

Acetic acid bacteria in oenology

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Resum

Els bacteris de l'àcid acètic han estat sempre considerats perjudicials en els àmbits enològics, com a principal font de problemes en el vi (fonamentalment per ser una font d'acidesa volàtil). El desenvolupament de noves tècniques de biologia molecular ha permès que la taxonomia i el coneixement del metabolisme d'aquests bacteris que, per les seves exigències nutricionals, són molt difícils de cultivar evolucionin ràpidament. En l'àmbit taxonòmic, s'ha produït el canvi de dos gèneres i cinc espècies el 1984 a deu gèneres i més de quaranta espècies al moment actual. De totes maneres, les poderoses eines moleculars utilitzades en taxonomia no són apropiades per a l'ús rutinari en estudis ecològics, on s'ha d'analitzar un gran nombre de mostres. Per tant, s'han desenvolupat noves tècniques moleculars que han permès la millora del seu coneixement i control a Enologia. Així mateix, han permès un avenç considerable en la producció de vinagre, procés en el qual aquests bacteris són imprescindibles, com s'ha posat de manifest en el projecte europeu WINEGAR.

Paraules clau: vi, vinagre, acetobacter, gluconobacter, gluconacetobacter

Abstract

Acetic acid bacteria have always been considered the bad microorganisms of oenology; responsible for wine spoiling (vinegary taint). The taxonomy and our knowledge of the metabolism of acetic acid bacteria are rapidly evolving, especially as new molecular biology techniques are applied to this fastidious group of microorganisms, which are still rather difficult to work with. The dramatic change that acetic acid bacteria taxonomy has undergone can be summarized by the transformation of 2 genera and 5 species in 1984 into 10 genera and over 40 species at present. The powerful molecular tools used in taxonomy are not appropriate for frequent use in identification and ecological studies; yet new molecular tools for routine analysis have also been developed. These provide new insights and means of controlling this microbial group. Furthermore, these advances have improved vinegar production; a process where the presence of acetic acid bacteria is essential. The WINEGAR European Project is evidence of these improvements in vinegar production.

Keywords: wine, vinegar, acetobacter, gluconobacter, gluconacetobacter

From grapes to wine: A changing environment

Yeasts, bacteria and filamentous fungi all contribute to the microbial ecology of wine production and the chemical composition of wine, although yeasts play the dominant role because they drive alcoholic fermentation [18]. Many factors affect the microbial ecology of wine production, of which the chemical composition of the grape juice and the fermentation processes are the most significant. In complex microbial ecosystems containing mixtures of different species and strains, interactions between microorganisms may occur and these may affect the final ecology [12, 18, 41]

Grape juice presents extreme conditions for the growth of microorganisms; a low pH (between 3 and 4) and a high sugar concentration. During alcoholic fermentation the sugar content is transformed into ethanol by yeasts, which supposes an additional restriction on the development of microorganisms. Such environmental changes are responsible for differences in the microbial ecology throughout the process; tolerance to high concentrations of ethanol and low pH are the main factors that select species occurrence in wine ecosystems [18]. Furthermore, the coexistence of different microorganisms in the medium generates competition for nutrients. The early growth of yeasts in grape juice decreases the nutrient content, thus making the resulting wine less favourable as an environment for any further microbial growth. In addition, such growth releases different metabolites into the medium, some of which could be toxic to other species. Another factor that affects the development of some microorganisms is carbon dioxide production which strips the medium of oxygen, thereby limiting the growth

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of aerobic species such as acetic acid bacteria (AAB). Therefore, if a vigorous onset of alcoholic fermentation by yeasts (or *Saccharomyces cerevisiae*) occurs, non-*Saccharomyces* yeasts and bacteria have little opportunity to grow [23]. However, if yeast growth is delayed, various species of lactic acid bacteria and acetic acid bacteria may flourish, inhibit the growth of yeast, and cause sluggish or stuck fermentations [19, 41].

Interactions among the different wine microorganisms may or may not favour a particular microbial group. For example, when the large amount of yeast biomass produced during fermentation dies, the autolysis releases amino acids and vitamins into the medium, which may encourage the growth of AAB and lactic acid bacteria species later in the process [19]. Another concept that must be considered is quorum sensing as a mechanism by which microbial cells communicate with each other and regulate population growth. The development of microorganisms during the winemaking process therefore depends on different parameters such as these microbial interactions, but also the composition of the medium and oenological practices.

One parameter that may affect the development of microorganisms during alcoholic fermentation is temperature. Recently there has been a preference for fermenting white wines at controlled temperatures (between 13 and 18 °C) in order to enhance the production and retention of flavour volatiles [32]. In red wine fermentation, temperature tends to be less controlled and allowed to reach temperatures of 25-30 °C. Fermentation

temperature affects the rate of yeast growth and, consequently, the duration of fermentation [52]. Therefore, a delay in yeast growth, such as occurs during low temperature fermentation, may encourage the development of indigenous yeasts and cause a sluggish fermentation [32]. However, the wine bacterial population is more sensitive to lower temperatures.

Finally, the addition of sulphur dioxide to grape juice and wine is a common winemaking practice used to control oxidation and prevent the growth of indigenous microbiota, such as indigenous non-*Saccharomyces* yeasts or AAB and lactic acid bacteria. The antimicrobial effectiveness of SO₂ is highly dependent on the pH and on the presence of reactive molecules that can bind to SO₂. At lower must and wine pH, there will be more SO₂ in free molecular form; which is the form that acts against microorganisms [41].

