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Molecular identification of *Acetobacter* isolates from submerged vinegar production, sequence analysis of plasmid pJK2-1 and application in the development of a cloning vector

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Abstract Three new *Acetobacter* strains were isolated from vinegar. By plasmid profiling they were recognized as genotypically different from each other. Sequencing of the genes for 16S and 23S rRNA and DNA–DNA hybridization of total DNA against DNA of all type strains of *Acetobacter* identified *Acetobacter* strains JK2 and V3 as *A. europaeus*, and *Acetobacter* strain JK3 as *A. intermedius*. In contrast to the type strain of *A. europaeus* (DSM 6160), *A. europaeus* JK2 and V3 do not require acetic acid for growth and can be successfully transferred between media with and without acetic acid. This phenotypic characteristic enables convenient handling of both strains in genetic studies. Plasmid pJK2-1 from *A. europaeus* JK2 was used as the basis for shuttle plasmid construction with the aim of developing an efficient vector system for these strains. The entire nucleotide sequence of pJK2-1 was determined. High amino acid identities were found for three open reading frames: Rep (replication protein); Dinj1 (DNA damage inducible enzyme); and Dinj2 proteins. A recombinant plasmid pUCJK2-1 (5.6 kb) consisting of the entire plasmid pJK2-1 and the entire plasmid pUC18 was successfully used in transformation experiments. Plasmid pJT2 (5.8 kb) was constructed from pUCJK2-1 with the aim of reactivating the *lacZ′* gene.

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Introduction

Processes which enable the production of vinegar with high productivity are performed by submerged technology in acetators (Ebner 1982). Sievers et al. (1992) isolated and described the new species *Acetobacter europaeus* as a main component of the microflora in that kind of industrial bioreactor in Europe. The type strain of *A. europaeus* and all investigated strains from German and Swiss vinegar factories have a strict requirement of acetic acid for growth (Sievers et al. 1992). Lately, the new species *A. intermedius* was isolated from Kombucha beverage as well as from industrial acetators and described (Boesch et al. 1998). *A. intermedius* does not need acetic acid for growth (Boesch et al. 1998).

The productivity and maximum concentration of acetic acid was reported to be increased 2- and 1.4-fold by transformation of *A. aceti* subsp. *xylinus* with a multi-copy recombinant plasmid harboring an aldehyde dehydrogenase gene from *A. polyoxogenes* (Fukaya et al. 1989). However, the maximum concentration of acetic acid produced by transformants was improved only to 96.6 g/l, which is lower than the natural productivity (139 g/l) of *A. europaeus* (Fukaya et al. 1989; Sievers and Teuber 1995). Therefore, this technological parameter could be improved additionally by multiplying the aldehyde dehydrogenase gene in *A. europaeus*. For such experiments a vector system is needed for introducing genes into *A. europaeus*. Vectors are also prerequisite tools for studying resistance to acetic acid and phages, both important features of the vinegar-producing strains.

No vector systems for *A. europaeus* or *A. intermedius* are known. *A. europaeus* strains JK2, V3 and *A. intermedius* JK3, all described in this work, are easily cultivated without acetic acid, can be successfully introduced back into media with acetic acid and ethanol, and possess plasmids which can be used for development of plasmid vectors. Therefore the aim of this work was to develop a transformation procedure and to construct

shuttle vectors for further genetic studies of these industrially important strains of *A. europaeus* and *A. intermedius*.

Materials and methods

Microorganisms and plasmids

Acetobacter strains JK2, JK3 and V3 were isolated from non-filtered vinegar produced in Frings acetators installed in Ljubljana. *A. europaeus* JK2 (ZIM B021) and *A. intermedius* JK3 (ZIM B022) were isolated from 5.5% (v/v) cider vinegar. *A. europaeus* V3 (ZIM B028) was isolated from 10% (v/v) red wine vinegar, the final concentration of acetic acid in that process being 11.5% (v/v). All three strains were deposited at the ZIM (Zbirka Industrijskih Mikroorganizmov, Ljubljana, Slovenia) culture collection under the accession numbers listed in brackets. Plasmids and their properties are listed in Table 1.

