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Metaproteomics and ultrastructure characterization of *Komagataeibacter* spp. involved in high-acid spirit vinegar production

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A B S T R A C T

Acetic acid bacteria (AAB) are widespread microorganisms in nature, extensively used in food industry to transform alcohols and sugar alcohols into their corresponding organic acids. Specialized strains are used in the production of vinegar through the oxidative transformation of ethanol into acetic acid. The main AAB involved in the production of high-acid vinegars using the submerged fermentation method belong to the genus *Komagataeibacter*, characterized by their higher ADH stability and activity, and higher acetic acid resistance (15–20%), compared to other AAB.

In this work, the bacteria involved in the production of high-acid spirit vinegar through a spontaneous acetic acid fermentation process was studied. The analysis using a culture-independent approach revealed a homogeneous bacterial population involved in the process, identified as *Komagataeibacter* spp. Differentially expressed proteins during acetic acid fermentation were investigated by using 2D-DIGE and mass spectrometry. Most of these proteins were functionally related to stress response, the TCA cycle and different metabolic processes. In addition, scanning and transmission electron microscopy and specific staining of polysaccharide SDS-PAGE gels confirmed that *Komagataeibacter* spp. lacked the characteristic polysaccharide layer surrounding the outer membrane that has been previously reported to have an important role in acetic acid resistance in the genus *Acetobacter*.

1. Introduction

Acetic acid bacteria (AAB) differ from other microorganisms by their capability of transforming alcohols and sugar alcohols into the corresponding organic acids. This characteristic has been exploited for the production of different fermented foods and beverages. Strains from the genera *Acetobacter* and *Komagataeibacter* are involved in the production of vinegar by the oxidative transformation of ethanol into acetic acid. This oxidation is a two-step reaction catalyzed by the two enzymes – alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) (Adachi et al., 1980; Ameyama and Adachi, 1982) that are located in the inner-cytoplasmic membrane.

Strains of the genus *Acetobacter* are damaged when acetic acid concentration reaches 7–8%. In contrast, the strains from the genus *Komagataeibacter* are able to resist up to 15–20% acetic acid (Yamada, 2003). Because of this property, *Acetobacter* strains are responsible for the traditional surface production of vinegar, in which the final acidity does not normally exceed 8%. In some cases, acetic acid fermentation by *Acetobacter* spp. may achieve a maximum acidity up to 9–10%, as in a recent report where *Acetobacter pasteurianus* could be used for acetic acid production using a two-stage aeration protocol and a maximum titer of 93.09 ± 0.24 g/L acetic acid was achieved (Qi et al., 2014). By contrast, *Komagataeibacter* strains are involved in fermentation processes that are carried out by the modern submerged methodology (Gullo and Giudici, 2008; Gullo et al., 2009; Vegas et al., 2010, 2013; Hidalgo

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et al., 2010; Fernández-Pérez et al., 2010a, 2010b; Andrés-Barrao et al., 2011a). The submerged fermentation process is currently favored in industrial vinegar factories, because of the rapid acetification rate and the high concentration of acetic acid obtained.

Ecological studies performed on running acetic acid fermentations have shown that the bacterial population involved in traditional surface processes are much more diverse than those responsible for industrial submerged processes (Vegas et al., 2010; Hidalgo et al., 2010; Fernández-Pérez et al., 2010a, 2010b; Hidalgo et al., 2013a; Hidalgo et al., 2013b). Although some species of *Gluconobacter* and *Komagataeibacter* were identified, most strains found in traditional fermentation processes belong to the genus *Acetobacter*. The AAB population varies during the fermentation in a dynamic manner that favors the development of the species that are better adapted to higher acetic acid concentrations (Vegas et al., 2010; Andrés-Barrao et al., 2011a). These studies suffered the drawback of the quick loss of cultivability of AAB growing in high-acid environments; more particularly from vinegars with >10% acetic acid (Trček et al., 2007; Fernández-Pérez et al., 2010a). In spite of the cultivability problems, several strains of the genus *Komagataeibacter* have been isolated during the production of high-acid vinegars by the submerged methodology, being *Komagataeibacter europaeus*, *Komagataeibacter oboediens*, *Komagataeibacter hansenii* and *Gluconacetobacter entanii* (species taxonomically included in the genus *Komagataeibacter*) among the most prominent species (Sievers et al., 1992; Schüller et al., 2000; Trček et al., 2000; Yamada et al., 2012).

Few studies have reported the modification of the expressed proteins in AAB growing in the presence or absence of acetate. The result led to the identification of 8 acetate-specific stress proteins (Aps) in either *Acetobacter aceti* or *Gluconobacter oxydans*, of which only 3 were found to be common to both species (Lasko et al., 1997). A recent study on the adaptation to acetate of *A. aceti* when grown in batch or continuous cultures revealed >50 specifically-induced proteins. These proteins were classified into the following three categories: (i) acetate adaptation proteins (Aaps) (induced by acetate in both the batch and continuous cultures), (ii) acetate-induced proteins found in either batch or continuous cultures and (iii) general stress proteins (Steiner and Sauer, 2001). Aconitase (Acn) (an enzyme of the TCA cycle) and a putative ABC-transporter were also identified as two of the most responsive proteins involved in the acetic acid resistance of *A. aceti* (Nakano et al., 2004, 2006).

Similarly, during acetic acid fermentation, researchers have reported modifications of the membrane polysaccharides in *Acetobacter* spp. (Deeraksa et al., 2005, 2006; Kanchanarach et al., 2010; Moonmangmee et al., 2002). Modification of the membrane lipids in *K. europaeus* and *Komagataeibacter intermedius* (Trček et al., 2007) has also been shown.

In this work, with the aim of investigating the main modifications of the bacteria physiology and main metabolic pathways active during a natural acetic acid fermentation, a spontaneous submerged acetic acid fermentation to produce high-acid spirit vinegar was surveyed and the bacteria involved were characterized at various levels. During the fermentation process, a number of physico-chemical parameters were routinely monitored, including ethanol and acetic acid content, aeration and acetification rate, as well as bacterial growth. The AAB population involved in the process was studied by using a culture-independent approach. The effect of adaptation to high-acidity in the bacterial physiology was analyzed by comparative proteomics using 2D-differential in-gel electrophoresis (2D-DIGE) (Andrés-Barrao et al., 2012). Finally, the ultrastructure of AAB cell wall was examined by scanning and transmission electron microscopy (SEM, TEM) and cell surface polysaccharides were analyzed by specific staining of SDS-PAGE electrophoresis.

2. Materials and methods

2.1. Acetic acid fermentation and culture conditions

Acetic acid fermentation was performed in a 10 L pilot acetator (Frings Co., Bonn, Germany), as described by Andrés-Barrao et al. (2011a). Four liters of spirit "seed vinegar" were collected from running acetic acid fermentation in an vinegar industry in Geneva (Switzerland) and used as inoculum (3.8% ethanol and 10% acetic acid). The microbial characteristics of the seed vinegar were unknown at this stage of the process. The fresh mash consisted of filter sterilized ethanol (Reactolab, Switzerland) and sterilized tap water containing 0.5 g/L of nutrient concentrate NUTRACET® complete (Cetotec Biotechnologie GmbH, Germany). The seed vinegar was mixed with sterile tap water and filter sterilized ethanol to obtain 5 L of starting fermentation broth with 4% ethanol (EtOH) and 8% acetic acid (AcH). During the preparation and the cyclic production phases, the process was carried out as described by Andrés-Barrao et al. (2011a). Fresh mash was added during the preparation phase and the working volume was gradually increased until the maximum capacity of the acetator, 8 L, was achieved, and the first cycle of the vinegar production phase started.