Acetic acid bacteria metabolism

AAB occur in sugar and alcoholized, slightly acid niches, such as flowers, fruits, beer, wine, cider, vinegar, souring fruit juices and honey. On these substrates they oxidize the sugars and alcohols, thus causing an accumulation of organic acids as final products (although more complex intracellular metabolism may occur, see Figure 1). When the substrate is ethanol, acetic acid is produced (Figure 2). However, AAB also oxidize glucose to gluconic acid, galactose to galactonic acid, and arabinose to arabinonic acid. Some of these transformations are of consider-

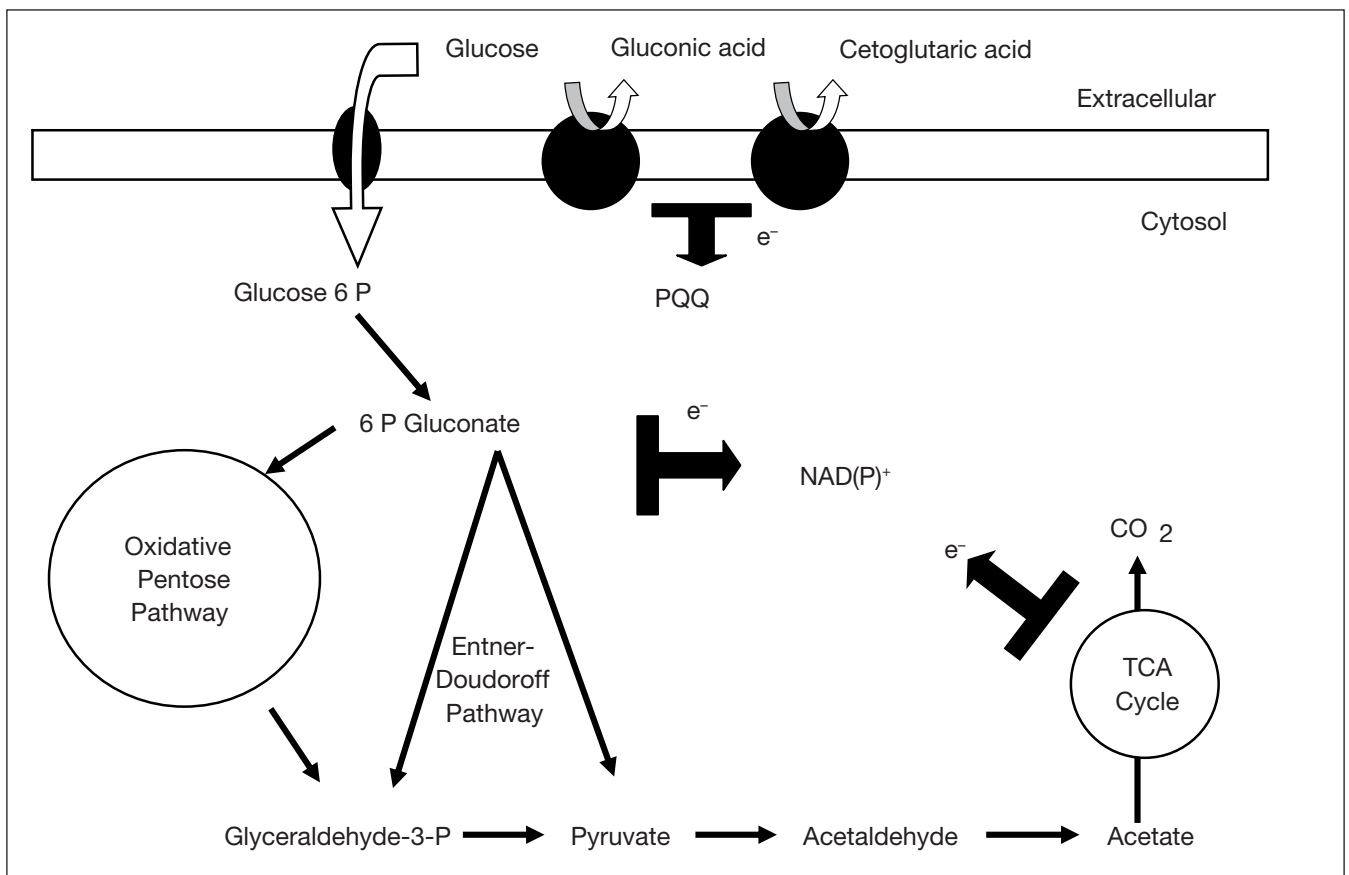


Figure 1. Carbohydrate metabolism in Acid acetic bacteria.

