techniques (colony and microscope counting) obtaining similar results with all the techniques. Optimised real time-PCR allows the enumeration of amounts of cells between 10^7 and 10^1 cel ml^{-1}, while with nested-PCR we were able to detect 3.4 cel ml^{-1}, although this last technique do not allow the enumeration, offer several advantages to routine laboratory analysis.

Introduction:

Acetic Acid Bacteria (AAB) can occur in sugar and alcoholised, slightly acid niches. Some of the transformations carried out by this bacterial group have a high interest for the biotechnological industry, such as sorbose and cellulose production. However, the most relevant industrial process in which they are involved is vinegar production. On the other hand, they play a negative role in wine making by increasing the volatile acidity by the production of acetic acid, which is considered objectionable at levels above 1.2 to 1.4 g l^{-1}, and is one of the main reasons of wine spoilage (Drysdale & Fleet, 1988). It has also been reported that an excessive growth of AAB on grape berries and musts can lead to incomplete alcoholic fermentation (Drysdale & Fleet, 1988). Despite the adverse conditions during alcoholic fermentation, AAB can survive and even grow. For the good quality of the wines is very important to keep AAB populations as low as possible (Du Toit & Pretorius, 2002). After the alcoholic fermentation, usual enological techniques such as pumping over and racking of wine may stimulate the growth of AAB (Joyeux et al., 1984; Drysdale & Fleet 1989) due to the intake of oxygen, which may lead to an undesirable production of acetic acid, acetaldehyde, ethyl acetate and dihydroxyacetone (Dupuy & Maugenet, 1963; Sponholz & Dittrich, 1984).

For any industrial process it is relevant the identification and quantification of the different species and strains involved in the biotechnological transformation, likely the absence of microorganisms in the final product is also an important control in the manufacture process. Because of the importance of this microbiological control, quick

and accurate procedures to detect and enumerate these bacteria are important from an industrial point of view.

The enumeration of AAB has traditionally been done quantifying viable colonies by platting in solid culture media. Some works have been done about selective culturing (Lisdiyanti *et al*., 2000), but the limitations of cultural methods are the time required and their inability to detect viable but non cultivable (VBNC) cells. To overcome this disadvantages of culturing, new techniques have been developed using molecular approaches such as FISH and epifluorescence (Blasco et al., 2003). The real-time PCR (rt-PCR) has evolved into a fast, sensitive and accurate tool for quantifying bacteria in environmental samples (Huijsdens *et al*., 2002; Harms *et al*., 2003). rt-PCR allows the determination of the initial template concentration and, therefore, an accurate estimation of cell number (Bleve et al., 2003) This method has been used to detect a diverse number of bacteria (Luo et al., 2004; Rousselon et al., 2004), but, so far no approaches have been done in the analysis of AAB.

As mentioned above, for some industrial processes (bottling, ageing of wines, etc), is important to detect presence or absence of a specific microorganism by a simple and accurate technique. A qualitative approach, widely used to diagnose infections, and sensitive enough to detect low DNA concentrations is nested-PCR. Nested-PCR is an effective method for detecting organisms in environmental samples where the presence of low concentrations of the DNA targets and high concentrations of contaminants could inhibit DNA amplification (Ciapina et al., 2003). Nested-PCR consists on a double amplification of a template DNA, using for the second amplification template the amplicon obtained in the first one, improving the sensitivity of the analysis.

The aim of the present study was to develop two techniques , nested- and rt-PCR for detection and quantification of AAB respectively. For that purpose we have designed specific primers based on the 16S rDNA gene. These primers were validated with reference strains as well as with contaminated samples from wine and vinegar.

Material and Methods:

Strains, samples and media:

Reference strains used in this study where: *A. aceti* (LMG 1261), *A. pasteurianus* (LMG 1262), *G. oxydans* (LMG 1408), *Ga. xylinum* (LMG 1515), *Ga. hansenii* (LMG 1527), *A. orleaniensis* (LMG 1545), *G. cerinus* (LMG 1366) and *K. baliensis* (DSM 14400). They were grown in Mannitol media (25 g l^{-1} D-mannitol; 5 g l^{-1} yeast extract; 3 g l^{-1} peptone) for 48h in 10 ml total volume. Solid media for AAB enumeration was prepared by the addition of agar (2% w/v) to the Mannitol media.

Red wine was sterilised by autoclaving and filtering and contaminated with different amounts of reference strains of AAB. Several different samples of commercial red wine and three of commercial vinegar were also used.