The progress of the acetic acid fermentation was monitored by measuring the residual alcohol (% EtOH) by ebulliometry (Dujardin-Salleron, France) and the total acidity by titration with 0.1 N NaOH. Total biomass during the process was determined by optical density at 600 nm (OD_{600}) in a Biowave WPA II UV/Visible Spectrophotometer (Biochrom, UK).

During the preparation phase as well as the cyclic production, 100 μl of fermentation broth were inoculated on YPM (0.5% yeast extract, 0.3% peptone, 2.5% mannitol, 1.5% agar) and RAE (1% yeast extract, 4% glucose, 1% peptone, 0.338% $Na_2HPO_4.2H_2O$, 0.15% citric acid$\cdot H_2O$, 2% (w/v) acetic acid, 1% (w/v) ethanol; bottom: 1% agar, top: 2% agar) media. Sampling points during the vinegar production phase included initial, middle and end points of each cycle. Reference *Acetobacter* and *Komagataeibacter* strains purchased from collection culture (*A. pasteurianus* LMG 1262^T, *A. pasteurianus* LMG 1513, *K. europaeus* LMG 18890^T, *K. europaeus* LMG 18494) and isolated in our laboratory (*A. pasteurianus* 3P3, *K. europaeus* 5P3, *K. europaeus* 4P2, *K. europaeus* TP1-23, *K. oboediens* 174Bp2), were also grown on YPM and RAE media.

To identify the AAB involved in the process, five vinegar samples were directly harvested throughout the acetic acid fermentation (Table 1) and analyzed by Denaturant Gradient Gel Electrophoresis (DGGE), Repetitive Sequence Based (rep-PCR) genomic fingerprinting and Multilocus Sequence Typing (MLST).

2.2. DNA extraction and PCR amplification reactions

Genomic DNA from bacteria directly harvested from vinegar and reference strains as well as isolates, was extracted using the GenElute™ Bacterial Genomic DNA Kit (Sigma–Aldrich, Switzerland), according to the manufacturer's instructions specific for Gram-negative bacteria.

PCR reactions were performed as follows: (DGGE)-PCR was performed using primers WBAC1 and WBAC2, specific to the V7–V8 variable region of the 16S rRNA gene of AAB (Table 2). To improve separation in DGGE conditions, a 40-GC clamp was added to the 5′-end of the WBAC1 primer and PCR reaction was performed as in Lopez et al. (2003). $(GTG)_5$-rep-PCR amplifications were carried out in a total volume of 25 μl comprising 22 μl of PCR mixture (15 pmol of GTG_5 primer (Table 2), 200 μM of each of the four dNTPs, 5 μl of 5XGB buffer (Rademaker et al., 1998; De Vuyst et al.,

Table 1
Spirit vinegar samples harvested during the fermentation process.

Sample	Time point (days)	Acetic acid fermentation phase	Acidity (%)	Ethanol (%)	Acetification rate (%/hr)
V1	10.02	Preparation	10.7	0.1	0.101
V2	12.50	Preparation	10.2	1.5	0.211
V3	14.54	End of preparation phase/Beginning of vinegar production	11.7	0.5	0.129
V4	25.65	Middle of vinegar production (cycle 4)	12.4	0.7	0.071
V5	31.77	End of vinegar production (cycle 7)	13.0	0.4	0.141

Table 2
Primers used in this study.

PCR program	Primer	Sequence (5′-3′)	Ref.
DGGE-PCR	WBAC1^{GC}	GTCGTCAGCTCGTGTCGTGAGA	Lopez et al., 2003
DGGE-PCR	WBAC2	CCCGGGAACGTATTCACCGCG	Lopez et al., 2003
GTG5-PCR	$(GTG)_5$	GTGGTGGTGGTGGTG	Rademaker et al., 1998
16S-PCR	16Sd	GCTGGCGGCATGCTTAACACAT	Ruiz et al., 2000
16S-PCR	16Sr	GGAGGTGATCCAGCCGCAGGT	Ruiz et al., 2000
HKG-PCR	dnaK-01-F	CTGCGCATCATCAACGAGCC	Cleenwerck et al., 2010
HKG-PCR	dnaK-02-R	CTCACGCTCGCCCTGATAGA	Cleenwerck et al., 2010
HKG-PCR	groEL-10-F	ACAAGTTCGAGAACATGGGC	Cleenwerck et al., 2010
HKG-PCR	groEL-11-R	TCCTTGCGCTCCTTCACCTC	Cleenwerck et al., 2010
HKG-PCR	rpoB-01-F	GATAACGGCACCTTCATCAT	Cleenwerck et al., 2010
HKG-PCR	rpoB-02-R	AGATTGTCGATATCGTCGAT	Cleenwerck et al., 2010

^{GC}GC-rich sequence (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3′).

2008), 5.4 μl of $MgCl_2$ 25 mM, 0.8 μl of BSA 10 mg/ml, 5 μl of DMSO and 2.5 U of Taq DNA polymerase (Sigma–Aldrich, Switzerland)) and 3 μl of DNA template (0.5–1.5 μg/μl). The thermocycler peqSTAR 96 Universal Thermocycler (PeqLab Biotechnologie GmbH, Switzerland) was programmed with an initial denaturation step at 94 °C for 5 min followed by 30 cycles with steps of 94 °C for 1 min, 40 °C for 1 min and 65 °C for 8 min, and a final extension of 16 min at 72 °C for final extension. The amplifications of the 16S rRNA gene were carried out using the specific primers 16Sd and 16Sr (Table 2), as described by Ruiz et al. (2000). Finally, the amplifications of the housekeeping genes *dnaK*, *groEL* and *rpoB* were carried out using the primers dnaK-01-F and dnaK-02-R, groEL-10-F and groEL-11-R, and rpoB-01-F and rpoB-02-R, respectively (Table 2), as described by Cleenwerck et al. (2010).

DGGE-PCR amplification products were analyzed by migration on denaturant 8% acrylamide-bisacrylamide 37.5:1, in TAE 1× buffer and a 35–65% urea-formamide gradient, in the Code Universal Mutation Detection System (BioRad, Hercules, CA). All other PCR products were detected on 0.8% agarose gel electrophoresis in TBE 1× buffer. Agarose gels were stained with GelRed™ or GelGreen™ (Biotium, US), visualized and photographed using the Safe Imager™ (Invitrogen, Switzerland).

2.3. DNA sequencing and phylogenetic analysis

The amplification products of 16S rRNA and housekeeping genes were purified using the Wizard® SV System Kit (Promega, USA), according to the manufacturer's instructions and Sanger sequencing was performed by Fasteris S.A. (Geneva, Switzerland) (www.fasteris.com). The obtained forward and reverse sequences were assembled and manually edited using BioEdit v7.1.3 (Ibis Bioscience, USA) (Hall, 1999). The final consensus sequences for each analyzed amplicon were deposited in GenBank/EMBL/DDBJ (Benson et al., 2011) under the accession numbers shown in the Fig. 3. To perform the phylogenetic analysis, the consensus sequences were imported into MEGA 5 package (Arizona State University) (Kumar et al., 2008; Tamura et al., 2011; Hall, 2013) and aligned with a set of reference sequences downloaded from public databases. Sequence alignment was done by using the MUSCLE multiple alignment algorithm (Edgar, 2004a, 2004b) and default parameters. Fifty-six 16S rRNA gene sequences from *Acetobacteraceae* strains and 37 sequences of the housekeeping genes *dnaK*, *groEL* and *rpoB* from *Komagataeibacter* strains were retrieved from the GeneBank database (www.ncbi.nlm.gov/nuccore). Unrooted phylogenetic trees were created by the Neighbor-Joining method (Saitou and Nei, 1987) and applying the Kimura 2-parameter correction model (Kimura, 1980). The reliability of the obtained groupings was evaluated by using the bootstrap test, with 1000 bootstrap replications (Felsenstein, 1985).

