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Genetic and restriction analysis of the 16S–23S rDNA internal transcribed spacer regions of the acetic acid bacteria

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Abstract

The 16S–23S rDNA internal transcribed spacer regions of the acetic acid bacteria were sequenced and evaluated for molecular identification of these bacteria. All the sequenced spacers contained genes for $tRNA^{Ile}$ and $tRNA^{Ala}$, and the antitermination element. The sequences revealed 56.8–78.3% similarity. By PCR amplification of the spacers from 57 strains of acetic acid bacteria, single products of similar sizes were produced. Digestion of the spacers by *Hae*III and *Hpa*II restriction enzymes resulted in 12 distinct groups of restriction types. All the restriction profiles obtained after analysis of microbial populations from vinegar matched one of the 12 groups. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Acetic acid bacteria; *Acetobacter*; *Gluconacetobacter*; *Gluconobacter*; 16S–23S rDNA spacer; Vinegar

1. Introduction

Acetic acid bacteria are widespread microorganisms in nature. An ability to oxidize different kinds of sugars and a strict requirement for an aerobic atmosphere stimulate their growth on various plants (flowers, herbs, fruits etc.). Insects, especially bees, coming into contact with plants disseminate the acetic acid bacteria [1]. The ability to oxidize ethanol to acetic acid enables growth of the acetic acid bacteria in wine, cider, sake and kombucha tea, resulting in the production of different kinds of vinegar and beverages. Another characteristic of the acetic acid bacteria, widely used for the production of Nata de Coco, is the ability to produce considerable amounts of extracellular polysaccharides. Besides producing the desired food, the acetic acid bacteria can also spoil beer, juice, wine and fruits [1].

The taxonomy of the acetic acid bacteria has been substantially changed in recent years [2,3]. The acetic acid bacteria are at present classified into five genera: *Acetobacter* (*A.*), *Gluconacetobacter* (*Ga.*), *Gluconobacter* (*G.*), *Acidomonas* (*Ac.*) and *Asaia* (*As.*). The phenotypic identification of the acetic acid bacteria, especially at the level of species, is difficult [1]. One of the reasons for this difficulty is the high frequency of spontaneous mutations, attributed to the presence of insertion elements in the acetic acid bacteria [4]. Another reason might be structural changes in crucial enzymes, such as alcohol dehydrogenase, causing transformation of the enzyme from the active to the inactive form and vice versa [5].

Since the acetic acid bacteria are involved in the production or spoilage of food, their species identity is important information for the technologist trying to control a bioprocess in the food industry. The identification methods, based on analysis of the phenotypic characteristics of the acetic acid bacteria, are not only inaccurate, but also very time-consuming. Therefore, the application of molecular methods, based on the identification/characterization of specific DNA segments, could be a proper solution for the quick and accurate identification of these microorganisms.

We present, on a model of 57 reference strains and natural isolates, the applicability of restriction fragment length polymorphism of the 16S–23S rDNA spacer region for genus and species delineation of the acetic acid bacteria. With the aim of finding the optimal restriction enzymes, the 16S–23S rDNA spacer regions from some of the type strains of the genera *Acetobacter*, *Gluconaceto-*

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Table 1
Bacterial strains, source of isolation, accession numbers of the 16S rDNA sequences and restriction types resulting from restriction analysis of the PCR-amplified 16S–23S rDNA ITS regions