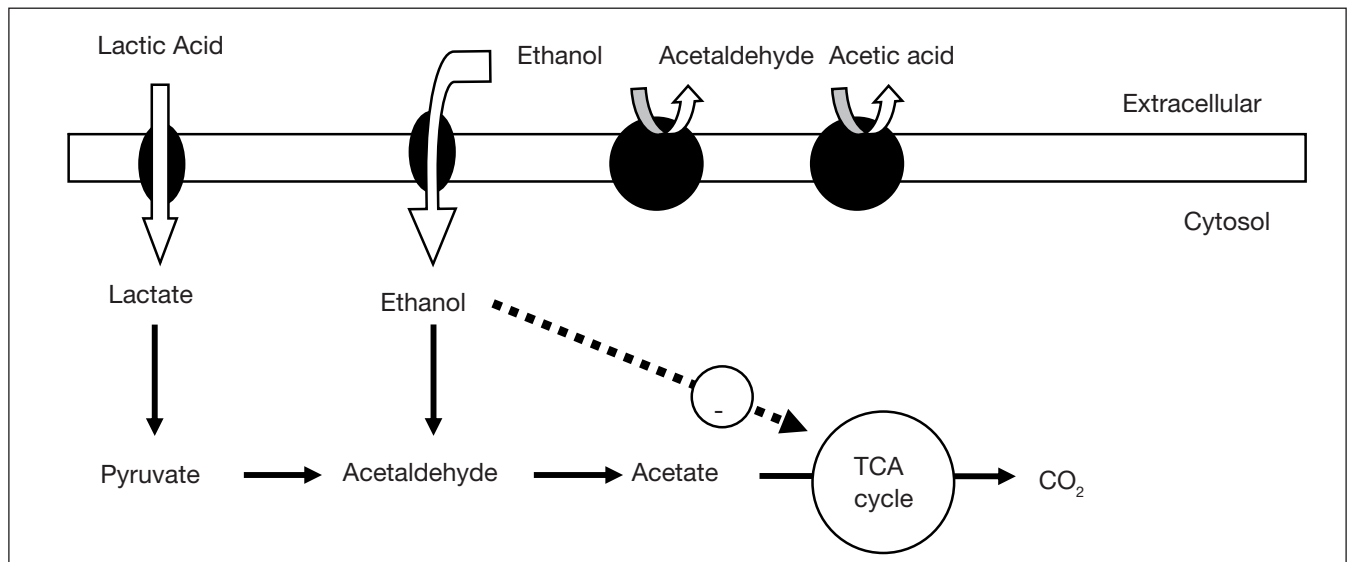


Figure 2. Ethanol metabolism in AAB.

able interest to the biotechnology industry. The best-known industrial application of AAB is vinegar production but they are also used to produce sorbose, from sorbitol, and cellulose. Despite this, interest in AAB and their possible applications has been limited because of the difficulties involved in growing them in solid media, and of their high mutability. However, the recent mapping of the entire *Gluconobacter oxydans* genome has opened up new possibilities concerning this bacterial group [39]

One of the most important characteristics of AAB is their ability to oxidize a wide variety of substrates and to accumulate metabolic products in different media while avoiding toxicity for themselves. However, this oxidation is often incomplete and the corresponding products (aldehydes, ketones and organic acids) are almost entirely excreted into the medium, largely because of dehydrogenase activity in cell membranes. Dehydrogenases are closely related to the cytochrome chain, which accumulates oxidized products in the medium and produces a proton-motive force, which is transformed into energy inside the cell [34]. Since the reactive centres of the oxidizing enzymes are oriented towards the periplasmic space, substrates and products need not to be transported into the cell. This facilitates the rapid accumulation of incompletely oxidized products in the medium. The capacity of AAB to accumulate substrates in the media makes them useful microorganisms for the biotechnology industry [7]. Furthermore, they are able to grow in highly concentrated sugar or ethanol solutions at low pH values.

The development of AAB is strongly dependent upon two basic components of the medium: molecular oxygen, and an adequate carbon source to sustain growth. The carbon source is typically ethanol, which is converted into acetic acid.

Although oxygen is not normally considered a nutrient, it is evident that the need for oxygen strongly limits the development and metabolism of AAB. Thus, AAB are strictly aerobic organisms that can survive in quasi-anaerobic conditions, but whose growth is normally restricted [13]. This is well known by wine-makers; exposure of wine surfaces to air, or the typical oenological practices of pumping, transfer and aging provide

enough oxygen to encourage AAB growth and spoil wines [30]. AAB metabolism does not stop under oxygen-deficient conditions, because AAB are able to use alternative electron acceptors such as quinones [11].

Oxygen is also one of the main factors in the industrial handling of AAB, and an oxygen diffusion system or generator must always be used to allow bacterial growth and achieve appropriate performance. Proper aeration of the growth media is a critical factor in the industrial production of vinegar and other industrial uses of AAB.

Isolation and identification

The physiological differences among microorganisms made it possible to develop differential culture media for isolating AAB with different carbon source, such as glucose, mannitol or ethanol. Some of these media can also incorporate CaCO₃ or bromocresol-green as acid indicators [6, 50]. Culture media are usually supplemented with pimaricin or similar antibiotics in the agar plates to prevent yeasts and moulds from growing, and with penicillin to eliminate Gram-positive acidophilic bacteria such as lactic acid bacteria.

Some of the most widely used culture media are GYC (5% D-glucose, 1% yeast extract, 0.5% CaCO₃ and 2% agar (w/v)) and YPM (2.5% mannitol, 0.5% yeast extract, 0.3% peptone and 2% agar (w/v)). These culture media are suitable for wine samples [3, 14] and plates need to be incubated for between 2 and 4 days at 28°C under aerobic conditions..

