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Molecular Characterization of Acetic Acid Bacteria Isolated from Spirit Vinegar

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Summary

Three different molecular techniques were used to characterize strains of the acetic acid bacteria isolated from spirit vinegar. Genomic DNA was analyzed by amplified rDNA restriction analysis (ARDRA), randomly amplified polymorphic DNA analysis (RAPD) and dot blot hybridization analysis with an IS1380 probe. Among 13 analyzed Acetobacter strains, the ARDRA method revealed three different profiles. Each of these profiles was comparable to one of the ARDRA profiles of the following reference strains – Acet. aceti^T, Acet. hansenii^T and Acet. xylinus^T. RAPD profiling was more discriminatory than ARDRA profiling, being able to distinguish between isolates with identical ARDRA profiles.

Key words: acetic acid bacteria, spirit vinegar, ARDRA analysis, RAPD analysis, IS*1380*

Introduction

Acetic acid bacteria are Gram-negative obligative aerobes composed of two genera *Acetobacter* (*Acet.*) and *Gluconobacter* (*Gluc.*) *(1)*. In Bergey's Manual of Determinative Bacteriology *(2)*, seven phenotypically different *Acet.* species and three *Gluc.* species are described. *Acet. europaeus* described by Sievers *et al. (3)* in 1992 has not (yet) been included in the afore mentioned publication. Acetic acid bacteria are used in different biotechnological processes such as vinegar production, cellulose production, sorbose production (partial reaction in the pathway of ascorbic acid production), *etc.*

The authors have classified strains isolated from industrial vinegar bioprocesses running in different parts of the world into various species according to their phenotypic features. Kittelmann *et al. (4)* phenotyped the strains isolated from bioreactors for the production of 90–150 g L^{-1} vinegar as *Acet. pasteurianus* and *Acet. hansenii*, Entani *et al. (5)* as »*Acet. polyoxogenes*« and Sievers *et al. (3)* as *Acet. europaeus*. Karova *(6)* identified the strains isolated from submerged bioreactors as *Acet. aceti* and *Acet. xylinus (7)* and Ohmori *et al. (8)* the strains isolated from a submerged bioprocess running at a high temperature as *Acet. aceti*.

Acetic acid bacteria are known for their high frequency of spontaneous mutations. All mutational mechanisms are not elucidated yet, however one of them was presented by Takemura *et al. (9)*. He has discovered IS*1380* in mutant strains of *Acet. pasteurianus* which have lost their ability to oxidize ethanol into acetic acid. The same element in high copy numbers was also found in *Acet. aceti* (IFO 3282), *Acet. xylinus* (NCIB 11664), »*Acet. polyoxogenes*« (NCI 1028) and *Acet. europaeus* (DSM 6160). In other *Acet.* species and *Gluc.* species DNA homology with IS*1380* was not detected *(9,10)*.

The purpose of the present work was to characterize the acetic acid bacteria isolated from spirit vinegar by molecular techniques, *i.e.* amplified rDNA restriction analysis (ARDRA), RAPD analysis and dot blot hybridization analysis with an IS*1380* probe. We were specially interested in comparing the discrimination capability of the ARDRA and RAPD techniques for the characterization of the acetic acid bacteria.

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Materials and Methods

Microorganisms

Fourteen strains of the acetic acid bacteria were isolated from the laboratory spirit vinegar producing bioreactor situated at the vinegar production plant in Ljubljana. The parameters of the bioprocess, the isolation protocol and the cultivation conditions are described in a previous publication *(11)*. Five reference strains of *Acetobacter* sp. were included in the molecular analysis for a comparative study: *Acet. aceti*^{T} (LMG 1261), *Acet. europaeus*^{T} (DSM 6160), *Acet. hansenii*^{T} (LMG 1527), *Acet. liquefaciens*^{T} (LMG 1382) and *Acet. xylinus*^{T} (LMG 1515).

