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Acetic acid bacteria population dynamics and natural imposition of *Gluconacetobacter europaeus* during submerged vinegar production

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Abstract

Acetic acid bacteria population dynamics and natural imposition of *Gluconacetobacter europaeus* during submerged vinegar production. – *The production of wine vinegar with the traditional as well as the submerged methods is generally started with an inoculum containing a heterogeneous population of acetic acid bacteria (AAB). In this study, two acetic acid fermentations were carried out with submerged, semi-continuous method, using starters that molecular analyses demonstrated composed by a mixture of two AAB species,* Acetobacter pasteurianus *and* Gluconacetobacter europaeus. *The results showed a high degree of similarity between the two processes, and molecular identification revealed the presence of only one AAB species at the end of both acetifications. The AAB species naturally imposed along the processes was* Gluconacetobacter europaeus, *more acidophilic or acetic acid resistant than* Acetobacter pasteurianus.

The morphological evolution of bacteria during vinegar production was also studied by using scanning and transmission electron microscopy. The results showed a major transformation in bacterial shape and size, ranging from ovoid cells (0.6-1.0 m) to rods (2.0-4.0 m). Additionally, an irregular amorphous layer surrounding bacteria, cytochemically identified as polysaccharides, was also observed.

Keywords: *Acetic acid bacteria, molecular identification, morphology, submerged vinegar production.*

Résumé

Dynamique d'une population de bactéries acétiques et imposition naturelle de *Gluconacetobacter europaeus* lors de la production de vinaigre par la méthode immergée. – *La production de vinaigre de vin aussi bien avec la méthode traditionnelle qu'avec la méthode dite immergée est généralement initiée avec un inoculum composé d'une population hétérogène de bactéries acétiques (BA). Dans cette étude, deux fermentations acétiques ont été réalisées selon la méthode immergée semi-continue en utilisant deux inocula dont les analyses moléculaires ont démontré qu'ils étaient composés d'un mélange de deux espèces de bactéries acétiques,* Acetobacter pasteurianus *et* Gluconacetobacter europaeus. *Les résultats obtenus ont montré un haut degré de similarité entre les deux processus d'acetification, mais l'identification moléculaire des bactéries présentes à la fin de l'acetification a permis de mettre en évidence une seule espèce.* Gluconacetobacter europaeus, *plus acidophile ou résistante à l'acide acétique qu'*Acetobacter pasteurianus, *est l'espèce qui s'est naturellement imposée à la fin du processus d'acetification.*

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En utilisant des techniques de microscopie électronique, les résultats de l'étude morphologique ont démontré principalement une transformation de la forme et de la taille des bactéries: ovoïdes (0.6-1.0 m) en bâtonnets (2.0-4.0 m). De plus, une couche externe, amorphe et irrégulière de nature polysaccharidique a été aussi mise en évidence par des méthodes cytochimiques.

Mots-clés : *Bactéries acétiques, identification moléculaire, morphologie, production de vinaigre, méthode immergée*

1. Introduction

Although vinegar has been known for thousands of years, its relation to microbes was not realized until 170 years ago, when Kützing (1837) reported that the conversion of ethanol to acetic acid was done by living microorganisms. Vinegar acetification is an oxidative process in which diluted ethanol is oxidized to acetic acid and water by acetic acid bacteria (AAB). This takes place in two steps. Ethanol (EtOH)is first oxidized to an intermediate product, the acetaldehyde, which is then oxidized to acetic acid (AcH). These sequential reactions are catalyzed by two enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), located at the outer surface of the cytoplasmic membrane of the AAB. Their function is linked to the respiratory chain of bacteria (Adachi et al. 1980; Ameyama and Adachi 1982; Matsushita et al. 2003). The strict aerobic metabolism, an ability to oxidize ethanol to acetic acid in acidic media, is unique to these organisms, and differentiates them from other bacteria. These properties are industrially exploited to produce vinegar.

Vinegar can be manufactured from almost any product capable of yielding alcohol by acetification. For the commercial production of vinegar, several methods have been used. These methods can be divided into two: the traditional surface culture (slow) and the modern submerged acetification (rapid). The surface culture process, normally called the Orléans or French method, is the oldest method of production of table vinegars. In this process, AAB form a thin film on the surface of the solution, which later becomes quite thick and gelatinous. This gelatinous material, which contains a large number of bacteria, is known as the "mother of vinegar". In the traditional surface culture, the mixture of heterogeneous microorganisms or a starter of "seed vinegar" is generally employed. Although this process produces vinegar of high quality, it is slow and involves high production costs. On the other hand, the industrial submerged method involves a rapid mixing of AAB starters with forced aeration into an Acetator (Arnol et al. 2002). The physico-chemical parameters that affect the acetic acetification process in modern industrial vinegar plants are: the raw material, temperature, pH, dissolved oxygen, biomass, acetic acid and ethanol contents, as well as media composition (Ebner et al. 1996). All these parameters are controllable during the submerged acetification.

During the last decade, several molecular methods have been developed for routine identification of AAB from wine and vinegar, involving the use of PCR reaction for amplification of different genomic regions. For identification at the species level, the most widely used technique is RFLP-PCR of the 16S rRNA gene and the 16S-23S rDNA intergenic transcribed spacer (ITS) region (Sievers et al. 1996; Poblet et al. 2000; Ruiz et al. 2000; Trček and Teuber 2002; González et al. 2004, 2005, 2006). Trček (2005) also studied the subunit I of the pyrroloquinoline quinone (PQQ)-dependent ADH (AdhA) as a possible target for identification purposes.

The main objective of this study was to compare the performance of the submerged semi-continuous process using two different starters or seed vinegars, with respect to lag phase length, acetification rate, maximum acetic acid concentration, cells viability, AAB population dynamics, bacterial growth or evolution, and other physical parameters. In order to obtain an unequivocal identification of the bacterial strains present, RFLP-PCR and sequence analysis of 16S rRNA gene, 16S-23S rDNA ITS region and partial *adhA* gene were carefully evaluated. Acetic acid bacteria population was also investigated by morphological characterization of selected samples from different stages of the vinegar acetification.

2. Materials and methods

2.1. Laboratory acetification

Red wine vinegar acetification was performed in a 10 litres pilot Acetator (Frings Co., Bonn, Germany), in a working volume of 8 litres. The aeration rate was 10 L/h during the adaptation phase and then increased progressively up to 60 L/h for the rest of the process (Sokollek and Hammes 1997; Frings 1998). Acetificaion was carried out semi-continuously at constant temperature of 30°C.

