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Acetic acid bacteria isolated from grapes of South Australian vineyards

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ABSTRACT

Acetic acid bacteria (AAB) diversity from healthy, mould-infected and rot-affected grapes collected from three vineyards of Adelaide Hills (South Australia) was analyzed by molecular typing and identification methods. Nine different AAB species were identified from the 624 isolates recovered: Four species from *Gluconobacter* genus, two from *Asaia* and one from *Acetobacter* were identified by the analysis of 16S rRNA gene and 16S–23S rRNA gene internal transcribed spacer. However, the identification of other isolates that were assigned as *Asaia* sp. and *Ameyamaea chiangmaiensis* required more analysis for a correct species classification. The species of *Gluconobacter cerinus* was the main one identified; while one genotype of *Asaia siamensis* presented the highest number of isolates. The number of colonies recovered and genotypes identified was strongly affected by the infection status of the grapes; the rot-affected with the highest number. However, the species diversity was similar in all the cases. High AAB diversity was detected with a specific genotype distribution for each vineyard.

1. Introduction

Acetic acid bacteria (AAB) are an important group of bacteria in the food and beverage industry, mainly due to their ability to oxidize ethanol to acetic acid. These bacteria are the key microorganisms in vinegar production, however conversely, in grape and wine production they are mainly spoilage microorganisms. AAB are found ubiquitously, including on grapes (Bartowsky and Henschke, 2008; Drysdale and Fleet, 1988; González et al., 2005; Joyeux et al., 1984; Valera et al., 2011). In the past, AAB were classified into two main genera, *Acetobacter* and *Gluconobacter*, but with more diversity studies and interest in the AAB taxonomy this has been revised to fourteen genera (Yamada et al., 2012).

Originally only three species, *Gluconobacter oxydans*, *Acetobacter aceti* and *Acetobacter pasteurianus*, were associated with grapes and wine (Barbe et al., 2001; Drysdale and Fleet, 1988; Joyeux et al., 1984), however, more recent studies using molecular based identification methods have extended the number of AAB species present on grapes and in wines to other ones never previously reported in these niches. Thus far, the additional *Gluconobacter* species identified on grapes and wine are *Gluconobacter japonicus*, *Gluconobacter cerinus*, *Gluconobacter thailandicus* and *Gluconobacter albidus* (Navarro et al., 2013; Valera et al., 2011) and within the *Acetobacter* genus, *Acetobacter oeni*, *Acetobacter cerevisiae*, *Acetobacter malorum*, *Acetobacter tropicalis*, *Acetobacter orleaniensis* and *Acetobacter syzygii* (Barata et al., 2012a; Silhavy and Mandl, 2006; Silva et al., 2006; Prieto et al., 2007; Valera et al., 2011). Furthermore, the ecological studies carried out in the last years have also revealed the presence of other species including *Kozakia baliensis* (Navarro et al., 2013), *Asaia siamensis* (Bae et al., 2006; Ruiz et al., 2010) and species of *Gluconacetobacter* genus, (Barata et al., 2012a; Du Toit and Lambrechts, 2002; González et al., 2004, 2005; Kato et al., 2011; Valera et al., 2011) several of them recently renamed as *Komagataeibacter* genus (Yamada et al., 2012).

The degree of grape spoilage is one of several factors to affect the grape microbiota, with damaged grapes harboring the highest AAB population (Barata et al., 2012b; Barbe et al., 2001; Fleet, 1999). The presence of damaged and infected grapes may compromise the vinification process and the final quality of wine (Bartowsky et al., 2003; Drysdale and Fleet, 1988; Fleet, 1999; Joyeux et al., 1984; Nisiotou et al., 2011). Therefore, the knowledge of how to control the ability of AAB to affect wine quality begins by determining the AAB diversity present in raw material, the grape.

Despite the importance of this group of bacteria, it has been less studied than yeast or lactic acid bacteria on grapes and/or wine; however, in recent years the AAB diversity has been the subject of renewed interest. Ecological studies of AAB have been carried out on grapes from world-wide wine regions (Barata et al., 2012a; Barbe et al., 2001; Du Toit and Lambrechts, 2002; González et al., 2005; Joyeux et al., 1984; Nisiotou et al., 2011; Prieto et al., 2007; Renouf et al., 2005; Valera et al., 2011). Several Australian AAB studies have examined grape musts and wines (Bartowsky et al., 2003; Bartowsky and Henschke, 2008; Drysdale and Fleet, 1985; Fleet, 1993) but none of them from healthy and unhealthy grapes.

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South Australia is one of the most important Australian wine regions; more than half of the total production comes from this state. In this state there are more than 15 wine-producing regions, including Adelaide Hills, where the present study was conducted. This region is located in Mount Lofty Ranges zone, 30 km east from Adelaide.

This study aims to investigate AAB diversity on several grape varieties and with different infection status from three Adelaide Hills vineyards. The analysis of both healthy and unhealthy grapes will provide a better knowledge of the AAB species present and could ultimately contribute to wine spoilage.

2. Materials and methods

2.1. Grape samples and AAB isolation conditions

During May of 2011 a total of 28 bunch samples of 12 different grapevine varieties (eight red and four white grapes) were selected from three commercial vineyards, "A", "B" and "C", of the Adelaide Hills region of Australia. The vineyards "B" and "C" were located in the hills closer to one another and approximately 20 km away from vineyard "A", which was located in a flatter area. These samples were collected from the bunches left on the vines after the harvest and, their infection status was classified as healthy, mould-infected and rot-affected (Table 1).