DNA extraction:

DNA extraction was performed by centrifugation of 1ml sample, the supernatant was discarded and the pellet resuspended in 1ml NaCl (1M). It was centrifuged again and the supernatant discarded. The pellet was then resuspended in 1ml water and frozen at –20ºC. An aliquot of the sample was heated for 15min at 95ºC and used for PCR

analysis. Extraction was also carried out using the method described by Rademaker & De Bruijn (http://www.msu.edu/user/debruijn).

Oligonucleotides design:

The oligonucleotide primers for the rt-PCR were designed by aligning sequences of the 16S rDNA gene present in the database www.pubmed.com by using clustalw program (Thompson et al., 1994). The sequences of following strains were considered for primer selection: GeneBank accession numbers X74066, D30768, AJ419840 (*A. aceti*), AJ419834, AB086016 (*A. pasteurianus*), Z21936, Y15289 (*Ga. europaeus*), AJ007698, X75619 (*Ga. xylinus*) X75620 (*Ga. hansenii*), X75617 (*Ga. liquefaciens*), X75618 (*Ga. diazotrophicus*), AB056321 (*K. baliensis*), AB110715 (*Acidomonas*), AB025931 (*As. bogorensis*) and X73820 (*G. oxydans*).

Primers were designed from the conserved parts of the gene using the Primer Express software, version 2.0 (Applied Biosystems, Foster City, USA) to amplify a product of 55 bases. Forward primer AQ1F(5' TCAAGTCCTCATGGCCCTTATG 3') and reverse primer AQ2R(5' TACACACGTGCTACAATGGCG 3').

Primers specificity were tested *in silico* in www.embl.org against databases. Primers were also tested against microorganisms normally present in wine such as lactic acid bacteria and yeasts in the laboratory with no positive results.

Real time PCR conditions:

The amplification reactions were carried out in a total volume of 25µl, containing 5µl of DNA solution, 12.5µl SYBR Green universal PCR master mix (Applied Biosystems)

5μM of each primer and 6μl of sterile H_2O. Reactions were run by triplicate on a GeneAmp 5700 Sequence detection system (Applied Biosystems). Reactions took place after 2 min at 50ºC, 10 min at 95ºC, followed by 40 cycles: 15s at 95ºC and 1 min at 60ºC. All tests were performed in triplicate in the same run.

Data analysis was carried out by GeneAmp 5700 software. The cycle threshold (C_T) was calculated as the cycle number at which concentration became exponential. The cycle threshold of each sample was then compared to a standard curve and the result was expressed as a numerical value of the number of bacteria in the sample. One no template sample was used always as a negative control.

Standard curve:

AAB were enumerated before DNA extraction by plating and microscope counting. The standard curves were generated by amplification of serial 10-fold dilutions of the different reference strains. Standard curves were designed by GeneAmp 5700 software (Applied Biosystems), the threshold cycle was determined to obtain optimal regression coefficient values (above 0.98).

Nested-PCR conditions:

The nested-PCR assay was performed using the following primers: Forward outer primer (5'-GCTGGCGGCATGCTTAACACAT 3'); Reverse outer primer (5'GGAGGTGATCCAGCCGCAGGT 3'); Forward inner primer (5' TCAAGTCCTCATGGCCCTTATG 3'); Reverse inner primer (5' TACACACGTGCTACAATGGCG 3'). First PCR was carried out in a final volume of 50 μl comprising 3 μl of DNA solution (ranging from 0.5 to 1.5 μg μl^{-1}), 5μl 10x

amplification buffer (ARK Scientific, Darmstadt, Germany), 15 pmol of each outer primer, 200 μM of each of the four dNTPs (Roche Diagnostics GmBh, Manheim, Germany), 3 mM $MgCl_2$, 0.4 μl of BSA (20mg ml^{-1}), 5 μl of DMSO and 2.5U of *Taq* Polymerase (Sigma-ARK). The reactions were carried out in Gene Amp PCR System 2700 (Applied Biosystems,). Samples were incubated for 5 min at 94ºC and then cycled 10 times at 94ºC for 1min, 58ºC for 1min and 72ºC for 2 min. The samples were incubated for 10 min at 72ºC for final extension and kept at 4ºC. Five ml of the amplified products were used for a second amplification of 40 cycle, using 15pmol of each of the inner primers, 5μl 10x amplification buffer (ARK Scientific), 200 μM of each of the four dNTPs (Roche Diagnostics GmBh, Manheim, Germany), 3 mM $MgCl_2$, 0.4 μl of BSA (20mg ml^{-1}), 5 μl of DMSO and 2.5U of *Taq* Polymerase (Sigma-ARK). Amplification products were solved on a 3% agarose gel, stained with ethidium bromide, and visualized by ultravioled light.

