Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S–23S rDNA intergenic spacer

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DNA corresponding to 16S rDNA and the 16S–23S rDNA intergenic spacer (ITS) from 22 reference strains of acetic acid bacteria, representing the diversity of the family *Acetobacteraceae*, and 24 indigenous acetic acid bacteria isolated from wine fermentations were analysed by PCR–RFLP. *Frateruria aurantia* LMG 1558^T and *Escherichia coli* ATCC 11775^T were included as outgroups. PCR-amplified products of about 1450 bp were obtained from the 16S rDNA of all the strains and products of between 675 and 800 bp were obtained from the 16S–23S rDNA ITS. PCR products were digested with 4-base-cutting restriction enzymes in order to evaluate the degree of polymorphism existing among these strains. Of the enzymes tested, *TaqI* and *RsaI* were the most discriminatory and showed no intraspecific variations in the restriction patterns. Restriction analysis of the 16S rDNA with these enzymes is proposed as a rapid and reliable method to identify acetic acid bacteria at the level of genus and species (or related species group) and its applicability to identification of indigenous acetic acid bacteria was demonstrated. The same degree of distinction as that for the 16S rDNA analysis was obtained within reference strains of acetic acid bacteria by PCR–RFLP of the 16S–23S rDNA ITS. However, 16S–23S rDNA ITS restriction patterns of strains isolated from wine did not match those of any of the reference strains. Thus, PCR–RFLP of the 16S–23S rDNA ITS is not a useful method to identify isolates of acetic acid bacteria at the species level, although it may be an adequate method to detect intraspecific differentiation.

Keywords: acetic acid bacteria, identification, PCR–RFLP, 16S rDNA, rDNA 16S–23S ITS

INTRODUCTION

Acetic acid bacteria are Gram-negative or Gram-variable, ellipsoidal to rod-shaped cells that have an obligately aerobic metabolism with oxygen as the terminal electron acceptor. They are classified in the family *Acetobacteraceae* as a branch of the acidophilic bacteria in the α -subdivision of the *Proteobacteria* (De Ley *et al.*, 1984; Sievers *et al.*, 1994). Members of the family are recognized by their unique ability to oxidize ethanol to acetic acid in neutral and acidic (pH 4.5) media. They include bacteria with relevant industrial applications in the production of vinegar (*Gluconacetobacter europaeus*), gluconate and sorbose (*Glu-*

conobacter oxydans) and paper from cellulose (*Gluconacetobacter xylinus*) due to their abilities to oxidize alcohols and sugars and to synthesize bacterial cellulose.

Genotypic relationships among acetic acid bacteria have been studied by DNA–DNA hybridization (Entani *et al.*, 1985; Mason & Claus, 1989; Micales *et al.*, 1985; Sievers *et al.*, 1992; Urakami *et al.*, 1989; Yamada *et al.*, 1984), DNA–RNA hybridization (Gillis & De Ley, 1980) and 5S and 16S rDNA sequence analysis (Bulygina *et al.*, 1992; Sievers *et al.*, 1994; Yamada *et al.*, 1997). The most recent approaches to classification within the family *Acetobacteraceae* (Gillis *et al.*, 1989; Mason & Claus, 1989; Urakami *et al.*, 1989; Yamada & Kondo, 1984; Yamada *et al.*, 1997) reveal the existence of four well-

Abbreviation: ITS, internal transcribed spacer.

Table 1. Reference strains used in this study

Sources of strains are abbreviated as: LMG, Laboratorium voor Microbiologie Universiteit Gent, Belgium; CECT, Colección Española de Cultivos Tipo, Valencia, Spain; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; ATCC, American Type Culture Collection, Manassas, VA, USA. NK, Not known.

