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Characterization of *Agrobacterium* species by capillary isoelectric focusing

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Abstract Tumorigenic and non-tumorigenic strains of *Agrobacterium tumefaciens*, *A. rhizogenes*, *A.rubi*, and *A.vitis* were examined using capillary isoelectric focusing, phenotypic determinative tests, PCR and fatty acid analysis. The isoelectric points (p*I*) of the 40 strains investigated clearly differentiated the strains according to their respective species. The different species were characterized with the following p*I* values: *A. tumefaciens* 2.2, *A. rhizogenes* 4.0, *A. rubi* 2.15, and *A. vitis* 2.6. This differentiation corresponded to the phenotypic, PCR and fatty acid characterizations. Strains with the similar chromosomal background but different plasmid content, e.g. *A.vitis* strain S4, and F2/5 gave the same p*I* values. Strains of *Rhizobium* species differed from *Agrobacterium* strains in their p*I* values. The advantage of capillary isoelectric focusing over the phenotypic determinative tests, PCR and fatty acid analysis is its speed (15 min), relative simplicity, and the very small amount of chemicals used. This rapid and simple method is a major improvement over the classical methods of separation of *Agrobacterium* species and should prove useful for rapid characterization of *Agrobacterium*-like colonies isolated from plant tumours for epidemiological and generic diversity studies.

Keywords *Agrobacterium radiobacter* · *A. tumefaciens* · *A. rhizogenes* · *A. rubi* · *A. vitis* · Species PCR · FAME · Crown gall

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Introduction

Agrobacteria may cause crown-gall or hairy root diseases in many plants. The most characteristic symptom of the disease caused by the tumorigenic strains of *Agrobacterium tumefaciens* and *A. rhizogenes* is a tumour-like growth on infected plants, often at the junction between the root and the shoot. Tumours are incited by the conjugative transfer of a DNA segment (T-DNA) from the bacterial tumour-inducing (Ti) plasmid. Strains of *A. rhizogenes* carry a distinct Ri (root inducing) plasmid, induce root tumours and, rarely hairy roots. *A. vitis* and *A. rubi* are host specialized species usually causing tumours on grapevine and *Rubus* sp. respectively. Non-tumorigenic environmental strains of *Agrobacterium* possess neither Ti or Ri plasmids. These strains may

be members of either *A. tumefaciens, A. rhizogenes* or *A. vitis*.

Although the genus *Agrobacterium* has been very well studied and its structure understood, two different nomenclature are applied to it. The traditional form uses species names to distinguish the distinct plasmid-associated pathogenic characters using the species names, *tumefaciens, rhizogenes* and *vitis*. Phenotypic differences (Kerr and Panagopoulos 1977; Süle 1978) are referred to a biovar nomenclature. However, Holmes and Roberts (1981), proposed the elevation of biovars to species and this classifcation has been repeatedly confirmed by sequence comparision (Willems and Collins 1993; Puławska et al. 2000) and fatty acid analysis (Bouzar et al. 1993; Tighe et al. 2000). According to this classification (Young et al. 2005), biovars 1, 2 and 3 are referred to the natural species, *A. tumefaciens, A. rhizogenes* and *A. vitis*, respectively. Pathogenicity is treated as a strain characteristic, strains being referred to as tumorigenic, rhizogenic or non-tumorigenic. This nomenclature is used here.

Traditionally, detection and enumeration of agrobacteria have been based largely on the use of selective culture and standard biochemical methods. To confirm the pathogenicity of the isolates, test plants must be inoculated and the results assessed after 2–4 weeks. Such methods suffer from a number of drawbacks. They are time consuming, and low throughput. Since the introduction of PCR methods many highly sensitive primers are available for the identification of pathogenic agrobacteria (Bini et al. 2008; Suzaki et al. 2004; Puławska et al. 2006). Recently, a new method capillary isoelectric focusing (CIEF) has been used for separation of bacteria, yeasts, phages etc. (Horká et al. 2010). The outer face of bacteria consists of a proteinaceous layer, a membrane composed of glycoproteins and lipid or a cell wall, which is electrically charged under most conditions. Exposed amino acid residues, acidic carbohydrate moieties, etc. are protolysed to a degree that depends on the pH of the solution. Under the influence of an electric field the cells move with a velocity related to their electrophoretic mobility enabling their separation and purification according to their isoelectric points (p*I*). This position depends on the characteristic surface features of individual species (Lantz et al. 2007; Rodriguez and Armstrong 2004). An ampholyte solution containing the untreated bacterial cells is injected directly into the capillary. Upon application of the voltage, a pH gradient is formed within the capillary due to the migration of ampholyte components. Cells migrate according to their surface charges until they reach the pH which equals their p*I*, at which point their mobility is lost and they focus into a sharp zone. A mobilization step is then used to force the cells past the detector for analysis (Horká et al. 2010). CIEF is a potentially simple, rapid, cost- and labour-effective technique for the characterization of microorganisms (Armstrong and He 2001; Desai and Armstrong 2003; Horká et al. 2010). In this study, CIEF, phenotypic characterization, PCR and fatty acid analysis were compared as characterization methods for tumorigenic and non-tumorigenic strains of *A. tumefaciens, A. rhizogenes, A. rubi*, and *A. vitis*. Rhizobia, the closest relatives of agrobacteria were included into the comparison for discrimination of the different *Agrobacterium* and *Rhizobium* species.