Results:

Primers specificity:

To evaluate specificity and effectiveness of the pair of primers AQ1F and AQ2R, conventional PCR was performed using bacterial DNA from the reference strains of AAB as well as DNA from yeasts (both *non-Saccharomyces* and *Saccharomyces*) and lactic acid bacteria, which are the most common microorganisms associated with wine or vinegar. A DNA fragment of the expected size (54bp) was found in all the AAB tested, while no amplification was produced from any of the outgroups tested.

Detection limit and standard curves:

DNA from known amounts of AAB of the different reference strains was used in 10-fold serial dilutions to generate the standard curves from 10^8 to 10^1 cel ml^{-1}. The DNA extraction was performed to all the diluted samples. C_T values decreased at least 3-fold on each dilution between values of 10^7 to 10^3 cel ml^{-1}. All standard curves ranged similar slope values (between -2.9 and -3.2). with regression coefficients values (r^2) always above 0.96, showing in all cases a good linearity within values of 10^7 to 10^3 cel ml^{-1}(fig 1), *Ga. xylinus* showed good linearity up to 10^8 cel ml^{-1}. No template samples were used in all cases as negative controls giving results below linearity C_T values. The values of C_T were similar to the ones obtained with the samples of less than 10^3 cel ml^{-1}.

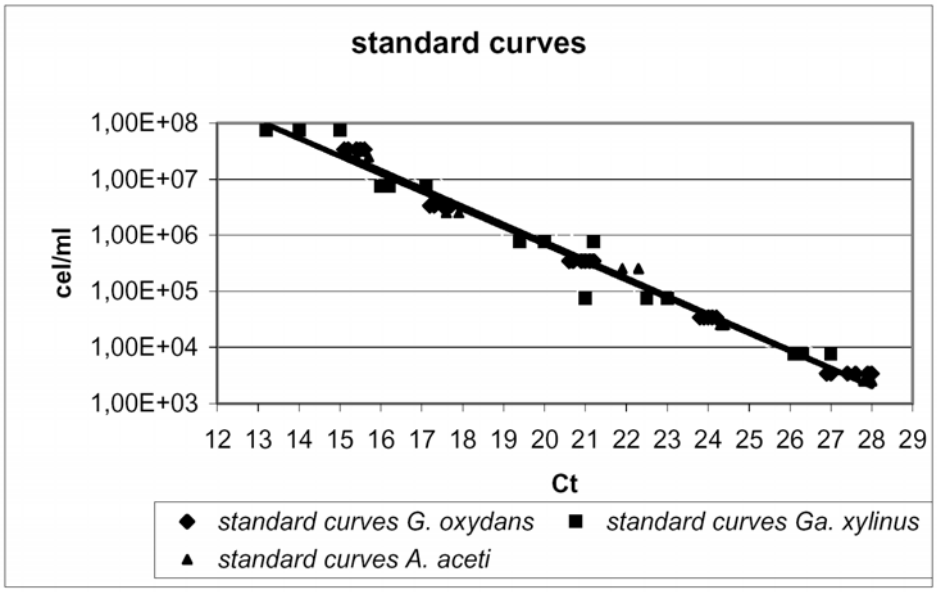

Fig. 1: Standard curves for *G. oxydans* (♦) ($y = 1E+12e^{-0.72x}$; R^2 =0.965), *Ga. xylinus* (■)($y = 2E+12e^{-0.72x}$; R^2=0.989) and *A. aceti* (•) ($y = 2E+12e^{-73x}$; R^2=0.989).

In order to decrease the minimum population we are able to detect with rt-PCR (10^3 cells ml^{-1}) we used higher volumes of the samples. Instead of centrifuging 1ml samples we used 100ml samples, resuspending them in 1ml of sterilized water, obtaining, in this

case, 100-folds concentration of the samples. We were able, then, to detect populations of 10 cells ml^{-1}, lowering 100-folds our detection limit, set now then in 10^1 cel ml^{-1}.

To determine the usefulness of the technique to detect AAB in complex samples and to evaluate the standard curves obtained, known amounts of different AAB strains were mixed in mannitol medium, and DNA extraction was performed. Those complex samples were amplified by triplicate together with different standard curves. Comparing the C_T values of the samples to any of the standard curves, very similar values were obtained, and, in all cases, matching the values obtained by plating and microscope counting, indicating that any of the standard curves could be used to quantify AAB in a complex sample (Table 1).

	Plating	rt-PCR				
		G. oxydans	*A. aceti*	*Ga. xylinum*	*A. pasteurianus*	*Ga. hansenii*
Mannitol mixture	9,40E+06	9,30E+06	8,10E+06	8,60E+06	9,60E+06	7,90E+06

Table 1 : Complex sample of AAB enumerated by plating and by rt-PCR. The enumeration by rt-PCR is done with standard curves from different reference strains. Results given in cel ml^{-1}.

