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Free flow and capillary isoelectric focusing of bacteria from the tomatoes plant tissues

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ABSTRACT

The means of the preconcentration and preseparation of selected species or pathovars of bacteria directly from the plant tissue suspension by free flow isoelectric focusing are introduced here. After the focusing, the resulting fraction of microorganisms, native or dynamically modified by the non-ionogenic tenside on the basis of pyrenebutanoate, was separated by capillary isoelectric focusing and/or cultivated and positively identified by gas chromatographic analysis of fatty acid methyl esters. Simultaneously, capillary isoelectric focusing with UV and fluorometric detection was used for the rapid estimation of unknown isoelectric points of the examined plant pathogenic species of genus *Clavibacter*, *Xanthomonas* and *Pseudomonas* prior to the preconcentration and preseparation. The microorganisms were of different origin, native and/or dynamically modified by the non-ionogenic tenside.

1. Introduction

At the determination of the etiological agents of plants the suspension of the infected plant tissue is usually plated on semiselective medium and subsequently the suspected colonies of microorganisms, MOs, are identified. Furthermore, the bacterial cell of the target must be isolated and their pathogenicity demonstrated for a positive diagnosis [1]. The application of the immunoisolation or immunomagnetic techniques permitted the separation of MOs of the target from naturally infected materials with higher sensitivity and faster than direct isolation on the semiselective medium currently used.

Free flow electrophoresis [2] was used for the separation of cells, organelles and membrane vesicles, purification proteins and peptides [3,4] or for the study of the oxidative response of neutrophil subpopulations [5] or for purifying mitochondria from *Saccharomyces cerevisiae* for subsequent proteome analysis [6,7]. Whole cells, e.g., gastric endocrine cells [8], cells of *Nitrosomonas eutropha* [9], the sperm [10], and their defined parts can be fractionated on the basis of their electrophoretic mobility [11,12]. The multicompartment electrolyzers have been developed for the isoelectric fractionation using either mixture of carrier ampholytes [13] or membranes with immobilized pH gradients [14]. They are now commercially available [15], e.g., MicroRotofor, from Bio-Rad Labs, was used for free solution isoelectric focusing of protein digests [16]. In the latter case the focusing process was monitored visually using colored isoelectric point, pI, markers. The hydrophilic and hydrophobic proteins of rat liver peroxisomes [17] were fractionated by free flow isoelectric focusing. The disadvantage of this technique is the large consumption of the expensive commercial carrier electrolytes. New media for fast generation of narrow- or broad-range pH gradients for free flow isoelectric focusing were developed, see Ref. [18] and used for the focusing of the human serum proteins. The mixture of simple defined buffers similar to the one described in Refs. [18,19–25] and modified to cover the pH range from 3 to 11 was used at preparative divergent flow isoelectric focusing of pI markers [22].

Since MOs belong to the amphoteric bio-particles, they are characterized by the isoelectric point [26–29] which is one of the potentially suitable markers for their identification [30]. Previously the capillary isoelectric focusing, cIEF, was used for the separation of MOs according to their pI [26–29,31,32]. Owing to its versatility, speed of the separation and the sensitivity of the fluorometric detection this technique could be possible to be applied especially in conjunction with the preconcentration and preseparation of bacteria by free flow isoelectric focusing from the real samples. The trace analysis of MOs is dependent mainly on the tagging of the cells by fluorophores [33,34] without significant change in the respective pI [35,36].

In this pilot project we examine, whether MOs can be preconcentrated from the plant tissue suspension by free flow isoelectric focusing and whether the collected fractions could be cultivated and subsequently examined and MOs identified by gas chromatographic analysis of fatty acid methyl esters [31,37] or directly

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Table 1
The strains of the plant pathogens included in this study, comparison of their isoelectric points, pI, and RSDs from three measurements of the migration times, *t*, for each from the strains.

