ELSEVIER

Journal of Chromatography A, 979 (2002) 277–284

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Capillary electrophoresis study of outer membrane proteins of *Pseudomonas* strains upon antibiotic treatment

Tamás Kustos[a], Ildikó Kustos[b,*], Edit Gonda[b], Béla Kocsis[b], György Szabó[a], Ferenc Kilár[c]

[a]*Department of Orthopaedics, Faculty of Medicine, University of Pécs, Szigeti út 12, H-7643 Pécs, Hungary*
[b]*Department of Medical Microbiology and Immunology, Faculty of Medicine, University of Pécs, Szigeti út 12, H-7643 Pécs, Hungary*
[c]*Central Research Laboratory, Faculty of Medicine, University of Pécs, Szigeti út 12, H-7643 Pécs, Hungary*

Abstract

Nosocomial wound infections by *Pseudomonas aeruginosa* strains have increasing importance in orthopaedic surgery. Outer membrane protein composition and cell-surface hydrophobicity of the bacteria have strong influence on adhesion to living tissues or artificial medical devices. Outer membrane proteins of five *Pseudomonas* strains (*KT 2, KT 7, KT 25, KT 28, KT 39*) isolated from orthopaedic patients' wounds and one standard strain *NIH Hungary 170000* isolated from pus were examined. The capillary electrophoretic patterns of the outer membrane proteins were characteristic to each bacterial strains possessing different relative ratios of major and minor proteins. Antibiotic treatment of bacteria with three antibiotics, cefotaximum, amoxicillinum/clavulinic acid and amikacinum (applied often in prophylaxis and treatment of patients) induced changes in the electrophoretic profiles showing that outer membrane protein composition was altered significantly. The most pronounced alterations in the electrophoretic patterns after antibiotic treatment were obtained in the cases of the strains *KT 2, KT 7* and *KT 28*. The amikacinum administration strongly decreased the relative percentage of the 38800 rel. mol. mass protein in *KT 2* (from 20 to 6%), while the relative amount of the same protein increased significantly in *KT 7* and *KT 28* after cefotaximum treatment (from 2 to 16% and from 12 to 28%, respectively). Decrease in cell-surface hydrophobicity was also observed by salt aggregation test. The results obtained can be used to determine the therapeutic concentrations of antibiotics to induce changes in the adhesion properties of bacteria.

Keywords: *Pseudomonas aeruginosa*; Antibiotics; Proteins

1. Introduction

Pseudomonas aeruginosa, a gram-negative bacterium, can be part of the normal microbial flora of the skin, throat and stool. It might be isolated more frequently from hospitalized patients, especially from the skin of burnt patients, from the gastrointestinal tract of patients receiving chemotherapy, or from the lower respiratory tract of ventilated patients [1]. The *P. aeruginosa* strains are frequently responsible for nosocomial infections, such as nosocomial pneumonias, septicaemias or surgical wound infections due to, e.g. implantation of artificial medical devices [2].

The adhesion and permanent attachment of bacterial cells to the surface of living tissues or artificial medical devices depend mainly on the cell-surface hydrophobicity and on specific interactions between microbial adhesins and surface receptors [3]. An

*Corresponding author. Tel.: +36-72-536-001-1905; fax: +36-72-536-253.
E-mail address: ildiko.kustos@aok.pte.hu (I. Kustos).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)01521-2

important group of these adhesins is the group of outer membrane proteins (OMPs), which are expressed in the cell envelope of all gram-negative bacteria. Changes in the outer membrane protein composition upon, e.g. antibiotic treatment can modify the surface properties, and hydrophobicity, thus the adhesion and pathogenicity of bacterial cells [2,4].