Nevertheless, some studies show that it is difficult to culture this bacterial group from some industrial samples, especially those originally from extreme media, such as vinegar [47]. This problem has been partially solved by introducing a double layer of agar (0.5% agar in the lower layer and 1% agar in the upper layer (w/v)) containing ethanol and acetic acid (such as AE medium) into the cultures and media in an attempt to simulate the atmosphere of the acetification tanks [17]. However, cultur-

ing AAB still hinders proper studies of this group of microorganisms that are critical for ecological studies. Culture-independent molecular techniques are therefore being developed to solve this problem.

The identification of AAB has gone hand-in-hand with changes in the taxonomy and classification of AAB. *Mycoderma* was first described by Persoon in 1822 and observed by Pasteur, Hansen and Beijerinck in the 19th century. The general consensus throughout the second half of the 20th century was that there were two AAB genera: *Gluconobacter* and *Acetobacter*. These two genera were described in the *Bergey's Manual of Systematic Bacteriology*, which for many years has collected together the key points regarding the taxonomy of bacteria. The 1984 edition [6] included such molecular techniques as fatty acid composition, soluble protein electrophoresis, percentage content of G + C, and DNA-DNA hybridisation.

The taxonomy of AAB microorganisms, initially based on morphological and physiological criteria, has varied and been reoriented continually, largely because of the application of molecular techniques. The most common techniques 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.
- 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. The *Bergey's Manual of Systematic Bacteriology* [6] includes these values to differentiate between *Acetobacteraceae* species.
- 16S rDNA sequence analysis. The 16S rDNA gene is a highly preserved region in which small changes characterize different species. Ribosomal genes are compared in most taxonomic studies of bacteria.

The *Acetobacteraceae* family is no exception to this reorganization of species and genera. AAB are considered to be a lineage within the *Acetobacteraceae* family, which is characterized by the ability to produce acetic acid, although some of them are very weak producers. Eight new AAB genera have been added to the two genera mentioned above: *Acidomonas*, *Gluconacetobacter*, *Asaia*, *Kozakia*, *Saccharibacter*, *Swaminathania*, *Neoasaia* and *Granulibacter* (Table 1 contains a list updates in 2007). As expected, some species have also been renamed (particularly some species of *Acetobacter* which were assigned to the *Gluconacetobacter* genus).

Molecular techniques for routine identification of AAB

The main objective of microbial classification is to identify an isolated microorganism at the species level. However, discriminating or typing the different strains or genotypes of a species is becoming increasingly important 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 between strains and to know how many species or strains are involved.

Depending on the degree of polymorphism provided by the various molecular markers, they are more suitable for inter-specific or for intra-specific discrimination. Therefore, we divided the molecular techniques into two main groups: those that can discriminate at the species level and those that can discriminate at the strain level.

One important aspect of these quick molecular techniques is that they can process a large number of samples in a reasonable period of time. However, this means that they are often limited to providing an orientational identification. These tech-

Table 1. Species of AAB. The species detected in grapes, wine or vinegar are in bold

<i>Acetobacter</i>	<i>Gluconacetobacter</i>	<i>Gluconobacter</i>	<i>Acidomonas</i>
A. aceti	Ga. liquefaciens	G. oxydans	<i>Ac. methanolica</i>
A. pasteurianus	<i>Ga. diazotrophicus</i>	<i>G. frateurii</i>	
<i>A. pomorum</i>	Ga. xylinus	<i>G. assaii</i>	
A. peroxydans	Ga. hansenii	<i>G. cerinus</i>	<i>Kozakia</i>
<i>A. indonesiensis</i>	Ga. europaeus	<i>G. albidus</i>	<i>K. baliensis</i>
<i>A. tropicalis</i>	Ga. oboediens	<i>G. thailandicus</i>	
<i>A. syzygii</i>	Ga. intermedius		
<i>A. cibirongenensis</i>	<i>Ga. sacchari</i>	<i>Asaia</i>	<i>Saccharibacter</i>
<i>A. orientalis</i>	Ga. entanii	<i>As. bogorensis</i>	<i>Sa. floricola</i>
A. orleaniensis	Ga. johannae	<i>As. siamensis</i>	
A. lovaniensis	<i>Ga. azotocaptans</i>	<i>As. krugthepensis</i>	
A. estuniensis	<i>Ga. swingsii</i>		<i>Neoasaia</i>
A. malorum	<i>Ga. kombuchae</i>		<i>N. chiangmaiensis</i>
A. cerevisiae	<i>Ga. nataicola</i>		
A. oeni	<i>Ga. rhaeticus</i>	<i>Swaminathania</i>	<i>Granulibacter</i>
<i>A. nitrogenifigens</i>	<i>Ga. saccharivorans</i>	<i>S. salitolerans</i>	<i>Gr. bethesdensis</i>