Materials and methods

Bacterial strains and growth conditions A total of 35 *Agrobacterium* and 5 *Rhizobium* strains used in this study are described in Table 1. This panel of strains represents members of *A. tumefaciens*, *A.rhizogenes*, *A.rubi* and *A. vitis*, isolated from different hosts in different geographical regions. Standard *Rhizobium* species were kindly provided by I. Dusha (BRC, Szeged), and were characterized solely by CIEF. The bacteria were stored in 25% glycerol at −70°C. Before use, strains were streaked for purity onto LB agar containing 1 g l^{-1} $MgSO_4$ at 28°C, and single colonies were selected for further use.

Identification using determinative tests Simplified methods were used to confirm species affiliation to *A. tumefaciens*, *A. rhizogenes*, *A. rubi* and *A. vitis*. Media and procedures used for biochemical characteristics were as previously described (Kerr and Panagopoulos 1977; Süle 1978). The strains were tested for production of 3-ketolactose, acids from erythritol and melezitose, alkali from malonate, and pectolytic activity. The drop collapse test was as previously described (Süle et al. 2009). β-galactosidase activity was tested on LB plate containing ready-made X-Gal Solution (Fermentas). Pathogenicity tests were performed on

Table 1 Strains used in this study

Strain	Source	Original host
A. tumefaciens		
B6	ATCC 23308	Apple
C58	USA (Dickey,R.)	Cherry
CG628, CG632	USA (Burr,T.J.)	Grape
T37	USA (Braun,A.C.)	*Juglans* sp.
3, 6	Hungary (S.Süle)	Cherry
15/6, 16/6, 18/3	Hungary (S.Süle)	Grape
32/1	Hungary (S.Süle)	Apple
41/10	Hungary (S.Süle)	Peach
A. rubi	ICMP6428^T	Boysenberry
A. rhizogenes		
K27, K47	Australia (Kerr,A.)	Peach
K84		
7/2, 9/2, 9/6, 51	Hungary (S.Süle)	Raspberry
30/2, 47/1	Hungary (S.Süle)	Peach
CG918	USA (Burr,T.J.)	Rose
A. vitis		
AB3, AB4, S4, Tm4	Hungary (Szegedi,E.)	Grape
AA25	Afganistan (Ercolani,G.L)	Grape
K306	Australia (Kerr,A.)	Grape
CG49, CG484	USA (Burr,T.J.)	Grape
CG964		
2/3, 3/5	Hungary (S.Süle)	Grape
F2/5	S.-Africa(Staphorst,J.)	Grape
Rhizobium species		
Rhizobium leguminosarum bv. trifolii WSM2304		I.Dusha (BRC, Szeged)
Rhizobium leguminosarum bv. viciae 3841		I.Dusha (BRC, Szeged)
Rhizobium galegae ATCC43677^T		I.Dusha (BRC, Szeged)
Mesorhizobium loti NZP 2037		I.Dusha (BRC, Szeged)
Sinorhizobium meliloti 41		I.Dusha (BRC, Szeged)

stems of 2-week old sunflower (*Helianthus annuus* L.) seedlings by puncturing them with sterile toothpicks that were first dipped into well-isolated colonies on agar medium. Tumour formation was recorded 4 weeks after inoculation.

PCR amplification Bacterial DNA was extracted by suspending 10^8 bacterial cells ml^{-1} in DNAzol Direct (MRC Inc.) and 0.5 μl was used for 25 μl of PCR mixture. The universal UFf, and species specific B1Rr, B2Rr, ArRr, and AvRr primers complementary for the 23S rDNA of agrobacteria (Puławska et al. 2006) were used to identify *Agrobacterium* species. VCF3/VCR3 primers complementary to the *Vir*C pathogenicity gene (Suzaki et al. 2004) were used to identify pathogenicity of the strains investigated. For the 23S rRNA gene PCR, amplification was performed in a DNA Thermal Cycler 2700 (Perkin-Elmer Cetus, Emeryville, Calif.) for 35 cycles by using the following parameters: denaturation at 94°C for 1 min, annealing at 67°C for 1 min, and extension at 72°C for 1.5 min. For strains S4 and CG964 of *A. vitis* the annealing temperature using UFf/AvRr primers was lowered to 52°C and for the *A. rubi* strain using UFf/ArRr primers to 60°C. For the *Vir*C gene PCR with VCF3/VCR3 primers, the amplification was performed for 35 cycles (94°C for 30 s, 52°C for 30 s, and 72°C for 1 min). The cycles were preceded by a