The electrophoretic patterns of both, the cell lysates and outer membrane preparations, are characteristic to the bacterial strains [4–6]. It has been shown that the effect of antibiotic treatment on *Escherichia coli*, *Shigella sonnei*, *Salmonella minnesota* and *Proteus penneri* bacteria can be investigated by capillary electrophoresis [4]. The pharmacodynamics of the antibiotics applied in treatment of patients is different. In orthopaedic surgery cefotaximum, the combination of amoxicillinum and clavulinic acid and amikacinum are often used. Both, cefotaximum and the combination of amoxicillinum and clavulinic acid are able to induce changes in the cell membrane, but the latter can inhibit β-lactamases, as well. The effect of amikacinum is the inhibition of protein synthesis [7].

In this work we studied the outer membrane protein patterns of *Pseudomonas aeruginosa* strains isolated from orthopedic wounds. The protein composition was studied by capillary electrophoresis and the cell-surface hydrophobicity of bacteria was determined by the salt aggregation method [8]. Effect of antibiotics on protein profile and hydrophobicity was also examined.

2. Experimental

2.1. Chemicals

Tris(hydroxymethyl)aminomethane, sodium dodecyl sulfate (SDS), sucrose, lyzozyme, EDTA and benzoic acid were purchased from Sigma (St. Louis, MO, USA). β-Mercaptoethanol and the run buffer of CE-SDS Protein Kit were obtained from Bio-Rad (Richmond, CA, USA). Pharmacia (Uppsala, Sweden) supplied the low molecular-mass calibration kit. Benzoic acid (used as internal standard in capillary electrophoresis) was obtained from Sigma.

2.2. Bacterial strains

Six *Pseudomonas aeruginosa* strains were analyzed, the Hungarian standard *NIH Hungary 170000* strain isolated from pus (as control), and five bacterial strains (*KT 2, KT 7, KT 25, KT 28, KT 39*) isolated from wounds of female patients. The strains were isolated and identified by standard bacteriological methods [9]. The *KT 2* and *KT 39* strains were isolated after acute infections from a 16-year-old patient (diagnosis: scoliosis, type of operation: postero-lateral fusion Cottrell-Dubousset) and a 74-year-old patient (diagnosis: tumour of the left proximal femur; operation: tumour resection and prosthetic replacement), respectively. The *KT 7* strain was isolated from the wound of a 38-year-old patient in chronic infection (diagnosis: spondylolysthesis lumbar V.; operation: implant removal). The *KT 25* and *KT 28* strains were isolated from a 87-year-old patient (diagnosis: hip osteoarthritis, infected total hip prosthesis; type of operation: prosthesis removal) in chronic infection.

Bacterial strains were cultivated with shaking (50 rpm) at 37 °C in 2000 ml of culture medium [1.667% (w/v) bacteriological peptone, 0.11% (w/v) Na_2HPO_4, 0.389% (w/v) glucose, 0.195% (w/v) beef extract, 0.023% (w/v) $MgCl_2$, pH 7.2] till they reached the logarithmic phase of their multiplication. Bacteria were then collected by centrifugation (5000 *g*, 15 min, 4 °C).

2.3. Antibiotic treatment of bacteria

Three antibiotics were applied: Claforan (Hoechst Marion Roussel, France) containing cefotaximum, Aktil (Richter, Hungary) containing amoxicillinum and clavulinic acid with a ratio 2:1 and Amikin (Bristol-Myers Squibb, USA) containing amikacinum. The minimum inhibitory concentrations (MICs) of the antibiotics were determined by the tube dilution method. Twofold dilution series from the antibiotic stock solutions (800 μg/ml) were prepared [10]. Then, 10 μl of the bacterial cultures (10^5 bacteria/ml) were added to each tube containing 1 ml antibiotic solution. MIC was regarded as the lowest antibiotic concentration where growth of the bacteria after 24 h incubation was still inhibited.

To examine the effect of antibiotic treatment

bacteria were cultivated in the culture medium described above. When the strains reached their logarithmic phase of multiplication, antibiotics were added at 0.5×MIC (sub-inhibitory concentration) or 2×MIC (supra inhibitory concentration). After incubation for 60 min at 37 °C the suspensions were centrifuged (5000 *g*, 15 min, 4 °C), and washed with physiological saline in order to remove the antibiotic from the culture media. Bacteria were finally resuspended in 25 ml of 0.75 *M* sucrose–10 m*M* Tris, pH 7.5.