Abbreviation in figs.	Strain	pI
C. michiganensis	*Clavibacter michiganensis* subsp. *michiganensis* CCM 1635	4.6
	Clavibacter michiganensis subsp. *michiganensis* VURV C254	4.6
	Clavibacter michiganensis subsp. *michiganensis* VURV 2/4/99	4.7
	Clavibacter michiganensis subsp. *michiganensis* VURV 5090	4.6
	Clavibacter michiganensis subsp. *michiganensis* VURV 5059	4.7
	Clavibacter michiganensis subsp. *michiganensis* VURV 7008	4.7
	Clavibacter michiganensis subsp. *michiganensis* VURV 7018	4.6
	Clavibacter michiganensis subsp. *michiganensis* VURV 7030	4.7
	C. michiganensis	pI = 4.7, RSD = 1.9%
X. vesicatoria	*Xanthomonas vesicatoria* CCM 2101	4.0
	Xanthomonas vesicatoria CCM 2102	4.1
	Xanthomonas vesicatoria VURV P-1-1	4.0
	Xanthomonas vesicatoria VURV P-6-1	4.1
	Xanthomonas vesicatoria LMG 2804	4.1
	Xanthomonas vesicatoria LMG 667	4.1
	X. vesicatoria	pI = 4.1, RSD = 0.7%
P. syringae	*Pseudomonas syringae pv. tomato* CFBP 5422	4.0
	Pseudomonas syringae pv. tomato CFBP 2212	4.0
	Pseudomonas syringae pv. tomato IVIA 1733.3	4.0
	P. syringae	pI = 4.0, RSD = 1.9%
P. corrugata	*Pseudomonas corrugata* CFBP 4901	2.4
	Pseudomonas corrugata CFBP 5465	2.4
	Pseudomonas corrugata CFBP 6663	2.4
	Pseudomonas corrugata IVIA 614.5.3	2.4
	P. corrugata	pI = 2.4, RSD = 0.9%

separated by cIEF with UV or sensitive fluorometric detection. For the experiments we have chosen the pathogens from the group of gram positive cells, *Clavibacter michiganensis* subsp. *michiganensis* (*C. michiganensis*), and gram negative cells, *Xanthomonas vesicatoria* (*X. vesicatoria*), *Pseudomonas syringae* pv. *tomato* (*P. syringae*) and *Pseudomonas corrugata* (*P. corrugata*), which belong to the disease-causing organisms of tomatoes. Some of these pathogens are on the list of quarantine harmful organisms. Early detection and exact identification can be helpful in effective and successful plant protection. However, till now only small number of microbial pI have been determined. The detection and isolation of *C. michiganensis* from seeds before field or greenhouse cultivation is difficult when the bacterium is at the low concentration and associated microbiota are present. Furthermore, *C. michiganensis* belong to the slow-growing MOs [1].

Therefore, the single strains, from the group of tomatoes disease-causing organisms, are separated here by cIEF and pI of the native various strains of each species of the bacteria and labeled MOs are determined and compared. MOs are dynamically modified by the non-ionogenic tenside based on pyrenebutanoate, poly(ethylene glycol) 4-(1-pyrene)butanoate (PB-PEG) [38]. The pH gradients are traced by the low-molecular-weight UV detectable and fluorescent pI markers.

2. Experimental

2.1. Plant pathogens

The strains included in this study, see Table 1, were obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic), from the Collection of Microorganisms of the Research Institute of Crop Production (VURV, Prague-Ruzyně, Czech Republic), from IVIA (IVIA, Valencia, Spain), from the BCCM, Laboratorium voor Microbiology (LMG, Universiteit Gent, Belgium) and from the Collection Francaise de Bactéries Phytopathogénes (CFBP, Angers, France).

2.2. Chemicals

The ampholyte high resolution, pH 2–4, and ampholyte pH 3–4.5, 2-morpholinoethanesulphonic (MES) acid monohydrate, 3-morpholino-propanesulphonic (MOPS) acid, N-[tris-(hydroxymethyl)-methyl]-3-amino-2-hydroxy-propanesulphonic acid (TAPSO) were from Fluka Chemie (Buchs, Switzerland). Poly(ethylene glycol) (M_r 400 and 10 000) and 4-(1-pyrene)butyric acid were from Aldrich (Milwaukee, WI, USA), Brij 35 from Sigma (St. Louis, MO, USA). The solution of synthetic carrier ampholytes, Biolyte, pH 3–10, was obtained from Bio-Rad Labs. (Hercules, CA, USA), L-aspartic (Asp) acid from LOBA Chemie (Vienna, Austria), N-(2-acetamido)-2-aminoethansulphonic (ACES) acid and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic (HEPES) acid from Merck, Darmstadt, Germany. The specifications [39,40] of the used spacers and simple ampholytes are described in Ref. [32]. All chemicals were of analytical grade.

Poly(ethylene glycol) pyrenebutanoate, fluorescein-based pI markers, pI = 1.8, 3.0, 4.0, 4.7, and 5.5, the low-molecular-mass pI markers, pI = 2.0, 2.7, 3.0, 3.9, 4.9, and 4-morpholinylacetic (MAA) acid [19] were synthesized in the Institute of Analytical Chemistry Academy of Sciences of the Czech Republic, Brno, Czech Republic. MAA was prepared by the reaction of morpholin and chloracetic acid, Sigma, and PB-PEG by the reaction of 4-(1-pyrene) butyric acid and PEG 400 [38].