Genomic DNA preparation

Isolates of the acetic acid bacteria were grown on the AE (acetic acid – ethanol) agar media up to 5 days at 30 °C and 92–96 % humidity. The reference strains *Acet. aceti*^{T}, *Acet. europaeus*^{T}, *Acet. hansenii*^{T}, *Acet. liquefaciens*^{T} and *Acet. xylinus*^{T} were grown at 30 °C in a liquid medium composed as described by LMG (Culture Collection of the Laboratorium voor Microbiologie, Rijksunversiteit) and DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen) culture collections, all of them up to 0.8–1.0 absorbance at 620 nm. After harvesting the biomass, DNA was isolated according to a modified Marmur procedure *(12)* as described previously *(11)*.

Amplification of the rDNA and restriction analysis

The polymerase chain reaction (PCR) was carried out in 20 μL containing 30–50 ng of DNA, 2 mM $MgCl_2$, 20 pmoles of each primer, 1.5 U of Ampli Taq DNA polymerase (Perkin Elmer), 200 μM each of dCTP, dGTP, dATP, dTTP (Perkin Elmer) and 2 μL 10× PCR buffer II (Perkin Elmer). A Perkin Elmer Gene Amp PCR System 2400 was used for amplification. The cycling programme started with initial denaturation of DNA at 94 °C for 5 min and continued with 35 cycles of 92 °C for 1 min, 55 °C for 2 min and 72 °C for 4 min. At the end a final extension at 72 °C for 7 min was performed, followed by cooling to 4 °C. The PCR product was electophoresed in 8 g L^{-1} agarose gels submerged in 1x Tris acetate running buffer. The DNA molecular weight marker X (Boehringer Mannheim) was used as a length standard. PCR products were visualized after staining with ethidium-bromide by transillumination and photographed with a Polaroid camera.

Oligonucleotide primers were derived from conserved regions in the 16S and 23S rDNA *(13,14)*. The sequences of the primers were 5'-GAGTTTGAT(C/T)(C/A)TGGCTCA-3' and 5'-CCTTTCCCTCACGGTACTGGT-3'. The gene fragment amplified (ca. 2400 bp) encompassed the 16S and 16S–23S spacer region and part of the 23S rDNA. Synthesized and purified oligonucleotides were obtained from Codon genetic systems GmbH (Wien, Austria).

The PCR products were digested with 11 different restriction enzymes following the instructions of the manufacturer (Boehringer Mannheim). Restriction fragments were analysed by agarose 20 g L^{-1} gel electrophoresis at 7 V/cm after 2 h.

RAPD analysis

A synthetic oligonucleotide primer with GC content 70 % (5'-AGCGGGCGTA-3') was selected. RAPD analysis was carried out as described in our previous publication *(11)*.

Dot blot hybridization analysis with an IS1380 probe

A PCR amplified probe (1.3 kbp) *(15)* complementary to an *IS1380* region of *Acet. pasteurianus*^{T}, was labelled with the ECL^{TM} random prime labelling system, Version II (Amersham), according to the manufacturer's instructions. Samples of DNA diluted at the same final concentrations (100 ng/L) in the TE buffer were denatured by heating for 5 min in a boiling water bath followed by cooling on ice. Volume (5 μL) of each DNA sample was transferred to a Hybond-N+ nylon membrane (Amersham) by dot blotting. The DNA was bound to the membranes by ultraviolet crosslinking. The membranes were prehybridized for 3 h at 65 °C in a buffer containing 5 × SSC (20 × SSC contains 0.3 M Na-citrate and 3 M NaCl) 1 g L^{-1} sodium dodecylsulphate (SDS), 50 g L^{-1} dextran sulphate and a 20-fold dilution of liquid block (supplied with the labelling system mentioned above). The probe was then added and hybridization was allowed to perform overnight at 65 °C. The membrane washing steps were: once in 1× SSC, 1 gL^{-1} SDS for 15 min at 60 °C and once in 0.5× SSC, 1 g L^{-1} SDS for 15 min at 60 °C. Chemiluminescent detection was performed by the ECL^{TM} detection system, Version II (Amersham), according to the manufacturer's instructions.

Results and Discussion

In this study, *Acetobacter* strains isolated from spirit vinegar and five reference strains of *Acetobacter* sp. were identified and characterized by different molecular approaches.