Culture media and growth conditions

Acetobacter strains were isolated on a modified acetate-ethanol (AE) agar medium (Sievers and Teuber 1995) composed of 0.3% yeast extract, 0.4% peptone, 0.75% glucose, 0.8% agar, 3% (v/v) acetic acid and 3% (w/v) ethanol. Bacteria were grown at 30 °C and 92–96% relative air humidity and were transferred every 2 weeks onto fresh medium. For long-term preservation, *Acetobacter* strains were harvested at exponential growth phase from glucose–yeast-extract (GY) broth containing 5% glucose and 0.5% yeast extract, resuspended in 25% (v/v) glycerol and stored at −70 °C. All type strains of *Acetobacter* and *Escherichia coli* XL1-Blue were maintained as described in the catalog of culture collections and by Sambrook et al. (1989), respectively.

Biochemical tests

Growth of *Acetobacter* strains without acetic acid was tested on GY agar medium. The ability to overoxidize acetic acid to CO_2 and H_2O was analyzed on Frateur and Carr agar media (Swings, 1992). Acetic acid tolerance [4 and 6% (v/v) of acetic acid] was determined on modified Entani agar medium, and tolerance to 8% and 10% (v/v) of acetic acid was determined in AE broth. 2- and 5-ketogluconic acids were analyzed as described by Gosselé et al. (1980) after cultivation in AE broth.

Plasmid profiles

A method for plasmid DNA isolation from *Acetobacter* described by Teuber et al. (1987) was modified as follows. *Acetobacter* was grown in 100 ml of AE or GY broth in the shaker flasks with baffle indents until the late exponential phase. Cells were harvested and washed twice with 50 mM Tris, 1 mM EDTA, pH 8.0 and once with 1 M Tris, pH 8.0. Biomass was resuspended in 750 µl Tris-sucrose (TS) solution composed of 50 mM Tris, pH 8.0 and 25% (w/v) sucrose. Lysis of cells was achieved by the addition of 500 µl lysozyme solution (10 mg TS/ml) and incubation at 37 °C for 15 min. After addition of 300 µl 0.25 M EDTA, pH 8.0 and 100 µl 10% (w/v) SDS, the mixture was incubated at room temperature for about 10–15 min until the solution cleared. The clear solution was vortexed three times for 1 s. DNA was denatured by the addition of 115 µl of 1 M sodium hydroxide to a final pH of 13.0. Renaturation of plasmid DNA was performed by the addition of 600 µl of 2 M Tris, pH 7.0 to a final pH of 8.5. After addition of 500 µl of 5 M sodium chloride, the suspension was kept for 2 h at 4 °C, and centrifuged at 12,000 rpm at 4 °C for 30 min. The resulting supernatant was extracted twice with 3 ml phenol saturated with 3% NaCl and 3 ml methylene chloride–isoamyl alcohol (24:1). Plasmid DNA was precipitated overnight with two volumes of cold (−18 °C) 99.8% ethanol and 1/10 volume of 3 M sodium acetate (pH 5.5). After centrifugation at 3,800 rpm at 4 °C for 20 min, the pellets were dried, dissolved in 50 µl 10 mM Tris, 1 mM EDTA, pH 8.0 and incubated for 15 min at 37 °C. RNA was digested with 10 µg RNase A/ml for 15 min at 37 °C.

DNA–DNA hybridization

The procedure of Boesch et al. (1998) was used with total DNA isolated from *A. europaeus* JK2, *A. europaeus* V3, *Acetobacter* type strains, *Gluconobacter oxydans* type strain and the radioactively labeled total DNA (restricted by *Eco*RV) of *A. europaeus* JK2 as a probe. This method is based on hybridizations between membrane-bound DNA, previously digested and electrophoretically separated in agarose gels, and [α-^{32}P]-labeled DNA, previously digested.

Hybridizations with radioactively labeled plasmid DNA as a probe and plasmid extracts of *Acetobacter* strains were performed as described above for total DNA, except that plasmid extracts and the *ccc*-form of plasmid pJK2-1 isolated from low-melting agarose were not digested.