Enumeration of AAB:

Different samples were used in order to compare traditional enumeration techniques such as colony and microscope counting with rt-PCR. Results obtained are shown in table 2. Samples were obtained by inoculation of sterilized wine with different AAB strains and different mixtures of them and samples of commercial wines and vinegars

All the samples for rt-PCR were amplified by triplicate, and the fluorescent signals detected were averaged compared to a standard curve generated with *G. oxydans* obtained at the same experiment. Vinegar samples were contaminated by yeasts

($8,4x10^5$ cel ml^{-1}), but their presence did not affect rt-PCR detection of AAB. The total numbers of bacterial cells were very similar using either microscope, platting or rt-PCR in all the samples, not detecting high differences , ranging differences of less than 10 folds.

Table 2: Comparison of enumaration by microscope counting, plating and rt-PCR. Results are given in cel ml^{-1}.

Sample	microscope *	plating *	rt-PCR *
inoculated wine:			
A. aceti	3,20E+06	3,10E+06	3,90E+06
G.oxydans	6,00E+06	6,00E+06	4,30E+06
Ga. hansenii	1,30E+07	1,10E+07	1,20E+07
A. pasteurianus	9,80E+06	9,80E+06	1,10E+07
Ga. xylinus	3,20E+06	3,00E+06	2,30E+06
Mixture of *A. acei*+ *G. oxydans*+*Ga. hansenii*	2,70E+07	2,50E+07	1,30E+07
Mixture of all 5 species	3,30E+07	3,30E+07	2,00E+07
vinegar1	3,80E+05	3,60E+05	2,00E+05
vinegar2	3,60E+05	3,50E+05	1,00E+05
wine 1	7,50E+03	7,20E+03	6,30E+03
wine 2	3,80E+03	3,10E+03	4,20E+03

Nested-PCR:

Both sets of primers, inner and outer primers, have already been proven to be specific for AAB. The outer primers have been used for the RFLP of the 16S rDNA (Ruiz *et al*., 2000, González *et al*., 2004) and the inner primers in this study.

Ten-fold serial dilutions of the reference strains were performed and amplified in order to test the technique. In the nested-PCR assays it was possible to detect $3,4x10^1$ bacterial cells ml^{-1}, reaching lower values than rt-PCR technique (fig. 2), proving the usefulness of the nested-PCR to detect low populations of AAB in samples. We reduced the number of cycles of the first amplification from 35 to 10 in order to avoid saturation of the amplification, although we are not able to quantify the AAB population because all the amplification products yielded the same intensity.

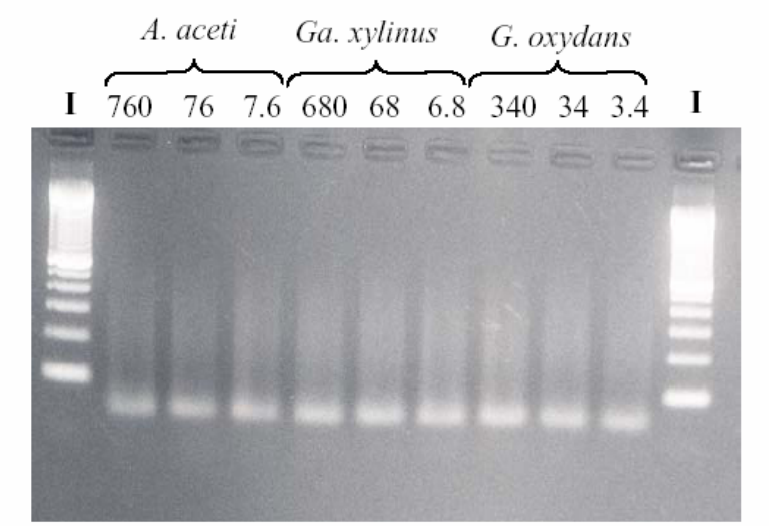

Fig. 2: Nested-PCR of different AAB species. Lane **I** is molecular size marker:mixture of λ phage DNA digested with *Hind*III-*Eco*RI and *Hind*III. Lane assessments correspond to the cells ml^{-1} of the samples.