2.3. Preparation of the microbial sample

Before each experiment, the strains were thawed quickly at 28 °C and cultivated on Nutrient Agar (Difco, Chemos, Prague, Czech Republic) at 28 °C, both for 24 and 48 h. The microbial cultures were resuspended in physiological saline solution (PSS). The concentration of the resuspended microorganisms was estimated by the measurement of the optical density of the suspension. It was measured by spectrophotometer at 550 nm, according to the calibration curve, which was defined by reference samples. These samples were prepared by resuspension of the microbial culture in PSS. The num-

bers of microorganisms in reference samples were measured by dilution and by plating 100 μL of suspension on Muller–Hinton's agar (Bio-Rad Labs.). After cultivation at 28 °C for 24 h the colonies were counted.

Plant tissue suspension was prepared by homogenizing 1 g of leaf of tomatoes washed 10 min under potable water in 10 mL of the sterile demineralized water with mortar and pestle.

2.4. Sample preparation

2.4.1. cIEF with UV detection

The segmental injection of the sample into the capillary [29] was employed here. The sample was injected in three parts—segment of the spacers, solution of the selected simple ampholytic electrolytes dissolved in the catholyte, Ca [32], and segment of the sample mixture of MOs and the segment of the mixture of commercial carrier ampholytes and low-molecular pI markers for the tracing of the used pH gradient in the pH range of 2.0–4.9. The height differences of the reservoirs at the injection of the segments, Δh, were 100 mm and the time of injection, t_{inj}, of the segment of spacers was 25 s, sample segment, 10 s, and segment of carrier ampholytes and pI markers, 35 s.

The second segment was composed of the suspension of plant pathogens (see Section 2.1), 2×10^7–8×10^8 cell mL^{-1}, dissolved in water solution of 3% (v/v) ethanol (EtOH), 2% (m/v) PEG 10 000 and 15×10^{-3} mol L^{-1} NaCl. The injected volume of the analytes was approximately 20 nL, which represents maximum 16×10^3 cells injected into the capillary.

The third segment contained of the water solution of pI markers, 25 μg mL^{-1}, and 5% (m/v) of synthetic carrier ampholytes, Biolyte, pH 3–10, ampholyte pH 3–4.5 and pH 2–4, in the ratio 1:2:5.

2.4.2. cIEF with fluorimetric detection

Similarly as in the paragraph above, the segmental injection of the sample into the capillary [29] was used here. The third segment, except the mixture of commercial carrier ampholytes, was composed of pI markers for the tracing of the used pH gradient in the pH range of 1.8–5.5.

The second segment was composed of the suspension of plant pathogens at the concentration 5×10^5 cell mL^{-1} dissolved in water solution of 4×10^{-4} mol L^{-1} PB-PEG, 3% (v/v) EtOH, 0.5% (m/v) PEG 10 000 and 15×10^{-3} mol L^{-1} NaCl. Both sample mixtures were stored for 15 min at 20 °C before use. The injected volumes of the pathogens were maximum 40 cells injected into the capillary.

The concentration of each pI marker in the water solution in the third segment was 5 μg mL^{-1} (injected amounts 0.4 ng).

2.4.3. Micropreparative isoelectric focusing

3000 μL of the tomatoes leaf suspension or water in the focusing chamber includes dissolved 2×10^{-4} mol L^{-1} PB-PEG, 15×10^{-3} mol L^{-1} NaCl, 600 μL of simple ampholytic electrolytes, *P. corrugata*, *P. syringae* and *C. michiganensis*, each of them 1×10^5–2×10^7 cells in 1 mL, or without resuspended cells, respectively. The incubation time was 15 min at 20 °C before injection for the purposes of the fluorometric detection; for the UV detection PB-PEG was replaced by 0.3% (m/v) Brij 35 and only in the preliminary experiments the low-molecular pI markers 2.0 and 5.5, each of them 35 μg mL^{-1}, were used for the tracing of the pH gradient in the pH range of 1–10.

2.5. Electrolyte systems

2.5.1. UV detection

At cIEF 3×10^{-2} mol L^{-1} sodium hydroxide and 0.1 mol L^{-1} ortho-phosphoric acid were used as the catholyte and the anolyte (An) solutions, respectively, with the addition of 1% (v/v) EtOH and 0.3% (m/v) PEG 10 000.