PCR of 16S and 23S rDNA

PCR of 16S rDNA 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), sterile double-distilled water, 100 pmol of each primer and 2.5 units of *Taq* DNA-Polymerase (Pharmacia). Amplification was performed in 0.2 ml tubes using the Genius Thermocycler (Techne). The universal primers bak11w and bak4 were used for the amplification of 16S rDNA (Dasen et al. 1998). The annealing temperature used for this set of primers was 56 °C. PCR of 23S rDNA was performed as described by Boesch et al. (1998).

DNA manipulations

Chromosomal DNA from *Acetobacter* strains was isolated as described previously (Trček et al. 1997). Plasmid pJK2-1 was isolated after electrophoresis from low-melting agarose, linearized with *Sal*I, ligated into pUC18 and electroporated into *E. coli* XL1-

Table 1 Bacterial plasmids used in this study

Plasmid	Relevant properties	Source
pJK2-1	Cryptic plasmid from *Acetobacter europaeus* JK2; 3.0 kb	This work
pUC18	ColE1 replicon; Ap^r; LacZ′; 2.7 kb	(Yanisch-Perron et al. 1985)
pUCJK2-1	*Sal*I digested pJK2-1 was cloned into *Sal*I digested pUC18; Ap^r; 5.6 kb	This work
pGEM-T	Ap^r; LacZ′; used for cloning of PCR products in *E. coli*; 3.0 kb	Promega
pJT1	The *Nde*I/*Pst*I fragment (0.2 kb) of pUCJK2-1 was removed, the overhanging sites filled up, the protruding sites modified to blunt ends and the plasmid religated; 5.4 kb	This work
pJT2	The *Xba*I/*Ssp*I fragment (0.6 kb) of LacZ′ was isolated from pUC18 and cloned into *Xba*I/*Cla*I (*Cla*I site modified to blunt end) sites of pJT1; Ap^r; LacZ′; 5.8 kb	This work

Blue. Restriction enzymes, ligase, DNA polymerase Klenow fragment, calf intestinal phosphatase and the corresponding buffers were supplied by Pharmacia. Agarose gel electrophoresis, restriction analysis, ligation, filling of recessed 3′-termini, removing of protruding 3′-termini, electroporation of *E. coli*, screening of recombinant *E. coli* cells, alkaline lysis of *E. coli* cells for mini plasmid DNA isolation and analysis of recombinant plasmids were performed as described by Sambrook et al. (1989).

Transformation of *A. europaeus* and *A. intermedius*

Competent cells of *A. europaeus* V3 and *A. intermedius* JK3 were made by the following procedure. Bacteria were cultivated in 100 ml GY broth in Erlenmayer flasks with baffle indents to the late exponential phase (OD_{620} of approximately 0.7) on a rotary shaker. A fresh 100 ml GY broth was inoculated with 1 ml cell culture and cultivated again to the late exponential phase. This procedure was performed three times. The flask with the biomass was kept at 4 °C for 20 min following centrifugation at 5,000 rpm for 10 min at 4 °C. The biomass was washed with 100 ml of 10% (v/v) glycerol, centrifuged, washed with 50 ml of 10% glycerol, centrifuged and washed once more with 2.5 ml 10% glycerol. After centrifugation, competent cells were resuspended in 250 µl of 10% glycerol and frozen below −70 °C. Electroporation was performed with 40 µl competent cells and 2 µl recombinant plasmid DNA (2.9 µg) in cold 0.2-cm electroporation cuvettes using a Bio-Rad Gene Pulser and a Pulse Controller apparatus at 2.3 kV, 200 Ω, 25 µF and pulse lengths of 2–4 ms. Pulsed cells were transferred into 1 ml GY broth in a McCartney flask and incubated with shaking at 30 °C for 3 h. Samples were spread onto GY agar medium containing either 30 or 90 µg ampicillin/ml to select transformants of *A. intermedius* JK3 and *A. europaeus* V3, respectively. Each of these ampicillin concentrations in GY agar inhibits growth of the wild types of *A. intermedius* JK3 and *A. europaeus* V3.

Direct sequencing of PCR products

Purification of PCR products, cycle sequencing reactions, primers for 16S and 23S rDNA sequencing, detection of sequenced samples and data analysis were performed as described by Boesch et al. (1998).