denaturation step at 94°C for 2 min, and at the end by an extension step at 72°C for 7 min. The total reaction volume was 25 μl, containing 0.5 μl of template DNA, 12.5 μl (2X) Dream Taq™ PCR Master Mix (Fermentas), and 0.5 μM of each primer. For detection of PCR products, 10 μl of the amplified DNA was separated by electrophoresis with a 1.0% agarose gel and the DNA band was visualized as UV fluorescence after staining with ethidium bromide. A negative control consisting of all reaction reagents necessary for PCR but with water as a template was included in all runs.

Fatty acid methyl ester (FAME) analysis The isolates were cultured on trypticase soy agar (Oxoid, Hampshire, UK), then the cells were harvested from the plates by scraping with a sterile plastic loop and used for FAME analysis. Saponification, methylation, and extraction were performed using the procedure described in the MIDI manual (Sasser 1990). The presence of characteristic fatty acids was compared by SHERLOCK Microbial identification system with a database of bacteria.

Preparation of the microbial sample for capillary isoelectric focusing (CIEF) Bacteria were cultivated by shaking for 48 h in Nutrient Broth supplemented with 0.1% saccharose. Cells were centrifuged (10,000 g 1 min) and resuspended in physiological saline solution. To avoid bacterial aggregates the centrifuge tubes were sonicated for 2–3 min in an ultrasound bath and their optical density measured at 550 nm using a spectrophotometer. Cell concentration was adjusted to 10^8 cells ml^{-1} by comparing the OD to a calibration curve.

Capillary isoelectric focusing (CIEF) The capillary isoelectric focusing experiments were carried out using the laboratory-made apparatus (Horká et al. 2006a) at constant voltage (-) 20 kV on the side of the detector supplied by high voltage unit Spellman CZE 1000 R (Plainview, NY, USA). The lengths of fused silica capillaries, 0.100 mm I.D. and 0.350 mm O.D. (Pliva-Lachema, Brno, Czech Republic) were 300 mm, 150 mm to the detector. The ends of capillaries were dipped in 3 ml-glass vials with the anolyte, 4×10^{-2} mol l^{-1}sodium hydroxide, or the catholyte, 0.1 mol l^{-1} ortho-phosphoric acid. In all these electrolytes 1% *v/v* EtOH and 0.6% *w/v* PEG 4 000 were dissolved. Before each injection the capillaries were rinsed for 10 min with ethanol and then back-flushed with the catholyte for 1 min. The on-column UV–Vis detector LCD 2082 (Ecom, Prague, Czech Republic), was connected to the detection cell by optical fibers (Polymicro Technologies, Phoenix, USA) at the wavelength 280 nm. The width of the detection window was 1 mm. Light absorption (optical density) of the microbial suspensions was measured using a DU series 520 UV/Vis spectrophotometer (Beckmann Instruments, Palo Alto, CA, USA) at 550 nm. Segmental injection of samples into the capillary (Horká et al. 2006b) was employed. Samples were injected in three parts: 1.—segment of the spacers, solution of the selected simple ampholytic electrolytes dissolved in the catholyte; 2.—segment of the sample mixture of microorganisms; and 3.—the segment of the mixture of commercial carrier ampholytes and low-molecular p*I* markers for the tracing of the appropriate pH gradient in the pH range 2.0–3.3 or 3.3–4.7. The height differences of the reservoirs at the injection of the segments were 100 mm and $t_{inj.}$ of spacer segment was 25 s, sample segment, 10–15 s, and segment of carrier ampholytes and p*I* markers, 35 s. The second segment was composed of 10^8 cells ml^{-1} suspended in 15×10^{-3} mol l^{-1} NaCl. The third segment contained the water solution of p*I* markers, 25 μg ml^{-1}, p*I* 2.0, 2.7, 3.3, 3.7 and 4.7, and 5% *w/v* of synthetic carrier ampholytes, Biolyte, pH 3–10, ampholyte pH 3–4.5 and pH 2–4, in the ratio 1:2:5 (pH gradient pH range 2.0–3.3) and 1:3:4 (pH gradient pH range 3.3–4.7). The detector signals were acquired and processed with a Chromatography data station Clarity (DataApex s.r. o., Praha, Czech Republic). The values of p*I*s of cells were calculated using the migration times of the selected p*I* markers and their isoelectric points. Each measurement was repeated at least three times. The values of the isoelectric points can also be deduced from the dependences of the isoelectric points of the used p*I* markers on their migration times (Fig. 1).