Wines and vinegars used in this study came from a vineyard in the Priorat region, in the northeast of Spain. The first acetification process used filter-sterilized wine (0.7% AcH/11.5% EtOH) and non-sterile vinegar to obtain a starter mixture with 6% AcH/4% EtOH. The second experiment used filter sterilized wine (0.9% AcH/13.7% EtOH) and high alcohol non-

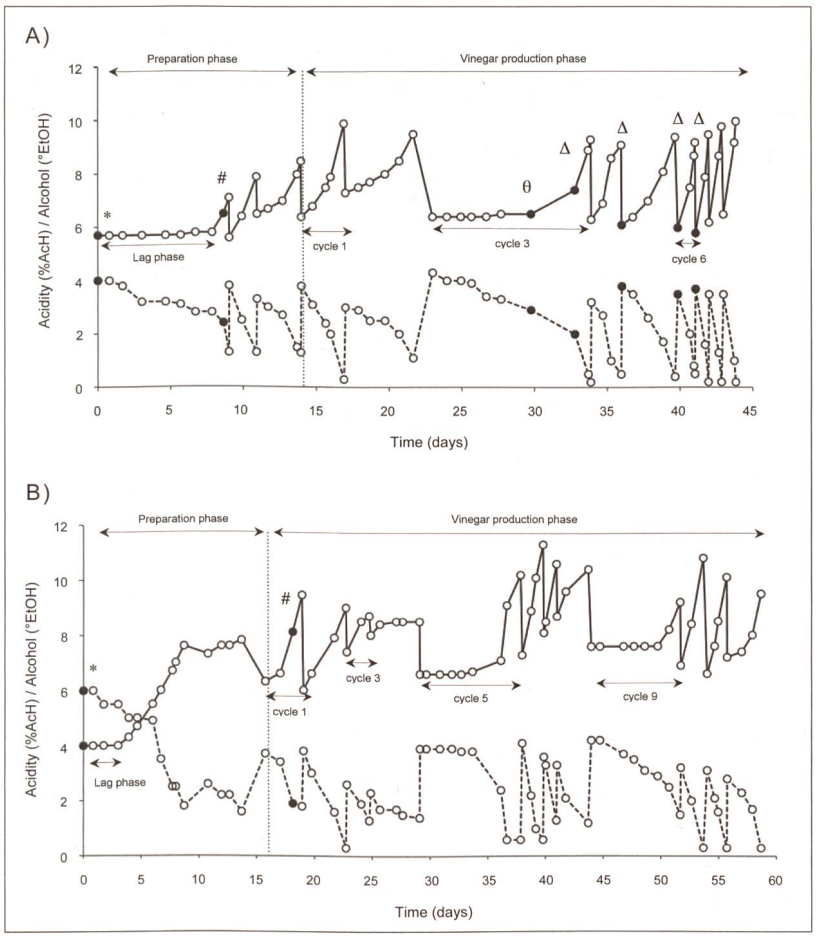

Fig. 1. General performance of the acetification process during experiment 1 (A) and experiment 2 (B). In both diagrams, continuous line represents the acidity (%AcH), and dotted line represents the alcohol content (%EtOH). In the two processes, circles represent the time points of vinegar sampling. Closed circles indicate the moments in which a mixture of Ga. europaeus *and* A. pasteurianus *was identified from agar plates, and the percentage of the last AAB species in the mixture was as follows: A) * 60%, # 4%, q 1%, D 0.5%, B) * 99%, # 88%.*

sterile vinegar to obtain a starter mixture with 4% AcH/6% EtOH. In this way, the only microorganisms acting in the oxidative process were AAB present in both seed vinegars, of which the species composition was a priori unknown.

During each preparation phase, whenever the residual ethanol concentration in the acetification broth came down to 0.5-1.0% EtOH, fresh filtered mash (wine) was added to maintain the initial alcohol concentration to 4% EtOH. This step was repeated until the desired working volume was reached and the production cycles started (De Ory et al. 2002) (Fig. 1). At the end of each production cycle, when the ethanol concentration had decreased to 0.2-0.5%, one third of the acetator content was discharged as end vinegar and replaced by fresh filtered mash. This operation restored the ideal starting conditions for each new cycle during the vinegar production process: 6-7% AcH/3-4% EtOH (De Ory et al. 2002) (Fig. 1). Whereas refilling of the acetator was done relatively slowly, the discharge of the volume was carried out as quickly as possible, to avoid complete depletion of the alcohol. Nine to twelve repeated cycles were thus carried out and samples were collected at three points during each cycle: beginning (6% AcH), middle (7-8% AcH) and end (9-10% AcH).

The aeration/air flow was kept at 10 L/h during the lag phase, raised to ~40 L/l during the rest of the preparation phase, and raised to further the maximum (~60 L/h) from the beginning of the producing cycles.

2.2. Strains, media and growth conditions

Reference strains used in this study where purchased from the Belgian Coordinated Collection of Microorganisms (BCCM/LMG), the German Collection of Microorganisms (DSMZ) and the Spanish Type Culture Collection (CECT), and are listed in Table 1.

Reference strains, except *Ga. europaeus* and *Ga. intermedius*, were grown on YPM (0.5% yeast extract, 0.3% peptone, 2.5% mannitol, 1.5% agar) and YGC (1% yeast extract, 10% glucose, 2% $CaCO_3$, 1.5% agar) media. *Ga. europaeus* and *Ga. intermedius* strains were grown on RAE (1% yeast extract, 4% glucose, 1% peptone, 0.338% $Na_2HPO_4.2H_2O$, 0.15% citric acid×H_2O, 2% (w/v) acetic acid, 1% (w/v) ethanol; bottom: 1% agar, top: 2% agar) medium. In order to reveal the widest spectrum of different bacteria, vinegar samples were inoculated in the general growth media YPM and YGC. Culture plates were incubated for 2-7 days at 30°C.

2.3. Counting of microorganisms

Direct epifluorescence method (DEM) (Mesa et al. 2003) was used to calculate the number of viable and non-viable cells. DEM was performed using the LIVE/DEAD® *Bac*Light bacterial viability kit (L-7012) (Molecular Probes, Eugene, OR, USA), according to Baena-Ruano et al. (2006).

Table 1. Reference strains used in this study.

Bacterial species	Strain	Growing medium	Source	Accession Number (AC) for the 16S rRNA gene sequence
Acetobacter pasteurianus	LMG 1262^T	YPM	Beer	X71863
Acetobacter pomorum	LMG 18848^T	YPM	Cider vinegar fermentation Celluloseless mutant 1 derived from	AJ419835, AJ001632
*Gluconacetobacter hansenii**	LMG 1527^T	YPM-YGC	NCIB 8745, itself isolated by M.Aschner, Jerusalem, vinegar	X75620
*Gluconacetobacter hansenii**	DSM 5602^T	YPM-YGC	Vinegar	
Gluconacetobacter liquefaciens#	LMG 1381^T	YPM-YGC	Diospyros sp., dried fruit	X75617
Gluconacetobacter liquefaciens#	DSM 5603^T	YPM-YGC	Dried fruit (Diospyros sp.)	
Gluconacetobacter xylinus¥	LMG 1515^T	YGC	Mountains ash berries	X75619
Gluconacetobacter xylinus¥	DSM 6513^T	YGC	Mountains ash berries	
Gluconacetobacter europaeus	LMG 18494	RAE	Red wine vinegar produced in submerged bioreactor	AJ012698
Gluconacetobacter europaeus	DSM 6160^T	RAE	Submerged culture vinegar generator	AB205220
Gluconacetobacter intermedius	LMG 18909^T	RAE	Commercially available tea fungus beverage (Kombucha)	Y14694
Gluconacetobacter intermedius	CECT 944	YPM-YGC	Beer	n. d.

^{T}: type strain *,#,¥: synonym strains n.d.: not available sequence

Samples harvested at several time points during different cycles along the acetification process were diluted with water at 1:5 for total cell counting in a special Neubauer chamber with 0.02 mm depth and rhodium-coated bottom, and examined under a Leica Orthoplan microscope equipped with an U-RFLT-100W mercury lamp and appropriate filters of LIVE/DEAD® *Bac*Ligth bacterial viability kit. Images were taken with an Olympus CF70 camera and Olympus C4040-zoom.