DNAs of five reference strains and 13 out of 14 *Acetobacter* isolates were successfully amplified with universal ribosomal primers. They all produced a single band of about 2400 bp. Eleven restriction enzymes were tested on four randomly selected PCR products. With some of the restriction enzymes we obtained no restriction products (*Bam*HI, *Eco*RV, *Hind*III, *Pst*I, *Sau*3A,), with some of them restriction profiles were not different (*Eco*RI, *Dde*I, *Hind*II, *Rsa*I). Only two enzymes (*Hae*III, *Scr*FI) gave polymorphic restriction profiles. With the *Hae*III restriction enzyme we identified three different ARDRA profiles among 13 analyzed *Acetobacter* strains (Table 1). We compared these three different profiles with those of *Acet. aceti*^{T}, *Acet. europaeus*^{T}, *Acet. hansenii*^{T}, *Acet. liquefaciens*^{T} and *Acet. xylinus*^{T} (Fig. 1). Each of the three profiles was identical to one of the profiles of the afore mentioned *Acetobacter* type species. This means that four out of the 13 *Acetobacter* strains isolated from spirit vinegar could be identified as *Acet. aceti*, four of them as *Acet. hansenii* and five of them as *Acet. xylinus*. It must be pointed out that with standard agarose gel electrophoresis that we used, the differences below approximately 300 bp could not be precisely evaluated.

Table 1. Strains of *Acetobacter* sp. examined in this study and types resulting from ARDRA, RAPD analysis and hybridization analysis with an *IS1380* probe

Species	Strain no.	Sources	ARDRA type	RAPD type	Hybridization with *IS1380* probe
Acet.[a] *aceti*^{T}	LMG 1261^{T}	Vinegar	2	8	nt[b]
Acet. europaeus^{T}	DSM 6160^{T}	Vinegar	5	6	+
Acet. hansenii^{T}	LMG 1527^{T}	Vinegar	1	10	–
Acet. liquefaciens^{T}	LMG 1382^{T}	Dried fruit	4	9	–
Acet. xylinus^{T}	LMG 1515^{T}	Mountain ash berries	3	7	+
Acet. sp.	SI/3	Vinegar	1	1	–
Acet. sp.	SI/4	Vinegar	3	3	+
Acet. sp.	SI/11	Vinegar	1	2	–
Acet. sp.	SI/12	Vinegar	1	2	–
Acet. sp.	SI/19	Vinegar	1	2	–
Acet. sp.	SI/23	Vinegar	nt	4	+
Acet. sp.	SII/1	Vinegar	2	5	+
Acet. sp.	SII/3	Vinegar	3	3	+
Acet. sp.	SII/6	Vinegar	2	5	+
Acet. sp.	SII/9	Vinegar	3	3	nt
Acet. sp.	SII/11	Vinegar	2	5	+
Acet. sp.	SII/16	Vinegar	3	3	+
Acet. sp.	SII/18	Vinegar	2	5	+
Acet. sp.	SII/23	Vinegar	3	3	+

[a]Acet. = Acetobacter; [b]nt = not tested

Further on, we subjected all *Acetobacter* strains to RAPD analysis. This analysis revealed ten different profiles (Fig. 2). None of the strains isolated from Slovene vinegar had an identical RAPD profile to those of *Acet. aceti*^{T}, *Acet. europaeus*^{T}, *Acet. hansenii*^{T}, *Acet. liquefaciens*^{T} and *Acet. xylinus*^{T}. Besides, strain *Acet.* SI/3 which was identified as *Acet. hansenii* by ARDRA profiling, had a different RAPD profile in comparison to those of the other three *Acet. hansenii* strains, SI/11, SI/12, SI/19 (Table 1).

We followed also the presence of the insertion sequence IS*1380* in *Acetobacter* strains. We used an approach which is easy and quick to perform, i.e. dot blot DNA hybridization. With a positive hybridization signal