2.4. Proteomic analysis

With the aim to characterize the molecular events that are associated with the adaptation to high-acetic acid during acetic acid fermentation, we compared the global proteins expression patterns of the present vinegar bacteria under low-acid and high-acid conditions. For that, bacteria from two vinegar samples were directly harvested from the running process at 8.2 and 14.2% AcH respectively (open arrows in Fig. 1). Cell pellets were washed twice with 1× PBS, once with 5 mM Tris–HCl pH 8, and re-suspended in 1 ml of Protein 2D Lysis Buffer (8 M urea, 2 M thiourea, 30 mM Tris–HCl pH 8.5, 4% (w/v) CHAPS) and were incubated for 1 h at 4 °C. The samples were then sonicated at "high" (H) power (320 W) for 30 min using cycles of 20 s "ON" – 30 s "OFF" in a Bioruptor™ UCD-200 (Diagenode SA, Liège, Belgium) and centrifuged for 20 min at 4 °C. Supernatants were taken as crude protein extracts and were quantified by using the Bradford Quick Start Reagent (BioRad, Reinach BL, Switzerland). Aliquots of 50 mg were kept at −80 °C until use. 2D-DIGE analysis was performed as described in Andrés-Barrao et al. (2012). For protein characterization, the 2D-DIGE gels were silver-stained after fluorescence image acquisition following a protocol that is compatible with mass spectrometry (Stochaj et al., 2007). Unique spots were picked manually and identified by MALDI-TOF/TOF analysis (Alphalyse, Denmark) (www.alphalyse.com).

2.5. Transmission and scanning electron microscopy

For transmission and scanning electron microscopy (TEM, SEM), cultured reference bacteria (*A. pasteurianus* LMG 1262^T,

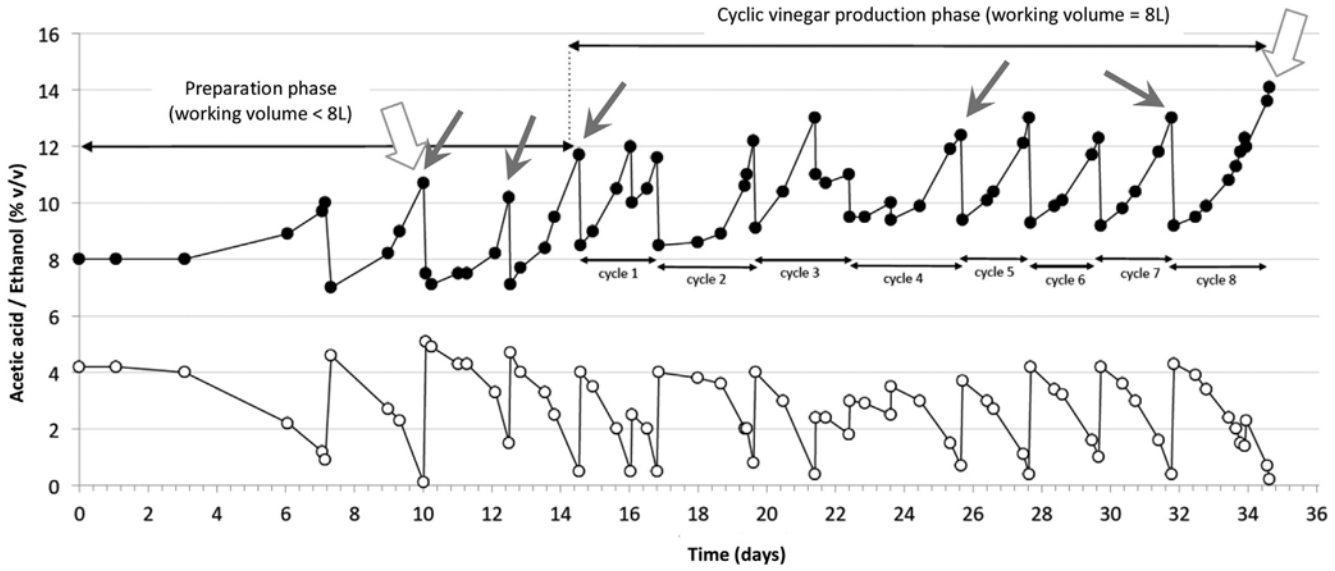

Fig. 1. Overview of the fermentation process. The closed circles line indicates the total acidity (%AcH), and the open circles line the alcohol strength (%EtOH) measured during the process. The preparation phase correspond to the period of time during which bacteria adapt to the fresh fermentation broth and start the acetification reaction, producing AcH and consuming EtOH. During this phase, when the ethanol content is between 0.5 and 0.2%, fresh alcoholic mass was added and the total reaction volume increased. The vinegar production phase started when the reaction volume reached a value of 8 L (in a 10 L fermentor). From this point, when ethanol content reached the minimum level, and before complete depletion, a third of the vinegar produced was withdrawn as final product and the volume was replaced by fresh mass, letting a new production cycle starts. Cyclic vinegar production was performed this way until the process was stopped at the end of cycle 8. Grey arrows indicate the vinegar sampling points for AAB molecular identification (Table 1). Open arrows indicate sampling points for 2D-DIGE analysis.

K. europaeus LMG 18890^T; *K. oboediens* 174Bp2), and vinegar samples were immediately fixed in 2.5% final concentration of glutaraldehyde for 1–2 h at room temperature. Samples were then processed as described in Andrés-Barrao et al. (2012). TEM sections were placed on gold grids and were stained for specific detection of polysaccharides using the periodic acid-thiocarbohydrazide-silver proteinate (PATAg) test (Thiéry, 1976). Ultrathin sections were examined at 120 kV in a TECNAI transmission electron microscope.

2.6. Cell surface polysaccharide analysis

Cell surface polysaccharides of frozen reference and vinegar bacteria samples were prepared by using the Quick Lysis method described in Le Quéré et al. (2006). Crude extracts were separated by 12% Trizma based SDS-PAGE (Sigma–Aldrich, USA) gels, which were then stained to visualize capsular polysaccharides (CPS) and lipopolysaccharides (LPS) (Corzo et al., 1991; Tsai and Frasch, 1982).

3. Results and discussion

3.1. Acetic acid fermentation and high-acid vinegar production

To ensure the maximal activity and fitness of bacterial cells, the spirit vinegar used as inoculum in the present study was harvested from a running industrial acetic fermentation during the exponential phase of an acetification cycle. As shown in Fig. 1, AAB started to produce acetic acid with the concomitant depletion of ethanol after an initial adaptation phase of about 3 days. Once the vinegar production phase started, after reaching a working volume of 8 L, acetification cycles were regular and reached acidity levels of up to 12–13%. The maximum acidity >14% was ultimately achieved at the end of cycle 8, when cell biomass showed a maximum of 4.10^5 cells/ml ($OD_{600} = 0.4$). The vinegar production was stopped at the end of this cycle.