2.4. Transmission and scanning electron microscopy

For scanning electron microscopy (SEM), bacteria from vinegar samples and colonies from agar media were fixed for 1-2 h at room temperature. In order to maintain the integrity of the culture and to keep any alteration owing to fixation at a minimum, a concentrated solution of the fixative was directly added to the medium to obtain the final concentration of 2% glutaraldehyde. After three successive washes in 66 mM Sörensen buffer ($KH_2/Na_2HPO_4.2H_2O$, pH 6.8), the cells were post-fixed in 1% osmium tetroxide (OsO_4) for 1 hour, dehydrated respectively in 50%, 70%, 90% and 100% ethanol, for 10 min each, and observed under a Zeiss 940A scanning electron microscope.

For transmission electron microscopy (TEM), glutaraldehyde was added to a final concentration of 2.5%. After three successive washes in 66 mM Sörensen buffer, the cells were post-fixed in 2% OsO_4 for 1 hour. After three successive washes in distilled water, samples were then agarised and treated with 2% uranyl acetate for 30 min at room temperature, protected from light. Following two washes with distilled water at 4°C, samples were dehydrated in 25%, 50%, 75%, 95% and 100% ethanol respectively, for 10 min each, then in 100% ethanol for 15 min, and finally in 100% for 30. Samples were then embedded with Spurr's resin in ethanol 100% 1:1 (v:v) and 1:3 (v/v) successively, for 30 min under agitation; then twice with pure Spurr's resin for 1 and 2 h respectively. Samples were finally immersed into pure Spurr's resin and polymerised for 12 h at 60-70°C. Ultrathin sections were taken on Au grids, stained for PATAg (see below), and examined at 60 kV in a Philips M400 transmission electron microscope.

2.5. Cytochemical techniques

Cytochemical detection of polysaccharides was performed by PATAg labelling (periodic acid, thiocarbohydrazide (TCH), silver proteinate) (Thiéry 1976). Thin sections placed on Au grids were treated with 1% periodic acid for 30 min, washed with distilled water four times for 5 min each and treated with 0.2% TCH in 20% acetic acid for 24 h. Sections were again washed four times in 20% and once in 10%, 5%, 2% and 1% acetic acid, for 5 min each, and then three times in distilled water for 20 min each. After 30 min incubation in 1% silver proteinate in the dark, sections were again washed several times in distilled water. The specificity of the reaction was assessed with control sections without periodic acid but with 5% hydrogen peroxide (H_2O_2) treatment.

2.6. Isolates selection

Vinegar samples were harvested along the two acetification experiments at several time points (Fig. 1A,B) and 100 µl were inoculated on agar plates.

Along the different production cycles, samples were harvested to represent the beginning, the middle and the end of each cycle.

50 colonies from experiment 1 and 19 from experiment 2 were randomly selected from agar plates and subjected to molecular identification at the species level by RFLP-PCR analysis. After the first clustering by RFLP analysis, 3 *Acetobacter* and 3 *Gluconacetobacter* representative isolates were selected for sequencing of the 16S rRNA gene, the 16S-23S ITS region and the partial *adhA* gene. Once established the relationship between the morphology and the AAB species, optical counting of grown colonies was used to calculate the ratio between the two identified AAB species over the experiments.

2.7. DNA extraction

DNA extraction from selected samples was performed using a modified version of the Ausubel CTAB method (Ausubel et al. 1992). Harvested cell pellets were resuspended in 520 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), then added 30 µl of SDS 20% and 6 µl of proteinase K (20 mg/ml) and incubated for 1 hour at 37°C. Subsequently, 150 µl of 5 M NaCl and 140 µl of 5% CTAB in 0.7 M NaCl were added and samples were re-incubated at 65°C for 10 minutes. They were then placed on ice for 15 min and the aqueous phase was extracted twice with 500 l phenol: chloroform: isoamyl alcohol (25:24:1) by centrifugation at 10'000 rpm for 10 min at 4°C. The aqueous phase was collected and DNA precipitated with 380 µl isopropanol by centrifugation at 10'000 rpm for 10 min at 4°C. Pellets were washed with 170 µl of 70% ethanol and dried using a vacuum pump. DNA was resuspended in 50 µl of TE buffer and rehydrated overnight at 4°C and stored at -20°C until analysed.

2.8. Restriction fragment length polymorphism analysis (RFLP-PCR)

PCR amplification of the 16S rRNA gene was carried out using specific primers designed by Ruiz et al. (2000): 16Sd, 16Sr (Table 2). Reactions were performed as described by Ruiz et al. (2000), using an Eppendorf Thermocycler Primus 25 (PeqLab, Switzerland), and samples were maintained at 4°C until analysis by agarose gel electrophoresis.

PCR amplification products were digested with *TaqI*, *MspI*, *HaeIII* (González et al. 2006) and *BccI* (Torija et al. 2010) endonucleases, according to the manufactures' instructions (Qbiogene, France; Promega, Switzerland; Biolabs, Switzerland).

Amplification products were detected on 0.8% agarose gel electrophoresis in 1X TAE buffer, whereas restriction fragments were analyzed on 3.5% agarose gel electrophoresis or, when necessary, on 8% polyacrylamide gel electrophoresis. Agarose gels were stained with SYBR® Safe (Invitrogen, Switzerland) and polyacrylamide gels with SYBR® Green (Sigma-Aldrich, Switzerland), visualized with the Safe Imager™ (Invitrogen, Switzerland) and photographed. The length of the restriction fragments was considered relative to that of DNA markers: 100 bp DNA TrackIt™ ladder and 50 bp DNA TrackIt™ ladder (Invitrogen, Switzerland) and compared with theoretical reference values (Table 3).

Available *Acetobacteraceae* 16S rRNA gene sequences from the GenBank database (http://www.ncbi.nlm.nih.gov) were aligned using the online ClustalW multiple alignment tool from the server of the Swiss EMBnet node (http://www.ch.embnet.org), and the consensus sequence was subjected to virtual amplification using the iPCR tool available on the same server. Theoretical restriction profiles were obtained by *in silico* digestion of the consensus amplification product, using the Genome Restriction Analysis tool available on the SGD server (http://www.yeastgenome.org).

2.9. Sequence annotation

Amplification products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and used as a template to obtain the par-

Table 2. Primers used in PCR amplification reactions in this study.

PCR primers		
Primer	**Sequence**	**Reference**
16Sd	5'– GCTGGCGGCATGCTTAACACAT– 3'	Ruiz et al. 2000
16Sr	5'– GGAGGTGATCCAGCCGCAGGT – 3'	Ruiz et al. 2000
ITS1	5'– ACCTGCGGCTGGATCACCTCC – 3'	Ruiz et al. 2000
ITS2	5'– CCGAATGCCCTTATCGCGCTC – 3'	Ruiz et al. 2000
ADHfor	5'– TSGATGCSAAGACCGG – 3'	Trček 2005
ADHrev	5'– CCCCAGCCCACTTCAAC – 3'	Trček 2005
Sequencing primers for the complete 16S rRNA gene		
Primer	**Sequence**	**Reference**
Pfor2	5'– GACTAGAGTGTGAGAGAG – 3'	This study
Prev2	5'– ATGTCAAGCCCTGGTAAG – 3'	This study
Pfor3	5'– TCAGACCAGCTATCGATC – 3'	This study
Prev3	5'– TGGGGATGACGTCAAGTC – 3'	This study
Pfor4	5'– CAACTAGCTAATCGAACG – 3'	This study
Prev4	5'– GAAGCCAGGTAGCGATCA – 3'	This study

Table 3. RFLP-PCR of 16S rRNA profiles (in base pairs, bp) and identification strategy. Identified species are in bold.