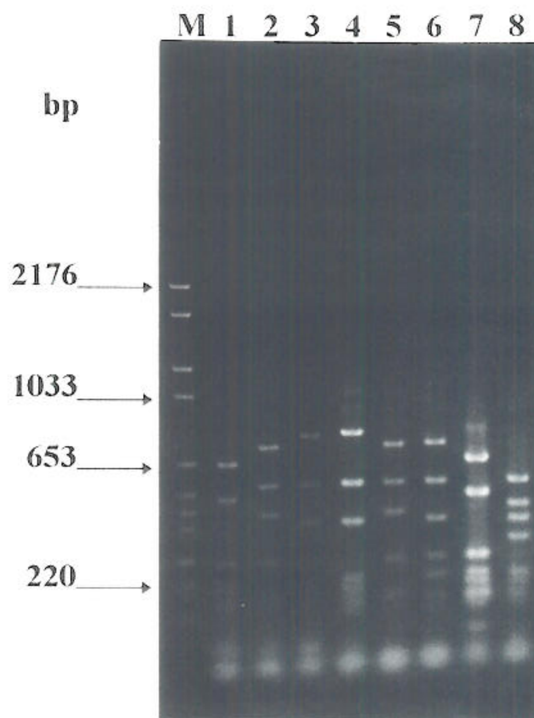

Fig. 1. Restriction profiles of PCR amplified rDNA, digested with *Hae*III. Lane 1 (type 1), lane 2 (type 2) and lane 3 (type 3) are representatives of three different profiles identified among 13 strains of *Acet.* sp. isolated from spirit vinegar. Lane 4 (type 3) shows the profile of *Acet. xylinus*^{T}, lane 5 (type 2) of *Acet. aceti*^{T}, lane 6 (type 4) of *Acet. liquefaciens*^{T}, lane 7 (type 1) of *Acet. hansenii*^{T}and lane 8 (type 5) of *Acet. europaeus*^{T}. Lane M contained molecular size marker VI (Boehringer, Mannheim)

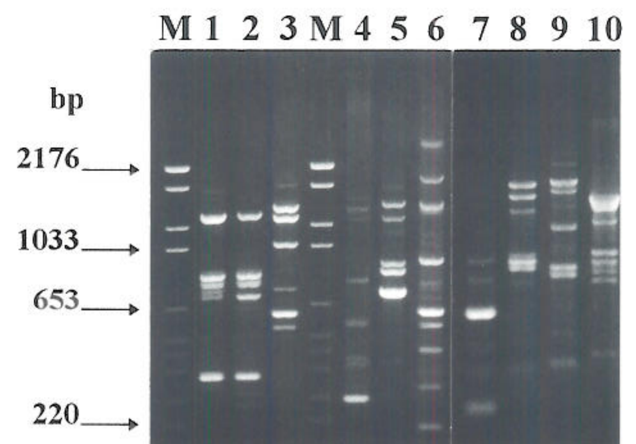

Fig. 2. RAPD profiles of *Acet.* sp. generated with primer 70 % GC. Lane 1 (type 1), lane 2 (type 2), lane 3 (type 3), lane 4 (type 4) and lane 5 (type 5) are representatives of five different profiles identified among 14 strains of *Acet.* sp. isolated from spirit vinegar. Lane 6 (type 6) shows the profile of *Acet. europaeus*^{T}, lane 7 (type 7) of *Acet. xylinus*^{T}, lane 8 (type 8) of *Acet. aceti*^{T}, lane 9 (type 9) of *Acet. liquefaciens*^{T} and lane 10 (type 10) of *Acet. hansenii*^{T}. Lane M contained molecular size marker VI (Boehringer, Mannheim)

we confirmed IS*1380* in 9 out of 13 *Acetobacter* strains. We detected IS*1380* also in *Acet. europaeus*^{T} and *Acet. xylinus*^{T}, but we did not detect it in *Acet. hansenii*^{T} and *Acet. liquefaciens*^{T}, which agreed with the findings of Takemura *et al. (9)*, Sievers and Teuber *(10)*. We did not detect IS*1380* in any of the *Acetobacter* isolates which had the same ARDRA profiles as *Acet. hansenii*^{T}.

Previously *(11)* we classified most of these 14 *Acetobacter* strains which were isolated from spirit vinegar into *Acet. europaeus* since they had an obligative requirement of acetic acid in isolation and cultivation media *(3)*. We subcultured these isolates every 14 days onto fresh, modified AE media and after six months we tried again to cultivate them on media not containing acetic acid. Despite the same cultivation conditions as before, all of them gained the ability to grow without the acetic acid (data not shown). In this study we demonstrated with the ARDRA method that these strains, in fact, should not be classified in *Acet. europaeus* since none of the ARDRA profiles of *Acetobacter* isolates was comparable to the profile of *Acet. europaeus*^{T}. This finding stresses the importance of using the genotypic methods for identification of the acetic acid bacteria.