2.5.2. Fluorometric detection

For cIEF experiments presented here, 2×10^{-2} mol L^{-1} sodium hydroxide and 0.1 mol L^{-1} ortho-phosphoric acid were used as Ca and An, respectively, with the addition of 7×10^{-5} mol L^{-1} PB-PEG, 1% (v/v) EtOH and 0.75% (m/v) PEG 10 000.

2.6. cIEF—equipment and procedure

The capillary isoelectric focusing experiments were carried out using the laboratory-made apparatus [32] 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 the fused silica (FS) capillaries, 0.1 mm I.D. and 0.35 mm O.D. (Pliva-Lachema, Brno, Czech Republic) were 350 mm, 200 mm to the detector, effective volumes of the columns ~1.6 μL, respectively. The ends of the fused silica capillary were dipped in 3 mL-glass vials with Ca or An. During the cIEF experiments, the current decreased from 40 to 60 μA at the beginning of the experiment down to 3 or 6 μA at the time of detection, depending on the sampling time interval and the sample solution.

The on-column UV-Vis detector LCD 2082 (Ecom, Prague, Czech Republic), connected to the detection cell by optical fibers (Polymicro Technologies, Phoenix, AZ, USA) at the wavelength 280 nm, was used. For the fluorometric detection the PU4027 Programmable Fluorescence detector (Philips Scientific, Cambridge, UK) was modified. The excitation wavelength, λ_{EX}, was 335 nm, the emission wavelength [38,41], λ_{EM}, was 480 nm. The width of the detection window was 1 mm. The 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.

The sample injection was accomplished by siphoning action obtained by elevating the inlet reservoir on the side of the anode relative to the outlet reservoir, side of the cathode. The height difference of the reservoirs for the sample injection was 100 mm, and the time of injection was from 10 to 35 s. The segmental injection of the sample pulse was used. The clusters of the microbial cells and the sample of the plant tissue suspension contamined with dispersed MOs were disrupted by the sonication of the microbial suspension in the ultrasound bath Sonorex (Bandelin electronic, Berlin, Germany). The sonication was processed for 1 min at 27 °C and at frequency 35 kHz. Between the separation runs the sample suspensions were vortexed (IKA Works, Wilmington, DE, USA).

The detector signals were acquired and processed with the Chromatography data station Clarity (DataApex, Prague, Czech Republic).

2.6.1. Rinsing procedure

Before each injection the capillaries were always rinsed with acetone/ethanol mixture (10:1, v/v) for 10 min, and then backflushed with catholyte for 2 min. The rinsing procedures were carried out hydrodynamically.

2.7. Micropreparative isoelectric focusing

The samples were focused on MicroRotofor device from Bio-Rad Labs. [17]. The separation was conducted using the PowerPac 3000 power supply. The voltage program was started at 50 V (30 min) and gradually increased to 400 V until reaching the power limit of 1 W. The run was terminated after 2 h, when the current stopped decreasing—typically after additional 25 min. The sample was fractionated in ten 250 μL compartments. The pH of the individual

fractions was measured with precision digital pH meter CyberScan pH 510 (Eutech Instruments, Singapore, China) equipped with micro pH electrode Slimtrode (Hamilton). After fractionation the photographs of the electrolysis cell were prepared by PowerShot G5 digital camera (Canon, Tokyo, Japan) with the help of the Gel Doc 2000 Gel Documentation System Bio-Rad Labs. equipped with trans UV illumination. The fluorescence of the individual fractions was measured using the PU4027 Programmable Fluorescence detector. After focusing the fractions were separated by cIEF or cultivated and subsequently identified by standard gas chromatography technique, see the sections below.

2.8. Gas chromatography of fatty acid methyl esters analysis

GC was accomplished according the published procedure, e.g., Ref. [42]. The cultures were grown on trypticase soy agar (Oxoid, Basingstoke, UK) for 24 h at 28 °C. The presence of characteristic fatty acids was compared by SHERLOCK Microbial identification system (MIDI Newark, DE, USA) with database of bacteria.

2.9. Safety

The potentially pathogenic microorganisms were separated here at cIEF and/or at micropreparative isoelectric focusing. Therefore, the strong safety procedures are necessary to adhere. All operations are performed according to the instruction for the labour with for plants infection materials. Care must be taken to avoid contact with either of these pathogens. The use of the gumgloves, the disinfection of the inner and the outer surface of the capillary, etc. after its contamination by the microorganisms is platitude.