Gluconacetobacter sp.			
Enzyme	**Experimental restriction pattern**	**Theoretical restriction pattern**	**Bacterial species**
TaqI	650, 360, 220, 180, 50	653, 362, 217, 177, 43	***Ga. europaeus***, *Ga. xylinus, Ga. hansenii, Ga. intermedius, Ga. oboediens*
HaeIII	540, 210, 210, 205, 180, 150, 80, 70	538, 206, 203, 179, 151, 75, 66, 34	***Ga. europaeus*** (DSM 6160^T)#, *Ga. xylinus, Ga. intermedius, Ga. oboediens*
MspI	450, 410, 210, 200, 100, 60	445, 415, 210, 206, 102, 53, 11	***Ga. europaeus***, *Ga. xylinus*
BccI	930, 400, 120	932, 402, 118	***Ga. europaeus***, *Ga. intermedius, Ga. oboediens*
Acetobacter sp.			
Enzyme	**Experimental restriction pattern**	**Theoretical restriction pattern**	**Bacterial species**
TaqI	500, 380, 360, 220	497, 376, 361, 216	***A. pasteurianus, A. pomorum***
HaeIII	540, 280, 180, 160, 150, 70	538, 280, 179, 158, 150, 66, 45, 34	***A. pasteurianus, A. pomorum***, *A. aceti, A. malorum, A. orleanensis, A. oeni, G. oxydans*
MspI	440, 420, 210, 120, 100, 60	444, 415, 210, 110, 106, 101, 53, 11	***A. pasteurianus, A. pomorum***, *Ga. intermedius, Ga. oboediens, Ga. hansenii*

The reference strain *Ga. europaeus* LMG 18494 showed a different restriction pattern: 538, 281, 203, 179, 151, 66, 34 (bp)

tial sequences of the three genomic region: 16S rRNA gene, 16S-23S ITS and partial *adhA*. (Fasteris S.A. sequencing service). The previous PCR primers were used for sequencing of the three genomic regions. Additionally to obtain the complete 16S rRNA gene sequence the use of internal primers were necessary (Microsynth sequencing service). PCR and internal sequencing primers are listed in Table 2. Sequencing reactions were performed in triplicate. Consensus sequences were deposited in GenBank with the following accession numbers: FJ715621, FJ715622, FJ715623, FJ715624, FJ715625, FJ715626, FJ715627, FJ715628, FJ715629, FJ715630, FJ715631, FJ715632, FJ865208, FJ865209, FJ865210, FJ865211, FJ865212, FJ865213.

2.10. Phylogenetic analysis

Acetobacteraceae 16S rRNA gene sequences were retrieved unaligned from the ribosomal database project (RDP) server (Cole et al. 2007), by selecting only those cultured type strains which were ≥1200 nucleotides long and of good quality. The 16S rRNA gene sequences of *Granulibacter bethesdensis* and *Neoasaia chiangmaiensis* were obtained from the EMBL database (Cochrane et al. 2009). Also from EMBL, *Acetobacter* and *Gluconacetobacter* sequences were retrieved for *adhA* and 16S-23S loci.

A multiple alignment was made for each of the three loci, which included the clones we sequenced as well as representative sequences of the *Acetobacteraceae* family (16S) or of the *Acetobacter* and *Gluconacetobacter* genera (16S-23S and *adhA*). The sequences were aligned using MUSCLE v3.7 (Edgar 2004) (16S and *adhA*) or ClustalX v2.0.10 (Larkin et al. 2007) (16S-23S) and the resulting multiple alignments were corrected manually when needed. The 16S-23S locus includes different regions of high divergence, which could not be aligned precisely even at the genus level. They were removed using Gblocks v0.91b (Castresana 2000) with the parameters set to "allow smaller final blocks" and "allow less strict flanking positions".

The phylogenetic trees were constructed with the PHYLIP v3.68 package (Felsenstein 1989). The goal here was not to decipher the evolutionary relationship of yet unclassified species, but to place our samples in a tree of characterized sequences. The three resulting dendograms were checked for being compatible with *Acetobacteraceae* evolution as described in literature (Yamada et al. 1997; Yamada and Yukphan 2008).

A first tree was obtained with the Neighbor-Joining algorithm, from the matrix of sequence distances calculated under the F84 nucleotide substitution model. The tree topology was assessed by the bootstrap re-sampling method with 1'000 replicates. Branch nodes having a bootstrap value >70% were considered as significant. The result was a tree in which the branch lengths were proportional to bootstrap values, not to evolutionary distances. A second tree was thus computed, using the Maximum-Likelihood algorithm and the topology of the tree obtained previously, and the significant bootstrap values were reported on it.

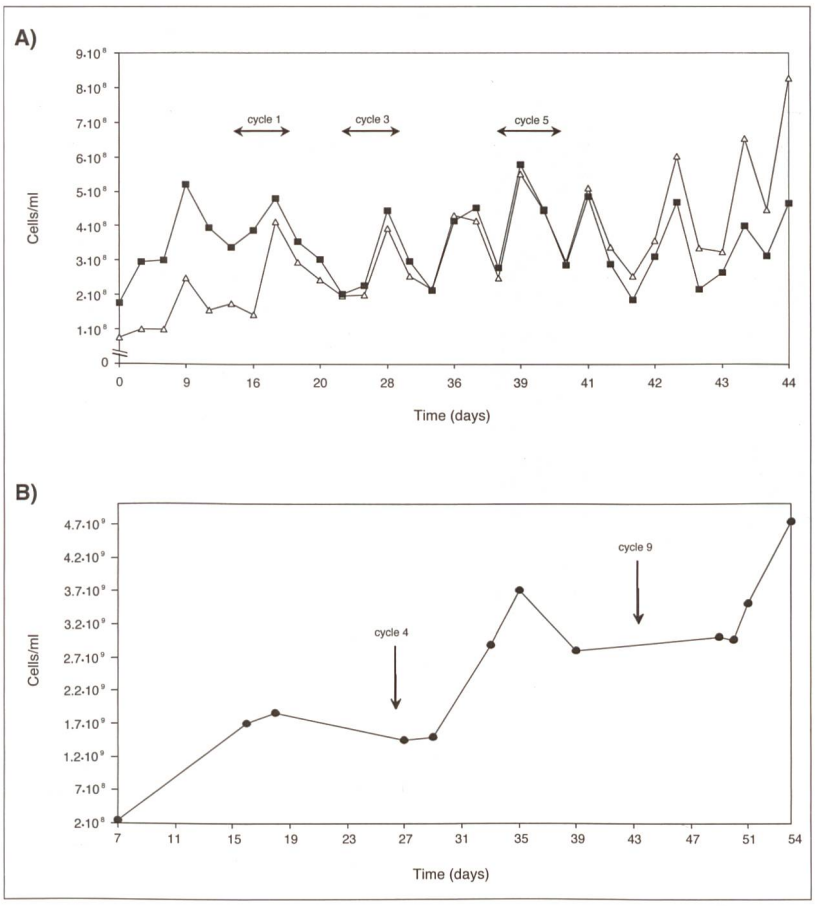

Fig. 2. A) Evolution of the cell viability during experiment 1. Closed squares correspond to non-viable cells, and open triangles correspond to viable ones. B) Evolution of the total cell number during experiment 2.