Our results demonstrated a characteristic profile for each of the five analysed reference *Acetobacter* species. Each of the ARDRA profiles of *Acetobacter* strains which we isolated from vinegar was comparable to one of the ARDRA profiles of reference *Acetobacter* sp. Therefore we think that the ARDRA method offers us the possibility for simple, quick and reliable identification of *Acetobacter* species. However, we found RAPD profiling to be more discriminatory than ARDRA profiling, being able to distinguish between isolates with identical ARDRA profiles. Therefore, to distinguish individual *Acetobacter* strains, the two methods should be considered complementary.

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References

1. J. De Ley, M. Gillis, J. Swings, *Acetobacteraceae*. In: *Bergey's Manual of Systematic Bacteriology, Vol 1*, N. R. Krieg, J. G. Holt (Eds.), Williams & Wilkins, Baltimore (1984) pp. 267–278.
2. J. G. Holt, N. R. Krieg, P. H. A. Sneath, J. T. Staley, S. T. Williams: *Bergey's Manual of Determinative Bacteriology*, Williams & Wilkins, Baltimore (1994) p. 126, p. 142.
3. M. Sievers, S. Sellmer, M. Teuber, *Syst. Appl. Microbiol. 15* (1992) 386–392.
4. M. Kittelmann, W. Stamm, H. Follmann, H. G. Trüper, *Appl. Microbiol. Biotechnol. 30* (1989) 47–52.
5. E. Entani, S. Ohmori, H. Masai, K. I. Suzuki, *J. Gen. Appl. Microbiol. 31* (1985) 475–490.
6. E. Karova: *Isolation Characteristics and Application of Acetic Acid Bacteria*, Ph. D. Thesis, Plovdiv, Bulgaria (1988).
7. J. P. Euzeby, *Int. J. Syst. Bacteriol. 47* (1997) 585.
8. S. Ohmori, H. Masai, K. Arima, T. Beppu, *Agric. Biol. Chem. 44* (1980) 2901–2906.
9. H. Takemura, S. Horinouchi, T. Beppu, *J. Bacteriol. 173* (1991) 7070–7076.
10. M. Sievers, M. Teuber, *J. Appl. Bacteriol. Symp. Suppl. 79* (1995) 84S–95S.
11. J. Trček, J. Ramuš, P. Raspor, *Food Technol. Biotechnol. 35* (1997) 63–67.
12. J. Marmur, *J. Mol. Biol. 3* (1961) 208–218.
13. M. Sievers, W. Ludwig, M. Teuber, *Syst. Appl. Microbiol. 17* (1994) 189–196.
14. M. Vaneechoutte, R. Rossau, P. De Vos, M. Gillis, D. Janssens, N. Paepe, A. De Rouck, T. Fiers, G. Claeys, K. Kersters, *FEMS Microbiol. Lett. 93* (1992) 227–234.
15. C. Boesch: *Vergleich von spezifischen DNA-Sequenzen in der Taxonomie von Essigsäurebakterien*, Graduate Thesis, ETH-Zürich, Switzerland (1993).

Molekularna karakterizacija bakterija octene kiseline izoliranih iz alkoholnog octa

Sažetak

Za karakterizaciju sojeva bakterija octene kiseline, izoliranih iz alkoholnog octa, primijenjena su tri različita postupka. Genomska DNA analizirana je amplificiranom rDNA restrikcijskom analizom (ARDRA), analizom nasumce amplificiranom polimorfnom DNA (RAPD) i analizom točkaste hibridizacije sa sondom IS1380.

Između 13 analiziranih sojeva Acetobacter, postupkom ARDRA, otkrivena su tri različita profila. Svaki od njih mogao se usporediti s jednim ARDRA profilom od referentnih sojeva – Acet. aceti^T*, Acet. hansenii*^{T} *i Acet. xylinus*^{T}*. RAPD profiliranje bilo je mnogo selektivnije jer se moglo razlučiti izolate s identičnim ARDRA profilima.*