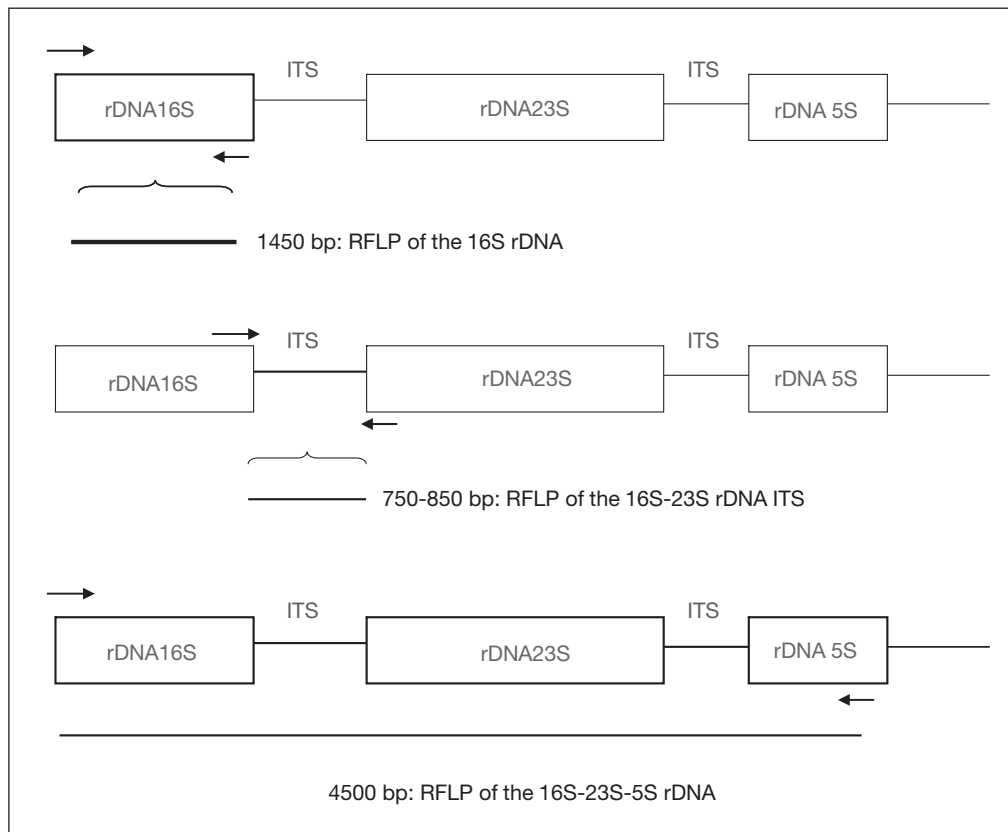


Figure 3. Structure of the ribosomal DNA with indication of the primers used to identify the different species of AAB.

niques actually allow effective groupings of microorganisms that can be associated with a given genera or species by sharing the same patterns as a given type or strain. To validate these groupings it is necessary to use techniques with a higher resolution, such as 16S rDNA sequencing, to identify selected representative group individuals [38]. Techniques based on the amplification of the rRNA coding genes (rDNA) followed by restriction enzyme digestion (PCR – restriction fragment length polymorphism [RFLP]) are frequently used for this. The different amplified fragments are indicated in Figure 3.

Species level:

- PCR-RFLP of the 16S rDNA. This technique was used by Poblet *et al.* (2000) [37] and Ruiz *et al.* (2000) [42] to identify AAB and is appropriate for differentiating and grouping microorganisms on the basis of their phylogenetic relationships. 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 of amplifying the 16S rDNA region and then digesting the amplified fragment with different restriction enzymes. The DNA fragments obtained are separated by electrophoresis. The resulting patterns make it possible to characterize almost all the AAB species. This technique has been used several times by our group [22, 23, 24, 38] and others [21]. One of the main limiting factors of this technique is that it does not always discriminate between all the species and further use of other techniques might be necessary [24].
- PCR-RFLP of the 16S-23S rDNA ITS. This technique was used by Sievers *et al.* (1996) [45], Ruiz *et al.* (2000) [42], Trcek & Teuber (2002) [54], Trcek (2005) [55], González *al.* (2006a) [24] and Prieto *et al.* (2007) [38] to characterize AAB species. The sequences and lengths of the 16S-23S ITS region vary considerably among the species, and this region also contains conserved sequences with functional roles such as tRNA genes and antitermination sequences [45]. This technique consists of amplifying a region of the 16S and 23S rRNA ITS and then digesting the amplified products with different restriction endonucleases. In other bacterial groups intergenic sequences are known to have higher variability than functional sequences, and they make it possible to distinguish beyond the species level. However, in AAB, the results obtained by Ruiz *et al.* (2000) [42] and Trcek & Teuber (2002) [54] only differentiated as far as the species level. Although it may have a higher resolution than the previous technique, in some cases produces more than one pattern for the same species and also some different, unknown patterns for known species.
- PCR-RFLP of the 16S-23S-5S sequences: Technique used by Gullo *et al.* (2006) [28] studying AAB in “traditional balsamic vinegar”. As in the previous two cases, this technique consists of amplifying part of the ribosomal DNA; in this case the region between the 16S, 23S and 5S rDNA genes, to generate an amplified product of around 4500 bp. This is then digested using *RsaI* as a restriction endonuclease. The results are similar to the previous techniques, although with higher polymorphism resolution. The length of the amplified fragment is the main limitation to this technique.
- Denaturing gradient gel electrophoresis (DGGE). DGGE separation of bacterial DNA amplicons is a common

method used to characterize microbial communities from specific environmental niches. This technique was used by López *et al.* (2003) [33] to characterize all of the microorganisms in wine (yeasts, lactic acid bacteria and AAB), by Haruta *et al.* (2006) [29] to analyse the fungal and bacterial population in rice vinegar production, and by De Vero *et al.* (2006) [8] to study the AAB population in vinegar (*aceto balsamico tradizionale*) production. It does not require isolation of the microorganisms. The most commonly used genes for the DGGE method are 16S and 23S rDNA because they are species specific. The band pattern obtained indicates the number of different species present in a sample. Each individual band can be recovered and used for sequencing, which can be an additional tool for species identification. One of the main limitations of this technique is that only the most important microorganisms present in the samples can be differentiated and resolution at the species level is often limited. However, it allows a quick identification of the most common genera present.