2.4. Outer membrane protein preparation

Preparation of outer membrane proteins (OMPs) was made according to Ref. [4]. Briefly: bacterial cell pellets were suspended in 25 ml of 0.75 *M* sucrose, 10 m*M* Tris, pH 7.5, then 250 μl of lysozyme (10 mg/ml) was added and the suspension was incubated for 2 min at 4 °C. The originally rod-shaped bacteria were converted to spheroplasts by slow addition of 50 ml cold 1.5 m*M* EDTA solution, pH 7.5 (within 10 min). Spheroplasts were lysed by sonication (MSE type, 400 W, 4 °C). The sonicated material was centrifuged to remove unbroken cells. The envelope fraction was collected by ultracentrifugation at 100 000 *g* for 1 h at 4 °C. The pellets were resuspended in 3 ml of 25% (w/w) sucrose in 5 m*M* EDTA, pH 7.5, and applied to a sucrose step gradient of 30–55% (w/w) in 5 m*M* EDTA solution, pH 7.5. The gradients were centrifuged at 100 000 *g* in a SW 28 rotor of a Spinco ultracentrifuge (Beckman) for 21 h at 4 °C. After centrifugation outer membrane (OM) and cytoplasmic membrane (CM) could be seen as separate turbid bands. The OM fractions were collected and washed by deionized water (Milli-Q, Millipore, Bedford, MA, USA), and centrifuged at 100 000 *g* for 2 h at 4 °C. Pellets were resuspended in 200 μl of sample buffer [0.125 *M* Tris–HCl, pH 6.8, 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol].

2.5. Capillary electrophoresis

The ''dynamic sieving'' capillary electrophoretic technique described in Refs. [4,5] was applied for the analysis and comparative studies of bacterial proteins. The commercially available CE-SDS Protein Kit Run Buffer of Bio-Rad (Richmond, USA) was used as background electrolyte. The sieving agent, a hydrophilic polymer, creates a dynamic network ensuring the separation of proteins on the basis of their molecular masses. The Biofocus 3000 System (Bio-Rad Labs., Hercules, CA, USA) was used in the experiments. A 24 cm×50 μm I.D., uncoated, fused-silica capillary was applied. Bacterial protein samples were prepared as described above and injected hydrodynamically with 10–20 p.s.i. s (1 p.s.i.= 6894.76 Pa). Separation was performed by constant voltage at 15 kV, current limit was 50 μA. The temperature was stabilized at 20 °C. The capillary was washed thoroughly by 0.1 *M* NaOH, 0.1 *M* HCl and deionized water between each run. UV detection was performed at 220, 254 and 280 nm. Data were collected and evaluated by the Biofocus Integration Software.

2.6. Quantitative evaluation of the capillary electrophoretic profiles

Relative molecular masses of proteins were determined as described previously in Ref. [5] using an internal standard (benzoic acid). The calibration curve was constructed by plotting the $\log M_r$ values of the six standard proteins of the low molecular-mass calibration kit (Pharmacia) vs. the relative migration times of peaks. Standard deviation of the molecular mass determination was similar to that in Refs. [4,5]. The relative amounts of major proteins were calculated according to the respective peak areas.

2.7. Determination of bacterial cell-surface hydrophobicity—Salt aggregation test

Hydrophobicity of bacterial cell surface was determined by the method of Lindahl et al. [8]. One drop (10 μl) of bacterial suspension (5×10^9 bacteria/ml) in 0.04 *M* sodium phosphate buffer (pH 6.8) was mixed with 10 μl of ammonium sulphate solution. The concentration of the ammonium sulphate solution varied between 0.05 and 4.00 *M*. The mixture was gently shaken on a glass for 1 min. The lowest concentration of ammonium sulphate at which aggregation of bacteria occurred was regarded to be

characteristic for the cell-surface hydrophobicity of the respective bacterial strain. The hydrophobicity test was repeated with untreated and antibiotic treated bacteria. Both, supra inhibitory (2×MIC) and sub-inhibitory (0.5×MIC) antibiotic concentrations were applied. Higher ammonium-sulphate concentration reflects lower cell-surface hydrophobicity.