Sequencing of cloned DNA

Plasmid DNA cloned into *E. coli* XL1-Blue was isolated from cells using the Wizard Plus Midipreps DNA Purification System (Promega). Nucleotide sequences of both strands were determined by the primer walking method. Primers were synthesized by Microsynth (Balgach, Switzerland). Sequencing reactions were performed with AutoRead Kit and Cy5-dATP Labeling Mix (Pharmacia) according to the manufacturer's instructions.

DNA sequence analysis

DNA sequences were analyzed using the GCG software package of the University of Wisconsin Genetics Computer Group (Madison, Wis., USA) and compared to information deposited in the GenBank/EMBL Database.

Analysis of plasmid stability

For *A. intermedius* JK3 and *A. europaeus* V3, generation times were determined from growth curves following cfu (colony forming units) after transferring an appropriately diluted sample of biomass from GY broth onto GY agar medium. OD_{620} in GY broth was also followed. Cells were grown to an OD_{620} of approximately 0.7 in GY broth without ampicillin. An appropriate volume was transferred to a new medium so that the initial OD_{620} could be restored overnight after approximately ten generations. Before each transfer, cells were diluted and plated onto GY agar medium with and without ampicillin. The plasmid stability was estimated by comparing the number of colonies appearing on antibiotic vs non-antibiotic supplemented plates. Plasmid profiles were analyzed from ten randomly selected transformants. The recombinant plasmid pUCJK2-1 was isolated from these profiles and its restriction profiles were compared to the restriction profile of the original plasmid pUCJK2-1.

Nucleotide sequence deposition numbers

The nucleotide sequences of 16S rDNA, 23S rDNA, plasmid pJK2-1 of *A. europaeus* JK2 and the plasmid construct pJT2 were deposited in the GenBank/EMBL Database under accession numbers Y15289, Y15288, AJ223503 and AJ009780, respectively.

Results

Taxonomic characterization of *Acetobacter* strains JK2, JK3 and V3

*Acetobacter s*trains JK2, JK3 and V3 were successfully isolated on a modified AE agar medium. On this medium, *Acetobacter* strain JK2 can show two types of colony with or without production of a cellulose pellicle. *Acetobacter* strain V3 does not form cellulose on AE agar medium; *Acetobacter* strain JK3 forms a soft slimy layer on AE agar medium. Several attempts were made with the aim of isolating acetic acid bacteria from the same samples of vinegar on GY, Frateur, GYC, Carr and YM agar media described by De Ley et al. (1984). None of these media without acetic acid supported the direct isolation of acetic acid bacteria from vinegar. Surprisingly, all strains were afterwards successfully transferred from AE agar onto GY agar medium. Growth (clearly seen as colonies of 1 mm diameter) was obtained with *Acetobacter* strain V3 after 1 week, with *Acetobacter* strain JK2 after 4 days, and with *Acetobacter* strain JK3 after 3 days. Strains were successfully transferred back to the media with acetic acid. Transfer from non-acetic acid medium to acetic acid medium was fastest if the biomass from GY agar medium was transferred to the modified AE broth enriched with glucose (3.5% final concentration), yeast extract (1% final concentration) and peptone (1% final concentration). This procedure enables growth of strains transferred from a medium without acetic acid to acetic acid-containing medium in 7–10 days.

Maximum acetic acid tolerance in AE broth is 10% (v/v) for *A. europaeus* V3, 6% (v/v) for *A. intermedus* JK3, and 8% (v/v) for *A. europaeus* JK2. All strains were classified into the genus *Acetobacter* because of their ability to overoxidize acetic acid to H_2O and CO_2. *A. europaeus* strains V3 and JK2 form 5-ketogluconic acid but not 2-ketogluconic acid from glucose, which is typical for the *A. europaeus* type strain (Kneubühler 1994). *A. intermedius* JK3 forms both types of acid from glucose. Long time preservation of strains was achieved by resuspending bacterial biomass in glycerol and stor-

age at −70 °C. After 6 months at −70 °C they were successfully brought back onto GY agar medium.