Species	Strain designation	Source of isolation	Identification based on 16S rDNA sequence (accession number in parentheses)	Restriction types obtained after restriction with:	
				*Hae*III	*Hpa*II
Group *A. aceti*					
A. aceti^T	DSM 3508	beech-wood shavings from a vinegar plant	*A. aceti* (X74066)[a]	A_1	B_1
A. aceti	LMG 1531	n.k.	*A. aceti* (AJ130729)	A_1	B_2
A. aceti	LMG 1512	film in fermenter of rice vinegar, Japan	*A. aceti* (AJ012545)	A_2	B_1
A. aceti	LMG 1496	n.k.	*A. aceti* (AJ012541)	A_1	B_1
A. aceti	LMG 1525	quick vinegar, The Netherlands	n.d.	A_1	B_1
A. aceti	LMG 1372	Nakano rice vinegar-producing company	n.d.	A_2	B_1
A. aceti	ZIM B034, SegI/9	submerged culture alcohol vinegar bioreactor, Slovenia	*A. aceti* (AJ130731)	A_1	B_1
A. aceti	ZIM B043, 105	submerged culture alcohol vinegar bioreactor, Slovenia	*A. aceti* (AJ012542)	A_1	B_1
Group *A. pasteurianus*/*A. pomorum*					
A. pasteurianus^T	LMG 1262	beer, The Netherlands	*A. pasteurianus* (X71863)[a]	P_1	R_1
A. pasteurianus	LMG 1543	vinegar brews, Africa	*A. pasteurianus* (AJ130728)	P_1	R_1
A. pasteurianus	LMG 1607	n.k.	n.d.	P_1	R_2
A. pasteurianus	LMG 1609	n.k.	n.d.	P_1	R_2
A. pasteurianus	ACM 2866	n.k.	n.d.	P_2	R_3
A. pomorum^T	LTH 2458	cider vinegar, Germany	*A. pomorum* (AJ001632)[b]	P_1	R_1
Group *Ga. europaeus*/*Ga. xylinus*					
Ga. europaeus^T	DSM 6160	submerged culture vinegar bioreactor, Germany	*Ga. europaeus* (Z21936)[a]	E_1	F_1
Ga. europaeus	DSM 6161	submerged culture vinegar bioreactor, Germany	*Ga. europaeus*[c]	E_1	F_1
Ga. europaeus	ZIM B059, S1	submerged culture vinegar bioreactor, Germany	*Ga. europaeus*[c]	E_2	F_1
Ga. europaeus	ZIM B058, S3	submerged culture vinegar bioreactor, Germany	n.d.	E_1	F_1
Ga. europaeus	SegI/4	submerged culture alcohol vinegar bioreactor, Slovenia	*Ga. europaeus* (AJ130727)	E_1	F_1
Ga. europaeus	ZIM B053, TSA4	vinegar-producing generator, Switzerland	*Ga. europaeus*[c]	E_1	F_1
Ga. europaeus	ZIM B028, V3	submerged culture red wine vinegar, Slovenia	*Ga. europaeus* (AJ012698)[d]	E_2	F_2
Ga. europaeus	DSM 13109, JK2	submerged culture alcohol vinegar bioreactor, Slovenia	*Ga. europaeus* (Y15289)[d]	E_2	F_1
Ga. xylinus^T	LMG 1515	mountain ash berries	*Ga. xylinus* (X75619)[a]	E_2	F_3
Ga. xylinus	DSM 46604	n.k.	*Ga. europaeus* (AJ316552)	E_2	F_1
Ga. xylinus	LMG 25	n.k.	*Ga. europaeus* (AJ316551)	E_2	F_4
Ga. xylinus	DSM 46603	n.k.	n.d.	E_2	F_1
Ga. xylinus	DSM 2004	vinegar brew, Kenya	n.d.	E_2	F_1
Ga. xylinus	DSM 2325	n.k.	n.d.	E_2	F_3
Group *Ga. intermedius*/*Ga. oboediens*					
Ga. intermedius^T	DSM 11804	kombucha beverage, Switzerland	*Ga. intermedius* (Y14694)[c]	I_1	J_1
Ga. intermedius	DSM 13111, JK3	submerged culture cider vinegar, Slovenia	*Ga. intermedius* (AJ012699)[d]	I_1	J_2
Ga. intermedius	ZIM B051, TSN3	vinegar-producing generator, Switzerland	*Ga. intermedius*[c]	I_1	J_2
Ga. intermedius	ZIM B074, E1	submerged culture vinegar bioreactor, Spain	*Ga. intermedius*[c]	I_1	J_2
Ga. intermedius	ZIM B062, ØSSPR	submerged culture vinegar bioreactor, Switzerland	*Ga. intermedius*[c]	I_1	J_2
Ga. hansenii	LMG 1517	n.k.	*Ga. intermedius* (AJ012464)	I_1	J_3
Ga. hansenii	LMG 1689	n.k.	*Ga. intermedius* (AJ316550)	I_1	J_3
Ga. xylinus	LMG 1510	vinegar, Denmark	*Ga. intermedius* (AJ316549)	I_1	J_1
Ga. oboediens^T	LTH 2460	red wine vinegar, Germany	*Ga. oboediens* (AJ001631)[b]	I_1	J_2
Group *Ga. hansenii*					
Ga. hansenii^T	LMG 1527	vinegar, Jerusalem	*Ga. hansenii* (X75620)[a]	H_1	K_1