3. Results

3.1. Antibiotic treatment of bacteria

Six strains of *Pseudomonas aeruginosa* (strain *NIH Hungary 170000* isolated from pus, and strains *KT 2, KT 7, KT 25, KT 28* and *KT 39* isolated from wounds of orthopaedic patients) were studied. The minimum inhibitory concentrations (MICs) of three different antibiotics were determined as described in the Experimental section. MIC values of cefotaximum was obtained to be 50 μg/ml (*NIH Hungary 170000*, *KT 7, KT 25* and *KT 39*) or 100 μg/ml (*KT 2* and *KT 28*). In the case of amikacinum the MIC was 1.56 μg/ml (*NIH Hungary 170000*, *KT 7, KT 25*), 3.12 μg/ml (*KT 28*), 6.25 μg/ml (*KT 39*) and 12.5 μg/ml (*KT 2*). The 2:1 combination of amoxicillinum and clavulinic acid inhibited the growth of the *Pseudomonas* strains at high concentrations, MIC was found to be: 200 μg/ml (*KT 25*), 400 μg/ml (*NIH Hungary 170000*, *KT 7, KT 28*, *KT 39*), and 800 μg/ml (*KT 2*).

Cell-surface hydrophobicity data of untreated and antibiotic treated *P. aeruginosa* strains determined by the salt aggregation test are summarized in Table 1. Values presented in the table correspond to the lowest ammonium sulfate concentrations at which bacterial aggregation was visible. A decrease of cell-surface hydrophobicity of the *P. aeruginosa* strains were observed after antibiotic treatment in most cases, but supra-inhibitory concentration (2×MIC) of antibiotics affected stronger the bacteria.

3.2. Capillary electrophoresis of outer membrane proteins

Capillary electrophoresis experiments of outer membrane proteins prepared from untreated and antibiotic treated *Pseudomonas aeruginosa* strains were performed as it is described in Experimental section. Treatment of bacteria was made at supra-inhibitory concentrations (2×MIC) of the antibiotics. Fig. 1 shows characteristic electropherograms of the outer membrane proteins of three strains before and after antibiotic treatment. The relative amounts of seven characteristic major proteins with molecular masses of 22 600, 25 800, 29 100, 34 400, 37 600, 38 800 and 46 600 were determined. Relative migration times of the bacterial proteins were calculated with the help of the internal standard. The relative amounts (ratios) of the bacterial proteins were determined by integration of the peak areas (see Experimental section). Two dominating protein peaks (rel. mol. masses: 22 600 and 34 400, respectively) were found in each patterns except in the case of *KT 25*. Fig. 2 shows the relative ratios of the major outer membrane proteins of untreated and

Table 1
Concentration of $(NH_4)_2SO_4$ resulting in aggregation of bacteria

P. aeruginosa strains	Ammonium sulfate concentration (mol/l)						
	Untreated	Treatment with antibiotic[a]					
		cefotaximum		amoxicillinum/clavulinic acid (2:1)		amikacinum	
		0.5×MIC	2×MIC	0.5×MIC	2×MIC	0.5×MIC	2×MIC
NIH 170000	2.0	2.0	2.5	2.0	2.0	2.0	2.5
KT 2	1.5	1.5	2.0	2.0	2.0	2.0	2.5
KT 7	3.0	3.5	3.5	3.5	3.5	3.0	3.0
KT 25	2.5	3.0	3.0	3.5	4.0	2.5	3.0
KT 28	2.5	3.5	3.5	2.5	3.0	3.0	3.0
KT 39	1.5	2.0	2.5	2.0	2.5	2.0	2.0

[a] Treatment was performed at sub-inhibitory (0.5×MIC) and supra-inhibitory (2.0×MIC) concentrations of the respective antibiotic.