In agreement with previous reports (Fernández-Pérez et al., 2010a; Sievers et al., 1992; Schüller et al., 2000; Trček et al., 2000; Entani et al., 1985; Sokollek et al., 1998), no bacterial growth on YPM or RAE agar plates was observed after incubation of samples harvested at various time intervals throughout the process. The lack of AAB growth in the present study, was probably due to the high acidity values observed from the beginning of the process (i.e. 8% in the starting mixture, 7–12% during the preparation phase, and 9–14% during the cyclic production phase). The high acidity together with the low pH and the high oxygenation rate typical of submerged cultures (~60 L air/min) must involved modifications of bacterial metabolism to adapt and survive in such extreme environment. The high acid and oxidative stress would not allow these sensitive microorganisms to grow outside the fermentation broth.

3.2. AAB molecular identification

To circumvent the absence of growth on agar plates, we used a culture-independent approach to identify and characterize the AAB species involved in the current acetic acid fermentation process. Samples at five time points were collected, at the preparation phase as well as at the beginning, middle and end of the vinegar production phase: V1–V5 (Table 1).

Unique DGGE amplification products were obtained from all the samples, V1 to V5, which were similar in size and distinct from those obtained from the reference strains: *A. pasteurianus* LMG 1262^T, *A. pasteurianus* 3P3, *K. europaeus* LMG 18890^T, *K. europaeus* LMG 18494, *K. europaeus* 5P3, *K. oboediens* 174Bp2 (Fig. S1 in Supplemental material). Further characterization by $(GTG)_5$-rep-PCR fingerprinting yielded similar fingerprints for all the samples (Fig. S2 in Supplemental material). These results suggested that the bacterial population responsible for the fermentation process was homogeneous and two possibilities arose: (i) a unique strain was consistently present throughout the acetic acid fermentation (clonal population) or (ii) a small number of closely related strains were stably maintained during the process.

In order to characterize vinegar bacteria to the genus/species

level, 16S-RFLP analysis of V1 to V5 samples was performed by enzymatic digestion using a set of 8 endonucleases. The result showed the same restriction pattern for all samples and identified AAB as *Komagataeibacter* spp (Fig. S3 in Supplemental material). The comparison of the observed endonuclease digestion pattern with those obtained *in silico* for reference AAB strains (Ruiz et al., 2000; González et al., 2006) tentatively identified the strain involved in the process as *Ga. entanii* or *K. hansenii* (Fig. S2 in Supplemental material). To verify this result and trying to unequivocally classify the AAB into one of the two former species, a phylogenetic analysis based on the 16S rRNA and MLST using concatenated sequences of *dnaK*, *groEL* and *rpoB* genes was performed. The analysis yielded congruent trees in which bacteria samples V1 to V5 formed a distinct branch within the genus *Komagataeibacter*, with *K. hansenii* and *Ga. entanii* as the closest relatives (Fig. 2).

So far, only few ecological studies have analyzed the bacterial population during submerged acetic acid fermentations by applying a culture-dependent approach. These reports identified *K. europaeus* as the only or main cultivable AAB species (Fernández-Pérez et al., 2010b; Andrés-Barrao et al., 2011a; Callejón et al., 2008). Several strains of *K. europaeus* and *K. intermedius* have also been reported during surface acetic acid fermentations, but in these conditions final acidities did not exceed 7–8% (Gullo et al., 2009; Vegas et al., 2010; Hidalgo et al., 2010; Callejón et al., 2008). Thus, to our knowledge, this is the first report using a culture-independent approach to identify AAB involved in high-acid submerged acetic acid fermentation for the production of spirit vinegar. Previous reports using culture-dependent techniques have described the occurrence of homogeneous mono-species populations of AAB in various wine vinegar industrial processes (Fernández-Pérez et al., 2010b). Through a culture-independent approach, our findings demonstrate the existence of such homogeneous populations also in high-acid spirit vinegar.

3.3. Differentially expressed proteins during acetic acid fermentation

To evaluate the metabolic changes of the identified *Komagataeibacter* spp. through the acetic acid fermentation, we focused in the study of the main proteins whose expression was induced during the process. Representative 2D-DIGE images of Cy3 or Cy5-labeled samples from the two selected conditions, 8.2% and 14.2% are shown in Fig. 3A and B. The analysis of the 2D-DIGE gel sets