Species	Strain	Other designations	Origin	Accession no.
<i>Gluconobacter oxydans</i>	LMG 1408 ^{T*}	DSM 3505 ^T	Beer	X73820
<i>Gluconobacter oxydans</i>	CECT 360 ^{T*}	—	Beer	X73820
<i>Gluconobacter oxydans</i>	LMG 1484	—	Amstel beer	—
<i>Gluconobacter oxydans</i>	LMG 1414	—	Grapes	—
<i>Gluconobacter frateurii</i>	LMG 1365 ^T	DSM 7146 ^T	<i>Fragaria ananassa</i>	—
<i>Gluconobacter asaii</i>	LMG 1390 ^T	DSM 7148 ^T	<i>Rheum rhabarbarum</i> , flower	—
<i>Acidomonas methanolica</i>	LMG 1668 ^T	DSM 5432 ^T	Fermentation of ethanol with <i>Candida</i> sp. (non-sterile)	—
<i>Acetobacter aceti</i>	LMG 1261 ^{T†}	DSM 3508 ^T	Beech-wood shavings from a vinegar plant	X74066
<i>Acetobacter aceti</i>	CECT 298 ^{T†}	DSM 3508 ^T	Beech-wood shavings from a vinegar plant	—
<i>Acetobacter aceti</i>	LMG 1505	—	Quick vinegar	—
<i>Acetobacter aceti</i>	LMG 1372	—	Nakano Rice-Vinegar Producing Company	—
<i>Acetobacter pasteurianus</i>	LMG 1262 ^T	DSM 3505 ^T	Beer	—
<i>Acetobacter pasteurianus</i>	LMG 1553	—	Spoiled beer	—
<i>Acetobacter pasteurianus</i>	LMG 1282	—	Beer	—
<i>Gluconacetobacter hansenii</i>	LMG 1527 ^T	DSM 5602 ^T	Vinegar	X75620
<i>Gluconacetobacter hansenii</i>	LMG 1511	—	NK	—
<i>Gluconacetobacter liquefaciens</i>	LMG 1381 ^T	DSM 5603 ^T	Dried fruit	—
<i>Gluconacetobacter liquefaciens</i>	LMG 1347	—	Fruit	—
<i>Gluconacetobacter xylinus</i>	LMG 1515 ^T	DSM 5615 ^T	Mountain ash berries	X75619, X89812
<i>Gluconacetobacter xylinus</i>	LMG 1518	—	NK	—
<i>Gluconacetobacter diazotrophicus</i>	DSM 5601 ^T	—	Sugar-cane roots	—
<i>Gluconacetobacter europaeus</i>	DSM 6160 ^T	—	NK	Z21936, X89771
<i>Frateruia aurantia</i>	LMG 1558 ^T	DSM 6220 ^T	<i>Lilium auratum</i>	—
<i>Escherichia coli</i>	ATCC 11775 ^T	—	NK	—

* Synonymous strains.

† Synonymous strains.

defined genera: *Acetobacter* (*Acetobacter aceti*^T and *Acetobacter pasteurianus*), *Acidomonas* (*Acidomonas methanolica*^T), *Gluconacetobacter* (*Gluconacetobacter liquefaciens*^T, *Gluconacetobacter hansenii*, *Gluconacetobacter xylinus*, *Gluconacetobacter europaeus* and *Gluconacetobacter diazotrophicus*) and *Gluconobacter* (*Gluconobacter oxydans*^T, *Gluconobacter asaii* and *Gluconobacter frateurii*). In addition, the new species *Acetobacter oboediens*, *Acetobacter pomorum*, *Acetobacter intermedius* and *Gluconacetobacter sacchari* have been reported recently (Sokollek *et al.*, 1998; Boesch *et al.*, 1998; Franke *et al.*, 1999).

RFLP analysis of PCR-amplified 16S rDNA is an appropriate technique for the differentiation and characterization of micro-organisms on the basis of their phylogenetic relationships (Carlotti & Funke, 1994). In eubacterial DNA, the rRNA genetic loci include 16S, 23S and 5S rRNA genes, which are separated by internally transcribed spacer (ITS) regions. It is known that DNA sequences in the

16S–23S ITS exhibit a great deal of sequence and length variation (Barry *et al.*, 1991; Navarro *et al.*, 1992) and conserved sequences with functional roles (tRNA genes, antitermination sequences) are also found within the ITS (Sievers *et al.*, 1996). It has been shown that PCR–RFLP analysis of the ITS is an appropriate method for differentiating species (Jensen *et al.*, 1993) and even strains of prokaryotes (Navarro *et al.*, 1992).