3. Results and discussion

3.1. The determination of pI's of pathogens by cIEF

The requested detection limits for different microbial pathogens are often very low. Therefore, the preseparation of monitored plant pathogenic bacteria from the associated microbiota [1] and their preconcentration from the real sample are necessary before acceptable detection in the electrolyzer, MicroRotofor. The values of pI of monitored MOs, gram positive *C. michiganensis* and gram negative *X. vesicatoria*, *P. syringae* and *P. corrugata* are not available. The single strains of examined MOs were separated by cIEF with UV detection for the determination of these values as shown in Fig. 1A. With respect to the potential hazardousness of the tomatoes pathogens cIEF technique enabling the rinsing and/or disinfection of the capillary between the each focusing run was used. Once more the segmental injection [29,32] was helpful for the achievement of the reproducibility and the linearity of the pH gradient.

According to our preliminary experiments pI of examined MOs lies within the pH range of 2.0–5.5, hence in this pH range the pH gradient was flattened [31]. The dependence of pI of MOs on their migration times, *t*, was measured for the verification of the linearity of the pH gradient, as shown in Fig. 1C, curve 1. The pH gradient was traced by the pI markers in the pH range from 2.0 to 4.9, closed circles. The closed stars on this curve are in accordance with the migration times and/or isoelectric points of detected MOs. The values of pI of MOs are calculated from the migration times of the selected pI markers and their isoelectric points. The values of pI in the used pH gradient (pH range 2–5), as shown in Table 1, were found to be not host-specific like as in Ref. [31]. Therefore, the average value of the isoelectric points of the examined strains was calculated from minimum three measurements for each of the strain from Table 1. The pI was determined as 4.7 for *C. michiganensis* (eight strains, RSD = 1.9%), 4.1 for *X. vesicatoria* (six strains, RSD = 0.7%), 4.0 for *P. syringae* (three strains, RSD = 1.9%) and 2.4 for *P. corrugata* (four strains, RSD = 0.9%).