3. Results

3.1. General performance of the acetic acid fermentations process

Submerged productions of vinegar carried out in a semi-continuous manner are presented in Figure 1. The first experiment started from a wine-vinegar mixture in ideal conditions: 6% AcH/4% EtOH and showed a lag phase of about 9 days (Fig. 1A). During this adaptation period no "fermentative" activity was observed. Production of acetic acid, as shown by a progressive increase in acidity, and the preparation phase took 5 additional days, until the first cycle was considered to begin. The following cycles showed an important increase in the acetification rate, reaching to a maximum value of 0.2-0.3% AcH h^{-1}, with a maximum acetic acid concentration of 9-10% AcH, at the end of the process. The acetification was manually stopped after cycle 9.

The second acetification experiment started from a mixture in a priori non-ideal conditions: 4% AcH/6% EtOH (De Ory et al. 2002), and surprisingly showed a shorter lag phase, of only 4 days (Fig. 1B). The maximum acetic acid concentration (11%) was reached at the end of cycle 6. This cycle presented also the highest acetification rate, of 0.16% AcH h^{-1}. This experiment was manually stopped after cycle 12.

Although non-viables cells were predominant in the raw materials and at the beginning of both acetifications, the number of viable ones progressively increased during the process. Viable cells were finally predominant from the latest cycles before the manual arrest of the processes (Fig. 2A). The number of total cells exhibited a stationary phase at the beginning of the process, but increased throughout the cycles, evolving from an order of 10^8 cell/ml at the beginning of the process, to an order of 10^9 cell/ml at the end (Fig. 2B). Cell count showed that non-viable cells were predominant at the beginning of both acetifications, during the adaptive phase, but the number of viable cells progressively increased during the process, and exceeded the non-viable ones at the moment of arrest (data not shown). The number of total cells exhibited a stationary phase at the beginning of the process, but increased throughout the cycles, evolving from an order of 10^8 cell/ml at the beginning of the process, to an order of 10^9 cell/ml at the end (data not shown).

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

PCR primers for the 16S rRNA gene (Table 2) led to an amplification product of ~1450 bp. Restriction of amplicons with the endonucleases *TaqI*, *MspI* and *HaeIII* (González et al. 2006) (Fig. 3), clustered all samples in two groups. 8 isolates from experiment 1 and 2 from experiment 2 clustered in a group formed by two AAB species: *Acetobacter pasteurianus/Acetobacter pomorum*. All other isolates clustered in the group formed by *Gluconacetobacter europaeus/Gluconacetobacter xylinus*. The additional restriction of the amplicons clustered in the genus *Gluconacetobacter* with *BccI* endonuclease (Torija et al. 2010), resolved them as belonging to the species *Ga. europaeus*. The restriction patterns obtained for both type of isolates perfectly matched with the ones of the correspondent reference strains (Table 3).

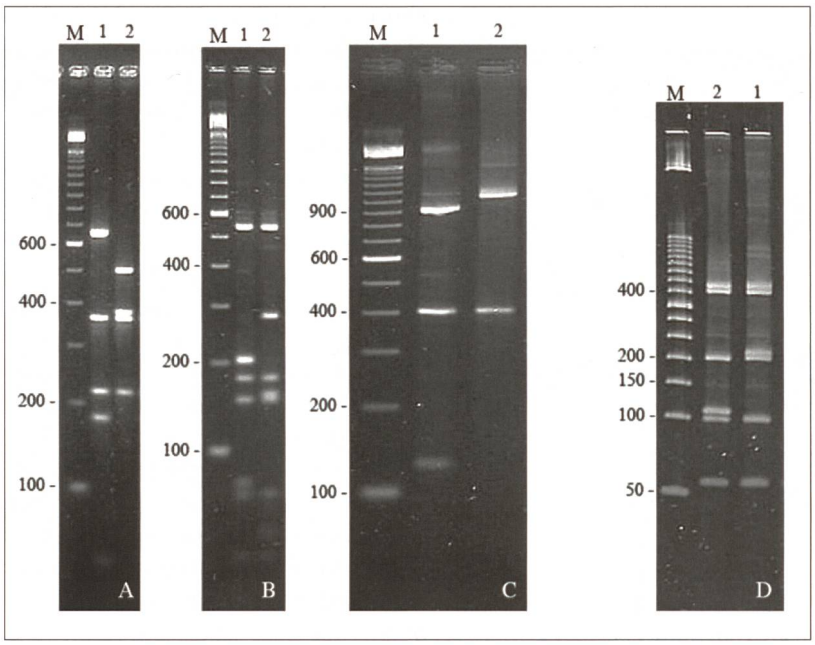

Fig. 3. RFLP-PCR analysis for the 16S rRNA gene. Restriction with TaqI (A), HaeIII (B), BccI (C) were visualized on 3% agarose gel electrophoresis. Restriction with MspI (D) was visualized on 8% polyacrylamide gel electrophoresis.
1 = Gluconacetobacter *type isolate.*
2 = Acetobacter *type isolate. M = 100 bp DNA TrackIt™ ladder (A, B, C) and 50 bp DNA TrackIt™ ladder (D).*

3.4. Analysis of 16S rRNA gene, 16S-23S ITS and partial adhA gene sequences

Three representative isolates belonging to each genus were selected to obtain the complete sequence of the 16S rRNA gene (~1450 bp), the partial 16S-23S ITS region (~750 bp), and the partial *adhA* gene (~600 bp).

All *Acetobacter* isolates (3P3e3, 7P3e3, 3P3e7) showed identical sequences for 16S rRNA and partial *adhA* genes, but for the 16S-23S ITS region 3P3e7 had more than 360 divergent nucleotides (Table 4). The 16S rRNA gene sequences were 99% identical to sequences of *A. pasteurianus* and *A. pomorum*. The partial *adhA* gene was also 95-100% identical to sequences of *A. pasteurianus* (Fig. 4A,B). Analysis of the 16S-23S ITS region seemed to separate isolates in two groups. 3P3e7 clustered in the *Acetobacter* branch showing a 98% similarity with *A. pasteurianus* sequences, whereas 3P3e3 and 7P3e3 clustered in the *Gluconacetobacter* group. The ITS sequences of these two isolates also shared 98% similarity with sequences of *Ga. europaeus* strains (Fig. 4B).

The three *Gluconacetobacter* isolates (1P1, 4P2, 5P3) shared identical sequence in all cases. The 16S rRNA gene sequence showed the highest degree of similarity with several sequences of *Ga. europaeus* and *Ga. xylinus*, with 98-99% of identical residues (Fig. 4A). The 16S-23S ITS sequence was 97% similar to different sequences of *Ga. euroapeus* (Fig. 3B). Finally, the partial *adhA* gene sequence obtained was 99%-100% similar to sequences of *Ga. europaeus* (Fig. 4C).

3.5. Population dynamics of acetic acid bacteria in fermenter

A quick imposition of the *Gluconacetobacter* species over the *Acetobacter* was observed in both processes. The starting mixture for the acetification experiment 1 was composed of *Ga. europaeus* and *A. pasteurianus* in a ratio of 1.5:1 (Fig. 1A,*), but this proportion changed in a drastic manner during the lag phase, leading to a predominance of *Ga. europaeus* (96%) after the start of the acetic acid production (Fig. 1A,#). *A. pasteurianus* was also observed after the acetification stoppage during the cycle 3, and again at the beginning of cycles 5, 6 and 7 (Fig. 1A,θ,Δ), in a very low proportion (0.5-1%). In contrast, the starting mixture for the second experiment was composed of 99% *A. pasteurianus* (Fig. 1B,*), descending to 88% at in the middle of the cycle

Table 4. Sequence difference count matrix for the 16S rRNA, 16S-23S ITS and ahdA loci of the three Acetobacter isolates. Matrix was obtained using the BioEdit Sequence Alignment Editor version 7.0.9.0 (Hall 1999).