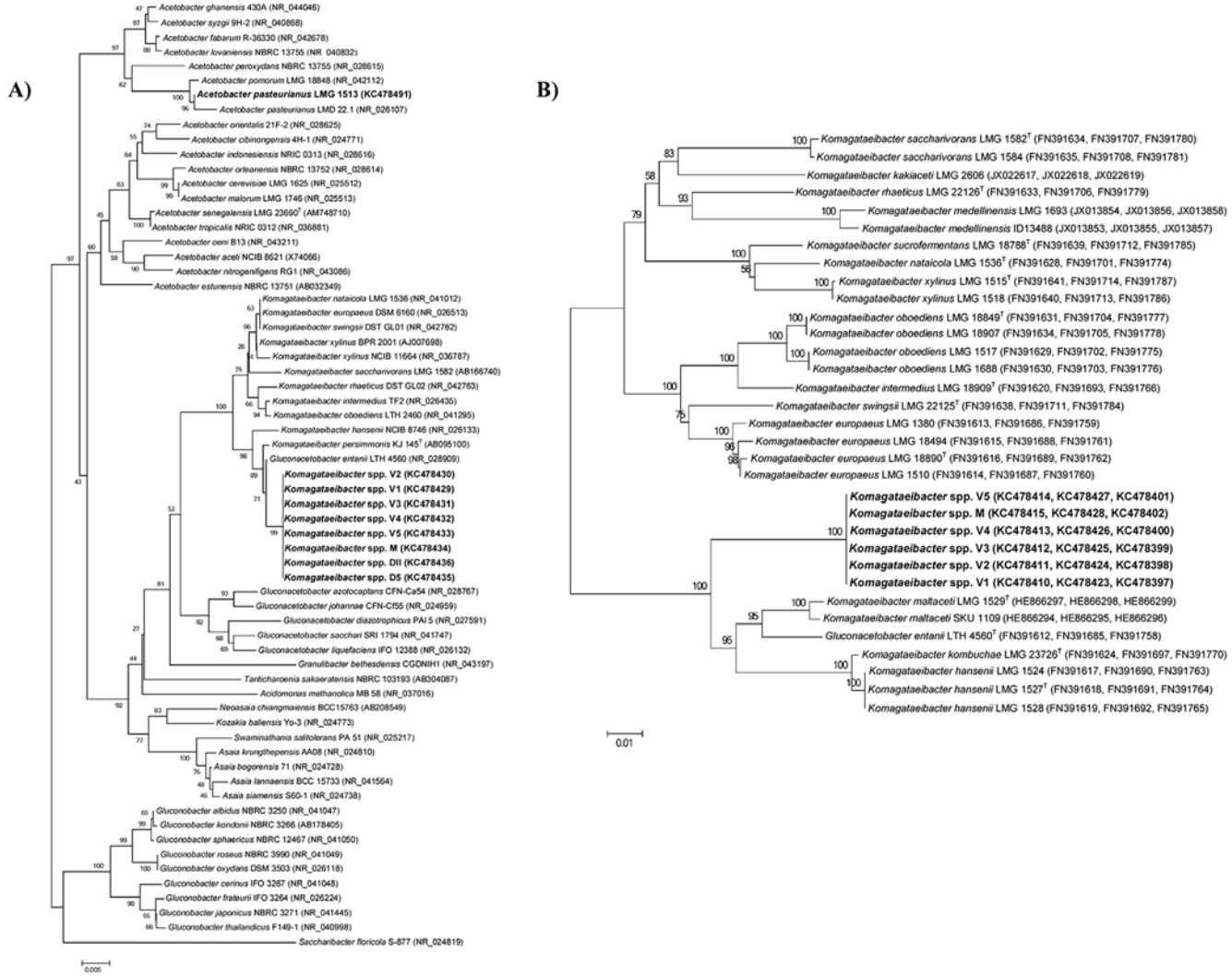

Fig. 2. Phylogenetic analysis based on the 16S rRNA gene sequence (A) and the MLSA of the three housekeeping genes *dnaK*, *groEL* and *rpoB* (B). The optimal Neighbor-Joining tree is shown. The bootstrap values are shown next to the branches. The evolutionary distances were computed using the Kimura-2-parameter method. All ambiguous positions were removed for each sequence pair. To construct the MLSA tree, individual sequences were cut to obtain blunt-end sequences before alignment.

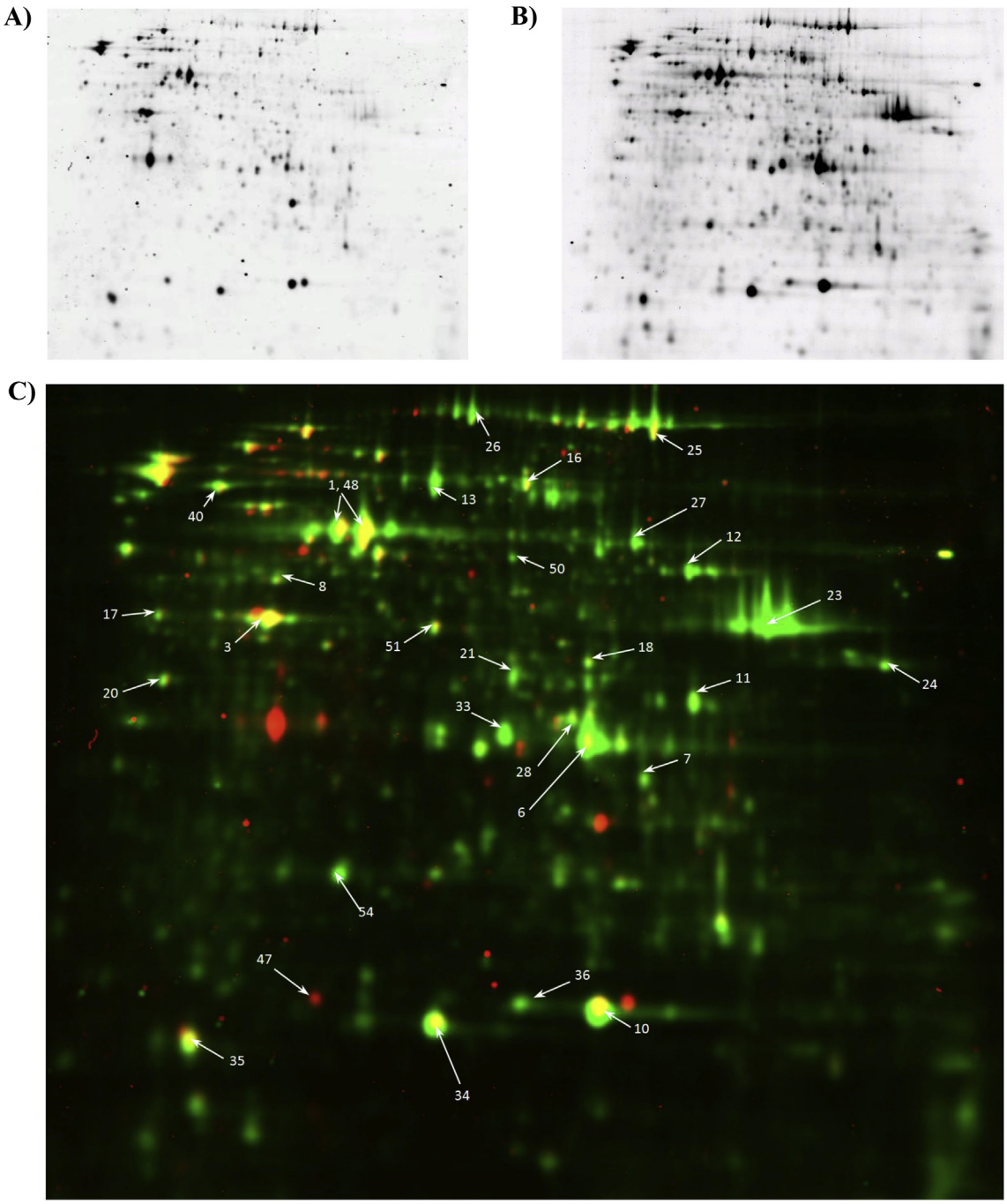

Fig. 3. 2D-DIGE analysis of bacteria harvested at low and high-acidity conditions. A) Single channel image of proteins labeled with Cy3 (red, 8.2% acidity). B) Single channel image of proteins labeled with Cy5 (green, 14.2% acidity). C) Fluorescence overlay image of the 2D gels. Green and red spots represent up- and down-regulated proteins respectively. Yellow spots correspond to non-regulated proteins. Main identified proteins are highlighted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

detected more than 2000 protein spots and, among them, 359 were consistently present in the two gel images and showed a differential expression statistically significant (ANOVA *P*-value < 0.05). Selecting 1.5-fold as the minimal level to consider a protein spot to be differentially expressed, we observed 52 protein spots that were up-regulated when bacteria were in high-acid condition. Among the protein spots that increased their expression level in 14.2%, compared to 8.2%, a set of interesting spots corresponding to the most abundant proteins was selected for identification by MALDI-TOF MS/MS analysis. When proteins were revealed as multiple spots, one or two spots from the group were selected, and a total of 32 proteins were successfully identified (Table 3, Fig. 3C). The identified proteins were mainly involved in (a) metabolic processes, (b) stress response, (c) protein folding, (d) oxidation–reduction processes and (e) biosynthetic processes.

Proteins showing the highest differential expression (DE) levels, from 5 to >13-fold, were enzymes involved in the TCA cycle: Citrate synthase (CS) (DE = 13.60), isocitrate dehydrogenase [NAD(+)] (DE = 13.42), dihydrolipoamide dehydrogenase (DE = 10.55), succinate dehydrogenase iron-sulfur subunit (DE = 8.56), fumarate

Table 3
Up-regulated proteins identified by MS/MS peptide fragmentation and MASCOT search.