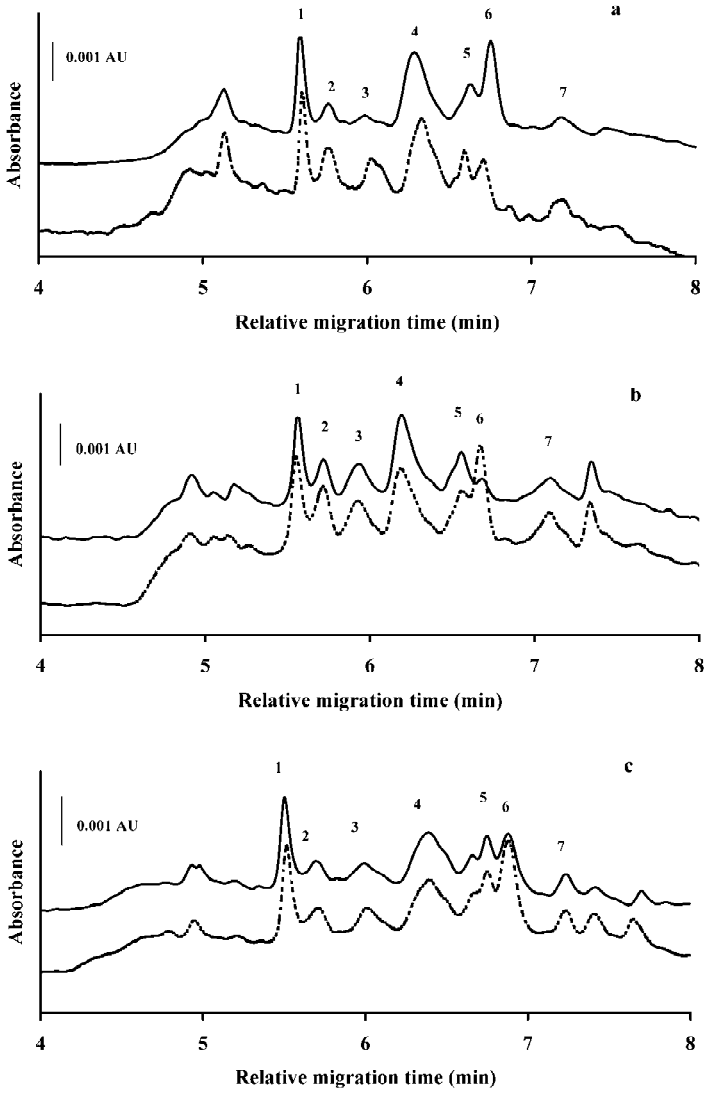

Fig. 1. Capillary electrophoresis of outer membrane proteins prepared from untreated (—) and antibiotic treated (- - -) *Pseudomonas aeruginosa* strains: (a) *KT 2*, (b) *KT 7*, (c) *KT 28*. Major protein components with relative molecular masses of 22 600, 25 600, 29 100, 34 400, 37 600, 38 800, 46 600 are marked with 1 to 7, respectively. Details of treatment (*KT 2* bacteria with 25 μg/ml amikacinum, *KT 7* bacteria with 100 μg/ml cefotaximum and *KT 28* bacteria with 200 μg/ml cefotaximum) is described in the text. Experimental conditions: buffer, run buffer of CE-SDS Protein Kit of Bio-Rad; sample injection, 10 p.s.i. s; voltage, 15 kV; current, 19 μA; detection, 220 nm; temperature, 20 °C; capillary, 24 cm×50 μm I.D., uncoated.

antibiotic treated bacteria. The relative standard deviations of the data are less than 9%. Since the capillary electrophoretic analysis of real samples a priori contains irreproducibility for a certain extent, changes in the protein relative ratios can be considered to be significant if the difference is higher than three times the standard deviation. The most pronounced alterations in the electrophoretic patterns after antibiotic treatment were obtained in the cases of the strains *KT 2, KT 7* and *KT 28*. The amikacinum administration strongly decreased the relative percentage of the 38 800 rel. mol. mass protein in *KT 2* (from 20 to 6%), while the relative amount of the same protein increased significantly in