Acetobacter	16S rRNA gene			16S-23S ITS region			*adh*A gene		
isolates	3P3e3	7P3e3	3P3e7fwd*	3P3e3	7P3e3	3P3e7fwd*	3P3e3	7P3e3	3P3e7fwd*
3P3e3	—	0	0	—	11	367	—	0	0
7P3e3	0	—	0	11	—	370	0	—	0
3P3e7fwd	0	0	—	367	370	—	0	0	—

*forward sequence retrieved after several sequencing reaction attempts

1 (Fig. 1B,#). This strain was no longer observed during the rest of the acetification process (Fig. 1B).

3.6. Morphological characteristics by electron microscopy

Scanning electron microscopy showed an amorphous layer surrounding the AAB, when they were directly harvested from wine or vinegar samples (Fig. 5A-D). This layer was absent in bacteria grown on synthetic media (Fig. 5E) and could be removed by the treatment with 0.6 N or 0.3 N NaOH solution (Fig. 5F), suggesting its polysaccharide nature. Transmission electron microscopy, following PATAg staining, showed a strong electron dense layer surrounding the plasma membrane, suggesting that it is composed of polysaccharides (Fig. 6A-D). As mentioned above, this electron-dense layer was absent in samples grown on synthetic media (Fig. 6E) and samples treated with NaOH solution (Fig. 6F).

Major morphological changes in the bacterial cells were also observed during acetification. The cells changed from ovoid cells (0.6-1.0 μm) at the beginning of the acetification, to rods (2.0-4.0 μm) at the end of the process. The analysis of colonies grown on solid culture showed no morphological differences between the two identified AAB species (data not shown).

4. Discussion

4.1. General performance and population dynamics

As shown in Figure 1, there is a high degree of similarity between the two acetification processes. Total and viable cell number increases with time in both cases. The maximum acetic acid concentration reached in the first and the second acetifications was very similar: 9-10% and 10-11% respectively (Fig. 1). This is consistent with the performance of the same AAB species in both cases, from the beginning of the cyclic vinegar production process. In our case, *Gluconacetobacter europaeus*.

The main difference between the two processes is the composition of the starting mixture. In the second experiment, the inoculum was not in the ideal conditions described by Sokollek and Hammes in 1997. Usually, an alcohol concentration of > 5.5% EtOH is toxic to most microorganisms, including AAB (Romero et al. 1994; De Ory et al. 2002; Mesa et al. 2003), and an acetic acid concentration of ~ 6% AcH

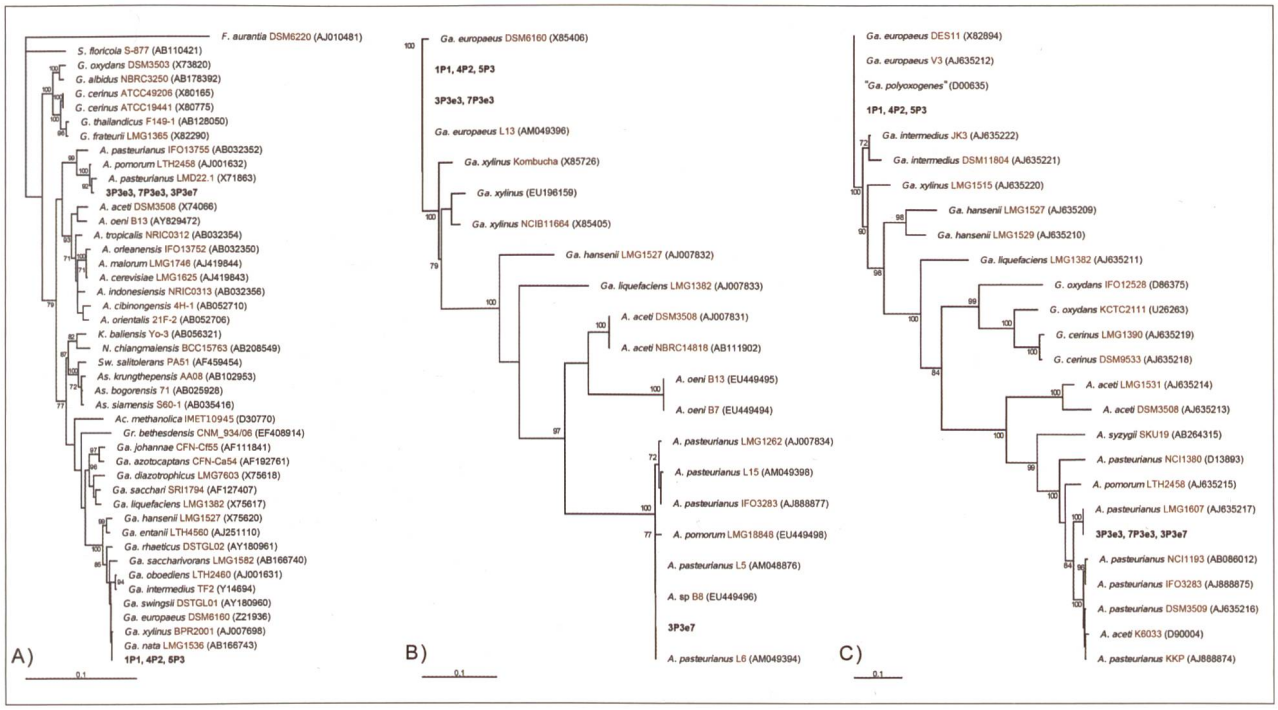

Fig. 4. Phylogenetic trees of AAB and vinegar isolates based on the 16S rRNA gene sequences from Acetobacteraceae *(A), 16S-23S ITS region from* Acetobacter *and* Gluconacetobacter *(B) and partial adhA gene from* Acetobacter *and* Gluconacetobacter *(C). Trees were constructed using the Neighbor-Joining and Maximum Likelihood algorithms. Accession numbers are in brackets. Significant bootstrap values (>70%) are indicated at branching points. The bar scale indicates the estimated sequence divergence in number of substitutions per position.*