Spot[a]	Identified protein[b]	Theoretical MW[c] (kDa)	Theoretical pI[d]	Mascot score	Sequence coverage[e] (%)	Differential expression	Accession number (UniProtKB/TrEMBL)	Accession number (UniParc)
33	Ketol-acid reductoisomerase	36.7	5.89	448	36	19.87	–	UPI000237EF84
23	Citrate synthase	47.9	6.68	357	29	13.60	F3SER3	UPI00020806A7
6	Isocitrate dehydrogenase (NAD(+))	36.4	6.16	561	44	13.42	D5QD55	UPI0001D2546B
13	Dihydrolipoamide dehydrogenase	60.1	5.53	120	13	10.55	D5QB40	UPI0001D24903
54	Succinate dehydrogenase iron-sulfur subunit	30.0	5.38	287	24	8.56	F3S4U5	UPI000208028B
36	Nucleoside diphosphate kinase	15.3	6.85	162	32	7.78	G2I2W5	UPI0002295033
27	Fumarate hydratase	60.6	6.05	985	37	7.37	–	UPI000237DAF6
12	Succinyl-CoA:coenzyme A transferase	54.7	6.29	276	22	7.04	F3SES0	UPI00020806AE
26	2-Oxoglutarate dehydrogenase, E1 subunit	105.3	5.71	614	20	6.75	D5QB37	UPI0001D24900
10	Superoxide dismutase	22.5	5.96	218	53	6.52	D5QCR3	UPI0001D25444
25	Aconitate hydratase	97.4	6.18	573	32	5.75	D5QB07	UPI0001D244A4
21	Fructose-1,6-bisphosphate aldolase	32.7	6.33	405	51	5.09	–	UPI000237F2BB
50	Aldehyde dehydrogenase	50.6	5.94	141	25	4.90	–	UPI000237DE7B
34	Alkyl hydroperoxide reductase subunit C	20.6	5.65	532	42	4.49	D5QFD6	UPI0001D22F42
11	Oxidoreductase	37.3	6.03	316	32	4.04	D5QJ40	UPI0001D222B0
1	Chaperonin GroEL	58.0	5.36	621	32	4.03	–	UPI000237EC18
24	Putative oxidoreductase	46.1	8.97	143	13	3.78	D5QJA6	UPI0001D226F4
7	Sugar isomerase (SIS)	34.3	5.80	155	15	3.56	D5QFE6	UPI0001D22F4C
47	Short-chain dehydrogenase/ reductase SDR	27.0	7.71	82	13	3.55	D5QHP7	UPI0001D25AAB
28	Aldo/keto reductase	36.9	6.09	106	19	2.92	–	UPI000237E9ED
28	Isocitrate dehydrogenase (NAD(+))	36.4	6.16	91	21	2.89	D5QD55	UPI0001D2546B
40	Heat shock protein 90 (HtpG)	70.1	7.94	275	16	2.61	–	UPI000237F195
49	Outer membrane protein	27.9	9.10	205	16	2.68	D5QAQ0	UPI0001D248D0
48	Chaperonin GroEL	58.2	5.43	187	23	2.66	D5QD05	UPI0001D24EB2
18	Flavohemoprotein	43.4	6.08	639	51	2.56	F3S263	UPI000207FF12
16	Threonyl-tRNA synthetase	73.7	5.8	104	23	2.48	G2I6F0	UPI0002294E5A
20	Isocitrate dehydrogenase	36.7	6.92	417	45	2.37	–	UPI000237EA6F
17	DNA-directed RNA polymerase subunit alpha	37.5	4.89	257	24	2.09	D5QH96	UPI0001D25A7E
35	Peroxiredoxin	20.1	4.96	306	37	2.03	D5QBF1	UPI0001D2492B
8	2,5-diketo-D-gluconate reductase	31.0	6.15	416	61	1.99	–	UPI000237EDC3
51	S-adenosyl-L-homocysteine hydrolase	49.5	5.76	242	20	1.75	A9HFJ7	UPI0001612637
3	Elongation factor Tu (EF-Tu)	43.1	5.21	487	39	1.74	F3SC86	UPI00020801F3

[a] Spot number shown in Fig. 3C.
[b] Mascot searches of sequenced peptides against UniProtKB/TrEMBL database. Best Mascot hits were against predicted protein from the genome sequence of *Komagataeibacter* strains.
[c] Theoretical molecular masses of proteins, calculated from amino acid sequences.
[d] Theoretical isoelectric points of proteins, calculated from amino acid sequences.
[e] Percentage of predicted protein sequence covered by matched peptides via MASCOT.

hydratase (DE = 7.37), succinyl-CoA:coenzyme A transferase (AarC/SCACT) (DE = 7.04), 2-Oxoglutarate dehydrogenase, E1 subunit (DE = 6.75), and aconitate hydratase (AcnA) (DE = 5.75). The TCA cycle related enzymes are known to be involved in the resistance of AAB to acetic acid (Nakano et al., 2004; Fukaya et al., 1990; Nakano and Fukaya, 2008). After the passive diffusion of the AcH molecule into the cytoplasm, the dissociated acetate anions are metabolized through the TCA cycle and acetyl-CoA synthase, and bacterial cells enter in what is called the overoxidation phase. The acetate is completely oxidized by this pathway linked with the respiratory chain to CO_2 and H_2O, providing energy (ATP) and detoxifying the cell.

A recent study reported the induced expression of genes coding for TCA enzymes, with higher up-regulation showed by citrate synthase (aarA/gltA/CS), aconitate hydrolase/aconitase (AcnA) and succinate dehydrogenase (sdhABCD) (Sakurai, Arai, Ishii, & Igarashi, 2012). Isocitrate dehydrogenase [NAD(+)], 2-oxoglutatare dehydrogenase E2 subunit and aconitase hydratase have been also observed to be up-regulated at the end of the ethanol oxidation phase in *A. pasteurianus* LMG 1262^T (Andrés-Barrao et al., 2012) (Table S1 in supplemental material). Aconitate hydratase, another enzyme of the TCA cycle, has been found to be up-regulated when growing *Acetobacter aceti* in 1% EtOH medium, and its over-expression remarkably increased the acetic acid resistance of the strain (Nakano et al., 2004). A specialized succinyl-CoA:coenzyme A transferase (AarC/SCACT) was identified as one of the first acetic acid resistance determinants (Mullins et al., 2008). This enzyme is synthetized by both genera *Acetobacter* and *Komagataeibacter* and replaces succinyl-CoA synthetase in *Acetobacter* spp. classified into the *A. pasteurianus* group (Azuma et al., 2009; Mullins and Kappock, 2012; Andrés-Barrao et al., 2011b; Ogino et al., 2011) (Table S2 in supplemental material). The role of SCACT enzyme in the TCA cycle is to transform succinyl-CoA to succinate at the same time that feed the cycle with a new molecule of acetyl-CoA from acetate, thus increasing the detoxifying effect. Additionally, two genes coding for the enzyme dihydrolipoyl dehydrogenase exist in both AAB genera, *Acetobacter* and *Komagataeibacter* (Table S2 in supplemental material). This enzyme catalyzes the transformation of

dihydrolipoamide into lipoamide. It takes part in the pyruvate dehydrogenase complex, in the glycolytic pathway; but also the 2-oxoglutarate complex, in the TCA cycle. The present result confirmed the importance of the TCA cycle in the strategy that confers AAB their inherent resistance to live and thrive in their natural aggressive environment, under high concentration of acetic acid, low pH and high aeration rate. This metabolic pathway allow the bacteria to metabolize the excess of acetate diffused into the cytosol and to produce energy in the form of ATP that will be used in the maintenance of other mechanisms involved in the stress resistance response.

Interestingly, the highest level of differential expression, 19.87-fold, and the lowest specific ANOVA value, 4.89647e-7 were showed by the ketol-acid reductoisomerase (ilvC). This enzyme is involved in the biosynthesis of the most hydrophobic branched-chain amino acids (BCAA), L-isoleucine and L-valine (steps 2 and 4 of the synthetic pathway, respectively) (Brosnan and Brosnan, 2006).