Discussion:

Rapid and sensitive detection and enumeration of microorganisms has always been a challenge for the food industry (Luo et al., 2004). In the world of enology, AAB have received less attention than the other microorganisms involved in wine processes, but, the presence of this bacterial group during alcoholic fermentation and during storage of the wine is a quite common problem in wineries. In the present study rt-PCR and nested-PCR, techniques already used with other bacterial groups (Lyons et al., 2000; Luo et al., 2004; Rousselon et al., 2004), were used to quantify and to detect respectively AAB. In this study, the set up of these techniques was done in order to get fast analysis. There is no need of culturing the samples, which might take, at least, 48h, and we do not perform DNA extraction, obtaining the same results with conventional DNA extraction and by breaking the cells by temperature shock (data not shown), which is much simple and faster.

rt-PCR:

We obtained specific set of primers for real-time amplification, with no positive results when testing them against the common microorganisms found in wine such as lactic acid bacteria and yeasts. Standard curves were generated with DNA from several AAB strains, obtaining, in all of the cases, similar results, and any one strain could be used for generating a standard curve for total bacteria. Because there are small shifts in the fluorescent signal from experiment to experiment, it is essential to generate a new standard curve for each set of measurements (Lyons et al., 2000). Linearity was obtained with population within 10^7 to 10^3 cells ml^{-1}, this quite high detection limit set at 10^3 cells ml^{-1} could be due to the presence of some bacterial genomic DNA not removed from the *Taq* polymerase preparation (Corless et al., 2000; Lyons et al., 2000), but this disadvantage can be solved by increasing the sample size and concentrating the sample by centrifugation. This improvement for lowering the detection limit allowed us to detect populations of 10 cells ml^{-1}. Increasing the volume sample is a preferred practice as far as the industrial processes volumes where AAB are involved are usually big enough (100-25000 l). The maximum amount of cells we were able to detect was 10^7 cel ml^{-1}, because with the populations tested over this level (up to 10^8 cel ml^{-1}), no linearity was obtained.

Enumeration of AAB:

All the samples analysed to compare the traditionally used techniques to enumerate AAB such as colony and microscope counting yielded the same results as being analysed by rt-PCR. No significant differences were found neither in sterilized and commercial wines nor in vinegar indicating that rt-PCR is a useful technique to enumerate AAB from commercial samples, even contaminated samples with other

microorganisms such as yeasts. In our case we were able to enumerate a contaminated sample with no inhibition by the presence of a high amount of yeasts.

Traditionally, enumeration of AAB from industrial processes has been done by plating on selective media, with the addition of antibiotics to avoid the growth of non-desirable microorganisms (yeasts, moulds and lactic acid bacteria), but, new evidences suggest the existence of VBNC state of this bacterial group in wine, indicating that under certain conditions this cells might not grow (Millet & Lonvaud-Funel, 2000). This fact was also observed by Du Toit et al. (2005) when studying the survival of *A. pasteurianus* in wine under anaerobic conditions, the cells counts by plating were lower than epifluorescence counts. In that sense, rt-PCR is a rapid and reliable method for enumeration, and avoid the problem of VBNC since there is no need of culturing the samples.

It should be interesting to be able to design specific primers to be able to differentiate among the species present in a sample, as well as to be able to analyse RNA instead of DNA to discriminate viable and dead bacteria.

Nested-PCR:

Nested-PCR is an effective method for detecting organisms in environmental samples where the presence of low concentrations of the DNA targets and high concentrations of contaminants should inhibit DNA amplification (Ciapina et al., 2003). In finished products such as wine or bottled vinegar, or during storage and ageing of the wines there is usually a low concentration of AAB and due to the presence of VBNC that can not be detected by colony counting, nested-PCR evolves into a sensitive and fast

technique to detect the presence of these microorganisms in the different samples without the need of high volume samples.

Several studies reveal the lower detection limits using nested-PCR than rt-PCR (Kawada et al., 2004; Gomez et al., 2004) as happens in our study, decreasing 1000-folds this limit detection, but with the disadvantage of not being able to enumerate the amount of cells in the sample, as it is only a qualitative technique, but, on the other hand can be very useful to detect in fast and simply the presence of AAB in a routinely laboratory analysis considering also that nested-PCR is a cheaper technique (there is no need of use of fluorescent dyes).

Further studies should be done in order to be able, not only to enumerate the total number of bacteria present in a sample, but also to be able to distinguish the different species present in the sample.

Acknowledgements:

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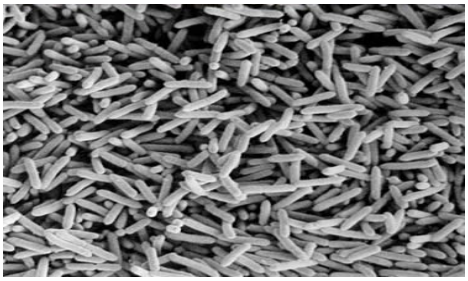

General Discussion.