Strain differentiation of all isolates by plasmid profiling (Fig. 1A) suggested that *Acetobacter* strains JK2, JK3 and V3 are genotypically different. Further comparison of the genomes of the three isolates was performed for species identification. 16S rDNA sequencing of *Acetobacter* strains JK2 and V3 gave the highest identity to *A. europaeus*^{T}, and of *Acetobacter* strain JK3 to *A. intermedius*^{T} (Table 2). The alignment of partially sequenced 23S rDNA genes of *Acetobacter* strains JK2, JK3 and V3 to the 23S rDNA gene of *A. europaeus*^{T}, *A. intermedius*^{T} and *A. xylinus*^{T} showed the highest identity of *Acetobacter* strains JK2 and V3 to *A. europaeus*^{T}, and of *Acetobacter* strain JK3 to *A. intermedius*^{T} (data not shown). Percentage DNA–DNA similarity obtained by DNA–DNA hybridization with *A. europaeus* JK2 to *A. europaeus* V3, *Acetobacter europaeus*^{T} and *Acetobacter intermedius*^{T} were 72%, 98% and 45% respectively. All other type species of *Acetobacter* and *G. oxydans* had less than 10% DNA similarity. However, *A. europaeus* strains V3 and JK2 have a special characteristic: independence from acetic acid for growth. *Acetobacter s*train JK3 is classified as *A. intermedius*.

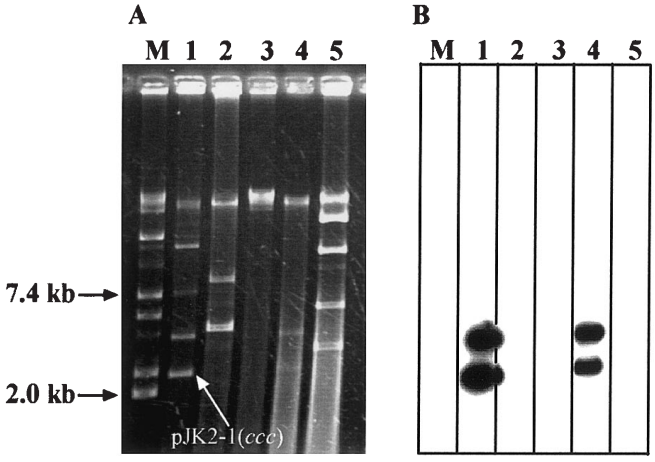

Fig. 1A, B Southern hybridization analysis of plasmid pJK2-1 with plasmid extracts from *Acetobacter* strains: **A** 0.65% agarose gel stained with ethidium bromide; **B** autoradiography of membrane-bound DNA hybridized with α-^{32}P-labeled pJK2-1. Lanes: *M* plasmid profile of *Lactococcus lactis* subsp. *cremoris* AC1 as plasmid DNA marker, *1* plasmid profile of *A. europaeus* JK2, *2* plasmid profile of *A. europaeus* V3, *3* plasmid profile of *A. intermedius* JK3, *4* plasmid profile of *A. intermedius*^{T}, *5* plasmid profile of *A. europaeus*^{T}

Characterization of plasmid pJK2-1

Plasmid pJK2-1 was isolated from *A. europaeus* JK2. Its entire nucleotide sequence was determined (2,954 bp). The plasmid has an overall G + C content of 56.3%. Plasmid pJK2-1 has no significant sequence similarity to plasmids previously characterized from *Acetobacter*, such as pAP12875 from *A. pasteurianus* (Fukaya et al. 1989) and pAEU601 from *A. europaeus*^{T} (Boesch, 1998). Analysis of the nucleotide sequence revealed five open reading frames (ORF), all of them preceded by putative ribosomal binding sites. ORF2, encoding a putative polypeptide of 181 amino acids (aa) had 46.4% aa-identity to a putative Rep (replication) protein (220 aa) localized on pBBR1 of *Bordetella bronchiseptica* (Antoine and Locht 1992). Upstream of ORF2, two 21-bp direct repeats (DR1), two 13-bp direct repeats (DR2) and a 9-bp inverted repeat (IV1) were identified. ORF3 (100 aa) and ORF4 (87 aa) had 34.5% and 33.7% aa-identity to DNA damage inducible proteins Dinj2 (92 aa) and Dinj1 (86 aa), respectively. *Dinj1* and *dinj2*, putative SOS genes, were found on the chromosome of *E. coli* (Ohmori et al. 1995). For ORF1 and ORF5 no significant similarity was found with sequences deposited in GenBank/EMBL Databank. The genetic map of pJK2-1 is presented in Fig. 2.