Table 1 (*continued*)

Species	Strain designation	Source of isolation	Identification based on 16S rDNA sequence (accession number in parentheses)	Restriction types obtained after restriction with:	
				*Hae*III	*Hpa*II
Ga. hansenii	LMG 1524	vinegar, Jerusalem	*Ga. hansenii* (AJ012543)	H_1	K_1
Ga. hansenii	LMG 1529	malt vinegar brewery acetifier	*Ga. hansenii* (AJ012544)	H_2	K_2
Ga. hansenii	ZIM B033, SegI/2	submerged culture alcohol vinegar bioreactor, Slovenia	*Ga. hansenii* (AJ130732)	H_1	K_1
Ga. hansenii	ZIM B040, SegII/12	submerged culture alcohol vinegar bioreactor, Slovenia	*Ga. hansenii* (AJ130726)	H_1	K_1
Ga. hansenii	ZIM B041, SegII/14	submerged culture alcohol vinegar bioreactor, Slovenia	*Ga. hansenii* (AJ130730)	H_1	K_1
Group *Ga. liquefaciens*					
Ga. liquefaciens^T	LMG 1382	dried fruit, Japan	*Ga. liquefaciens* (X75617)[a]	L_1	Q_1
Ga. liquefaciens	SRI 244	sugar cane, Australia	*Ga. liquefaciens* (AF127391)[e]	L_2	Q_2
Ga. liquefaciens	SRI 1994	mealy bug, Australia	*Ga. liquefaciens* (AF127395)[e]	L_2	Q_2
Group *Ga. diazotrophicus*					
Ga. diazotrophicus^T	LMG 7603	*Saccharum officinarum*, Hawaii	*Ga. diazotrophicus* (75618)[a]	D_1	C_1
Group *Ga. sacchari*					
Ga. sacchari^T	SRI 1794	sugar cane, Australia	*Ga. sacchari* (AF127407)[e]	S_1	V_1
Group *Ac. methanolica*					
Ac. methanolica^T	LMG 1668	ethanol fermentation process, Germany	*Ac. methanolica* (X77468)[a]	M_1	B_1
Group *G. oxydans*					
G. oxydans^T	DSM 3503	beer	*G. oxydans* (X73820)[a]	G_1	O_1
G. oxydans	DSM 3504	n.k.	n.d.	G_1	O_1
G. oxydans	DSM 50049	Amstel beer, The Netherlands	n.d.	G_1	O_1
G. oxydans	DSM 46616	n.k.	n.d.	G_1	O_1
G. oxydans	CECT 4009	n.k.	n.d.	G_1	O_1
Group *G. asaii*					
G. asaii^T	LMG 1390	*Rheum rhabarbarum*, flower, Japan	*G. asaii* (X80165)[a]	G_2	T_1
Group *G. cerinus*/*G. frateurii*					
G. cerinus^T	LMG 1368 t2	cherry, Japan	*G. cerinus* (X80775)[a]	G_2	U_1
G. frateurii^T	DSM 7146	*Fragaria ananassa*, Japan	*G. frateurii* (X82290)[a]	G_2	U_1

Superscript T, type strain; n.k., not known; n.d., not determined; SRI, Sugar Research Institute, Mackay, Australia; LMG, Laboratorium voor Microbiologie, Gent, Belgium; for the abbreviations DSM, ZIM, CECT, LTH and ACM see Section 2.

[a]Data taken from Sievers et al. [9].

[b]Data taken from Sokollek et al. [24].

[c]Data taken from Boesch et al. [7].

[d]Data taken from Trček et al. [25].

[e]Data taken from Franke et al. [26].

bacter and *Gluconobacter* were initially sequenced and the sequences analyzed in more detail.