UNIVERSITAT ROVIRA I VIRGILI

TARRAGONA, 2005

General discussion

In general terms little is known about the behaviour of AAB in the environments in which they develop, particularly in wine and vinegar processes. In oenology, AAB have received less attention than yeasts or lactic acid bacteria, the other microorganisms involved in wine-making. However, the increase in acetic acid and other spoiling metabolites as a consequence of the growth of AAB is a common problem for wineries (Du Toit and Lamberchts, 2002). Industrially it is of vital importance to know the number of bacterial cells, their enumeration, and the species involved in the various processes It is also important to know the strains that carry out the processes, and whether they are beneficial (as in vinegar production) or detrimental (as in wine spoilage).

One of our main goals here is to increase our knowledge of how they develop and how they behave in the different processes in which they are involved, and how they are affected by different conditions. Although some studies have already been made about these aspects (Du Toit & Lamberchts, 2002; Joyeux *et al*., 1984), some of the techniques used for enumerating or identifying AAB are not reliable, fast or sensitive enough. They have traditionally been identified by studying the physiological and chemotaxonomic properties of the AAB (Buchanan & Gibbons, 1974). For this purpose, we applied molecular techniques to identify AAB at species level (PCR-RFLP of the 16S rDNA and ITS region). These studies were initially made by Ruiz et al. (2000) and we tested their usefulness during alcoholic fermentation processes. The appearance of new AAB species meant that this technique had to be applied to these bacteria together with RFLP of the 16S-23S ITS rDNA, which has already been used by Trcek and Teuber (2002) and Ruiz *et al*. (2000) with some strains of AAB.

PCR-RFLP of the 16S rDNA and 16S-23S ITS rDNA, with different restriction endonuclease combinations, made it possible to identify the AAB species in a short period of time, and proved to be appropriate routine techniques for the laboratory and population studies in which large amounts of sample must be analysed.

Most AAB species could be differentiated using only PCR-RFLP of the 16S rDNA with the combination of a few restriction enzymes, but PCR-RFLP of the 16S-23S ITS rDNA had to be used to distinguish among the group of AAB *A. malorum*, *A. cerevisiae* and *A. orleaniensis*, and the pair *Ga. xylinus* and *Ga. europaeus*. The similarity of these species at molecular levels such as % GC content or 16S rDNA sequences justifies the need to use the second technique.

We were also unable to discriminate between the pair *A. pasteurianus* and *A. pomorum* with PCR-RFLP of the 16S rDNA or 16S-23S ITS rDNA. Sokollek et al. (1998) proposed to separate these two species using DNA-DNA hybridisation, 16S rDNA sequencing and phenotypic characterization. Cleenwerck et al. (2002) disagreed with almost all the results obtained previously and also with the methods used by Sokollek et al. (1998). Our results, together with those of Cleenwerck et al. (2002), suggest that the separation of those two species should be revised.

With the previous work made by our group (Ruiz *et al*., 2000) and the work presented in chapter 1 of this thesis, we are able to diferentiate the AAB currently described. The techniques have proved to be less time-consuming than other techniques for distinguishing among species, such as DNA-DNA hybridisation or 16S rDNA sequencing, which are neither appropriate for routine laboratory investigation nor for large amounts of samples. The techniques are also straightforward and cheap in comparison to the techniques mentioned above.

Our second objective was to be able to identify AAB at strain level. Genotypic differences between strains of the same species give them different phenotypic characteristics. The strains of one species do not all have the same ability to survive under the same conditions, in terms of ethanol or SO_2 tolerance and neither do all the strains have the same characteristics, or are adapted to the same conditions. Therefore, there is a strain-dependency in terms of sensitivity (Du Toit & Lamberchts, 2002). For this reason it is important not only to know which species are involved in the various processes, but also the strains. This could be applied in vinegar production finding the main strains carrying the process, or in wine, trying to avoid the presence of the most resistant strains to the conditions set during wine-making.

So we chose rapid and reliable molecular techniques such as ERIC- and REP-PCR. ERIC and REP elements are consensus sequences derived from highly conserved palindromic inverted repeat regions found in enteric bacteria (Pooler *et al*., 1996). These techniques have already been used to generate specific fingerprints of several bacterial groups (Pooler *et al*., 1996; Beyer *et al*., 1998; Guinebretier *et al*., 2001), but they have not always yielded strain-specific banding patterns (Wieser & Busse, 2000).

Nanda et al. (2001) have already used ERIC-PCR to identify AAB from vinegar samples by. We tested the technique with reference AAB strains, and obtained exclusive patterns for every strain. REP-PCR also yielded strain specific patterns, except for the group *Ga. liquefaciens, Ga. xylinus* and *Ga. europaeus*.

The usefulness of both techniques was tested by using them to identify and type wine isolates and they both proved to be suitable for our purposes. We were able to differentiate AAB at both species and strain levels with reliable and fast techniques which enabled us to carry out ecological studies about the origin of AAB as well as about their behaviour in different processes under the influence of common industrial practices, as recommended by Drysdale & Fleet (1988).