Construction of plasmid pJT2 containing a multiple cloning site

A recombinant plasmid pUCJK2-1 (5,640 bp) consisting of the entire plasmids pJK2-1 and pUC18, was constructed by the ligation of *Sal*I restricted pJK2-1 and *Sal*I restricted pUC18. A new recombinant plasmid pJT2 was constructed from pUCJK2-1 in two steps. In the first step, pJT1 (5.4 kb) was constructed by re-

Table 2 16S rRNA gene sequence identities of *Acetobacter europaeus* strains JK2, V3 and *A. intermedius* JK3 to type strains of *Acetobacter* and *Gluconobacter oxydans*

Organism	% 16S rRNA gene sequence identity		
	A. europaeus JK2	*A. europaeus* V3	*A. intermedius* JK3
A. europaeus^T	99.9	99.5	98.5
A. xylinus^T	99.6	99.2	98.2
A. intermedius^T	99.5	99.1	99.0
A. hansenii^T	98.5	98.1	97.3
A. liquefaciens^T	97.2	96.8	96.0
A. diazotrophicus^T	96.6	96.2	95.3
A. methanolicus^T	95.7	95.3	94.7
A. aceti^T	95.4	95.0	94.6
A. pasteurianus^T	95.0	94.6	94.3
G. oxydans^T	94.6	94.3	94.1

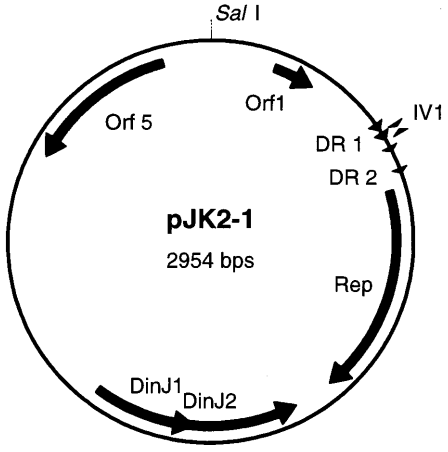

Fig. 2 Genetic map of plasmid pJK2-1 from *Acetobacter europaeus* JK2. Symbols represent: *Orf* open reading frame, *Rep* putative replication protein, *Din* putative DNA damage inducible protein, *DR* direct repeat, *IV* inverted repeat. *Arrows* depict direction of transcription. (Drawn from EMBL accession number AJ223503)

moving the *Nde*I/*Pst*I (0.2 kb) fragment from pUCJK2-1. In the second step, pJT2 (5.8 kb) was constructed by reincorporating the missing part of the *lacZ′* gene into the *Xba*I/*Cla*I (*Cla*I site modified to blunt end) sites of pJT1. The missing part of *lacZ′* gene was isolated as a 0.6 kb *Xba*I/*Ssp*I fragment from pUC18. Plasmid pJT2 has a functional *lacZ′* gene with *Bam*HI, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, *Sma*I and *Xba*I single restriction sites, which were demonstrated experimentally. Outside the *rep*, *oriEC*, *bla* and *lacZ′* genes, *Bgl*II, *Bss*HII, *Dsa*I, *Eco*RV, *Nsi*I and *Ssp*I single restriction sites were demonstrated. The genetic map of pJT2 is presented in Fig. 3.