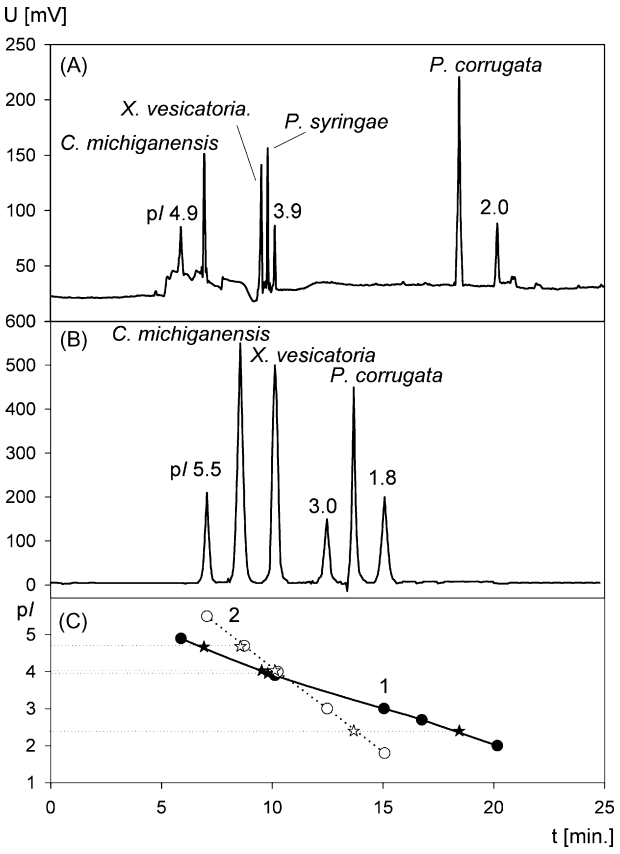

Fig. 1. Separation of the tomatoes pathogens by cIEF with UV (A) and fluorometric (B) detection in the pH gradient 2.0–4.9 and 1.8–5.5, respectively and the linearity of the pH gradient (C); conditions: FS capillary 0.1 mm I.D., 0.35 mm O.D., length 350 mm, 200 mm to the detection cell; applied voltage (−) 20 kV; An, 1×10^{-1} mol L^{-1} H_3PO_4; in both Ca and An dissolved 1% (v/v) EtOH; segmental injection: Δh, 100 mm; t_{inj}, spacer segment [32] (dissolved in Ca), 25 s, segment of the sample, 10 s, carrier ampholytes and pI markers together, 35 s; composition: segment of carrier ampholytes, 5% (m/v) solution of Biolyte pH 3–10, ampholyte, pH 3–4.5 and pH 2–4 in the ratios 1:2:5; (A) Ca, 3×10^{-2} mol L^{-1} NaOH, 0.3% (m/v) PEG 10 000; wavelength: λ = 280 nm; sample segment of MOs (for abbreviation see Table 1)—*P. corrugata*, *P. syringae*, *X. vesicatoria* and *C. michiganensis* (8×10^8 cell mL^{-1}), resuspended in water solution of 3% (v/v) EtOH, 2% (m/v) PEG 10 000 and 15×10^{-3} mol L^{-1} NaCl; pI markers: pI, 2.0, 3.9 and 4.9; (B) see (A), catholyte, 2×10^{-2} mol L^{-1} NaOH, 7×10^{-5} mol L^{-1} PB-PEG and 0.75% (m/v) PEG 10 000; λ_{EX} = 335 nm, λ_{EM} = 480 nm; sample segment of MOs—*P. corrugata*, *X. vesicatoria* and *C. michiganensis* (5×10^5 cell mL^{-1}), resuspended in water solution of 4×10^{-4} mol L^{-1} PB-PEG, 3% (v/v) EtOH, 0.5% (m/v) PEG 10 000 and 15×10^{-3} mol L^{-1} NaCl; pI markers: pI, 1.8, 3.0 and 5.5; *t*, migration time (min); (C) see (A) (curve 1, closed circle), (B) (curve 2, open circle); the dependence of pI on *t*; closed (curve 1) and open (curve 2) stars—*t* of the cells; before each injection the capillaries were rinsed for 10 min with the mixture of the acetone/ethanol, 10:1 (v/v), and then back-flushed with the catholyte for 2 min.

The isoelectric point of the native phytopathogens and those dynamically modified by the non-ionogenic fluorescent tenside based on pyrenebutanoate [26,31] was necessary trade off. cIEF separation of the cells *C. michiganensis*, *X. vesicatoria* and *P. corrugata* dynamically modified by PB-PEG with fluorometric detection is depicted in Fig. 1B. The pH gradient pH range from 1.8 to 5.5 was traced by fluorescent pI markers, as seen from Fig. 1B and C, curve 2 and open circles. The positions of the open stars on these curves are determined by the migration times and by the calculated isoelectric point of the labeled MOs. The isoelectric points of the labeled pathogens were found comparable with pI of the native compounds; the closed stars on the curve 1 and the open stars on the curve 2 are closed to the calculated values

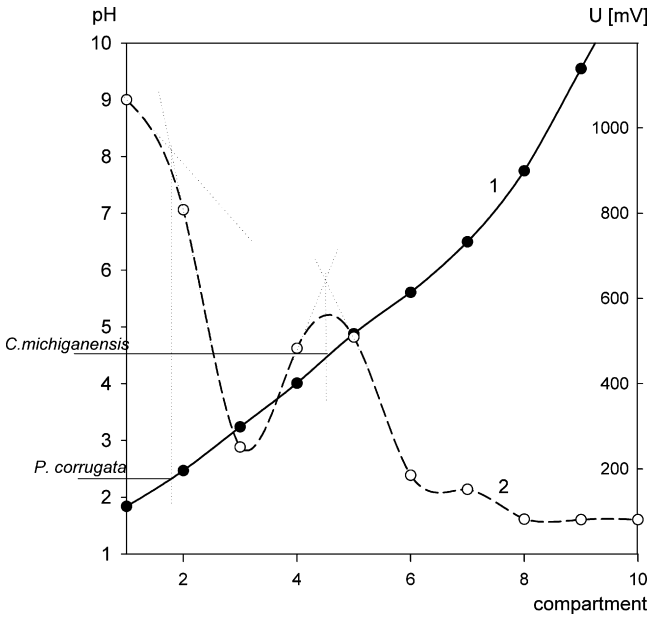

Fig. 2. Plot of the pH values (curve 1) and fluorescence measured (curve 2) in the collected fraction from chambers 1 to 10. Conditions and designations, see Fig. 1 and Supplementary Fig. S1B; pH values, see Supplementary Fig. S1, water solution without cells.

of pI for identical strains independently on their migration times.

3.2. The multicompartment electrolyzer

In cases when a relative large amount, order of millilitres, of the plant tissue suspension contamined by pathogens have to be rapidly preconcentrated and preseparated according to their pI. For further analysis the usage of the multicompartment electrolyzer seems to be possible solution. The multicompartment electrolyzer can preseparate the sample in 10–250 μL compartments, as seen in Supplementary Fig. S1. The simple ampholytic electrolytes were used here as carriers instead of the commercial polyampholytes [22]. The mixture of simple defined buffers similar to those described previously [18,19–25] was used in the broad pH range from 1 to 10.