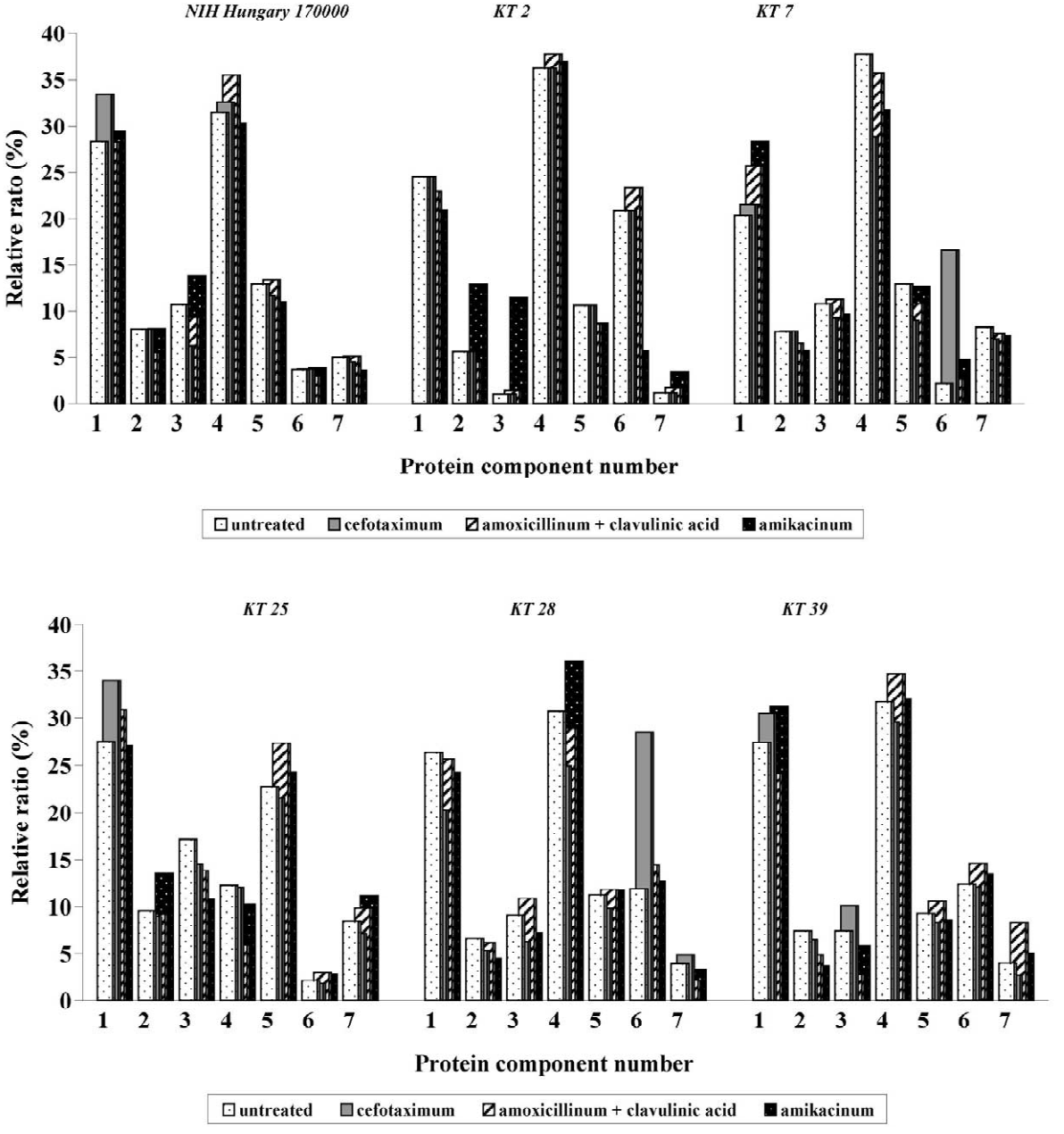

Fig. 2. Relative ratio percentages of the seven major components (respective mol. masses 22 600, 25 600, 29 100, 34 400, 37 600, 38 800 and 46 600) of the outer membranes of *Pseudomonas aeruginosa* strains (*NIH Hungary 170000; KT 2; KT 7; KT 25; KT 28; KT 39*). Proteins were prepared from untreated and antibiotic treated bacteria. Supra-inhibitory concentrations of Claforan (containing cefotaximum), Aktil (containing amoxicillinum and clavulinic acid with a ratio 2:1) and Amikin (containing amikacinum) were applied. The relative ratio values were determined from the capillary electrophoretic analyses of outer membrane proteins.

KT 7 and *KT 28* after cefotaximum treatment (from 2 to 16% and from 12 to 28%, respectively).

4. Discussion

Capillary electrophoresis is an effective, and quantitative method used increasingly in clinical diagnostics. In this study the applicability of the ''dynamic sieving'' capillary electrophoresis on the analysis of bacterial outer membrane proteins from clinical (orthopaedic) samples was proven. Capillary electrophoretic patterns of the outer membrane proteins of *Pseudomonas* strains were characteristic for the genus and differed markedly from the profiles of other bacterial genera, such as *Salmonella minnesota*, *Shigella sonnei*, *Escherichia coli*, *Proteus penneri* [4].

Outer membrane proteins of *Pseudomonas* strains play important role in several bacterial functions. They serve as receptors, induce immunological response in the host, and take part in the attachment process to living tissues. The most well-known outer membrane proteins are the porins, which construct pores in the outer membrane of bacteria. *Pseudomonas* outer membrane proteins form significantly larger pores than those of enteric bacteria and allow the diffusion of larger hydrophilic molecules (up to rel. mol. mass of 6000) [11]. However, these pores are more often in a closed state than those of *Enterobacteriaceae* [12]. Consequently, the total area of pores available for diffusion is considerably reduced in *P. aeruginosa*. This low outer membrane permeability explains the high natural resistance of *P. aeruginosa* to hydrophilic antibiotics [13–15].