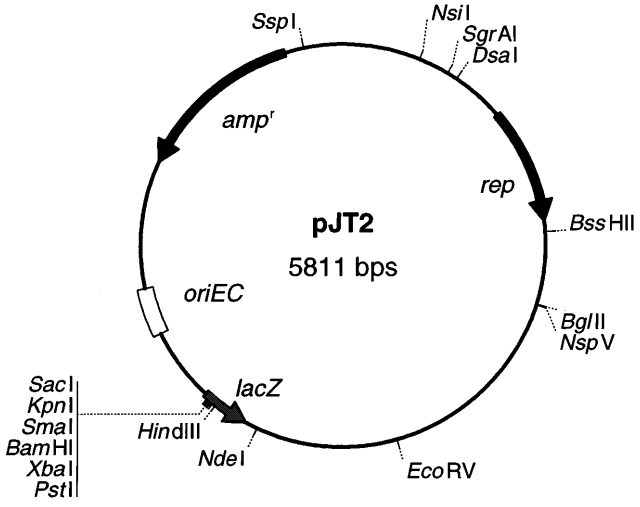

Fig. 3 Genetic map of plasmid construct pJT2. Symbols represent: *lacZ′* β-galactosidase α-peptide gene from pUC18, *oriEC* origin of replication from pUC18, *rep* replication gene from pJK2-1, *amp*^{r} ampicillin resistance gene from pUC18. *Arrows* depict direction of transcription. Only single restriction sites are represented. (Drawn from EMBL accession number AJ009780)

Transformation of *A. europaeus* V3 and *A. intermedius* JK3

Plasmid pUCJK2–1 was successfully electroporated into *A. europaeus* V3, *A. intermedius* JK3 and *E. coli* XL1-Blue with an efficiency of transformation of 4.8×10^2 cfu/μg, 7.0×10^2 cfu/μg and 5.0×10^4 cfu/μg of plasmid DNA, respectively. Single restriction sites for *Bam*HI, *Cla*I, *Eco*RV, *Hind*III, *Kpn*I, *Pst*I, *Sac*I, *Sca*I, *Sma*I and *Xba*I were experimentally demonstrated in pUCJK2-1 which was isolated from *Acetobacter* transformants grown on GY agar with ampicillin. Segregational stability of pUCJK2-1 in *A. intermedius* JK3 and *A. europaeus* V3 was 100% at the end of the 220th generation of growth in GY broth. Restriction profiles of pUCJK2-1 after 220 generations in *A. intermedius* JK3 and *A. europaeus* V3 did not change.

Plasmid pJT2 (Fig. 3) was successfully introduced into *A. intermedius* JK3 and *E. coli* XL1-Blue with an efficiency of transformation of 3.2×10^2 cfu/μg and 4.5×10^4 cfu/μg of plasmid DNA, respectively. The plasmid profile of *A. intermedius* JK3 before and after transformation with pJT2 is presented in Fig. 4.

Discussion

Variability of a strict requirement of acetic acid for growth of *A. europaeus*

All previously described *A. europaeus* strains isolated from German and Swiss vinegar bioreactors had a strict requirement of acetic acid for growth (Sievers and Teuber 1995). This was one of the recognized phenotypic characters for a differentiation of this species from all other *Acetobacter* species. *A. europaeus* JK2 and V3

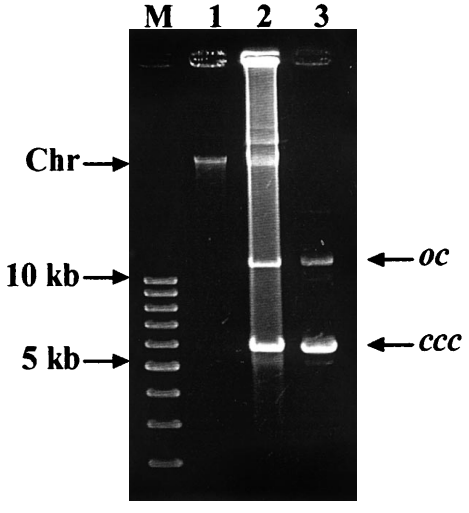

Fig. 4 Plasmid profiles of *Acetobacter intermedius* JK3 and its transformant. Lanes: *M* 10 kb supercoiled DNA ladder (Promega), *1 A. intermedius* JK3, *2* ampicillin-resistant transformant of *A. intermedius* JK3, *3* plasmid pJT2 (see Fig. 3)

isolated from different samples of Slovenian vinegar do not need acetic acid for growth but are genotypically identified as *A. europaeus* species. However, none of the strains described in this paper could be isolated on a medium without acetic acid initially. We noticed also that sometimes under the same conditions of transferring strains from acetic acid-containing to acetic acid-free medium, growth of 1-mm-diameter colonies could be seen within 3 days and sometimes only after up to 3 weeks. The mechanism for these differences remains enigmatic. These findings plus previously reported observations (Trček and Raspor 1999) suggest that a strict requirement of acetic acid for growth is a variable phenotypic character in *A. europaeus* strains and cannot be used for delineating *A. europaeus* from other *Acetobacter* species. Since a reliable phenotypic character is not yet known for the phenotypic identification of *A. europaeus*, the genotypic identification of acetic acid bacteria is the only way for accurately delineating *A. europaeus* from other species of the genus *Acetobacter*.