The objective of the present study was to evaluate the usefulness of PCR–RFLP analysis of the 16S rDNA and 16S–23S intergenic spacer in order to characterize acetic acid bacteria as well as to identify natural isolates belonging to this group.

METHODS

Strains, media and growth conditions. Reference strains used in this study are listed in Table 1. They were grown on solid GYC medium (5% D-glucose, 1% yeast extract, 0.5%

CaCO₃ and 2% agar, w/v). Twenty-four indigenous strains were isolated selectively by plating wine samples on the same medium at pH 4.5, without CaCO₃ and supplemented with pimaricin (100 mg l⁻¹) to inhibit the growth of yeasts and moulds and penicillin (3 U ml⁻¹) to inhibit lactic acid bacteria. Both antibiotics were added to the culture medium from stock solutions after sterilizing the medium. Plates were incubated at 28 °C for 2–4 d under aerobic conditions.

Biochemical identification tests. Some biochemical identification tests were performed in order to validate the data obtained by PCR–RFLP. The following principal characteristics were used for differentiation: (i) the ability or inability to degrade lactate distinguished *Acetobacter* from *Gluconobacter*; (ii) within *Acetobacter*, the ability or inability to oxidize glycerol distinguished *Acetobacter aceti* from *Acetobacter pasteurianus*. *Gluconacetobacter hansenii* was distinguished by its inability to grow with ethanol as carbon source. All the tests were performed as described previously (Buchanan & Gibbons, 1984; Drysdale & Fleet, 1985; Joyeux *et al.*, 1984).

DNA extraction. For each strain studied, a single colony was picked from a fresh culture on GYC medium and resuspended in 50 µl sterile deionized water (MilliQ) in a 0.5 ml tube. The mixture was vortexed for 15 s and then incubated at 95 °C for 15 min. After centrifugation (5000 g, 4 °C, 5 min), the supernatant containing the released DNA was used as the source of target DNA for PCR amplification.

Oligonucleotide primers. PCR primers used to amplify the 16S–23S rDNA spacer region were selected on the basis of the sequences of 16S (3'-end; its1, 5'-ACCTGCGGCTG-GATCACCTCC-3') and 23S (5'-end; its2, 5'-CCGAATGCCCTTATCGCGCTC-3') rRNA genes conserved among acetic acid bacteria. Primers for the PCR amplification of 16S rDNA were selected from conserved regions of the 5'-end (16Sd, 5'-GCTGGCGGCATGCTTAACACAT-3') and the 3'-end (16Sr, 5'-GGAGGTGATCCAGCCGCGAGGT-3') of the 16S rDNA. Primers its1 and 16Sr were complementary.

All multiple sequence alignments were performed with CLUSTAL W (Higgins & Sharp, 1988). The design of the primers was carried out by following criteria described previously (Bej *et al.*, 1991).

PCR amplification. PCR amplification was carried out in 50 µl samples consisting of 5 µl bacterial extract and 45 µl amplification mixture, which contained 15 pmol of each primer, 200 µM of each of the four dNTPs, 5 µl 10 × amplification buffer (ECOGEN; ARK Scientific), 3 mM MgCl₂ and 2.5 U *Taq* DNA polymerase (ECOGEN; ARK Scientific).

The reaction was performed in a GeneAmp PCR System 2400 (Perkin-Elmer). For the amplification of the 16S–23S intergenic spacer, the samples were incubated for 5 min at 94 °C to denature the target DNA and then cycled 35 times at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min. The samples were then incubated for 7 min at 72 °C for a final extension and were maintained at 4 °C until tested. For amplification of the 16S rDNA, samples were incubated for 5 min at 94 °C and then cycled 35 times at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min. The samples were then incubated for 10 min at 72 °C for a final extension and maintained at 4 °C until tested.