2. Materials and methods

2.1. Microorganisms

All microorganisms used in this study are listed in Table 1. They were purchased from the German Collection of Microorganisms and Cell Cultures (DSM), the Belgian Coordinated Collections of Microorganisms (BCCM), the Slovene Collection of Industrial Microorganisms (ZIM), the Spanish Type Culture Collection (CECT), the Institute for Food Science and Technology, University of Hohenheim (LTH) and the Australian Collection of Microorganisms (ACM). They were maintained as described in the catalogues of the culture collections.

2.2. DNA isolation

DNA was isolated as described previously [6] or by temperature lysis of cells (cooking at 95°C for 10 min and subsequent cooling to 4°C).

2.3. Amplification of the 16S–23S rDNA and restriction analysis

PCR of the 16S–23S rDNA spacer regions was performed in 100 μl solution containing 15–20 ng of DNA, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 10 μl 10×standard PCR buffer (Pharmacia Biotech), sterile double-distilled water, 100 pmol of each primer and 2.5 U *Taq* DNA polymerase (Pharmacia Biotech). The sequences of the primers were 5′-TGCGG(C/T)TGGATCACCTCCT-3′ (position 1522–1540 on 16S rDNA, *Escherichia coli* numbering) and 5′-GTGCC(A/T)AGGC-

Table 2
Sequencing primers for the 16S–23S rDNA spacer region

Primer	Sequence	Target site	Reference
Uni1392	5′-GTACACACCGCCCGTCA-3′	16S rDNA, 1392–1408[a]	[27]
b-1	5′-ATACGGGGCTATCACCCG-3′	23S rDNA, 340–323[a]	[27]
TAlaf	5′-AGAGCACCTGCTTTGCAA-3′	16S–23S rDNA, 285–300[b]	This study
TAlar	5′-ACCCCCTGCTTGCAAA-3′	16S–23S rDNA, 311–296[b]	This study

[a] *E. coli* position according to Brosius et al. [28]
[b] *Ga. hansenii* position (accession number AJ007832)

ATCCACCG-3′ (position 38–22 on 23S rDNA, *E. coli* numbering). PCR products used for the sequencing analysis were obtained by the following primers: 5′-CGTGTCGTGAGATGTTGG-3′ (position 1071–1087 on 16S rDNA, *E. coli* numbering) and 5′-CGGGGTGCTTTTCACCTTTCC-3′ (position 488–468 on 23S rDNA, *E. coli* numbering). The synthesized oligonucleotide primers were obtained from Microsynth (Balgach, Switzerland). Amplification was performed in 0.2-ml tubes using Genius thermocycler (Genius, Techne). The cycling program started with initial denaturation of DNA at 94°C for 5 min and continued with 30 cycles of 92°C for 30 s, 56°C for 45 s and 72°C for 1 min. The polymerization time was increased to 2 min when primers for the amplification of the rDNA fragment for direct sequencing were used. At the end a final extension at 72°C for 7 min was performed, followed by cooling down to 4°C. PCR products were electrophoresed in 1.0% w/v agarose gels submerged in 1×Tris-acetate running buffer. The 1-kb plus DNA ladder (Life Technologies) was used as a length standard.

PCR products were digested separately with *Hae*III and *Hpa*II restriction enzymes following the instructions of the manufacturer (Life Technologies). Restriction fragments were analyzed by 2.5% w/v agarose gel electrophoresis in 1×Tris-acetate running buffer. The length of the restriction fragments was calculated relative to that of DNA marker by linear regression of the semilogarithmic curve (mobility vs. logarithm of DNA fragment length).

2.4. Amplification and sequencing of the 16S rDNA

The genes encoding 16S rRNA were amplified in vitro using oligonucleotide primers 5′-AAATTGAAGAGTTTGATC(A/C)TGGC-3′ (position 1–23 on 16S rDNA, *E. coli* numbering) and 5′-AGGAGGTGATCC(A/G)CCGCA-3′ (position 1540–1522 on 16S rDNA, *E. coli* numbering). PCR was performed as described above for the amplification of the 16S–23S spacer regions using an appropriate polymerization time (1.5 min) for this set of primers. Direct sequencing of the PCR products was performed as described below for the sequencing of the 16S–23S spacer regions using the primers described by Boesch et al. [7].