Several data can be found in the literature about *Pseudomonas aeruginosa* outer membrane proteins. Hostacka and Karelova described four significant outer membrane proteins with relative molecular masses of 23 000, 35 000, 38 000 and 45 000 by SDS–polyacrylamide gel electrophoresis (PAGE) [2]. Hancock and Nikaido found four major protein bands with rel. mol. masses of 17 000, 21 000, 35 000 and 37 000 [11] in the outer membrane fraction of *P. aeruginosa PAO1* (separated in the absence of EDTA). In our capillary electrophoretic patterns of *Pseudomonas* strains seven major proteins were observed, and we also considered the proteins with molecular masses of 22 600, 34 400 and 37 600 as major components, similarly to the previous studies. The relative amounts of proteins, however, were not identical in each profile, and some major proteins in certain patterns appeared in low amounts.

Administration of antibiotics can modify the outer membrane protein composition of bacteria (see our previous results in Ref. [4]). The three antibiotics (cefotaximum, amoxicillinum/clavulinic acid (2:1), and amikacinum)—applied most often in our Department for prophylaxis and treatment of patients—affected the outer membrane composition unevenly. Significant changes, i.e. differences in the relative amounts of proteins higher than three times the standard deviation were found in several cases (Fig. 1), but smaller variations were also observed. The bacterial strains responded diversely to the different antibiotics, as can be seen in Fig. 2. Since the antibiotics may influence the protein synthesis or enzymes' functions the observed changes in the outer membrane composition upon antibiotic treatment are not surprising.

The noteworthy changes induced by cefotaximum in the membrane compositions of *KT 7* and *KT 28* strains can be in connection with the changes in hydrophobicity. Simultaneously, amikacinum altered the outer membrane profiles mostly in the *KT 2* strain, where a strong decrease in hydrophobicity was observed upon the treatment. The disproportion between the relative amounts of the proteins induced by amikacinum, which is protein-synthesis inhibitor, must be further investigated.