One of the main functions of these BCAAs is the protein synthesis. They play a crucial role in determining the structures of globular proteins as well as the interaction of the transmembrane domains of membrane proteins with the phospholipid bilayer (Brosnan and Brosnan, 2006). Their oxidation provides metabolic energy and they are precursors in the synthesis of amino acids, mainly alanine and glutamine in catabolic states (Jin et al., 2012). Recent studies have reported the up regulation of BCAA biosynthetic genes, such as *ilvC* and *ilvE* (coding for ketol-acid reductoisomerase and BCAA aminotransferase, respectively), in *Streptococcus mutants* and *Bifidobacterium longum* under acid stress (Jin et al., 2012; Santiago et al., 2012). In the case of AAB, our results suggest the importance of the metabolic switch from the central sugar metabolic pathway towards the synthesis of BCAA (the synthesis of L-isoleucine and L-valine start from pyruvate), in order to produce enough energy to survive in industrial high-acid spirit vinegar. This metabolic switch would induce a decrease of acid stress by reducing acid end products and assisting the maintenance of intracellular pH (Santiago et al., 2012). We can therefore suggest that the addition of BCAA to the fermentation broth during the industrial production of vinegar could improve acetic acid resistance of AAB, leading to final products with even higher concentrations of acetic acid.

3.4. Ultrastructure of bacterial cells: membrane polysaccharides

Among the mechanisms to help AAB to resist acetic acid, the production of a polysaccharide pellicle has been reported (Kanchanarach et al., 2010). To study the membrane polysaccharides of *Komagataeibacter* spp. involved in the current acetic acid fermentation, we performed the morphological and ultrastructure characterization of the individual cells using electron microscopy. SEM observations of high-acid vinegar samples harvested at the end of the process, at 14% AcH, showed the cell surface of *Komagataeibacter* spp. extremely rough, and the presence of an extracellular material in the form of cellulose-like fibers (Fig. 4A). To highlight surface polysaccharides, TEM sections were stained with PATAg. No electron dense layer was detected surrounding the plasma membrane of *Komagataeibacter* spp. cells. Instead, electron dense granules were detected inside the cells, suggesting glycoproteins (Fig. 4F).

SEM and TEM observations of the current spirit vinegar samples were similar to the results obtained from a previous acetic acid fermentation performed in our laboratory (Fig. 4B and G). In that occasion, limited bacterial growth was observed during the preparation phase (6–8% AcH), and AAB were identified as *K. oboediens*, being the strain *K. oboediens* 174Bp2 successfully isolated. When

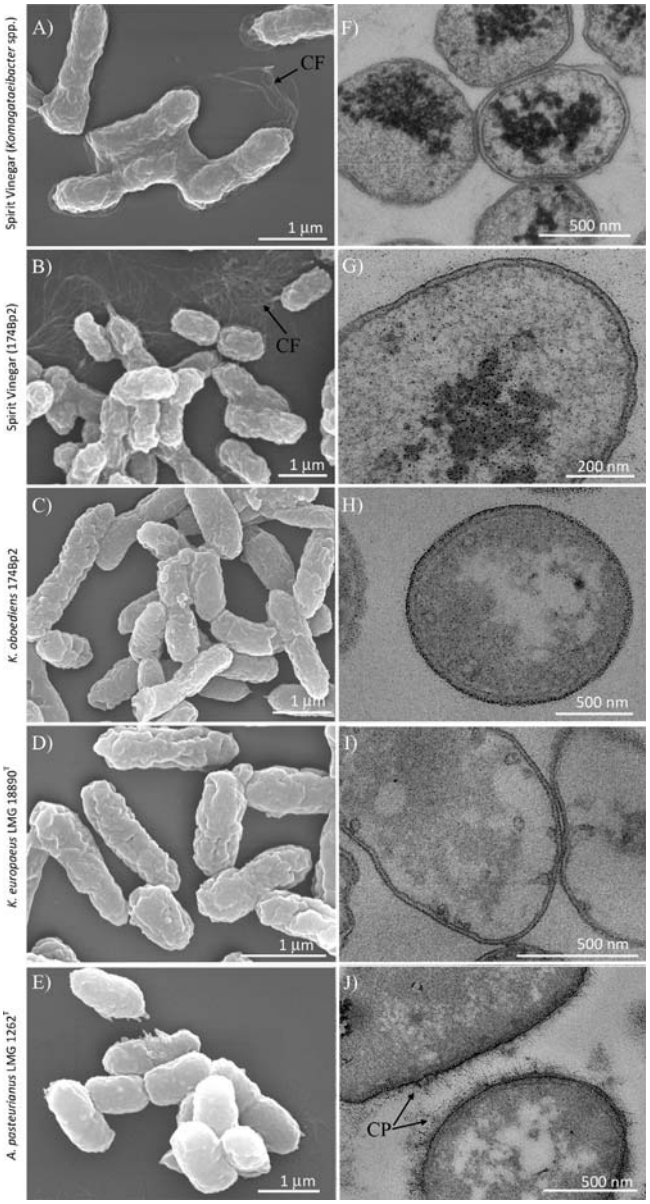

Fig. 4. SEM (A–E) and TEM (F–G) micrographs. A, F) Spirit vinegar bacteria harvested at the end of the current acetic acid fermentation, at 14% AcH; B,G) Spirit vinegar bacteria harvested from a previous acetic fermentation C,H) *K. oboediens* 174Bp2 grown on RAE 1a/2e (1% acetic acid and 2% ethanol). This strain was isolated from the spirit vinegar in figures B and G; D, I) *K. europaeus* 18890^T grown on RAE 1a/2e; E, J) *A. pasteurianus* LMG 1262^T grown in liquid RAE 1a/2e. CP = capsular polysaccharides, CF = cellulose-like fibers (exopolysaccharides).

growing in synthetic medium under mild conditions (1% AcH), the cell surface of this isolate showed similar characteristics with the former vinegar samples; a high roughness but no cellulose-like fibers were observed (Fig. 4C). TEM micrographs of *K. oboediens* 174Bp2 grown on synthetic medium (1%AcH) showed a thin layer of electron dense deposits around the cells, but no cytoplasmic deposits were observed (Fig. 4H). Samples of the reference strains *K. europaeus* LMG 18890^T grown in synthetic medium (1% AcH) showed the same phenotypic characteristics that *K. oboediens* 174Bp2 (Fig. 4D and I).

For comparative purposes, SEM and TEM preparations were set up from *A. pasteurianus* LMG 1262^T growing under the same mild conditions (1%AcH). In contrasts with *Komagataeibacter* spp., whether wild-type (vinegar) or cultured in synthetic medium, cells

of *A. pasteurianus* 1262^T showed a smooth surface and were surrounded by an thick electron dense material compatible with the presence of capsular polysaccharides (Schouls et al., 2008) (Fig. 4E and J).

The observation of cellulosic fibers in submerged vinegar samples is consistent with previous reports of *Komagataeibacter* strains being able to produce cellulose or cellulose-like exopolysaccharides and harvesting several types of the cellulose synthase operons (Andrés-Barrao et al., 2011b). The synthesis of such fibrous-extracellular material appeared to be repressed when bacteria were grown in synthetic media, out of their natural environment.