2.5. Sequencing of the 16S–23S rDNA spacer regions

Direct sequencing of the purified PCR products was performed by the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP according to the manufacturer's instructions (Amersham). The Cy5-labeled sequencing primers (Table 2) were obtained from Microsynth (Balgach, Switzerland). Cycle sequencing was performed on a Biometra Personal Cycler (Biometra, Göttingen, Germany) during 25 cycles of the following parameters: 95°C for 30 s, 50°C for 30 s and 72°C for 1 min. Detection was performed on an ALF-Express automatic DNA sequencer (Pharmacia Biotech).

2.6. Nucleotide sequence deposition numbers

All the nucleotide sequences were deposited in the EMBL/GenBank/DDBJ databases. Nucleotide sequences of the 16S–23S rDNA spacer region of *A. aceti*^{T}, *A. pasteurianus*^{T}, *Ga. hansenii*^{T}, *Ga. liquefaciens*^{T}, and *G. oxydans*^{T} were deposited under the accession numbers AJ007831, AJ007834, AJ007832, AJ007833, and AJ007763, respectively. The accession numbers of the 16S rRNA gene sequences are listed in Table 1.

3. Results and discussion

3.1. Sequence comparison of the 16S–23S rDNA spacers

Using primers constructed from the conserved regions of the genes encoding 16S and 23S rRNA, the 16S–23S

Table 3
Percentage of nucleotide similarity among the acetic acid bacteria based on 16S–23S rDNA ITS regions

Species	Sequence similarity (%)					
	1	2	3	4	5	6
1. *A. aceti*						
2. *A. pasteurianus*	67.8					
3. *Ga. europaeus*	64.1	67.3				
4. *Ga. hansenii*	56.8	69.7	69.4			
5. *Ga. liquefaciens*	69.3	65.6	60.0	61.4		
6. *Ga. xylinus*	59.6	64.5	78.3	64.1	58.7	
7. *G. oxydans*	62.7	59.2	61.4	61.6	62.6	56.8

E. coli	T	G	C	**T**	**C**	**T**	**T**	**T**	A	A	C	**A**
A. aceti	T	A	T	**T**	**C**	**T**	**T**	**T**	G	T	C	**A**
A. pasteurianus	T	G	A	**T**	**C**	**T**	**T**	**T**	G	T	C	**A**
Ga. europaeus	T	G	C	**T**	**C**	**T**	**T**	**T**	G	A	T	**A**
Ga. hansenii	T	G	T	**T**	**C**	**T**	**T**	**T**	G	T	C	**A**
Ga. liquefaciens	T	G	T	**T**	**C**	**T**	**T**	**T**	G	T	C	**A**
Ga. xylinus	T	G	C	**T**	**C**	**T**	**T**	**T**	G	A	T	**A**
G. oxydans	G	G	G	**T**	**C**	**T**	**T**	**T**	T	A	G	**A**

Fig. 1. Alignment between the box A-like sequences from the 16S–23S rDNA regions of *E. coli* [12], *A. aceti*^{T}, *A. pasteurianus*^{T}, *Ga. europaeus*^{T}, *Ga. hansenii*^{T}, *Ga. liquefaciens*^{T}, *Ga. xylinus*^{T} and *G. oxydans*^{T}.

rDNA spacers of the type strains of *A. aceti*, *A. pasteurianus*, *Ga. hansenii*, *Ga. liquefaciens* and *G. oxydans* were specifically amplified. By direct sequencing of the PCR products, 724, 724, 725, 704, and 653 bp long spacers were determined for *A. aceti*, *A. pasteurianus*, *Ga. hansenii*, *Ga. liquefaciens* and *G. oxydans*, respectively. Alignment of these sequences and the sequences from type strains of *Ga. europaeus* and *Ga. xylinus* [8] exhibits 56.8–78.3% similarity (Table 3). The much lower similarities of these sequences in comparison with the 94.2–99.6% similarities of the 16S rDNA sequences [9] are a result of greater evolutionary rates in the rDNA spacers [10]. On the other hand, two highly conserved regions (96–100% similarity), encoding $tRNA^{Ile}$ (77 bp) and $tRNA^{Ala}$ (75 bp), were found in the spacers of all acetic acid bacteria. These sequences are believed to have an important role in the process of rRNA formation [11]. The 3′-end terminal sequence CCA normally present in mature tRNA was observed in both tRNAs. The antitermination elements, box A-like sequences (Fig. 1), have two to four mismatches with the corresponding sequence of *E. coli* [12]. Another highly conserved region of 17 nucleotides, following the box A element, has been found in the spacers of all acetic acid bacteria (data not shown).

