Mitteilungen Klosterneuburg 56 (2006): 102-107

Acetobacter tropicalis in spontaneously fermented wines with vinegar fermentation in Austria

KARIN SILHAVY and KARIN MANDL

Höhere Bundeslehranstalt und Bundesamt für Wein- und Obstbau
A-3400 Klosterneuburg, Wiener Strasse 74
E-mail: Karin.Silhavy@hblawo.bmlfuw.gv.at

Acetic acid bacteria are known for their ability to spoil wines irreversibly. To get to know the species diversity in Austria, 84 bacterial strains were isolated from spontaneously fermented wines with following acetic fermentation. The strains were examined with biochemical and molecular-biological methods such as RFLP analysis of PCR-amplified DNA fragments, for their affiliation to the family of acetic acid bacteria. Furthermore, the DNA of some of the strains were sequenced. One of the isolated strains showed 99% similarity in the sequenced 16S rDNA region of the type strain of Acetobacter tropicalis*.*
Keywords: Wine, acetic acid bacteria, DNA analysis, 16S rDNA, *Acetobacter tropicalis*

Acetobacter tropicalis in spontan vergorenem österreichischen Wein mit anschließender Essigsäuregärung. *Essigsäurebakterien sind bekannt dafür, dass sie Wein irreversibel verderben können. Um die Artendiversität in Österreich kennen zu lernen, wurden 84 Bakterienstämme aus spontan vergorenem Wein mit anschließender Essigsäurefermentation isoliert. Die Stämme sind mit biochemischen und molekularbiologischen Methoden, wie RFLP-Analyse von PCR-amplifizierten DNA-Fragmenten, auf ihre Zugehörigkeit zur Familie der Essigsäurebakterien untersucht worden. Weiters wurde die DNA von einigen Stämmen sequenziert. Einer dieser Stämme zeigte eine 99% ige Übereinstimmung der sequenzierten 16S rDNA Region mit der des Typstammes von* Acetobacter tropicalis
Schlagwörter: Wein, Essigsäurebakterien, DNA-Analyse, 16S rDNA, *Acetobacter tropicalis*

La détection en Autriche d'*Acetobacter tropicalis *dans du vin fermenté spontanément et transformé en vinaigre. *Afin d'étudier la population de bactéries acétiques dans des vins autrichiens, 84 souches bactériennes ont été isolées de vins fermentés spontanément puis soumis à une fermentation acétique. L'appartenance des souches à la famille des bactéries acétiques a été étudiée par des méthodes biochimiques et biomoléculaires, telles que l'analyse RFLP de fragments d'ADN amplifiés par PCR. En outre, l'ADN de quelques souches ont été séquencées. La région séquencée 16S rADN d'une de ces souches présentait une concordance de plus de 99 % de avec celle de la souche type d'*Acetobacter tropicalis*.*
Mots clés : vin, bactéries acétiques, analyse d'ADN, 16S rADN, *Acetobacter tropicalis*

Acetic acid bacteria taxonomically belong to the family *Acetobacteraceae*. This family consists of 18 genera (GARRITY et al., 2004). The three genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* with the strains *Acetobacter aceti*, *A. pasteurianus*, *Gluconacetobacter hansenii*, *Ga. liquefaciens*, *Ga. xylinus* and *Gluconobacter oxydans* are those which are mostly responsible for wine spoilage. *G. oxydans* is mainly found on sound, undamaged grapes and in juice, because it prefers sugar-rich substrates. *Acetobacter* and *Gluconacetobacter*, however, are found on damaged grapes or in wines beginning from the later stage of alcoholic fermentation because they prefer alcohol as a carbon source (DE LEY et al., 1984).