The SDS-PAGE gels polysaccharide pattern obtained for different isolates of both genera confirmed the lack of capsular polysaccharides in *Komagataeibacter* spp. The specific staining for capsular polysaccharides (CPS) showed an intense band on low molecular weight of *Acetobacter* strains, which was in contrast absent in *Komagataeibacter* strains (Fig. 5). Additionally, the gel stained for lipopolysaccharides (LPS) clearly showed the presence of complete smooth LPS (sLPS) including the O-antigen in *Acetobacter* spp. The O-antigen was in contrast absent in *Komagataeibacter* samples; grown in synthetic media or directly harvested from vinegar. *Komagataeibacter* spp. were then characterized by having rough LPS (rLPS).

Electron micrographs and SDS-PAGE specific staining revealed a clear difference in the ultrastructure of the cell surface between the genera *Acetobacter* and *Komagataeibacter*. The capsular polysaccharides produced by *Acetobacter* spp., clearly observed in TEM micrographs, is consistent with previous reports (Deeraksa et al., 2005; Kanchanarach et al., 2010; Moonmangmee et al., 2002; Andrés-Barrao et al., 2012). These CPS has been involved in the resistance against acetic acid, by acting as a barrier that prevents the entrance of the molecule inside the cell (Deeraksa et al., 2005; Kanchanarach et al., 2010). Interestingly, our results demonstrate that *Komagataeibacter* spp. do not synthetize these CPS, indicting that a different strategy must be used to guarantee the viability and stability of the bacterial cell under high-acid conditions.

Analysis of these data indicated that, under the studied conditions, *Komagataeibacter* strains do not synthesize CPS nor the LPS O-chain. This suggests that the positive PATAg staining might possibly be due to the reaction with Kdo residues found in the core of LPS or the polysaccharide moiety of glycoproteins. All together, these results strongly support the hypothesis that surface polysaccharides are not involved in the strategy used by *Komagataeibacter* spp. to resist high concentrations of acetic acid.

4. Conclusions

Although the resistance of AAB to acetic acid has been studied for more than 60 years, little is still known on the molecular mechanisms that confer this characteristic property to such microorganisms. In this study we have shown that a bacterial population is most probably composed of a single or few closely related strains that were responsible for the production of high-acid spirit vinegar during a submerged process. This specialized AAB was identified as a member of the genus *Komagataeibacter* and, on the basis of the sequenced loci, appears to be sufficiently distinct from reference strains to form a new species. Yet, further characterization of this particular microorganism at the species level may not be possible unless proper conditions for growth on agar plates are achieved. Proteomic analyses confirmed the importance of the TCA cycle during acetic fermentation and resistance to acetic acid in *Komagataeibacter* spp., and also highlighted the important role of BCAA during these processes. In the light of these results, it appears that an in-depth characterization of the metabolic and physiological properties, as well as composition of surface polysaccharides, of members of the genera *Acetobacter* and *Komagataeibacter* are

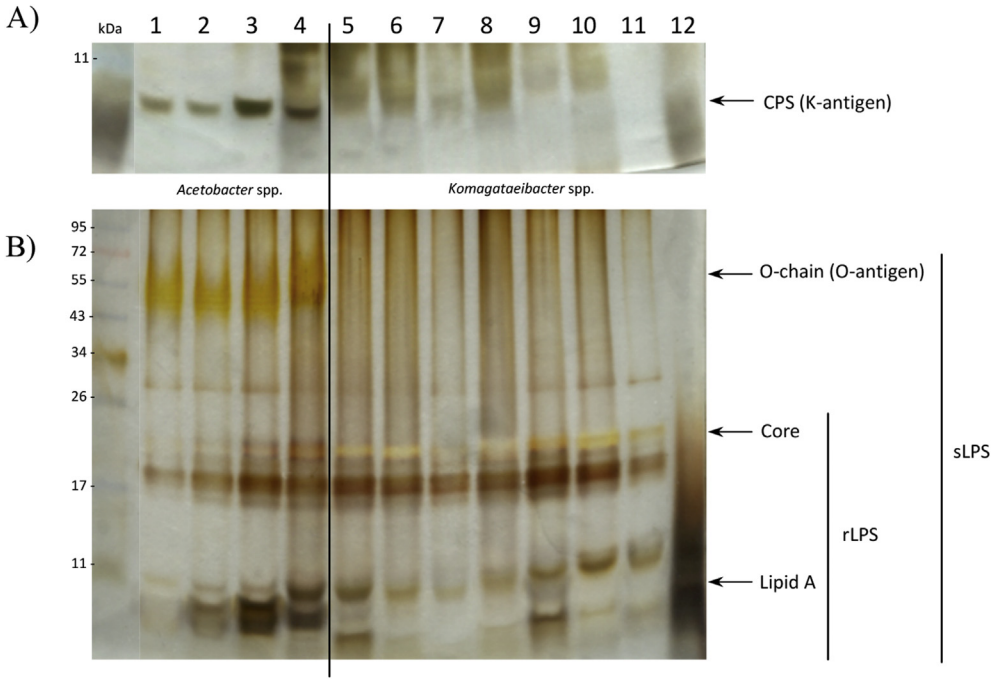

Fig. 5. SDS-PAGE images of specifically stained CPS (A) and LPS (B). *A. pasteurianus* 3P3 grown in YGC, YPM and RAE 1a/2e were loaded in lines 1, 2 and 3, respectively; *A. pasteurianus* LMG 1262^T grown in RAE 0a/0e (liquid basal medium with no acetic acid or ethanol added) was loaded in line 4; *K. europaeus* 5P3 grown in RAE 0a/0e, YGC, YPM and RAE 1a/2e were loaded in lines 5, 9, 10 and 11, respectively; *K. europaeus* 4P2 grown in RAE 0a/0e was loaded in line 6; *K. europaeus* TP1-23 grown in RAE 0a/0e was loaded in line 7; *K. oboediens* 174Bp2 grown in RAE 0a/0e was loaded in line 8; spirit vinegar bacteria from the end of the current acetic acid fermentation (14% AcH) was loaded in line 12. YCG medium is composed by yeast extract 1%, D-glucose 5%, Ca_3CO_2 3% and agar 1.5%. YPM and RAE media are described in Andrés-Barrao et al., 2011a. sLPS and rLPS are smooth and rough form of the lipopolysaccharide molecule, respectively.

essential steps towards a better understanding of the molecular basis for resistance to acetic acid. Ultimately this knowledge will not only improve fermentation processes required for the production of vinegar, but also those required for the industrial production of various important foods and beverages, including chocolate, kefir, or kombucha. Additionally, the polysaccharide layer that surrounds *Acetobacter* strains has been proposed to be part of the general resistance strategy of these bacteria to acetic acid (Deeraksa et al., 2005; Kanchanarach et al., 2010; Moonmangmee et al., 2002). Interestingly, we found that *Komagataeibacter* strains, which show higher acetic acid production and resistance than *Acetobacter* bacteria, do not possess such polysaccharide layer. Although these two genera of AAB are involved in the industrial production of vinegar, this difference in cell surface composition illustrates the variations in strategies used by *Komagataeibacter* and *Acetobacter* to resist high concentrations of acetic acid.

This finding gives new insights into the understanding of the physiology of the acetic acid bacteria, and help to clarify some acid resistance mechanisms that are active in these highly-resistant bacteria. *Acetobacter* and *Komagataeibacter* strains are widely used for the production of vinegar all around the world. The elucidation of the characteristics and metabolic pathways specific for both genera will facilitate more advance uses of these bacteria in fermentation industries. More effective and sophisticated.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2015.10.012.

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