The sequences of the 16S rRNA of the acetic acid bacteria are very similar to each other [3]. This causes problems in delineating all the species of the acetic acid bacteria on the basis of restriction fragment length polymorphism of the 16S rDNA [13]. Much higher variability in sequence composition of the spacer regions prompted us to test the applicability of the restriction fragment length polymorphism of the PCR-amplified spacer region for easy and quick species differentiation of the acetic acid bacteria. This approach was already successfully used for the strain classification and identification of many bacteria. Recently, also a database for 16S–23S rDNA sequences was established (http://ulises.umh.es/RISSC) enabling comparison of these sequences from different bacteria [14]. To be able to evaluate the above-mentioned approach for species identification of the acetic acid bacteria, exact species identity is obligatory information. Therefore, the species identity of the acetic acid bacteria was checked by sequencing of the genes encoding 16S rRNA.

3.2. Sequence analysis of the genes encoding 16S rRNA

For most of the strains described in Table 1, the 16S rDNA sequences were entirely or partially determined and aligned with previously described 16S rDNA sequences of the acetic acid bacteria. The strain was classified into the species with which its 16S rDNA sequence exhibited the highest percentage of similarity. All the sequences were deposited into the EMBL/GenBank/DDBJ databases; the accession numbers are listed in Table 1.

Gosselé et al. [15] have reclassified strains *A. aceti* subsp. *xylinus* LMG 1517 (NCIM 4940) and *A. aceti* subsp. *xylinus* LMG 1689 (LMD 29.8) into *Ga. hansenii* on the basis of their phenotypic features and protein gel electrophoregrams. Our results of partial sequencing of genes encoding 16S rRNA could not place these strains into *Ga. hansenii*, nor strain *Ga. xylinus* LMG 1510 into species *Ga. xylinus* (Table 1). 16S rDNA sequences from *Ga. hansenii* LMG 1517, *Ga. hansenii* LMG 1689 and *Ga. xylinus* LMG 1510 matched best with the 16S rDNA sequence of *Ga. intermedius*. For all three strains additional arguments exist suggesting their misclassification. Strains *Ga. hansenii* LMG 1517 and *Ga. hansenii* LMG 1689 showed very low DNA–DNA similarity (8% and 14%) with *Ga. hansenii* type strains as described by Navarro et al. [16]. For strain *Ga. xylinus* LMG 1510, Boesch [17] has shown below 15% DNA–DNA similarity with the type strains of *Ga. xylinus* and *Ga. europaeus*.

3.3. Size comparison of the 16S–23S rDNA spacer regions

Small length polymorphism exists among the PCR-amplified spacers of the acetic acid bacteria, but the differences are difficult to recognize accurately and reliably by standard agarose gel electrophoresis. However, precise length evaluation of the PCR products shows that the products from the genus *Gluconobacter* are smaller than those from the other examined acetic acid bacteria (data not shown). Also the nucleotide sequence of the spacer from *G. oxydans* type strain is about 50 bp smaller in comparison to the spacers from other analyzed acetic acid bacteria.

Sievers et al. [8] have estimated that four copies of the 16S–23S spacer region are present on the chromosome of *Ga. xylinus* and *Ga. europaeus*. In this study the copy number of the spacer regions was not studied. However, the single amplified PCR product of all 57 analyzed acetic acid bacteria suggests no length polymorphism among copies of the 16S–23S spacer region.

3.4. Restriction analysis of the 16S–23S rDNA spacer regions

Fifty-seven strains of acetic acid bacteria, which were obtained from different culture collections, were analyzed. In Table 1 strains are grouped according to their species

identity recognized by sequencing of the gene for 16S rRNA.