- Real-time PCR. This technique identifies and enumerates bacterial species without culturing. It is a fast and reliable identification and enumeration method. It has been successfully used to enumerate total populations of AAB in both wines and vinegars [25]. It is expected that this technique could be developed to simultaneously determine and quantify the most important species in wines or vinegars. However, this is a single-target identification technique, where the single target may be a family group, genera or species: it does not detect other microorganisms.
- PQQ-dependent alcohol dehydrogenase gene targeting. This technique has been used to detect both generic AAB and specifically *Acetobacter aceti* from cider vinegar [55]. The variable and conserved segments in partial *adhA* sequences allow the construction of a generic PCR set of primers for all the AAB species and a specific PCR primer for the detection of *A. aceti*. Trcek in 2005 claims that the analysis of partial *adhA* sequences shows that this region is more discriminative for AAB species than 16S rRNA gene is, but less so than the 16S-23S rRNA intergenic regions are [55].
- Fluorescence in situ hybridization (FISH). This technique has been used to detect *Ga. sacchari* [20] and other wine-related microorganisms such as lactic acid bacteria [4]. FISH directly identifies and quantifies bacterial species at the microscopic level without previous cultivation. It consists of fluorescent-labelled DNA probes that specifically hybridize each of the species or genera. The high content of different binding compounds in wine or vinegar can quench fluorescence and also limit the resolution.

Strain level:

- Random amplified polymorphic DNA-PCR (RAPD-PCR). The RAPD fingerprint amplifies the genomic DNA with a single primer of arbitrary sequence, 9 or 10 bases in length, which hybridizes with sufficient affinity to chromo-

somal 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. The technique was first used with AAB by Trcek *et al.* (1997) [53] in spirit vinegar, and later by Nanda *et al.* (2001) [36] to characterize rice vinegar AAB. Bartowsky *et al.* (2003) [3] also used this technique to differentiate strains in spoiled wines and Prieto *et al.* (2007) [38] to differentiate AAB strains in grapes.

- 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. However, these sequences seem to be widely distributed through the genomes of various bacterial groups. The amplification of the sequences between these repetitive elements has generated DNA fingerprints for several microbial species. ERIC-PCR has already been used by Nanda *et al.* (2001) [36] to identify AAB strains isolated from vinegar. Both techniques have been applied to AAB in wines (González *et al.* 2005) [23] and used to follow the AAB population dynamics before and during alcoholic fermentation.
- A similar technique based on repetitive element genomic fingerprinting was recently proposed by Camu *et al.* (2007) [9] using (GTG)₅ primers. Multiple copies of these non-coding sequences are present in the genomes of most Gram-negative and several Gram-positive bacteria. The preliminary report suggests a good discrimination method with a high degree of polymorphism in AAB [9].

Ecological studies

Grape and wine

AAB in grapes and musts

As grapes mature the amount of the sugars in them (glucose and fructose) increases and improves the chances for AAB growth. In healthy grapes, the predominant species is *Gluconobacter oxydans*, and the most common populations are around 10²–10⁵ cfu ml⁻¹ [14, 23, 30, 38, 40]. *Acetobacter* species have also been isolated from unspoiled grapes, albeit in very small amounts [14, 22, 38]. Damaged grapes contain larger AAB populations [2] which mainly belong to the *Acetobacter* species (*Acetobacter aceti* and *Acetobacter pasteurianus*). Under these conditions, the sugars released from the spoiled grapes can be metabolized by yeasts into ethanol, which is a preferred carbon source of the *Acetobacter* species that overgrow *Gluconobacter* [23, 26, 31]. However, the descriptions of new AAB species will increase the number of species isolated from this substrate. In fact, the recently described *Acetobacter cerevisiae* [5] has already been isolated from Chilean grapes [38].

Grape processing in the cellar (pressing, pumping, racking, etc.) may contaminate the must since there is contact with cellar equipment, which contains resident AAB, mostly made up of *Acetobacter* species [23]. However, in the literature it is possible to find some exceptions to this ubiquitous presence of AAB. Subden *et al.* (2003) [49] was not able to find AAB among the bacteria isolated from ice wine musts. Curiously, the predominant species isolated from this substrate was *Pantoea agglomerans*, which had never been reported as a contaminant in grape musts.

Most studies of AAB in winemaking have focused on the evolution of species during the process. Recently, we have also classified the different AAB isolates from grapes to wine at the strain level [23]. We found important strain diversity in grapes (calculated as the percentage of different strains in the total isolates analysed) which ranged from 45 to 70%. A few of these grape strains were continually isolated throughout alcoholic fermentation. Prieto *et al.* (2007) [38] have also classified isolates from Chilean grapes, confirming the high diversity of AAB strains in grapes, in particular among the *Gluconobacter oxydans* isolates.