Each sample comprised of one or two bunches which were randomly and aseptically collected in a sterile seal plastic bag, representing the different states of infection. The samples were transported to The Australian Wine Research Institute (AWRI) (Adelaide, Australia) and processed immediately. They were hand crushed and the obtained grape juices were plated onto GY medium (1% yeast extract (Oxoid), 5% glucose (Oxoid), 2% agar (Oxoid) (w/v)) supplemented with pimaricin (100 mg/L) (Sigma-Aldrich, Australia) to suppress fungal growth. After incubation at 28 °C for 3–5 days, 40 to 50 colonies were randomly picked and streaked on GYC (GY medium supplemented with 3% $CaCO_3$ (Oxoid)) and those, up to 25 colonies per sample, that produced a clear halo on this medium were subjected to a catalase test. The catalase positive colonies were considered as putative AAB isolates and analyzed by molecular methods.

Furthermore, chemical analysis of glucose, fructose and ethanol from all the samples was carried out by high-performance liquid chromatography (HPLC) using a BioRad HPX87H column as described previously (Nissen et al., 1997).

Table 1
Source and description of grape samples used in this study.

Vineyard	Type of grape	Varieties	Infection status (number of samples) [a]
"A"	Red grape	Raboso	Healthy (1)
		Canonazo	Rot-affected (1)
		Tinta Amarela BVRC VC	Rot-affected (1)
		Tinto Cão 894	Rot-affected (1)
	White grape	Muscat Gard Blanco	Rot-affected (1)
		Semillon 32	Rot-affected (2)
		EM (root stock)	Healthy (1)
		1202 (root stock)	Healthy (1)
"B"	Red grape	Shiraz	Healthy (2)/mould-infected (5)/rot-affected (1)
	White grape	Sauvignon Blanc	Mould-infected (2 samples)/rot-affected (2)
		Chardonnay	Mould-infected (5)/rot-affected (1)
"C"	Red grape	Pinot Noir	Rot-affected (2)

[a] Mould-infected grapes contained gray mould while rot-affected grapes underwent decomposition mainly attacked by sour rot.

2.2. AAB molecular analysis

Genomic DNA of isolates was extracted using the CTAB method (Cetyltrimethylammonium bromide) as described Ausubel et al. (1992). Genotyping of these isolates was carried out at AWRI (Adelaide, Australia) while fingerprinting analysis and the species identification of different genetic profiles was performed at the Universitat Rovira i Virgili (Tarragona, Spain); after the DNA samples were received from AWRI in less than four days.

2.2.1. AAB typing

All the isolates were subjected to fingerprinting techniques of ERIC-PCR and $(GTG)_5$-PCR. Primers ERIC1R (5′-atgtaagctcctggggattcac-3′) and ERIC2 (5′-aagtaagtgactggggtgagcg-3′) described by Versalovic et al. (1991) were used for ERIC-PCR as described by González et al. (2004) and one primer (5′-gtggtggtggtggtg-3′) was applied for $(GTG)_5$-PCR (De Vuyst et al., 2008). The PCR amplified DNA fragments obtained were separated on a screening cartridge with the QIAxcel capillary electrophoresis system (QIAGEN).

The different genotypes were analyzed by BioNumerics software program (Version 6.5, Applied Maths, Sint-Martens — Latem, Belgium) to determine their phylogenetic relationships. Comparison was performed with Dice coefficient of 1% of band position tolerance and Unweighted Pair Group Method with Arithmetic average (UPGMA). In addition, all band profiles were carefully checked by visual inspection to be correctly marked.

Diversity was calculated using Simpson's biodiversity index, which shows the probability that two randomly selected isolates are different genotypes. The index was calculated using $1 - \Sigma p_i^2$, where p_i is equal to the number of isolates of the same genotype divided by the total number of isolates.

2.2.2. AAB identification

The different fingerprinting profiles were identified at species level by amplifying and sequencing the 16S rRNA gene and 16S–23S rRNA gene internal transcribed spacer (ITS). For 16S rRNA gene amplification, 16Sd (5′-gctggcggcatgcttaacacat-3′) and 16Sr (5′-ggaggtgatccagccgcaggt-3′) primers were used and, for 16S–23S rRNA gene ITS amplification, with primers its1 (5′-acctgcggctggatcacctcc-3′) and its2 (5′-ccgaatgcccttatcg cgctc-3′) (Ruiz et al., 2000); PCR conditions were as previously described by Ruiz et al. (2000) using a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, USA). The amplicons were purified and sequenced using an ABI3730 XL automatic DNA sequencer by Macrogen Inc. (Seoul, South Korea). Sequences were compared with those present in the GenBank database using the *Basic Local Alignment Search Tool* (BLAST).

Phylogenetic analyses with all sequences were performed using MEGA version 4 software (Tamura et al., 2007). Dendrograms were constructed based on the neighbor joining and Kimura 2-parameter methods (Kimura, 1980).

3. Results

Grapes were collected from three vineyards representing 12 different grape varieties (Table 1) and were graded according to infection state and analyzed for chemical and microbial composition. Grape must samples had pH values between 3.0 and 4.1, increasing with the highest infection rate. The average sugar (glucose and fructose) content was 176 g/L (range from 137 to 265 g/L). Ethanol was detected in all the samples; 3.2–40 g/L. The total bacterial population ranged between 1.2 and 6.4 log CFU/mL, increasing in number as the infection state increased, however, putative AAB isolates were recovered from all the grape samples collected. A total of 624 AAB isolates were analyzed by typing and identification molecular techniques.