Materials and chemicals Ampholyte high resolution, pH 2–4, and ampholyte pH 3–4.5, 2-morpholinoethanesulphonic acid monohydrate (MES), 3-morpholino-propanesulphonic acid (MOPS), *N*-[tris-(hydroxymethyl)-methyl]-3-amino-2-hydroxy-propansulphonic acid (TAPSO) were from Fluka Chemie (Buchs, Switzerland). PEG-4000 was from Aldrich (Milwaukee, WI, USA). The solution of synthetic

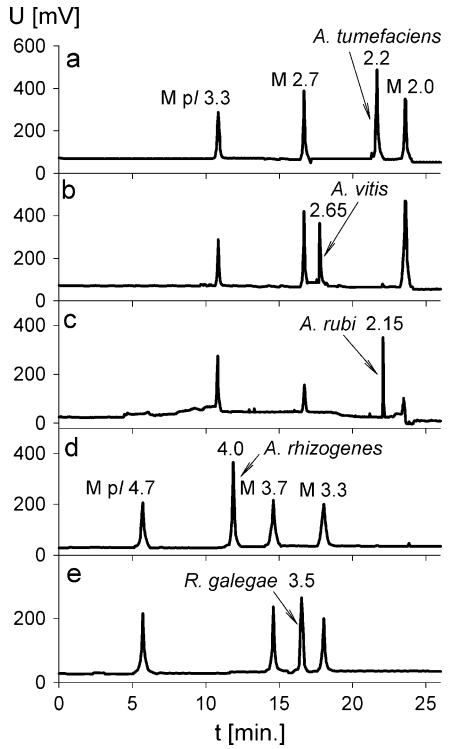

Fig. 1 Capillary Isoelectric Focusing (CIEF) of representative strains of *A. tumefaciens* (**a**: strain B6), *A.vitis* (**b**: AB3), and *A. rubi* (**c**: ICMP6428), in the pH gradient 2 –3.3 (**a**,**b**,**c**), *A. rhizogenes* (**d**: strain K27), and *Rhizobium galegea* (**e**: strain ATCC43677) in the pH gradient 3.3 –4.7. Peaks with numbers are p*I* markers (M)

carrier ampholytes, Biolyte, pH 3–10 were obtained from Bio-Rad laboratories (Hercules, CA, USA). The specifications of the spacers used and simple ampholytes, were as previously described (Horká et al. 2006a, b). All chemicals were analytical grade. The UV detectable p*I* markers, p*I* = 2.0 (Šťastná and Šlais 2005), 2.7, 3.0, 3.7, 4.7 (Šťastná et al. 2005), 3.3 (Šťastná and Šlais 2003), were synthesized in the Institute of Analytical Chemistry Academy of Sciences of the Czech Republic, v. v. i., Brno.

Results

Species identification determined by phenotypic methods Initially, strains were classified by biochemical tests (Table 2) as *A. tumefaciens*, *A. rhizogenes*, *A. rubi*, and *A. vitis*. Each species was represented by a well characterized homogeneous group (Table 1). In each species there were non-tumorigenic strains which clustered together with tumorigenic strains. All *A. tumefaciens* strains were 3-ketolactose positive, produced acid from melezitose, grew in 1.5% NaCl; failed to grow on *meso*-erythritol and malonate containing media; were negative in drop collapse, polygalacturonase and β-galactosidase assays. All *A. rhizogenes* strains did not produse 3-ketolactose and polygalacturonase; gave positive reactions in meso-erythritol, malonate, and β-galactosidase tests; did not utilize melezitose; failed to grow in 1.5% NaCl; and were negative in drop collapse assay. The *A. rubi* strain was negative in 3-ketolactose, drop collapse, and polygalacturonase tests; produced β-galactosidase, utilized malonate, melesitose, did not utilize m-erythritol and grew in 1.5% NaCl. All *A. vitis* strains utilized malonate; grew in 1.5% NaCl; failed to grow on *meso*-erythritol and melezitose; did not produce 3-ketolactose, but were positive in the drop collapse test, and the polygacturonase and β-galactosidase assays. These conventional methods clearly distinguished the four major species and corresponded the original descriptions of biovars and the Bergey's Manual (Young et al. 2005). The new drop collapse and β-galactosidase assays supported the original distinguishing characters.