AAB during fermentation

Studies of the evolution of AAB species through wine fermentations have established certain general trends (Table 2): *Glu-*

conobacter oxydans is usually the dominant species in fresh must and the initial stages of fermentation but it is rarely isolated from wines, while *Acetobacter aceti* is the most common AAB species in the final stages of fermentation [11, 23, 30]. However, we have also found *Gluconobacter oxydans*, *Gluconacetobacter liquefaciens* and *Gluconacetobacter hansenii* in higher percentages, as well as *Acetobacter pasteurianus* in the final stages of fermentation [22] and recently, *Acetobacter oeni* has been proposed as a new species isolated from wine [46]. Thus, this pattern of species evolution seems somewhat reductionist and may depend on multiple oenological factors such as SO₂, pH, ethanol, low temperature and yeast inoculation. All these factors have been reported as inhibitors of AAB growth, yet they can also modify the species distribution during the process. For instance, different studies have suggested that *Acetobacter pasteurianus* is more resistant to SO₂ [14], ethanol [6], and low temperature than *Acetobacter aceti* is. The growth of AAB during alcoholic fermentation may also be linked to the number of bacteria and yeast in the must at the start of fermentation [56]. It seems that the initial population of AAB, before alcoholic fermentation starts, may determine the number of cells that survive during and after fermentation [14]. If AAB grow significantly during the initial stages of alcoholic fermentation, this may become stuck or sluggish, which in turn might enhance the

Table 2. Summary of publications that indicate the presence of AAB in grapes, musts and wines

Reference	Source	Harvest year	Grapes/ Must		Mid Fermentation		End Fermentation	
			cfu ml ⁻¹	Identified AAB Species	cfu ml ⁻¹	Identified AAB Species	cfu ml ⁻¹	Identified AAB Species
Joyeux <i>et al.</i> (1984a)	White wine Semillon botrytized grapes		10 ⁶	<i>G. oxydans</i> <i>A. pasteurianus</i>	10 ⁵	<i>A. pasteurianus</i> <i>A. aceti</i>	10 ²	<i>A. aceti</i> <i>A. pasteurianus</i>
	Red wine Cabernet-Sauvignon		10 ⁴	<i>G. oxydans</i>	10 ²	<i>G. oxydans</i> <i>A. pasteurianus</i>	10 ¹	<i>A. pasteurianus</i> <i>G. oxydans</i>
Barbe <i>et al.</i> (2001)	Botrytized grapes	1995		<i>Gluconobacter sp.</i>				
		1996	10 ⁶	<i>A. pasteurianus</i>				
		1997		<i>A. aceti</i>				
Du Toit and Lambrechts (2002)	Cabernet Sauvignon	1998	10 ⁶ -10 ⁷	<i>G. oxydans</i>	10 ³ -10 ⁴	<i>A. pasteurianus</i> <i>A. aceti</i>	10 ² -10 ³	<i>A. pasteurianus</i>
		1999	10 ⁴ -10 ⁵	<i>G. oxydans</i> <i>A. pasteurianus</i>	10 ² -10 ⁴	<i>A. pasteurianus</i> <i>Ga. liquefaciens</i> <i>G. oxydans</i> <i>A. aceti</i>	10 ² -10 ³	<i>A. pasteurianus</i> <i>Ga. liquefaciens</i> <i>A. hansenii</i>
Bartowsky <i>et al.</i> (2003)	Bottled red wine						10 ² -10 ³	<i>A. pasteurianus</i>
González <i>et al.</i> (2004)	Red Grenache	2001	10 ³	<i>G. oxydans</i>	10 ³	<i>A. aceti</i> <i>Ga. liquefaciens</i> <i>Ga. hansenii</i>	50	<i>A. aceti</i>
González <i>et al.</i> (2005)	Red Grenache	2002	10 ⁴ -10 ⁵	<i>G. oxydans</i> <i>A. aceti</i>	10 ⁴ -10 ⁵	<i>A. aceti</i>	10 ² -10 ³	<i>A. aceti</i>
Prieto <i>et al.</i> (2007)	Carmenere, Cabernet Sauvignon Other varieties Chile	2004		<i>G. oxydans</i> <i>A. cerevisiae</i>				

growth of AAB during wine storage, with a corresponding reduction in the quality of the wine [31]. Otherwise, inoculation with a high population of yeasts, which is a common practice in winemaking, will produce a rapid onset of alcoholic fermentation and a concomitant decrease in the AAB population [27]. However, little is known about the impact of these oenological factors, or of how they interact with other wine microorganisms, on the selection and evolution of AAB species during wine fermentation.

Even less is known about the development of AAB during malolactic fermentation, or how they interact with lactic acid bacteria (the most important microorganisms during this process). Joyeux *et al.* (1984a) [30] reported constant cell counts of AAB of approximately 10^2 - 10^3 cfu ml⁻¹, which consisted mainly of *Acetobacter pasteurianus*, throughout malolactic fermentation. In contrast, we detected a major increase in the AAB population up to approximately 10^6 cfu ml⁻¹ during this process, with *Acetobacter aceti* being the species most common in this environment [27]. This increase in the AAB population did not interfere with the simultaneous development of the lactic acid bacteria population up to cell densities of approximately 10^8 cfu ml⁻¹. A possible synergic mechanism between the two bacterial groups may emerge from this result. In our studies on classifying AAB strains and monitoring strain evolution during alcoholic fermentations, we were able to conclude that: i) the origin of the strains isolated during wine fermentation are both the grapes and the wine cellar environment; ii) the high strain diversity detected at the beginning of the process decreased significantly during the final stages of the process, the anaerobic conditions and increasing ethanol concentration clearly selected the most resistant strains; and iii) regardless of the degree of genotype diversity, there were clearly dominant genotypes at all stages [22, 23].