For the first ecological study we analysed the diversity of AAB at both species and strain level throughout a spontaneous red wine alcoholic fermentation during the 2001 vintage. We analysed 120 AAB isolates with ERIC-, REP-PCR and PCR-RFLP of the 16S rDNA and the population kinetics were determined by selective plating.

Low populations were expected in must samples, because the grapes where exceptionally healthy, and we found a population of $3x10^3$ cfu ml^{-1}. In spoiled, damaged grapes, populations are much higher, and numbers can reach around 10^6 cfu ml^{-1} (Drysdale & Fleet, 1988). The conditions set during alcoholic fermentation seem to be unsuitable for AAB development, although some studies reveal that these bacteria grow considerably during fermentation (Du Toit & Lamberchts, 2002,; Du Toit et al., 2005). In our case a slight increase in AAB was detected in the first stages of the process, but when yeast activity was maximum and conditions were anaerobic, the population decreased drastically.

Joyeux *et al*. (1984) mentioned the predominance of *G. oxydans* in must and the first stages of the fermentation. In our case G. oxydans represented 85% of the total colonies analysed. A minor presence of *A. aceti* was observed during the first days (5% in must), but increased during the process to reach values of 100% by the end of the fermentation.

The diversity of genotypes (strains) was high at the beginning of the fermentation but decreased drastically as the process continued, and by the end there were only three different strains. This must be due to the anaerobic conditions and the increase in ethanol concentration, which only allows the most resistant or adapted strains to survive.

The second ecological study was carried out in the 2002 vintage. The grape must was fermented under four different conditions: yeast inoculation and SO_2, inoculation and no SO_2, no inoculation and SO_2 and no inoculation and no SO_2. Two of these practices (yeast inoculation and SO_2 addition) are widespread in the oenological industry. The AAB population was also previously analysed during grape ripening and throughout the different fermentations with the same techniques as in the first study. The first thing to be said is that the climatic conditions during this vintage caused some damage and spoilage to the grapes. The AAB populations in the grapes were 2.2 10^5 cfu ml^{-1}, while in the previous year the population was 3 10^3 cfu ml^{-1}. Some studies reported the predominance of *A. aceti* and *A. pasteurianus* in spoiled grapes (Joyeux *et al*., 1984), but, in our case, we detected the presence of *G. oxydans* and *A. aceti* as the predominant species. Du Toit and Lamberchts (2002) and Barbe *et al*. (2001) also isolated *G. oxydans* in spoiled grapes and in *Botrytis*-infected grapes respectively.

The population in the must is ten times higher than in grape samples from the same day. This increase can be accounted for by *A. aceti*, which suggests that there is a considerable presence in the cellar of resident AAB, which should be more adapted to the wine-making conditions. It should be taken into account that the winery was in the middle of harvesting and, although general hygienic practices were followed, there is always some contaminating grape must in the equipment. Populations during the fermentations are drastically reduced, and only in the spontaneous fermentation without SO_2 can we find populations over 10^3 ufc ml^{-1} at the end of the process.

A. aceti was the dominant species in every stage of the four fermentations. Du Toit and Lamberchts (2002) suggested that *A. pasteurianus* is more resistant to SO_2 than *A. aceti*, but, in our case, small percentages of *A. pasteurianus* are only found in the final stages of the fermentation in which no SO_2 was added. Unlike the previous year *G. oxydans*, *Ga. liquefaciens* and *Ga. hansenii* did not represent a high percentage of the species found in any of the fermentations.

Only a few strains of the grapes can be found in must or during the fermentations, most of which belong to *A. aceti*. In fact, only one strain, previously isolated from grapes, was able to survive. This suggests that some AAB strains can adapt to changing conditions. The predominant strains at the end of the 2001 vintage did not appear in either must or in the first stages of the fermentation. Instead, in the 2002 vintage, one of the most predominant strains in three out of four of the fermentations was already present in must, but not in grapes, which suggests that this strain is a cellar resident that has adapted well to the harsh conditions of wine-making. As in the 2001 vintage, there is a clear reduction in the strains found at the end of the process, indicating that the conditions in the medium are very extreme and not suitable for most AAB strains.

There is still a need for further investigation into the physiological and genetic characteristics of the strains so that the resistance of some strains to wine-making conditions can be explained. However, it seems that *A. aceti* is the strain that is best adapted to the harsh conditions of fermentation and that it can survive and grow under these conditions.