Restriction of amplified DNA. Five microlitres of each PCR-amplified product was digested with restriction endo-

nucleases as recommended by the manufacturer. Restriction enzymes were obtained from Boehringer Mannheim.

Gel electrophoresis. Amplified DNA was detected by electrophoresis on a 0.8% (w/v) agarose gel in 1 × TBE buffer, while the restriction fragments were separated on a 3% (w/v) agarose gel. Short restriction fragments (<400 bp) were resolved better on a 5% (w/v) agarose gel. The gels were stained with ethidium bromide and photographed. The lengths of both amplified products and restriction fragments were estimated by comparison against a 100 bp DNA ladder (Gibco-BRL).

RESULTS

PCR–RFLP of the 16S rDNA: characterization of reference strains

PCR-amplified products corresponding to the 16S rRNA gene were obtained specifically from all strains. The PCR products contained approximately 1450 bp (results not shown), while the expected length was 1458 bp. Co-migration of amplified rDNA from all strains indicated an identical size.

Eight 4-base restriction endonucleases (*Taq*I, *Cfo*I, *Hae*III, *Alu*I, *Hinf*I, *Mbo*I, *Msp*I and *Rsa*I) were tested and cut the amplified 16S rDNA. RFLPs were observed with all the enzymes (Fig. 1) and different

Fig. 1. *Msp*I restriction patterns of PCR-amplified 16S rDNA. Lanes: M, 100 bp DNA ladder (Gibco-BRL); 1–3, *Gluconobacter oxydans* LMG 1408^T, LMG 1484 and LMG 1414; 4, *Gluconobacter frateurii* LMG 1365^T; 5, *Gluconobacter asaii* LMG 1390^T; 6–8, *Acetobacter aceti* LMG 1261^T, LMG 1505 and LMG 1372; 9–10, *Acetobacter pasteurianus* LMG 1262^T and LMG 1553; 11–12, *Gluconacetobacter liquefaciens* LMG 1381^T and LMG 1347; 13–14, *Gluconacetobacter hansenii* LMG 1527^T and LMG 1511; 15–16, *Gluconacetobacter xylinum* LMG 1515^T and LMG 1518; 17, *Acetobacter pasteurianus* LMG 1282; 18, *Gluconacetobacter europaeus* DSM 6160^T; 19, *Gluconacetobacter diazotrophicus* DSM 5601^T; 20, *Acidomonas methanolica* LMG 1668^T; 21, *F. aurantia* LMG 1558^T; 22, *E. coli* ATCC 11775^T.

Table 2. Sizes of PCR–RFLP fragments obtained from the 16S rDNA of acetic acid bacteria

Genera are abbreviated as: *Ab.*, *Acetobacter*; *Ac.*, *Acidomonas*; *Ga.*, *Gluconacetobacter*; *Gb.*, *Gluconobacter*.