AAB during ageing and wine maturation

Once alcoholic fermentation has finished, the pumping over and racking of wine may stimulate the growth of AAB and can lead to populations of up to 10^8 cfu ml⁻¹ [10, 31] owing to the intake of oxygen during these operations. During storage and ageing, the most common AAB species found belong to *Acetobacter* (*Acetobacter aceti* and *Acetobacter pasteurianus*). AAB have been isolated from the top, middle and bottom of tanks and barrels, suggesting that AAB can actually survive under the semi-anaerobic conditions occurring in wine containers [16]. This can be explained by the ability of AAB to use such compounds as quinones and reducible dyes as electron acceptors [15]. The numbers of bacteria usually decrease drastically after bottling, because of the relatively anaerobic conditions within a bottle. However, excessive aeration during bottling can increase the number of AAB [35]. Furthermore, bottle position during storage, poor storage conditions or spoiled corks may facilitate AAB growth. Wine spoilage in the bottle by AAB has been reported, mostly due to *Acetobacter pasteurianus* [3]. It should be pointed out that the number of AAB in wine after fermentation may be underestimated because the counting of colonies grown in solid media does not allow for viable but not culturable (VBNC) status [35].

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 was known about the role played by microorganisms in vinegar production, vinegar was mainly produced from wine, alcohol and rice. Nowadays, knowledge is much more advanced, particularly 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 it is still not very clear which species and strains are responsible for vinegar production. There are currently three different biotechnological processes for producing vinegar: Orleans, Schutzenbach and submerged methods.

Although interest in microorganisms and vinegar production has been constant since Pasteur's times, the fact that the microorganisms are difficult to isolate and characterize, and the changes in their taxonomy make it difficult to give a clear indication of the microorganisms involved. In addition, the existence of VBNC AAB has made these surveys more difficult [21]. Initial studies associated the genus *Acetobacter* [1], especially *A. aceti*, *A. pasteurianus* and *A. hansenii*, with the production of vinegar [6]. However, several subspecies have since become separate species. Although the *A. aceti*, *A. pasteurianus* and *Ga. hansenii* species are still detected in vinegar production [29, 36, 51], several new species have been isolated from vinegars. It must be emphasized that only culturable AAB strains have been characterized and the samples originating in acetifiers have high mortalities because they have developed in a highly oxygenated medium. In fact, it is well known by vinegar makers that even a short period (minutes) without oxygenation dramatically decreases the activity and cell population in acetifiers. In these submerged systems, *Ga. europaeus* was proposed as a new characteristic species [44] and later recovered from the acetifiers [48, 55]. Schuller *et al.* (2000) [43], working with the same submerged systems but with greater acidity, suggested that *Ga. entanii* was the only species that could be recovered after plating. Sokollek *et al.* (1998a) [47] suggested that *A. pomorum* and *Ga. oboediens* were new species in the acetifiers. In more traditional systems, Giudici *et al.* (2003) [21] identified *Ga. xylinus* as being chiefly responsible for *aceto balsamico* production, although *A. aceti*, *A. pasteurianus*, and *A. malorum* were also isolated [8, 28]. It is clear that the new techniques available will identify more new species.

The WINEGAR Project

The WINEGAR Project (Wood solutions to excessive acetification length in traditional vinegar production) is a European Cooperative Project within the 6th Framework programme that emphasizes the cooperation between Small and medium-sized enterprises (SME) and research centres. The main objective of the project is the reduction of acetification time while maintaining traditional superficial methods of vinegar production. To ful-

fil this objective different approaches have been taken. On one hand, work is performed on the barrels, and on the other, on the microorganisms. Several parameters have been considered to optimise the exposure of the acetic acid bacteria to oxygen: wood type, wood thickness and barrel design. With regard to the microorganisms, several starter cultures have been isolated from the participating SMEs and tested for vinegar production. *Acetobacter pasteurianus* and *Gluconacetobacter europaeus* are the most important species isolated from traditional vinegar plants. Individual strains of *Acetobacter pasteurianus* have been successfully tested for vinegar production at the collaborating SMEs. The final reduction in acetification time obtained by the right combination of barrel design and selected starter culture is between 50% and 90%. Individual detailed results of the project will be published at the end of the project (November 2007). The partners in the WINEGAR Project are the *Universitat Rovira i Virgili* (Spain, coordinator), *Università degli Studi di Modena e Reggio Emilia* (Italy), *Universidad de Sevilla* (Spain), and *Université de Geneve* (Switzerland) as research centres, and *Boteria Torner and Viticultors Mas d'en Gil* (Spain), *Acetaia Cavalli* (Italy) and *Vinaigrierie La Guinelle* (France).

Acknowledgements

This work was supported by grant AGL2004-07494-C02-02/ALI from the *Ministerio de Educación y Ciencia*, Spain, and the WINEGAR Project; UE, Cooperative Research Project number 017269.

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