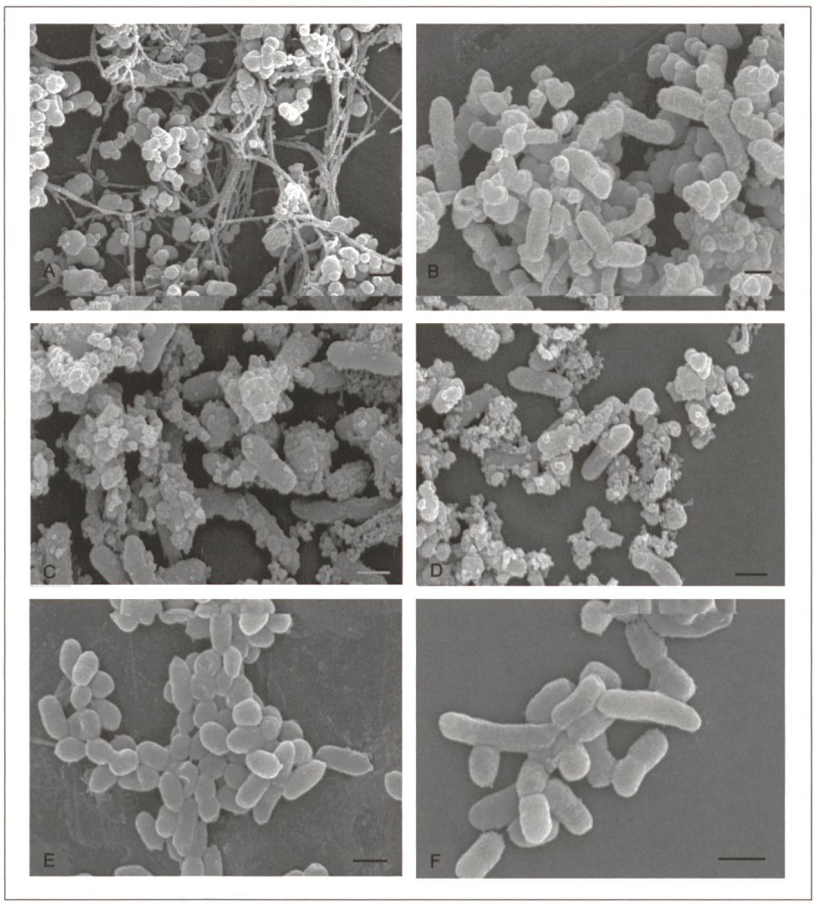

Fig. 5. Scanning electron microscopy of bacteria from different stages of the acetic acid fermentation processes. Starter vinegar: 8.2 %AcH/1.1 %EtOH (A), end of lag phase: 8.5 %AcH/1.3 %EtOH (B), beginning of a cycle: 7.2 %AcH/2.8 %EtOH (C), and end of a cycle: 10.2 %AcH/0.6 %EtOH (D). Acetic acid bacteria grown on solid media (E). Vinegar sample after treatment with 0.3 N NaOH solution (F). Cellulose-like fibres in starter vinegar are indicated by an arrow. Bar: 1µm.

is considered necessary to start the submerged vinegar production (Sokollek and Hammes 1997; Frings 1998). The 4% AcH and the 6% EtOH of the mixture led us expect a longer lag period before the start of the "fermentative" activity. But although both experiments started with the same total cell number, in a similar state of viability, surprisingly, in the second acetification, bacteria took half the time to initiate acetic acid production than in the first process. Due to the control and invariability of other physical parameters, this different behaviour must be assigned to the nature of the AAB strains involved in each acetification experiment. Different AAB strains have different physiological characteristics and do not behave in the same manner under the same physico-chemical conditions.

Sokollek and Hammes (1997) established that inocula to be used as starter material in industrial submerged vinegar productions had to have an ideal acid and alcohol content of 6% and 4% respectively. Additionally, AAB taking part in industrial vinegars were identified as belonging to the genus *Gluconacetobacter*. More specifically, *Ga. europaeus* was described as the main component of industrial vinegar fermenters in central Europe (Sievers et al. 1992). This species is usually related with high acid vinegars produced by the submerged method, with a final acidity up to 10-15% AcH (Sievers et al. 1992; Yamada 2003; Trček et al. 2007; Fernández-Pérez et al. 2010). On the contrary, *A. pasteurianus* is usually related with wine-making, found on grapes, musts, and also in traditional surface vinegars of low acidity, up to 6-7% AcH (Joyeux et al. 1984; Drysdale and Fleet 1988; Du Toit and Lambrechts 2002; Du Toit and Pretorius 2002; Yamada 2003; González et al. 2004; Bartowsky and Heschke 2008, Vegas et al. 2010). This alcohol tolerant strain should not resist the high acetic acid concentrations of industrial vinegars. A similar population dynamics was observed by Hidalgo et al. (2010) during the submerged production of vinegar.

Our results suggest that the "ideal" composition of the starting material, regarding acetic acid and ethanol content, must be revised and related to the AAB strain taking part in the process. A starting mixture at 6% AcH/4% EtOH can be suitable for acetifications using *Gluconacetobacter* strains, but industrial processes could also be launched from mixtures at 4% AcH /6% EtOH if *Acetobacter* strains are used.

The second acetification experiment was designed from a raw vinegar that has originally a high alcohol content (5.6% AcH/7.1% EtOH). The alcoholic degree of this vinegar is consistent with the extremely high proportion of *A. pasteurianus* (99%) in the starter mixture. The more suitable explanation for the shorter lag phase in this process is the fermentative activity due to this alcohol tolerant AAB in the starter mixture. The high alcohol content in this case would favour the metabolic activity of this alcohol tolerant strain, which would rapidly start the production of acetic acid.

During both ongoing processes, *A. pasteurianus* would stress because of the increasing acidity and would slow down its metabolic activity, whereas the activity of the *Gluconacetobacter* strain would

increase with the increasing acidity. The enzymatic activity of the pyrroloquinoline quinone (PQQ)-ADH in *Ga. europaeus* is higher than the activity of this enzyme in *Acetobacter* strains (Trček et al. 2007).

The cultivability of AAB from vinegar has always been a very hard task, especially when they are harvested from submerged high acid vinegar (Entani et al. 1985; Sievers et al. 1997; Schüller et al. 2000). In general, cell viability expressed in CFU/ml decrease concomitantly with the increase in acetic acid content (Matsushita et al. 2005b). Because of that, it seems reasonable to hypothesize that when the acetic acid content increases in the acetification broth, *A. pasteurianus* enters a non-viable or non-culturable state. This strain, which grows more slowly than *Ga. europaeus* in high acid conditions, would become unculturable, thus unidentified by culture dependent techniques. It would finally be washed up after successive removals of final product and additions of fresh sterilized wine. Surprisingly, the organism seems to have enough energy to reactivate its metabolism when acidity decreases at the beginning of several cycles, when acidity decreases below 7% AcH. This way, it seems logical to hypothesize why *A. pasteurianus* was not found along the vinegar production cycles of the acetification experiment 2, because the beginning of all cycles showed an acidity > 7.2AcH.

4.2. Molecular identification

As already reported (Boesch et al. 1998; Sokollek et al. 1998; Trček and Teuber 2002; Trček 2005; González et al. 2006) RFLP-PCR or sequence analysis of the 16S rRNA gene is not a suitable strategy to resolve some AAB species which share a high degree of similarity in their sequences (>99%). Some examples are *A. aceti/A. cerevisiae*, *A. pasteurianus/A. pomorum*, *Ga. europaeus /Ga. xylinus*, *Ga. intermedius/Ga. oboediens*. To resolve these groups of species, the use of other additional DNA based targets is required.