Strain	<i>TaqI</i>	<i>RsaI</i>	<i>AluI</i>	<i>MspI</i>
<i>Gb. oxydans</i> LMG 1408 ^T	350, 190, 175, 160, 120, 120, 110	400, 400, 400, 150, 90	550, 290, 210, 190, 125	425, 300, 225, 160, 125, 125
<i>Gb. oxydans</i> CECT 360 ^T	350, 190, 175, 160, 120, 120, 110	400, 400, 400, 150, 90	550, 290, 210, 190, 125	425, 300, 225, 160, 125, 125
<i>Gb. oxydans</i> LMG 1484	350, 190, 175, 160, 120, 120, 110	400, 400, 400, 150, 90	550, 290, 210, 190, 125	425, 300, 225, 160, 125, 125
<i>Gb. oxydans</i> LMG 1414	350, 190, 175, 160, 120, 120, 110	400, 400, 400, 150, 90	550, 290, 210, 190, 125	450, 425, 225, 125, 125
<i>Gb. frateurii</i> LMG 1365 ^T	350, 190, 175, 160, 120, 120, 110	400, 400, 300, 150, 130	550, 290, 210, 190, 125	450, 310, 225, 125, 125
<i>Gb. asaii</i> LMG 1390 ^T	350, 190, 175, 160, 120, 120, 110	400, 400, 300, 150, 130	550, 290, 210, 190, 125	450, 310, 225, 125, 125
<i>Ab. aceti</i> LMG 1261 ^T	850, 350, 210	500, 400, 300, 150, 125	310, 290, 240, 210, 190, 125	450, 310, 225, 125, 125
<i>Ab. aceti</i> CECT 298 ^T	850, 350, 210	500, 400, 300, 150, 125	310, 290, 240, 210, 190, 125	450, 310, 225, 125, 125
<i>Ab. aceti</i> LMG 1505	850, 350, 210	500, 400, 300, 150, 125	310, 290, 240, 210, 190, 125	450, 310, 225, 125, 125
<i>Ab. aceti</i> LMG 1372	850, 350, 210	500, 400, 300, 150, 125	310, 290, 240, 210, 190, 125	450, 310, 225, 125, 125
<i>Ga. hansenii</i> LMG 1511*	850, 350, 210	500, 400, 300, 150, 125	310, 290, 240, 210, 190, 125	450, 310, 225, 125, 125
<i>Ab. pasteurianus</i> LMG 1262 ^T	500, 350, 330, 210	500, 400, 300, 150, 125	450, 310, 290, 190, 190	450, 425, 225, 125, 125
<i>Ab. pasteurianus</i> LMG 1553	500, 350, 330, 210	500, 400, 300, 150, 125	450, 290, 190, 190, 190, 140	450, 425, 225, 125, 125
<i>Ga. hansenii</i> LMG 1527 ^T	650, 350, 210, 175	500, 400, 400, 150	790, 480, 125	450, 425, 225, 125, 125
<i>Ga. liquefaciens</i> LMG 1381 ^T	500, 350, 210, 175, 160	500, 400, 400, 150	790, 275, 210, 125	450, 425, 225, 225, 125
<i>Ga. liquefaciens</i> LMG 1347	500, 350, 210, 175, 160	500, 400, 400, 150	790, 275, 210, 125	450, 425, 225, 225, 125
<i>Ga. xylinus</i> LMG 1515 ^T	500, 350, 210, 175, 160	500, 400, 400, 150	790, 275, 210, 125	450, 425, 225, 225, 125
<i>Ga. xylinus</i> LMG 1518	500, 350, 210, 175, 160	500, 400, 400, 150	790, 275, 210, 125	450, 425, 225, 225, 125
<i>Ab. pasteurianus</i> LMG 1282†	500, 350, 210, 175, 160	500, 400, 400, 150	790, 275, 210, 125	450, 425, 225, 225, 125
<i>Ga. europaeus</i> DSM 6160 ^T	500, 350, 210, 175, 160	500, 400, 400, 150	790, 275, 210, 125	450, 425, 225, 225, 125
<i>Ga. diazotrophicus</i> DSM 5601 ^T	500, 350, 210, 175, 160	500, 400, 250, 150, 150	790, 275, 210, 125	450, 310, 225, 225, 125, 125
<i>Ac. methanolicus</i> LMG 1668 ^T	450, 190, 175, 160, 120, 110	500, 400, 400, 150	550, 275, 225, 210, 190	450, 425, 225, 225, 125
<i>F. aurantia</i> LMG 1558 ^T	600, 350, 210, 175, 125	450, 400, 350, 150	350, 300, 225, 210, 175, 125	450, 425, 175, 140, 125, 90
<i>E. coli</i>	750, 350, 210	500, 450, 400, 150	350, 350, 225, 210, 100	450, 310, 175, 175, 150, 125, 125, 90

* Reclassified in this study as *Acetobacter aceti*.

† Reclassified in this study as *Gluconacetobacter liquefaciens*/*Gluconacetobacter xylinus*/*Gluconacetobacter europaeus*.

information was obtained with each one. As expected, the eight enzymes distinguished the acetic acid bacteria from the outgroups. Of all the enzymes tested, *TaqI* and *RsaI* were chosen for the identification. They distinguished the largest number of species of acetic acid bacteria without intraspecific variations, as seen in Table 2 in comparison with other restriction enzymes (*AluI* and *MspI*).