Our second objective was to detect and quantify AAB without having to culture because of the limitations of cultural methods and their inability to detect viable but non-cultivable (VBNC) cells. There is evidence to suggest that the VBNC state exists in this bacterial group in wine (Millet & Lonvaud-Funel, 2000). This seems to be confirmed by Du Toit *et al*. (2005) who observed that *A. pasteurianus* survived under anaerobic conditions. On the other hand, rapid and sensitive detection and enumeration of microorgansism has always been a challenge for the food industry (Luo *et al*., 2004).

We selected rt-PCR for the enumeration and nested-PCR for the detection of AAB, both of which have already been used by other bacterial groups (Lyons *et al*., 2000; Luo *et*

al., 2004). We tested the usefulness of rt-PCR in comparison to colony and microscope counting, and the results were similar in all cases. This showed that the technique can be useful in this bacterial group. The detection limit of the technique was set at 10^3 cel ml^{-1}, which is quite high considering that healthy grapes normally have AAB populations around the same level. In order to reduce the detection limit, we used higher amounts of sample (100 ml) and we reconcentrated the sample 100-fold by centrifugation, in those cases in which we were able to detect populations of 10^1 cel ml^{-1}. The volumes usually used in oenology and vinegar production make it possible to use higher amounts of samples without damaging the processes.

In finished products such as wine and vinegar, or while the products are in storage, the levels of AAB are usually very low, and the only important thing is to have a fast, sensitive method for detecting their presence or absence. Nested-PCR is an adequate technique for the fast and reliable detection of these bacterial group. We achieved a detection limit of 3.4 cel ml^{-1}. Several studies have revealed that nested-PCR had lower detection limits than rt-PCR (Kawada *et al*., 2004; Gomez *et al*., 2004). One of its disadvantages, however, is that it cannot enumerate, although it is more appropriate for routine laboratory assays and large samples since there is no need for special equipment, unlike rt-PCR, which requires a special thermocycler.

The next step should be to develop specific primers for differentiating the species in the various processes that use rt-PCR. This would allow species to be enumerated and identified in only one step. Another step forward would be to use an RNA template instead of a DNA template, which would enable the viable cells to be enumerated, not the total cells.

To sum up, we have developed sensitive, fast and reliable techniques for identifying AAB at both species and strain level, and for quantifying and detecting their presence without the need for culturing. These were two of the main aims of our study of this bacterial group

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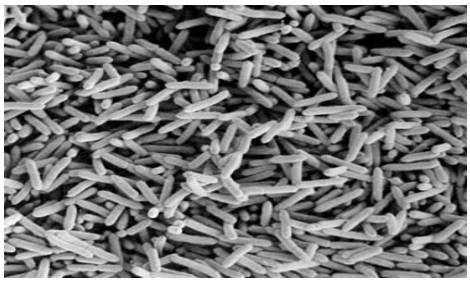

General conclusions.

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General conclusions

- Molecular techniques such as PCR-RFLP of the rDNA 16S and 16S-23S ITS have shown to be fast, sensitive and reliable techniques for the differentiation of AAB in routine analysis and have allowed us the differentiation at the species level of the AAB with the exception of the pair *A. pasteurianus* and *A. pomorum*.

- Both techniques have demonstrated to be less time-consuming than the techniques used to distinguish among species such as DNA-DNA hybridisation or 16S rDNA sequencing.

- We have successfully applied ERIC- and REP-PCR for the identification and typing of AAB at strain level. We obtained exclusive patterns for all the reference strains tested with ERIC-PCR. REP-PCR also yielded specific patterns except for the group *Ga. liquefaciens*, *Ga. xylinus* and *Ga. europaeus*.

- The usefulness of both techniques was tested by using them to identify and type wine isolates and they both proved to be suitable for our purposes.

- In the first survey study, done in the 2001 vintage, the population of acetic acid bacteria in must was relatively small because the grapes were exceptionally healthy.

- A total amount of 33 different strains were found out of 120 colonies analysed.In each stage of the fermentation one predominant strain was found. The strain diversity decreases along the process. In the first stages the predominant specie was *G. oxydans*, while in middle stages appeared *Ga. hansenii*. In the last stages of the alcoholic fermentation only 3 strains were isolated, all of them belonging to *A. aceti*.

- In the second survey study, done in the 2002 vintage, higher populations of AAB were found in grape, caused by the damage and the spoilage of the grapes

due to the bad climatic conditions. The major species found were *G. oxydans* and *A. aceti*.

- Populations increased 10 times from grape to must, suggesting the importance of the cellar resident AAB as they can be more adapted to the wine-making conditions.

- *A. aceti* was the dominant species in all the fermentations and only a few strains coming from must or grapes were able to survive until the end of one fermentation, indicating the ability of AAB to adapt to the changing conditions.

- We are able, using rt-PCR and nested-PCR, to enumerate and detect respectively AAB without the need of culturing, which makes possible to detect the presence of VBNC cells.