PCR amplification Primers UF/B1R amplified a 190 bp region at the 5′ end of 23S rDNA of all *A. tumefaciens* strains. *A. rhizogenes* and *A. vitis* strains did not produce any bands with these primers. Primers UF/B2R produced a band at appr. 1,000 bp with *A. rhizogenes* strains. None of the *A. tumefaciens* and *A. vitis* strains examined produced any bands with UF/B2R primers. UF/AvR primers were used to differentiate *A. vitis* strains from other species of the *Agrobacterium* genus. For these amplifications, the annealing temperature had to be optimized. DNA of *A. vitis* strains S4 and CG964 was amplified at an annealing temperature of 52°C. Irrespective of this, all *A. vitis* strains were amplified with the UF/AvR primer pair, and no bands were amplified by strains of *A. tumefaciens* and *A. rhizogenes*. The UF/ArRr primer pair amplified the *A. rubi* strain at 60°C annealing temperature, although at that temperature *A. rhizogenes* strains also were weakly positive with this primer pair. The *Vir*C specific primers VCF3/VCR3 (Suzaki et al. 2004) amplified the template DNA in all pathogenic strains irrespective of their species. Strains

Table 2 Phenotypic characterization of *Agrobacterium* strains as compared to known strains

Strains	Determinative tests[a]									
	3-kl[b]	NaCl[c] 1.5%	Mel[d]	Ery[d]	Mal[d]	Drop[b]	PG[b]	βGal[b]	Path	PCR[e]
A. tumefaciens										UF/B1R
B6^T, C58, CG628,	+	+	+	−	−	−	−	−	+	+
CG632, T37, 3,	+	+	+	−	−	−	−	−	+	+
6, 15/6, 16/6, 41/10	+	+	+	−	−	−	−	−	+	+
18/3, 32/1	+	+	+	−	−	−	−	−	−	+
A. rubi										UF/ArR
ICMP6428	−	+	+	−	+	−	−	+	NT	+
A. rhizogenes										UF/B2R
K27, K47, 7/2,	−	−	−	+	+	−	−	+	+	+
9/2, 9/6, 47/1,	−	−	−	+	+	−	−	+	+	+
51, 30/2, CG918,	−	−	−	+	+	−	−	+	+	+
K84	−	−	−	+	+	−	−	+	−	+
A. vitis										UF/AvR
AB3, AB4, S4, 3/5,	−	+	−	−	+	+	+	+	+	+
Tm4, AA25, K309^T,	−	+	−	−	+	+	+	+	+	+
CG49, CG964, 2/3,	−	+	−	−	+	+	+	+	+	+
F2/5, CG484	−	+	−	−	+	+	+	+	−	+

[a] 3-kl = 3-ketolactose, [b] += produced, −not produced; [c] NaCl, + tolerate, −not tolerate; Mel = melezitose, [d] +used, −not used; Er = *meso*-erythritol, [d] +used, −not used; Mal = sodium malonate, [d] +used, −not used; Drop = drop collapse, [b] += produced, −not produced; PG = polygalacturonase, [b] += produced, −not produced; βGal = β-galactosidase, [b] += produced, −not produced ; Path = pathogenicity on sunflower seedling, + tumor, −no tumor; PCR = polymerase chain reaction primers (Puławska et al. 2006), [e] +single band in agarose gel; NT = not tested

that did not induce tumours on the test plants (sunflower) were also negative in the PCR test. These data support the suitability of this primer-pair for preliminary screening of putative pathogenic isolates. PCR amplifications using these primers for differentiation of *Agrobacterium* species were in agreement with the phenotypic methods. The ability of PCR to group strains according to species demonstrates the value of this method for rapid identification of agrobacteria at species level. This is a major improvement over the classical methods of identification for *Agrobacterium* species.

Fatty acid methyl ester (FAME) analysis Results of cellular fatty acid analysis varied quantitatively and qualitatively between the *Agrobacterium* strains investigated and separated them into four groups according to their FAME profiles. Ranges and means of fatty acid amounts for each species are listed in Table 3. Fatty acid compounds detected in all investigated species included 16:0, 16:0 3-OH, 18:0, 18:1 w7c, and summed feature 2. *A. tumefaciens* was characterized by fatty acids 18:1 w7c and 19:0 10-methyl, and the absence of 18:1 2-OH. The amount of 18:1 w7c in all *A. tumefaciens* strains was around 75% except C58 where it was just 69%. *A. rhizogenes* could be differentiated from the two other species by the presence of 10:0 3-OH, 15:0 iso 3-OH, and 17:0 iso. *A. vitis* did not have 10:0 3-OH, 15:0 iso 3-OH, and 17:0 iso, but had 18:1 2-OH fatty acids. The Similarity Indexes (Sherlock MIS) from FAME analysis according to the existing library were the following: *A. tumefaciens*: 0.742–0.872 (identified as *A. radiobacter*), *A. rhizogenes*: 0.37–0.584 (identified as *Phyllobacterium* sp.), *A. rubi*: 0.792 (identified as *A. rubi*) and *A. vitis*: 0.44–0.674 (identified as *A. radiobacter*). These results indicated that the fatty acid analysis was in agreement with the conventional biochemical methods and PCR grouping of *Agrobacterium* species. Strains belonging to *A.tumefaciens, A. rhizogenes* and *A. vitis* on the basis of fatty acids formed discrete clusters, different from each other according to the current classification of agrobacteria at the species level. Although the fatty acid profiles in this study were not quite in agreement with previous reports (Bouzar et al. 1993; Tighe et al. 2000) in each fatty acid, the analysis grouped the strains according to their respective species. In accordance with the previous reports the presence or absence of a Ti plasmid did not influence the fatty acid profile.