It is noteworthy that strain LMG 1282, classified as *Acetobacter pasteurianus*, did not give the same patterns as the type strain, LMG 1262^T. This strain grouped with strains of *Gluconacetobacter liquefaciens*, *Gluconacetobacter xylinus* and *Gluconacetobacter europaeus* with all the enzymes tested. In the same way, patterns for *Gluconacetobacter hansenii* LMG 1511 did not match those of *Gluconacetobacter hansenii* LMG 1527^T. The former strain showed the same restriction patterns as strains of *Acetobacter aceti*.

PCR–RFLP of the 16S–23S rDNA ITS: characterization of reference strains

Specific amplification of the intergenic sequence spanning the 16S and 23S rDNAs was achieved by using primers *its1* and *its2*, with the exception of *Gluconobacter asaii* LMG 1390^T, *Frateruia aurantia* LMG 1558^T and *Escherichia coli* ATCC 11775^T. As primer 16Sr was effective in amplifying the 16S rDNA of these strains and primer *its1* is complementary to primer 16Sr, the lack of amplification of the ITS sequence of these strains is probably due to failure of primer *its2* to anneal.

Conservation of the length of the 16S–23S rDNA ITS was observed at the species level, but this length varied from 675 to 800 bp among some species. Digestion with the eight restriction endonucleases yielded practically the same degree of species differentiation as in the 16S experiment (Table 3).

Strain LMG 1511, formerly classified as *Gluconacetobacter hansenii*, yielded a different pattern from that of *Gluconacetobacter hansenii* LMG 1527^T in all cases, but, in contrast to the 16S experiment, also showed restriction patterns that were different from those of the *Acetobacter aceti* strains. *Acetobacter pasteurianus* LMG 1282 again showed patterns the same as those of the *Gluconacetobacter liquefaciens*/*Gluconacetobacter xylinus*/*Gluconacetobacter europaeus* group.

Identification of indigenous acetic acid bacteria

Twenty-four colonies isolated from wine were tested for their 16S rDNA restriction patterns with *TaqI* and *RsaI*. The patterns obtained with these endonucleases were the same as those of some reference strains, thus allowing the identification of the bacteria at the species level. Three strains were identified as *Acetobacter aceti*, one strain as *Gluconacetobacter hansenii* and 17 strains as *Gluconobacter oxydans*.

The length of the 16S–23S rDNA ITS was not conserved at the species level in the indigenous isolates, in contrast with the results obtained with the reference strains. Amplified ITS products were digested with *CfoI* and *HaeIII*. These enzymes were chosen ran-

Table 3. Sizes of the amplified 16S–23S rDNA ITS and restriction fragments obtained from acetic acid bacteria

Genera are abbreviated as: *Ab.*, *Acetobacter*; *Ac.*, *Acidomonas*; *Ga.*, *Gluconacetobacter*; *Gb.*, *Gluconobacter*.