Acetic acid bacteria are aerobic, therefore their growth is inhibited during fermentation because of lack of oxygen and presence of free sulphur dioxide. But there are studies (DRYSDALE and FLEET, 1989) which report that they are able to survive under such conditions. If there is a small amount of oxygen, then they will start with the production of acetic acid. They are also known to produce other compounds besides acetic acid, for ex-

ample dihydroxyaceton from glycerol, which may influence the quality of wine (Drysdale and Fleet, 1989). The traditional characterisation procedure with biochemical tests is not completely trustworthy for the unequivocal identification of acetic acid bacteria. Therefore nowadays these methods are complemented by different molecularbiological techniques such as DNA-DNA hybridization (Sievers et al., 1992), sequence analysis (Yamada et al., 1997), RFLP of PCR amplified 16S rDNA (Poblet et al., 2000; Ruiz, 2000), SDS-PAGE numerical analysis of total cell proteins (Du Toit and Lambrechts, 2002), and RAPD-PCR (Bartowsky et al., 2003).
For this study phenotypic tests as well as PCR-RFLP (Restriction fragment length polymorphism) of the 16S and the 16S-23S ITS (Internal Transcribed Spacer) region and sequence analysis of the 16S region were used.

Material and Methods

Bacterial strains and cultivation

Additionally the new isolates the following type strains from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were used:
Gluconobacter sp. (DSM 3504)
Gluconobacter oxydans subsp. *suboxydans* (DSM 50049)
Acetobacter aceti (DSM 3508)
Acetobacter pasteurianus (DSM 3509)
Gluconacetobacter hansenii (DSM 5602)
Gluconacetobacter liquefaciens (DSM 5603)
Gluconacetobacter xylinus (DSM 2325)

Isolation of strains

After finalisation of the alcoholic fermentation samples were taken and incubated at 28°C. During the acetic fermentation samples were drawn and plated on different culture media (see „Phenotypic analysis"). The plates also were incubated at 28°C for ten days. Representative colonies were purified by repeated streaking on AAB-medium (1.5% malt extract, 0.5% yeast extract, 1.5% agar, 3% ethanol) (Back, 2000) and modified GYC-medium (1% yeast extract, 2% D-glucose, 2% CaCO3, 2% agar) (Trek, 2002).

Phenotypic analysis

The Gram behaviour was tested by applying the potassium-hydroxide method as described by Back (2000). The activity of catalase was determined by observing the production of oxygen after addition of 5% hydrogen peroxide. Ketogenesis from glycerol was tested on glycerol-agar (2% glycerol, 2% agar) using the method of Back (2000). Over-oxidation from acetic acid to carbondioxide and water was conducted on Frageur-medium (1% yeast extract, 2% CaCO3, 2% (w/v) ethanol 96%, 2% agar) (De Ley et al., 1984) and on Carr-medium (3% yeast extract, 2% (w/v) ethanol (96%), 0.0022% bromocresol green, 2% agar). The ability of using mannitol as carbon source was tested on YPM medium (0.5% yeast extract, 0.3% peptone, 2.5% mannitol, 1.5% agar) (De Ley et al., 1984). The oxidation of glucose to gluconic acid was detected on modified GYC medium. The capability to produce acid from D-fructose was tested with an *Acetobacter* medium (1% yeast extract, 1% D-fructose, 0.004% bromocresol purple) (Back, 2000).

Extraction of DNA

The genomic DNA of the bacterial strains was isolated with the „GFX Genomic Blood DNA Purification Kit" from Amersham Biosciences. The quantification of the extracted DNA was done by electrophoresis in a 1% (w/v) agarose gel in TBE-buffer. The ethidium bromide stained DNA was visualized under UV light, photographed and compared with commercial available length standards (Sambrook and Russell, 2001).

Primer Design

The primers were designed with the help of the program Primer3 (Rozen and Skaletsky, 2000). The original sequence (S000380829) was that of the 16S rDNA of *E. coli* (Brosius et al., 1978). Two new primers (615R and 1358F) were chosen, after they had been verified in the database of the RDP (Ribosomal Database Project-II, Probe Match) (Cole et al., 2005).