Table 3 Mean fatty acid concentration of *Agrobacterium* species analysed in this study

Fatty acid compound	*A. tumefaciens*	*A. rhizogenes*	*A. vitis*	*A. rubi*
10:0 3-OH	ND	1.18 (0.56)[a]*	ND	0.80
14:0	ND	ND	ND	0.22
15:0 iso 3-OH	ND	4.14 (0.29)	ND	ND
16:0	7.52 (0.90)	6.44 (0.77)	5.91 (1.16)	6.48
16:0 3-OH	4.04 (0.46)	5.79 (0.33)	2.11 (0.24)	2.93
17:0 iso	ND	1.08 (0.12)*	ND	ND
18:0	2.84 (0.33)	4.68 (0.45)	4.30 (0.49)	0.74
18:1 2-OH	ND	2.04 (0.37)	2.75 (0.50)	ND
18:0 3-OH	0.59 (0.18)*	2.98 (0.23)*	1.98 (0.22)*	ND
18:1 w7c	74.41 (2.34)	62.11 (5.93)	72.90 (2.2)	1.00
19:0 cyclo w8c	1.66 (0.98)	9.66 (2.02)	ND (2 +)	ND
19:0 10-methyl	1.18 (0.23)	ND	ND (3 +)	ND
Summed feature 2[b]	6.02 (0.32)	3.33 (0.49)	6.54 (0.54)	5.73
Summed feature 3[c]	2.18 (0.56)	ND (3 strains +)	4.49 (0.87)	8.26

Compounds marked with an asterisk did not occur in all strains

ND not detected

[a] Values are percentage of the total amount of fatty acid compounds present for that species. Number in parentheses indicate standard deviation

[b] Summed feature 2 = 12:0(aldehyde?), unknown (ECL 10.928),16:1 iso I/14:0 3-OH

[c] Summed feature 3 = 16:1 w7c/16:1 w6c

Capillary isoelectric focusing Based on our preliminary studies, the narrow pH gradients pH range 2.0–3.3, and 3.3–4.7 (Fig. 1), were used for the separation of *Agrobacterium* and *Rhizobium* strains. The pH gradients were traced by the UV-detectable p*I* markers, p*I* 2.0, 2.7, 3.3, 3.7 and 4.7. Good linearity were achieved in the ranges of the pH gradients (correlation coefficient R, 0.99). The strains of *A. vitis* and *A. tumefaciens* were separated by CIEF in the pH gradient range 2.0–3.3. Small insignificant differences in p*I* values were found between the non-tumorigenic strains of *A. vitis* (F2/5 and CG484; p*I*~2.6) and all

Table 4 The isoelectric points (p*I*), of the *Agrobacterium* strains used in this study

Strains	Isoelectric point(p*I*)
A. tumefaciens	
B6, C58, CG628, CG632, T37, 15/6, 16/6, 18/3, 41/10, 3, 6, 32/1	2.2
A. rubi	
ICMP6428[T]	2.15
A. rhizogenes	
K27, K47, K84, 7/2, 9/2, 9/6, 30/2, 47/1, 51, CG918	4.0
A. vitis	
AA25, AB3, AB4, S4, Tm4, K306, CG49, CG964, 2/3, 3/5 CG484, F2/5	2.65
Rhizobium leguminosarum bv. trifolii WSM2304	3.8
Rhizobium leguminosarum bv. viciae 3841	3.85
Rhizobium galegae ATCC43677	3.5
Mesorhizobium loti NZP 2037	3.3
Sinorhizobium meliloti 41	2.4

tumorigenic strains of the species (p*I*~2.65) and between the non-tumorigenic strain of *A. tumefaciens* (32/1; p*I*~2.25) and all tumorigenic strains of the species (p*I*~2.2; Table 4). *A. rhizogenes* strains were separated by CIEF in the pH gradient pH range 3.3–4.7. Their p*I*s were about pH 4.0. There may be small differences between the isoelectric points of tumorigenic and non-tumorigenic strains but it is not clear if these are significant. CIEF confirms the conventional phenotypic, PCR, and FAME identifications of species in *Agrobacterium*. The three species of *A. tumefaciens, A. rhizogenes*, and *A. vitis* could be clearly differentiated by all methods. The p*I* of the type strain of *A. rubi* (ICMP6428) was 2.15. This value is very close to the p*I* of *A. tumefaciens* (2.2–2.25) indicating that phylogenetically the two species are very close (Young et al. 2005). CIEF can also differentiate strains of *Agrobacterium* and *Rhizobium* species and therefore can be an alternative, quicker method to characterize and group isolates from their respective host plants.