Strain	ITS (bp)	<i>TaqI</i>	<i>RsaI</i>	<i>AluI</i>	<i>MspI</i>
<i>Gb. oxydans</i> LMG 1408 ^T	700	410, 290	700	190, 190, 175, 110	350, 210, 140
<i>Gb. oxydans</i> CECT 360 ^T	700	410, 290	700	190, 190, 175, 110	350, 210, 140
<i>Gb. oxydans</i> LMG 1484	700	410, 290	700	190, 190, 175, 110	350, 210, 140
<i>Gb. oxydans</i> LMG 1414	700	410, 290	700	350, 110, 110	350, 210, 140
<i>Gb. frateurii</i> LMG 1365 ^T	700	410, 290	500, 200	190, 190, 175, 110	550, 150
<i>Ab. aceti</i> LMG 1261 ^T	750	375, 325	750	290, 130, 110, 90	750
<i>Ab. aceti</i> CECT 298 ^T	750	375, 325	750	290, 130, 110, 90	750
<i>Ab. aceti</i> LMG 1505	750	375, 325	750	290, 130, 110, 90	750
<i>Ab. aceti</i> LMG 1372	750	375, 325	750	290, 130, 110, 90	750
<i>Ga. hansanii</i> LMG 1511*	800	405, 395	800	325, 190, 110, 90	450, 350
<i>Ab. pasteurianus</i> LMG 1262 ^T	750	375, 325	750	290, 130, 110, 90	440, 310
<i>Ab. pasteurianus</i> LMG 1553	750	375, 375	650, 100	290, 130, 110, 90	440, 310
<i>Ga. hansanii</i> LMG 1527 ^T	775	425, 190, 160	775	475, 130, 90	450, 160, 100
<i>Ga. liquefaciens</i> LMG 1381 ^T	725	390, 375	425, 300	425, 210, 110	550, 125, 90
<i>Ga. liquefaciens</i> LMG 1347	725	390, 375	425, 300	425, 210, 110	550, 125, 90
<i>Ga. xylinus</i> LMG 1515 ^T	725	390, 375	425, 300	425, 210, 110	550, 125, 90
<i>Ga. xylinus</i> LMG 1518	725	390, 375	425, 300	425, 210, 110	550, 125, 90
<i>Ab. pasteurianus</i> LMG 1282†	725	390, 375	425, 300	425, 210, 110	550, 125, 90
<i>Ga. europaeus</i> DSM 6160 ^T	725	390, 375	425, 300	425, 210, 110	550, 125, 90
<i>Ga. diazotrophicus</i> DSM 5601 ^T	790	395, 375	790	435, 210, 110	650, 125
<i>Ac. methanolica</i> LMG 1668 ^T	775	410, 350	700, 175	400, 225, 110	440, 275

* Reclassified in this study as *Acetobacter aceti*.

† Reclassified in this study as *Gluconacetobacter liquefaciens*/*Gluconacetobacter xylinus*/*Gluconacetobacter europaeus*.

domly, since all the enzymes tested provided similar information on the reference strains. Six different restriction patterns were obtained with *CfoI* and seven with *HaeIII*. All of these patterns were different from those obtained with the reference strains.

DISCUSSION

PCR-RFLP of the 16S rDNA

The methodology used allowed us to differentiate the four genera of acetic acid bacteria as well as to identify natural isolates at the genus level. Also, identification at the species (*Gluconobacter oxydans*, *Acetobacter aceti*, *Acetobacter pasteurianus*, *Gluconacetobacter hansanii*, *Gluconacetobacter diazotrophicus* and *Acidomonas methanolica*) or related species group (*Gluconobacter frateurii*/*Gluconobacter asaii* and *Gluconacetobacter liquefaciens*/*Gluconacetobacter xylinus*/*Gluconacetobacter europaeus*) level was possible. Such identification has traditionally been performed by studying physiological and chemotaxonomic properties (Buchanan & Gibbons, 1984), but the methods are not completely reliable and are often time-consuming. PCR-RFLP of the 16S rDNA with *TaqI* and *RsaI* allows identification of acetic acid bacteria colonies in one working day.

Gluconobacter frateurii and *Gluconobacter asaii* have been reported as different species on the basis of

DNA–DNA hybridization (Mason & Claus, 1989) and their species status is supported phenotypically by their differential growth on nicotinate, ribitol and arabitol (Buchanan & Gibbons, 1984). These features have not been reflected in our 16S rDNA analysis, as they could not be differentiated. In a previous study, *Gluconacetobacter liquefaciens*, *Gluconacetobacter xylinus* and *Gluconacetobacter europaeus* showed a high percentage of similarity between their 16S rDNA sequences (more than 97%; Sievers *et al.*, 1994). A study by Yamada *et al.* (1997) based on a 156 bp partial sequence of 16S rDNA revealed slight nucleotide differences between these species (4 bp at most). Obviously, these differences were not detected with the restriction enzymes used in this study.