Description of plasmid pJK2-1

Besides plasmid pAEU601 (Boesch 1998), the plasmid pJK2-1 is the only other plasmid of *A. europaeus* completely sequenced, but there is no significant sequence similarity between them. The calculated overall G + C content of pJK2-1 (56.3%) is in agreement with the 56–58% G + C content determined for the total genomic DNA of *A. europaeus* (Sievers et al. 1992). A high aa identity to the putative Rep protein of pBBR1 (Antoine and Locht 1992) indicates that ORF2 of pJK2-1 could be involved in initiating replication. Additional arguments in favor of this assumption are direct repeats, an inverted repeat and a 72-bp A + T-rich region (74%) upstream of ORF2. These elements are frequently located at the origin regions of many well-studied replicons of plasmids in Gram-negative bacteria (Kües and Stahl 1989). ORF3 and ORF4 overlap for 32 bp, implying that they are coupled in translation. High similarity with SOS genes (*dinJ1*, *dinJ2* of *E. coli*) were found. SOS box consensus sequences normally precede SOS genes in *E. coli* (Lewis, 1994), but upstream of ORF4 this consensus box could not be found. Missing hybridizations of pJK2-1 DNA with plasmids from *A. intermedius* JK3, *A. europaeus* V3 and *A. europaeus*^{T} (Fig. 1B) might explain compatibility of pJK2-1 with plasmids from the mentioned strains. This was the reason why we did not need to use plasmid-cured cells of *A. intermedius* JK3 and *A. europaeus* V3 for successful transformation experiments. No DNA–DNA hybridizations between pJK2-1 and bacterial chromosomes were obtained for the above-mentioned strains, which might indicate there is no DNA exchange between plasmid and chromosome at the present stage (Fig. 1). This finding was another supporting argument for using pJK2-1 as a base for vector construction. Plasmid pJK2-1 remains cryptic because no phenotypic properties are yet known.

Transformation of *A. europaeus* V3 and *A. intermedius* JK3

Using the chemical method described by Grones and Turňa (1992) for transforming *A. pasteurianus* we did not obtain recombinant colonies with *A. europaeus* V3 and *A. intermedius* JK3. Therefore, an electroporation procedure based on the standard transformation protocol for *E. coli* (Sambrook et al. 1989) was successfully used for the newly isolated and characterized strains. This is the first procedure described for the transformation of *A. europaeus* and *A. intermedius*. By modifying electroporation parameters, transformation efficiency might be further improved.

The plasmid profile of the ampicillin-resistant *A. intermedius* JK3 contains two bands above the chromosomal DNA region, neither of which is seen in the plasmid profile of the wild type of *A. intermedius* JK3 (Fig. 4). It might be that they present additional physical forms (*ccc*-, *oc*-, linear-form, dimers, tetramers etc.) of the transferred plasmid.

Description of vectors pUCJK2–1 and pJT2

Plasmid pUCJK2–1 is the first described *A. europaeus* – *A. intermedius* – *E. coli* shuttle vector. It is stably maintained in all three species, having an ampicillin resistance marker and a number of single restriction sites, but lacking a marker for DNA insertion. Therefore, a new vector pJT2 was constructed with the ability to use X-gal selection in *E. coli* and having a multiple cloning site (MCS) from pUC18. It was successfully introduced into *E. coli* and *A. intermedius*. All the restriction sites in MCS are single cutting sites, except the *Eco*RI, *Sal*I and *Sph*I sites. Because its complete sequence is known, further reconstructions will be easily performed. The use of this vector to transfer and express further genes (in addition to ampicillin resistance and *lacZ*) is presently under investigation.

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