In the preliminary experiments the quality of the separation was initially evaluated by the addition of UV detectable pI markers 2.0 and 5.5, each of them 35 μg mL^{-1}, for the tracing of the pH gradient. We have measured the pH in the individual compartments of the MicroRotofor electrolysis cell; the results are shown in Fig. 2, curve 1. The focused zones of the pI markers 2.0 and 5.5 were visibly minimum in two or three compartments, respectively.

Subsequently the cells of *P. corrugata* (pI ~2.4) or *P. syringae* (pI ~4.0), each of the 2×10^7 cells in 1 mL, resuspended in 3000 μL of the water solution of 15×10^{-3} mol L^{-1} NaCl and 600 μL of simple ampholytic electrolytes were separated in the MicroRotofor. The presence of MOs in the samples from compartments 1 to 5 was examined by CIEF with UV detection in the pH gradient pH range of 2.0–4.9 under the conditions mentioned in Section 3.1. MOs were detected in each of the sample from the compartments 1 to 5. MOs are strongly adsorbed onto the plastic surface of the chamber. Therefore, before the separation of the cells from the tomatoes leaf suspension 0.3% (m/v) Brij 35 was added into the sample. At the subsequent control of the contents from the compartments 1 to 5 by cIEF with UV detection, as seen in Fig. 3, the maximum peak for *P. corrugata*, see electropherogram A, was found in the compartments 1 and 2 and for *P. syringae*, as seen in electropherogram B, in the compartments 3 and 4. The presence of the tomatoes leaf suspension in the samples from the compartments causes the higher signal noise at the test cIEF, see Fig. 3, electropherograms A and B and curve 1 vs. curve 2.

For the preconcentration and the preseparation of the low concentration of the cells from the tissue suspensions the cells, *P. corrugata* (pI ~2.4) and *C. michiganensis* (pI ~4.7), were resuspended into 3000 μL of the tomatoes leaf suspension with dissolved 2×10^{-4} mol L^{-1} PB-PEG, 15×10^{-3} mol L^{-1} NaCl and with the addition of 600 μL of the simple ampholytic electrolytes. The incubation time was 15 min at 20 °C before their separation in the McroRotofor electrolysis cell. After the termination of fractionation of the microbial samples in the electrolyzer the pictures of the electrolysis cell were taken by digital camera with the help of the Gel Doc 2000 equipped with trans UV illumination, as seen in Supplementary Fig. S1A and B.

The number of cells, *P. corrugata* and *C. michiganensis*, were 1×10^5 in 1 mL in the separation run A. The maximum intensity of the fluorescence we can see in the area of the *C. michiganensis* focusing, the compartments 3–6, but in the area of *P. corrugata* focusing, compartments 1 and 2, fluorescence is invisible to the naked eye. The "green stained" compartments 1–3 by the tomatoes leaf suspension are visible only. The sample from compartment 5