Some likely classification errors were found. Strain LMG 1511, formerly classified as *Gluconacetobacter hansanii*, showed the same restriction patterns as the *Acetobacter aceti* strains. Biochemical tests performed on this strain showed its capacity to grow with ethanol as a sole carbon source, and this feature does not correspond to a strain of *Gluconacetobacter hansanii*, while growth under these conditions is one of the characteristics of *Acetobacter aceti* (Buchanan & Gibbons, 1984). These properties demonstrate that this strain should be classified as *Acetobacter aceti*. In the same way, strain LMG 1282, formerly classified as *Acetobacter pasteurianus*, yielded the same pattern as

Gluconacetobacter liquefaciens/*Gluconacetobacter xylinus*/*Gluconacetobacter europaeus* with all of the enzymes tested. Moreover, this strain was positive for ketogenesis from glycerol, which is positive in members of the genus *Gluconacetobacter* and negative in *Acetobacter pasteurianus* (Buchanan & Gibbons, 1984). From these data, strain LMG 1282 should be considered as a member of the genus *Gluconacetobacter*.

Application of this method to colonies of acetic acid bacteria isolated from wine allowed us to identify them at the species level by using *TaqI* and *RsaI*. Biochemical identification tests performed on these strains confirmed the PCR-RFLP results (data not shown). The species found were *Gluconobacter oxydans*, *Acetobacter aceti* and *Gluconacetobacter hansenii*. The presence of these species of acetic acid bacteria in wines has been reported widely (Joyeux *et al.*, 1984; Drysdale & Fleet, 1985).

PCR-RFLP of the 16S-23S rDNA ITS

Two to 11 copies of the rRNA loci are present on the chromosomes of most bacterial species and, in some bacterial groups, the different copies of the intergenic spacer regions show extensive length variation (Dolzani *et al.*, 1995; Lagatolla *et al.*, 1996). A minimum of four copies of the *rrn* locus have been described in *Gluconacetobacter xylinus* and *Gluconacetobacter europaeus*, all of them having identical length and sequence (Sievers *et al.*, 1996). In our hands, only one amplified ITS fragment was obtained for each strain, confirming that there is no length variation in the ITS region within each strain of acetic acid bacteria. Moreover, in all the strains studied, the sum of the lengths of the restriction fragments obtained with the eight restriction endonucleases equalled the length of the corresponding PCR-amplified ITS sequence, indicating that all of the ITS copies within each strain have very similar sequences.

Intergenic rDNA sequences are known to have higher variability than functional sequences of the *rrn* cistrons, allowing the distinction of bacteria below the species level in some cases (Navarro *et al.*, 1992). On this basis, a higher degree of differentiation was expected from PCR-RFLP of the 16S-23S rDNA intergenic spacer than was observed in the 16S analysis, even below the species level. Differentiation to the species level was observed for acetic acid bacteria isolated from wine, which yielded neither the same amplified sizes nor the same restriction patterns as the reference strains. These results indicate that the higher variability of the ITS sequence and length in acetic acid bacteria can be used to identify strains or related groups of strains, while it is not extensible to rapid species identification, because of the lack of pattern identity among strains belonging to the same species.

In contrast, in the reference strains, practically the same degree of differentiation was obtained as in the 16S analysis. This indicates that the non-distinguished

reference strains are very closely related and this could be because these strains have common original substrates or because of the long period spent in the culture collections.

As in the 16S experiment, reference strains belonging to the species *Gluconacetobacter liquefaciens*, *Gluconacetobacter xylinus* and *Gluconacetobacter europaeus* could not be distinguished, despite the high sequence and length variability observed in the ITS. This result and the fact that the 16S rRNA genes of these species have a high percentage of homology (up to 97%; Sievers *et al.*, 1994) raises questions about maintaining them as separate species.

Strain *Acetobacter pasteurianus* LMG 1282 again showed the same patterns as the reference strains belonging to *Gluconacetobacter liquefaciens*, *Gluconacetobacter xylinus* and *Gluconacetobacter europaeus*, confirming the conclusions derived from the 16S analysis. However, strain *Gluconacetobacter hansenii* LMG 1511 did not share the same patterns as the *Acetobacter aceti* strains, as it did in the 16S experiment, representing the only case of variability at the species level within the reference strains.

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