PCR-RFLP-analysis of the 16S rDNA and the ITS between 16S and 23S

The PCR-RFLP analysis of the 16S rRNA gene was done as published by Poblet et al. (2000).
The ITS region between 16S and 23S rRNA was amplified by PCR with the following primer: Its1Ac (Ruiz, 2000) and 488R (Trček, 2002). The amplification of an aliquot of DNA was performed in a 50 µl reaction mixture containing 10x PCR buffer, 20 pmol forward primer, 20 pmol reverse primer, 100 µM of each dNTP (Sigma, Steinheim, Germany) and 2.5 units Taq DNA polymerase (Eppendorf, Germany) in a MasterCycler®

gradient (Eppendorf, Germany) and the following temperature program: 94°C for 5min, following sequence which is repeated 30 times: 94°C for 30s, 65°C for 1min and 72°C for 2min and finally 72°C for 8min. Afterwards the PCR products were digested with the enzyme TaqI and loaded on a 2.5% (w/v) agarose gel for fragment size analysis. As size marker a 100 bp DNA ladder (New England Biolabs, Frankfurt, Germany) was used.

Amplification and sequencing of the 16S rRNA gene

An aliquot of the rDNA was amplified in a 50 µl reaction mixture containing 10x PCR buffer, 20 pmol forward primer, 20 pmol reverse primer, 100 µM of each dNTP (Sigma, Steinheim, Germany) and 2.5 units Taq DNA Polymerase (Eppendorf, Hamburg, Germany). The following two primers were used for the amplification of the 16S rDNA: 1F (Trček, 2002) and 1541R (Seearunruangchai et al., 2004). The reactions were performed in a MasterCycler® Gradient (Eppendorf, Hamburg, Germany) and the samples were incubated at 94°C for 5min to denature the target DNA and then cycled 30 times at 94°C for 30sec, 62°C for 1min and 72°C for 2min. For final extension the samples were incubated for 8min at 72°C. The PCR products were purified using the Sephaglas™ BandPrep Kit (Amersham Biosciences), according to the manufacturerś instructions. The purified DNA was quantified after electrophoresis on an ethidium bromide stained 1% (w/v) agarose gel with the UVP Labworks software (UVP, Cambridge, UK). The sequencing was done by the company Ibl (Vienna, Austria) on an ABI PRISM™ model 3100 Genetic Analyzer with the ABI PRISM™ Big Dye Terminator Cycle Sequencing Kit. The following seven primers were used: 9F (Seearunruangchai et al., 2004), 520F (Yukphan et al., 2004), 615R (5'-CGGGGATTTCACATCTGACT-3', position 615 through 596), Ac3 (Poblet et al., 2000), 785F (Seearunruangchai et al., 2004), 1358F (5'- TCAG(A/C)ATGCC(A/G)CGGTGAATA-3', position 1358 through 1377), 1541R (Seearunruangchai et al., 2004).

Results

From the five wines 84 strains have been isolated, and were incubated at 28°C. They were examined with biochemical and molecular-biological methods for their affiliation to the family of the acetic acid bacteria. After completion of the biochemical tests the results showed, that all strains were Gram negative and catalase-positive rods, which belong to the family of the acetic acid bacteria. All strains had the ability to oxidize ethanol to acetic acid and further to carbon dioxide and water. Therefore the strains belong either to the genus *Acetobacter* or *Gluconacetobacter*.

Due to the results of the other tests described in Material and Methods, 37 strains belonged to *Acetobacter aceti*, *Gluconacetobacter liquefaciens* or *Gluconacetobacter xylinus*. A more precise statement was not possible, because these three species could not be distinguished with the applied phenotypic tests made. Thirty two strains belonged to *Acetobacter pasteurianus*, five to *Gluconacetobacter hansenii* and ten could not be assigned to a certain species (Tab.1).