Discussion

Currently, a variety of conventional, and molecular methods (Moore et al. 2001; Palacio-Bielsa et al. 2009; Costechareyre et al. 2010) exist for the characterization and identification of the species in the *Agrobacterium* genus, for example 3-ketolactose production, use of erythritol and malonate, PCR, and comparison of nucleic acid sequences. However, many of these approaches are costly and time-consuming because of the number of the experimental steps involved in the process. Instrumental techniques like PCR, FAME analysis, and the CIEF demonstrated in the present study not only simplify, but largely reduce the required time for the identification. Previous studies (Horká et al. 2006a) with this method demonstrated that mixed cultures of microorganisms (*Escherichia coli, Candida albicans, Staphylococcus aureus* etc.) could be separated by the CIEF method. In this paper, CIEF, was firstly applied for agrobacteria resulted in p*I* values 2.2, 4.0, 2.15 and 2.6 for *A. tumefaciens*, *A. rhizogenes, A. rubi* and *A. vitis* strains, respectively. The three major species can be well separated using CIEF. However, the differences between *A. tumefaciens* and *A. rubi* strains are too small and the two species cannot be discriminated on the basis of their p*I* values. Due to the single available *A. rubi* strain, no valid conclusions can be made. However, it is known that the *A. rubi* strains are phylogenetically very close to *A. tumefaciens* strains according to the 16S rRNA sequence data (Young et al. 2004, 2005), and the small difference in p*I* values may also indicate the close relationship between *A. tumefaciens* and *A. rubi* strains. Comparisons of 35 *Agrobacterium* strains using CIEF confirmed the results of phenotypic tests, PCR, and FAME analysis that strains of *A. tumefaciens, A. rhizogenes* and *A. vitis* form distinct species clusters. Grouping *Agrobacterium* species with CIEF agrees with the classical phenotyping and molecular methods (Moore et al. 2001; Palacio-Bielsa et al. 2009; Costechareyre et al. 2010). Although CIEF provides a rapid and reliable complementary method for the characterization of strains isolated from tumorigenic galls, at present it is not foreseeable how far this method can be used for the rapid evaluation of changes on bacterial surfaces. Further studies are needed to discover if these surface properties characterized by the p*I* values have any biological importance in the host-pathogen relationships. Armstrong and He (2001) proved by staining that microorganisms separated by capillary electrophoresis were still viable. Recently, Petr *et al.* (2009), and Horká *et al.* (2009) demonstrated the cultivation of *Escherichia coli* and plant pathogenic bacteria in sterile growth media after CIEF, proving that bacterial cells can survive the electrophoretic process. Further studies are also needed to investigate how agrobacteria remain alive during the CIEF and if the method is useful for isolation of different bacteria from mixed infections.

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References

Armstrong, D. W., & He, L. (2001). Determination of cell viability in single or mixed samples using capillary electrophoresis laser-induced fluorescence microfluidic systems. *Analytical Chemistry, 73*, 4551–4557.

Bini, F., Kuczmog, A., Putnoky, P., Otten, L., Bazzi, C., Burr, T. J., et al. (2008). Novel pathogen-specific primers for detection of *Agrobacterium vitis* and *Agrobacterium tumefaciens*. *Vitis, 47*, 181–189.

Bouzar, H., Jones, J. B., & Hodge, N. C. (1993). Differential characterization of *Agrobacterium* species using carbon-source utilization patterns and fatty acid profiles. *Phytopathology, 83*, 733–739.

Costechareyre, D., Rhouma, A., Lavire, C., Perrine, P., Chappulliot, D., Bertolla, F., et al. (2010). Rapid and efficient identification of *Agrobacterium* species by *recA* allele analysis: Agrobacterium RecA diversity. *Microbial Ecology, 60*, 862–872.

Desai, M. J., & Armstrong, D. W. (2003). Separation, identification, and characterization of microorganisms by capillary electrophoresis. *Microbiology and Molecular Biology Reviews, 67*, 38–51.

Holmes, B., & Roberts, P. (1981). The classification, identification and nomenclature of agrobacteria. *Journal of Applied Bacteriology, 50*, 443–467.

Horká, M., Růžička, F., Holá, V., & Šlais, K. (2006). Capillary isoelectric focusing of microorganisms in the pH range 2–5 in a dynamically modified FS capillary with UV detection. *Analytical and Bioanalytical Chemistry, 385*, 840–846.

Horká, M., Růžička, F., Horký, J., Holá, V., & Šlais, K. (2006). Capillary isoelectric focusing of proteins and microorganisms in dynamically modified fused silica with UV detection. *Journal of Chromatography. B, 841*, 152–159.