The minimum inhibitory concentration of the combination of amoxicillinum and clavulinic acid was very high compared to the other antibiotics. Treatment with sub-inhibitory (results not shown) or supra-inhibitory concentration did not cause considerable changes in the outer membrane composition, although some minor changes could be seen in the latter case (Fig. 2). The hydrophobicity of the bacteria, however, decreased at both concentrations (Table 1).

Higher antibiotic concentration affected stronger the hydrophobicity of the bacteria, but changes in the cell-surface hydrophobicity were more often detected after antibiotic treatment than changes in the capillary electrophoretic profiles. This might be explained

by the fact, that hydrophobic characters are regulated by more factors, e.g. fimbrial proteins, lipopolysaccharides, lipids, etc. [16,17].

The results show, that adhesion of bacteria to living tissues and arteficial medical devices can be modified by antibiotics. Since both, the outer membrane protein composition and hydrophobicity might change upon treatment, we propose to investigate the bacteria isolated from infected wounds. However, further investigations are necessary to clarify the correlation between protein composition and cell-surface hydrophobicity.

References

[1] M. Pollack, in: G.L. Mandell, J.E. Bennett, R. Dolin (Eds.), 5th edition, Principles and Practice of Infectious Diseases, Vol. 2, Churchill Livingstone, Philadelphia, PA, 2000, p. 2310.
[2] A. Hostacka, E. Karelova, Chemotherapy 43 (1997) 118.
[3] G.D. Christensen, L. Baldassarri, W.A. Simpson, Methods Enzymol. 253 (1995) 477.
[4] I. Kustos, B. Kocsis, I. Kerepesi, F. Kilár, Electrophoresis 19 (1998) 2324.
[5] I. Kustos, B. Kocsis, I. Kerepesi, F. Kilár, Electrophoresis 19 (1998) 2317.
[6] I. Kustos, V. Tóth, B. Kocsis, I. Kerepesi, L. Emõdy, F. Kilár, Electrophoresis 21 (2000) 3020.
[7] G.W. Amsden, C.H. Ballow, J.S. Bertino Jr., in: G.L. Mandell, J.E. Bennett, R. Dolin (Eds.), 5th edition, Principles and Practice of Infectious Diseases, Vol. 1, Churchill Livingstone, Philadelphia, PA, 2000, p. 253.
[8] M.A. Lindahl, A. Faris, T. Wadström, S. Hjertén, Biochim. Biophys. Acta 677 (1981) 471.
[9] D.L. Kiska, P.H. Gilligan, in: P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Tenover, R.H. Yolken (Eds.), Manual of Clinical Microbiology, 7th edition, ASM, Washington, DC, 1999, p. 517.
[10] J.H. Jorgensen, J.D. Turnidge, J.A. Washington, in: P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Tenover, R.H. Yolken (Eds.), Manual of Clinical Microbiology, 7th edition, ASM, Washington, DC, 1999, p. 1526.
[11] R.E. Hancock, H. Nikaido, J. Bacteriol. 136 (1978) 381.
[12] B. Lugtenberg, in: T.K. Korhonen, E.A. Dawes, P.H. Mäkelä (Eds.), Enterobacterial Surface Antigens—Methods for Molecular Characterisation, FEMS Symposia Ser. Vol. 25, Elsevier, Amsterdam, 1986, p. 3.
[13] R.E. Hancock, K. Poole, R. Benz, J. Bacteriol. 150 (1982) 730.
[14] A. Hostacka, E. Karelova, Pharmazie 52 (1997) 238.
[15] A. Hostacka, E. Karelova, Folia Microbiol. 42 (1997) 565.
[16] R.J. Doyle, Microbes Infect. 2 (2000) 391.
[17] J.N. Fletcher, J.R. Saunders, H. Embaye, R.M. Odedra, R.M. Batt, C.A. Hart, J. Med. Microbiol. 46 (1997) 67.