The results of the PCR with the primer combination Ac1/Ac3 showed with all samples a unique PCR fragment of 869 bp as described by Poblet et al. (2000). This is the characteristic fragment length for acetic acid bacteria. After digest with the enzyme TaqI most of the strains could be assigned to *Gluconacetobacter hansenii* (results not shown).

The PCR-RFLP-analysis of the ITS region showed a completely different result. None of the patterns corre-

Tab. 1: Phenotypic characteristics used for the identification of the acetic acid bacterial isolates

Biochemical / physiologic test	No. of positive strains	Acetobacter		Gluconacetobacter			Glucon
		aceti	pasteurianus	liquefaciens	xylinus	hansenii	oxy
Catalase	84	+	+	+	+	+	-
Ketogenesis from glycerol	41	+	-	+	+	+	-
Acid production:							
Ethanol	84	+	+	+	+	+	-
D-Glucose	63	+	+	+	+	+	-
D-Fructose	8	-	-	-	-	-/+	-
Growth on YPM	55	weak +	weak +	weak +	weak +	weak +	-
Overoxidation	84	+	+	+	+	+	

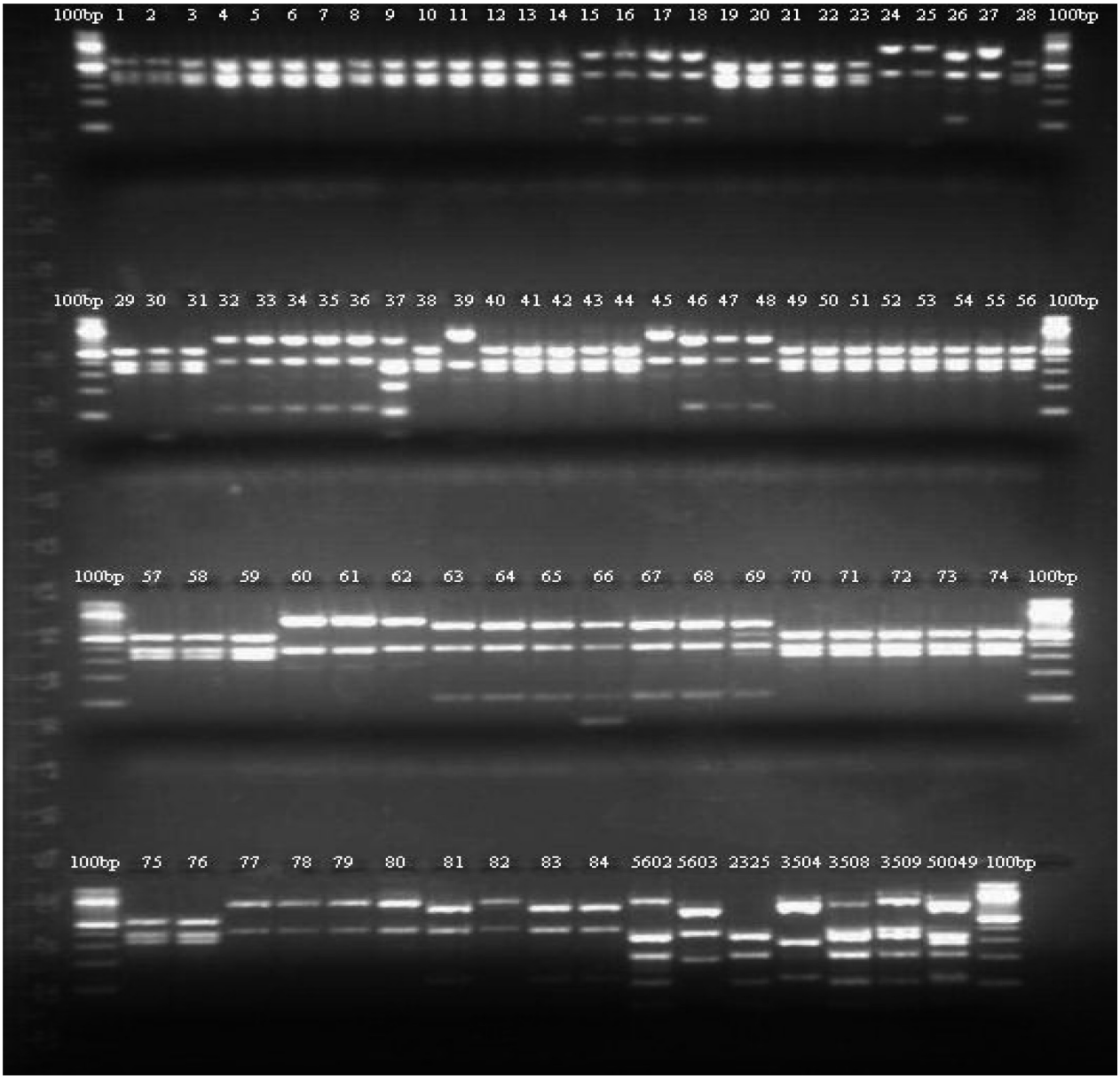

Fig.1: PCR-RFLP of the ITS-region

sponded with those of the reference strains (Fig.1). Therefore the samples with different restriction patterns were sequenced. The sequences were aligned with the BioEdit Sequence Alignment Editor (HALL, 1999) and the obtained sequences were put into Mega-BLAST program (http://www.ncbi.nlm.nih.gov/blast/megablast.shtml) for comparison with the known bacteria sequences. One of these strains could be identified as *Acetobacter tropicalis*.

Discussion

The result of the biochemical tests showed that the isolated strains from wines with acetic acid fermentation only belonged to the genus *Acetobacter* or *Gluconacetobacter.* This result was expected, since *Gluconobacter* occurs mainly on sound grapes and in juice and is rarely found in wine, because wine does not belong to its preferred nutritive solutions due to the low sugar content. *Acetobacter tropicalis* is an until quite recently unknown species of the genus *Acetobacter.* This species

Tab. 2: Results of genetic comparisons of the new strains with the MegaBLAST program

Strain	Identity (%)	Alignment length	Mis matches