Horká, M., Horky, J., Matoušková, H., & Šlais, K. (2009). Free flow and capillary isoelectric focusing of bacteria from tomatoes. *Journal of Chromatography. A, 1216*, 1019–1024.

Horká, M., Horký, J., Kubesová, A., Mazanec, K., Matoušková, H., & Šlais, K. (2010). Electromigration techniques—a fast and economical tool for differentiation of similar strains of microorganisms. *Analyst, 135*, 1636–1644.

Kerr, A., & Panagopoulos, C. G. (1977). Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. *Phytopathologische Zeitschrift, 90*, 172–179.

Lantz, A. W., Bao, Y., & Armstrong, D. W. (2007). Single-cell detection: test of microbial contamination using capillary electrophoresis. *Analytical Chemistry, 79*, 1720–1724.

Moore, L. W., Bouzar, H., & Burr, T. (2001). Agrobacterium. In N. W. Schaad (Ed.), *Laboratory guide for identification of plant pathogenic bacteria* (pp. 16–36). St Paul: APS.

Palacio-Bielsa, A., González-Abolafio, R., Álvarez, B., Lastra, B., Cambra, M. A., Salcedo, C. I., et al. (2009). Chromosomal and Ti plasmid characterization of tumorigenic strains of three *Rhizobium* species isolated from grapevine tumours. *Plant Pathology, 58*, 584–593.

Petr, J., Ryparova, O., Ranc, V., Hinnerova, P., Znaleziona, J., Kowalska, M., et al. (2009). Assessment of CE for the identification of microorganisms. *Electrophoresis, 30*, 444–449.

Puławska, J., Maes, M., Willems, A., & Sobiczewski, P. (2000). Phylogenetic analysis of 23S rRNA gene sequences of *Agrobacterium, Rhizobium* and *Sinorhizobium* strains. *Systematic and Applied Microbiology, 23*, 238–244.

Puławska, J., Willems, A., & Sobiczewski, P. (2006). Rapid and specific identification of four *Agrobacterium* species and biovars using multiplex PCR. *Systematic and Applied Microbiology, 29*, 470–479.

Rodriguez, M. A., & Armstrong, D. W. (2004). Separation and analysis of colloidal/nano-particles including microorganisms by capillary electrophoresis: a fundamental review. *Journal of Chromatography. B, 800*, 7–25.

Sasser, M. (1990). *Technical note 102: Tracking a strain using the Microbial Identification System*. North Newark: MIS.

Šťastná, M., & Šlais, K. (2003). Dynamics of gel isoelectric focusing with ampholytic dyes monitored by camera in real-time. *Journal of Chromatography. A, 1008*, 193–203.

Šťastná, M., & Šlais, K. (2005). Colored p*I* standards and gel isoelectric focusing in strongly acidic pH. *Analytical and Bioanalytical Chemistry, 382*, 65–72.

Šťastná, M., Trávníček, M., & Šlais, K. (2005). New azo dyes as colored isoelectric point markers for isoelectric focusing in acidic pH region. *Electrophoresis, 26*, 53–59.

Süle, S. (1978). Biotypes of *Agrobacterium tumefaciens* in Hungary. *Journal of Applied Bacteriology, 44*, 207–213.

Süle, S., Cursino, L., Zheng, D., Hoch, H. C., & Burr, T. J. (2009). Surface motility and associated surfactant production in *Agrobacterium vitis*. *Letters in Applied Microbiology, 49*, 596–601.

Suzaki, K., Yoshida, K., & Sawada, H. (2004). Detection of tumorigenic *Rhizobium* strains from infected apple saplings by colony PCR with improved PCR primers. *Journal of General Plant Pathology, 70*, 342–347.

Tighe, S. W., de Lajudie, P., Dipietro, K., Lindström, K., Nick, G., & Jarvis, D. D. W. (2000). Analysis of cellular fatty acids and phenotypic relationships of *Agrobacterium, Bradyrhizobium, Mesorhizobium*, *Rhizobium* and *Sinorhizobium* species using the Sherlock Microbial Identification System. *International Journal of Systematic and Evolutionary Microbiology, 50*, 787–801.

Willems, A., & Collins, M. D. (1993). Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences. *International Journal of Systematic Bacteriology, 43*, 305–313.

Young, J. M., Park, D.-C., & Weir, S. B. (2004). Diversity of 16S rDNA sequences of Rhizobium spp. implications for species determinations. *FEMS Microbiology Letters, 238*, 125–131.

Young, J. M., Kerr, A., & Sawada, H. (2005). Genus II. *Agrobacterium* Conn 1942. In Brenner, Krieg, Staley, & Garrity (Eds.), *The proteobacteria, Bergeyś manual of systematic bacteriology, vol. 2* (2nd ed., pp. 340–345). New York: Springer-Verlag.