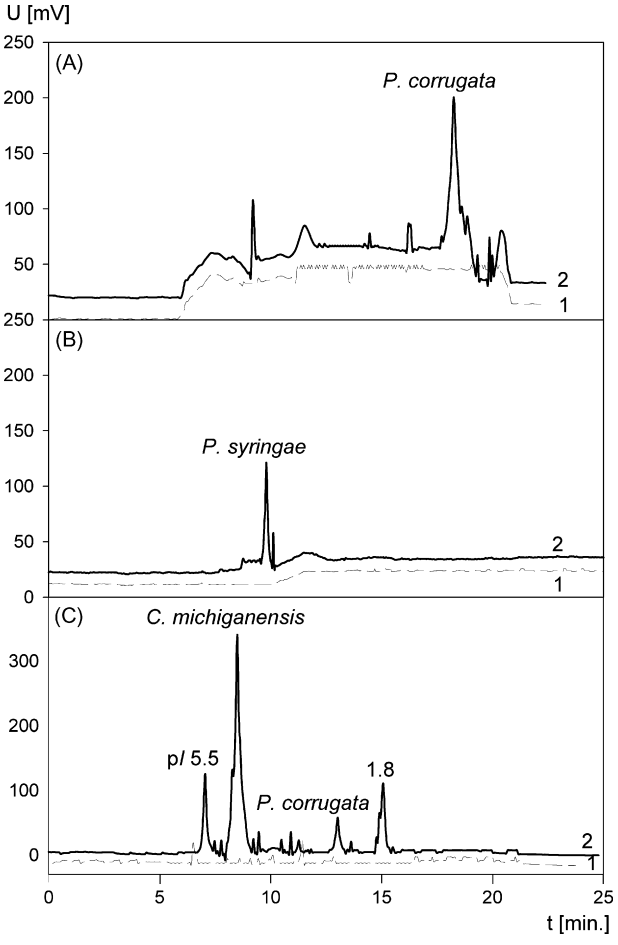

Fig. 3. Separation of MOs by cIEF with UV (A and B) or fluorometric detection (C) after fractionation in the electrolysis cell from the tomatoes leaf suspension. Conditions and designations, see Fig. 1 and Supplementary Fig. S1; curve 1, blank sample of the suspension; curve 2 suspension spiked by MOs; (A and B) PB-PEG in the focusing chambers was replaced by 0.3% (m/v) Brij; the start number of cells in the sample, *P. corrugata* and *P. syringae* each of them 2×10^7 cells in 1 mL; the samples were injected from compartment 2 (A) or from compartment 4 (B); (C) the start number of cells in the sample, *P. corrugata* and *C. michiganensis* 1×10^5 cells in 1 mL; the sample was injected from compartment 5.

was analyzed by cIEF with the fluorometric detection, as seen in Fig. 3C. After direct injection of the sample with dispersed plant tissue suspension the levels of the backgrounds are relatively low (see the electropherograms 1 vs. 2) like as in Ref. [31]. Therefore, at cIEF the pH gradient in the pH range from 1.8 to 5.5 was traced by the fluorescent pI markers. Together with *C. michiganensis* the low number of cells *P. corrugata* were detected also, but they are not detected at cIEF with UV detection.

In Supplementary Fig. S1B the number of cells of *C. michiganensis* and concentration of PB-PEG in the sample were the same and the number of cells of *P. corrugata* was increased on 1×10^6 cells in 1 mL. Now the sample in the compartments 1 and 2 was visible fluorescing and less fluorescing in the compartment 5. The results are graphically depicted in Fig. 2 as curve 2. From the cross points of the tangents of the curves 1 and 2 the approximate values of the isoelectric points for *P. corrugata* and *C. michiganensis* were possible estimate as ~2.4 and ~4.6, respectively, which can be relatively good preliminary information about pI of the preseparated MOs before their cultivation and/or subsequent identification, e.g., by cIEF.

3.3. The control of the quality of preseparation of MOs by their cultivation and by gas chromatography of fatty acid methyl esters

The presence of examined MOs, *P. corrugata* and *C. michiganensis*, which were subsequently separated by cIEF with fluorometric detection, was verified by the cultivation of the samples taken from the individual compartments 1–10 of the electrolyzer, as shown in Supplementary Fig. S1A and B, after focusing run. The standard method for the identification of MOs based on identification fatty acid methyl esters analysis by gas chromatography [31] was used after cultivation. The accuracy of the identification of the strains from the library is expressed as the probability, %. The cells of gram positive *C. michiganensis* were identified with the probability 63–84% from the compartments 3 to 6 according to the three independent experiments. The gram negative cells of *P. corrugata* and/or the cells of genus *Pseudomonas* were identified not only in the compartments 1 and 2 (maximum number of pathogens) but in all compartments. The cells of gram negative *Pseudomonas* are probably adsorbed more onto the inner surface of the plastic chamber than the gram positive *C. michiganensis*.

4. Conclusions

The isoelectric points of the plant pathogens—different origin, gram positive *C. michiganensis* subsp. *michiganensis*, and gram negative cells, *X. vesicatoria*, *P. syringae* pv. *tomato* and *P. corrugata* were determined by cIEF with UV detection. The values of isoelectric points of MOs were found to be not host specific and the estimated isoelectric points of the plant pathogens dynamically modified by PB-PEG and separated by cIEF with fluorometric detection were found to be comparable to the pI of native ones. Nevertheless, the pH gradient would be necessary more flatten in suitable pH rang for exact measurement of the isoelectric points of MOs. With respect to these results the selected pathogens from the plant tissue suspension, modified by PB-PEG, were preseparated and preconcentrated by free solution isoelectric focusing. The MOs harvested from the compartments were separated by cIEF and/or cultivated and subsequently identified by GC of fatty acid methyl esters. The utilization of the free flow isoelectric focusing for the preseparation and preconcentration of the real microbial samples and subsequently the possibility to cultivated these sample before the another microbial or analytical techniques seems to be very useful for the real microbial praxis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2008.12.024.

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