LMG 1663; AJ419842	99,38	1449	2
NRIC 0312; No.3; AB032354 (T)	99,24	1450	4
NRIC 0312; No.39; AB032355 (T)	99,44	1432	2
Y-1BM; AB052716	99,72	1414	2

was described by LISDIYANTI et al. (2000) for the first time. This acetic acid bacterium was isolated in Indonesia from coconut (*Coccos nucifera*).

Since we could identify *Acetobacter tropicalis* for the first time in Austrian vinegar and especially in spontaneously fermented and spoiled wine it is interesting to look at the reasons, why it has not been detected in earlier works. On one hand the diagnostic methods are getting more progressive, e.g. the technique of sequencing the 16S rDNA region. This method has already made contribution to find new acetic acid bacteria for example with the strains *A. orleanensis*, *A. indonesiensis* and *A. tropicalis* (LISDIYANTI et al., 2000). By means of the new techniques it is possible that some bacteria strains, which are identical according to phenotypical analysis, appear to be different because of differences found with genotypical analysis. Therefore it could be possible, that some strains, which were identified in vinegar with conventional methods, caused wrong results and not until sequencing of the 16S region it cannot be decided which bacteria it really will be.

On the other hand a lot of research on acetic acid bacteria has been done in recent years in Asia (LISDIYANTI et al., 2000). Thereby some new species were discovered and published, and these new data are now free available in online databases and can be used for comparison with individual results.

The presented results with the first identification of *Acetobacter tropicalis* in Austria show that there will be a lot of possibilities for identification of bacteria in wines in the future. It can be foreseen, that additional acetic acid bacteria will be identified with the help of analysis of the 16S and the 23S region, therefore it can be assumed that indigenous European acetic acid bacteria carry a lot of scientific potential for the future.

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Received May 3, 2006