The sequences of 16S–23S ITS regions of the acetic acid bacteria were subjected to theoretical restriction analysis using the WebCutter program. Two enzymes, producing two to three restriction products and giving different restriction profiles among all species of the acetic acid bacteria, were selected and applied for restriction analysis of the reference strains. To avoid ambiguities due to the poor visibility and resolution limitations of the standard agarose gel electrophoresis, only fragments bigger than 100 bp were taken into account for the interpretation of restriction patterns. The same letter and number were given to the same type of restriction patterns. For each strain, the patterns obtained with both enzymes were combined, resulting in the following 12 different restriction groups: *A. aceti, A. pasteurianus*/*A. pomorum, Ga. europaeus*/*Ga. xylinus*, *Ga. intermedius*/*Ga. oboediens*, *Ga. hansenii*, *Ga. liquefaciens*, *Ga. diazotrophicus, Ga. sacchari, Ac. methanolica, G. oxydans, G. asaii, G. cerinus*/*G. frateurii*. Each group exhibits up to four different *Hae*III and *Hpa*II restriction types (Tables 1, 4 and 5). None of the restriction profile from one restriction group was observed in the other restriction group, and more of the same restriction profiles were generated in the same restriction group. The only exception is profile B_1, which is actually a result of no restriction site in the spacers of *A. aceti* and *Ac. methanolica*.

To prove that the presented collection of the restriction profiles might be used as a database to which the restriction profile of an unknown isolate from vinegar might be compared, strains were isolated from industrially produced wine vinegar. Six of them were randomly chosen for PCR amplification of spacer regions and subsequent restriction analysis. Comparison of their restriction profiles to the restriction profiles of the reference strains placed all isolates (restriction type E_2F_1) in the group *Ga. europaeus*/*Ga. xylinus*. The same result was obtained when total DNA isolated directly from biomass harvested from alcohol vinegar was used as a template in the PCR reaction.

Table 4
Molecular sizes of 16S–23S rDNA restriction fragments of different restriction types obtained with *Hae*III

Restriction type	Molecular size (bp) of 16S–23S rDNA restriction fragments
A_1	500, 290
A_2	310, 290, 190
P_1	470, 300
P_2	300, 280, 180
E_1	330, 250, 210
E_2	540, 250
I_1	510, 260
H_1	400, 200, 100
H_2	370, 240, 100
L_1	490, 250
L_2	430, 250
D_1	310, 210, 150
S_1	500, 210
M_1	260, 230, 190
G_1	520, 120
G_2	520, 230

Table 5
Molecular sizes of 16S–23S rDNA restriction fragments of different restriction types obtained with *Hpa*II

Restriction type	Molecular size (bp) of 16S–23S rDNA restriction fragments
B_1	800
B_2	500, 210
R_1	450, 330
R_2	380, 330, 110
R_3	560, 210
F_1	450, 270, 100
F_2	400, 270, 100
F_3	390
F_4	470, 140
J_1	420, 150, 100
J_2	370, 270, 100
J_3	420, 270, 100
K_1	350, 170, 100
K_2	330, 170, 100
Q_1	550, 120
Q_2	430, 160, 120
C_1	400, 170
V_1	380, 210
O_1	340, 220, 160
T_1	480, 160, 100
U_1	570, 160

Besides other characteristics, each genus of the acetic acid bacteria typically possesses a distinctive feature, which can be easily used for identification on the genus level. The genus *Gluconobacter* is characterized by inability to overoxidize acetic acid to CO_2 and H_2O [1]. The genus *Acidomonas* is characterized by its ability to grow on methanol [1] and the genus *Asaia* by its inability to grow on a 0.35% acetic acid-containing medium [3]. The two other genera, *Acetobacter* and *Gluconacetobacter*, can be differentiated from each other on the basis of ubiquinone Q-9 and ubiquinone Q-10 contents [2]. Further identification of the acetic acid bacteria at the species level is very time-consuming and cumbersome [18,19]. From our experiences almost each strain of the acetic acid bacteria has a unique growth requirement. That induces problems in using an appropriate minimal medium which is a prerequisite for further biochemical identification of isolates. In this study, a molecular approach has been studied with the aim to make a database of 16S–23S rDNA restriction profiles of the acetic acid bacteria. By comparing the restriction profile of an unknown isolate of an acetic acid bacterium with the restriction profiles presented here, the isolate can be quickly and simply assigned to one of 12 presented groups. The approach presented here was successfully used for identification of the acetic acid bacteria from vinegar.

4. Note added in proof

During the preparation of this manuscript new species of the acetic acid bacteria were described [20–23].

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