With the exception of the $tRNA^{Ile}$ and $tRNA^{Ala}$ coding genes and the antitermination boxB element, the 16S-23S ITS region is highly divergent among AAB species (Sievers et al. 1996; Trček and Teuber 2002; Krevotá and Grones 2005; Kommanee et al. 2008). The 16S-23S ITS region is more divergent than the 16S rRNA gene and its analysis has been demonstrated to be suitable in many cases for the differentiation of very closely related species (Trček and Teuber 2002; González et al. 2005; Trček 2005). The sequence analysis of this region in the selected *Acetobacter* isolates produced an unexpected result. The 16S-23S ITS sequence of 3P3e3 and 7P3e3 were similar to *Ga. europaeus* species and clustered together in the phylogenetic tree (Fig. 4B), whereas 3P3e7 presented several difficulties in the sequencing reaction for this region. Several sequencing attempts resulted in a forward sequence that clustered the sample in the *A. pasteurianus* branch (Fig. 4B), but in a reverse sequence that included many unde-

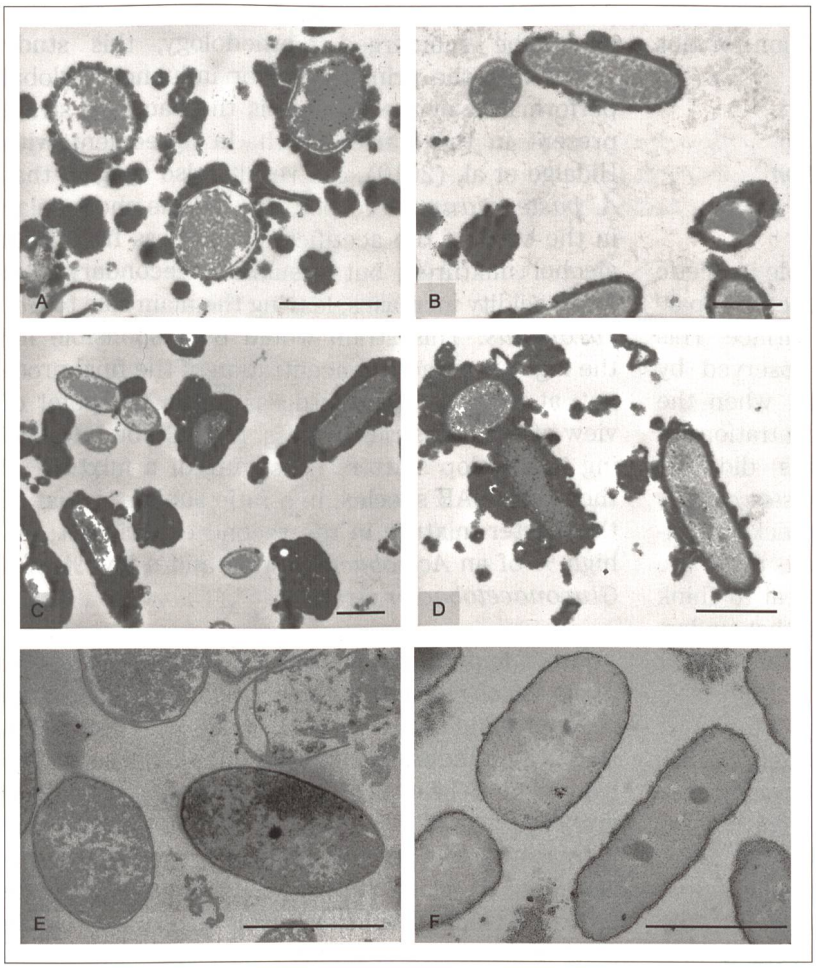

Fig. 6. PATAg staining of transmission electron microscopy sections of bacteria from different stages of the acetic acid fermentation processes. Samples include natural material as well as bacteria grown on solid media. Starter vinegar: 5.6 %AcH/7.1 %EtOH (A), end of lag phase: 8.5 %AcH/1.3 %EtOH (B), beginning of a cycle: 6.2 %AcH/3.5 %EtOH (C), and end of a cycle: 9.5 %AcH/0.4 %EtOH (D). Acetic acid bacteria grown on solid media (E). Vinegar sample after treatment with 0.3 N NaOH solution (F). Bar: 1 μm.

termined positions (N), giving an indeterminate sequence pattern that was consistent with a mixture of templates. The reason why sequencing did not work might be the formation of secondary structures in template. The analysis of this region is helpful when it shows sufficient degree of divergence, but when exhibits excessive sequence variability, the situation might also be complicated due to the presence of different spacer types within the same organism. In our particular case, the most suitable explanation to the former 16S-23S ITS sequence analysis is that the 3P3e7 *Acetobacter* isolate must have at least two types of intergenic sequences and at least two copies of the RNA operon. The ITS sequence in the first type operon should be similar to that of *A. pasteurianus* species, whereas the spacer region in the second type operon should have a sequence similar to that described for *Ga. europaeus* species. Additional analysis would be necessary to accurately establish whether it is indeed the case. The rest of selected *Acetobacter* and *Gluconacetobacter* isolates seem to share the same 16S-23S ITS sequence, highlighting a certain degree of interspecies conservation for this genomic region.

4.3. Morphological characteristics by electron microscopy

A variable that may play an important role in acetic acid resistance is the morphology of bacteria, particularly in relation to the membrane surface. This change in morphology was already observed by Trček et al. (2007) in *Ga. europaeus*, when the strain was subjected to increasing concentrations of acetic acid. However, theses authors did not observe morphological changes in *A. pasteurianus* under the same conditions. Due to the quick imposition of the *Gluconacetobacter* strain over the *Acetobacter* in our study, it seems logical to think that it is the *Gluconacetobacter* strain that undergoes the morphological change observed in vinegar samples.

Our hypothesis is that the increase in cell surface allows bacteria to augment the number of membrane transporters that have been proposed to participate, among other mechanisms (Fukaya et al. 1990, 1993; Sievers et al. 1997; Nakano et al. 2004; Mullins et al. 2008), in the acetic acid resistance in the *Acetobacteraceae* family. Once acetic acid is produced, it tends to accumulate in the broth and diffuse into the cell. A proton motive-force-dependent efflux system and a putative ABC transporter (Matsushita et al. 2005a; Nakano et al. 2006) have been described to take part in the export of the excess of acetic acid accumulated into the bacterial cytoplasm.

The irregular amorphous layer that surrounds bacteria when harvested directly from vinegar, identified cytochemically as polysaccharides, has already been reported by our group (Barja et al. 2003; Bey-Ruiz et al. 2006). The fact that this layer is absent when bacteria have been grown on synthetic media may indicate their implication in the mechanism of resistance to acetic acid. The relationship between capsular polysaccharides (CPS) and acetic acid resistance has also been proposed by Deeraksa et al. (2005), and preliminary results from our group demonstrated that *Ga. europaeus* has a higher amount of CPS than *A. pasteurianus*, when both strains were grown under the same conditions (Andrés-Barrao and Barja 2008). This property can contribute to the different innate resistance to acetic acid presented by these AAB strains.

5. Conclusion

Among the parameters affecting vinegar production using the submerged methodology, this study shows that the principal factor influencing global performance of the process is the bacterial strain present in the starter broth. In agreement with Hidalgo et al. (2010), our results also suggest that *A. pasteurianus* is responsible for the short delay in the start of the acetification process from high alcohol mixtures, but assume a secondary role when acidity increases, leaving the main role to *Ga. europaeus*. This strain would be responsible for the high acetic acid concentration of the final product, at the end of the process. From the point of view of the industrial process, it would be interesting to develop starters consisting of a mixture of these two AAB species, in a ratio similar to that of the starter mixture in the second experiment; i.e. high % of an *Acetobacter* strain and a low % of a *Gluconacetobacter* strain.

Another important observation is that *A. pasteurianus* becomes undetectable when the acetic acid concentration in the broth increases; but detectable again when the acetic acid content decreases, at the beginning of the cycles. Taking into account the general reduction of the cultivable cell number with the increasing acetic acid concentration, we can hypothesize that *A. pasteurianus* enters a metabolic state of arrest leading to a non-viable or non-culturable state in conditions of acidity >6.5-7%.

We have also shown that the increase in acetic acid concentration causes a change in the bacterial morphology, probably due to changes in the membrane composition, leading to the augmentation of the membrane surface and the increment in the efflux-pump system. Finally, our observations suggest that

the polysaccharide layer synthesized for bacteria in the "wild" environment should provide a resistant barrier against the aggressiveness of the high acetic acid concentration in the medium.

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