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Differentiation of polyvalent bacteriophages specific to uropathogenic *Proteus mirabilis* strains based on the host range pattern and RFLP*

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Urinary tract infections (UTIs) caused by *P. mirabilis* are difficult to cure because of the increasing antimicrobial resistance of these bacteria. Phage therapy is proposed as an alternative infection treatment. The aim of this study was to isolate and differentiate uropathogenic *P. mirabilis* strain specific polyvalent bacteriophages producing polysaccharide depolymerases (PDs). 51 specific phages were obtained. The plaques of 29 bacteriophages were surrounded by halos, which indicated that they produced PDs. The host range analysis showed that, except phages 58B and 58C, the phage host range profiles differed from each other. Phages 35 and 45 infected all *P. mirabilis* strains tested. Another 10 phages lysed more than 90% of isolates. Among these phages, 65A, 70, 66 and 66A caused a complete lysis of the bacterial lawn formed by 62% to 78% of strains. Additionally, phages 39A and 70 probably produced PDs. The phages' DNA restriction fragment length polymorphism (RFLP) analysis demonstrated that genomes of 51 isolated phages represented 34 different restriction profiles. DNA of phage 58A seemed to be resistant to selected EcoRV endonuclease. The 33 RFLP-EcoRV profiles showed a Dice similarity index of 38.8%. 22 RFLP patterns were obtained from single phage isolates. The remaining 12 restriction profiles consisted of 2 to 4 viruses. The results obtained from phage characterization based on the pattern of phage host range in combination with the RFLP method enabled effective differentiation of the studied phages and selection of PD producing polyvalent phages for further study.

Key words: *P. mirabilis*, UTI, bacteriophage, phage typing, RFLP

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INTRODUCTION

Proteus mirabilis is a Gram-negative opportunistic pathogen, causing a variety of diseases, urinary tract infections being the most common (Jacobsen & Shirtliff, 2011). These bacteria play an important role in catheter associated urinary tract infections (CAUTIs) in long term catheterized patients (more than 30 days), in humans with structural and/or physiological abnormalities in the urinary tract or after surgery (Stickler, 2014). In the infection processes, the virulence factors of *P. mirabilis* rods, especially urease, haemolysins, fimbriae and flagella are involved (Różalski *et al.*, 2007; Jacobsen and Shirtliff, 2011; Torzewska *et al.*, 2014). Furthermore, *P. mirabilis* strains are able to form biofilm, a structure resistant to commonly used antibiotics, host defense and disinfectants. Epidemiological data indicate that *P. mirabilis* accounts for 12% of complicated UTIs, being the third most common UTI cause and the second (15%) most common cause of catheter-associated bacteriuria in long term catheterized patients (Warren, 1996). *P. mirabilis* infections are recurrent and difficult to treat due to the drug resistance of these bacteria (Schneider *et al.*, 2014). Moryl *et al.* (2013) tested the sensitivity of 22 *P. mirabilis* strains, isolated from urinary catheters, to 13 drugs and observed 80% resistance to gentamicin, cotrimoxazole and amoxicillin with clavulanic acid, and 41% resistance to ciprofloxacin. Among the studied strains, 14% were resistant to all drugs used. Comparative studies of sessile and planktonic forms of *P. mirabilis* susceptibility to amikacin, ceftriaxone, norfloxacin, ciprofloxacin, gatifloxacin, cefepime revealed that minimum biofilm eliminating concentrations (MBECs) were often more than 1000-fold higher than minimum inhibitory concentrations, MICs (Moryl *et al.*, 2013).

Currently, several antimicrobial agents, as an alternative to antibiotics, are being tested. Many studies demonstrated that phages, as natural and self-amplifying antibacterial agents, could be used to effectively treat or prevent many human diseases caused by bacteria, including UTIs (Abedon *et al.*, 2011). An increasing number of studies focused on phage enzymes as potential agents in the fight against infections, the elimination of food or plant pathogens and as a diagnostic tool in pathogen detection (Drulis-Kawa *et al.*, 2012). One example are endolysins, highly efficient molecules produced by phages to digest the bacterial cell wall for virus progeny release. Yet another type of enzymes, polisaccharide depolymerases, causes degradation of bacterial polysaccharides including EPS, which is a dominant component of bacterial biofilms. Published data indicate that bacteriophages producing polysaccharide depolymerases could become part of an effective strategy to control and remove *Staphylococcus epidermidis*, *Enterobacter agglomerans* or *Pseudomonas putida* biofilms, and represent a new class of antibiofilm agents (Hughes *et al.*, 1998; Tait *et al.*, 2002; Cornelissen *et al.*, 2012; Gutiérrez *et al.*, 2012).

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Abbreviations: PD, polysaccharide depolymerase; PFU, plaque forming unit; RFLP, restriction fragment length polymorphism; UTI, urinary tract infection

From a medical or industrial point of view, it is very important to recognize different types of phages. The International Committee of Taxonomy of Viruses (ICTV) indicates about 70 features of viruses, which could help to differentiate bacteriophages. The most important characteristics, which are a virus phenotype and a nucleic acid type, allow assigning a phage to a family or identifying a new one (Ackermann, 2009). The high phage specificity is one of the main problems of phage therapy because it forces the examination of susceptibility of bacteria isolated from a patient. Therefore, bacteriophages selected for therapeutic purposes should preferably possess a wide host range. A spot test is a commonly used method for determining a bacteriophage's host range in a large bacterial collection (Kutter, 2009). This assay is also useful for bacteria typing (Dera-Tomaszewska & Tokarska-Pietrzak, 2012), as well as virus differentiation. Nowadays, very precise and sensitive tools for the differentiation of phages are molecular biology methods including RFLP. This technique differentiates phages based on a pattern of DNA fragments cut by specific endonucleases and analyzed on the agarose gel (Soykut & Tunail, 2014). The restriction profile analysis shows the degree of similarity between the viruses. RFLP is a relatively simple method with a great differentiation potential.

Therefore, the study presented here was designed to isolate uropathogenic *P. mirabilis* strain specific polyvalent bacteriophages producing polysaccharide depolymerases and differentiate them using two methods: standard – spot test, and molecular – RFLP technique.

MATERIALS AND METHODS

Bacterial strains. Fifty uropathogenic strains of *P. mirabilis* and one strain of *P. vulgaris* were used in this study (Table 1). These strains were isolated from biofilms formed on urological catheters obtained from long-term catheterized patients that were treated in two outpatient clinics in Lodz (Moryl *et al*., 2013), or from urine of patients treated in the Wards of Neurosurgery, Nephrology, Neurology Rehabilitation, The Children's Memorial Health Institute in Warsaw. Bacteria were stored at -70°C in L-Broth (BTL) supplemented with 10% DMSO.

Isolation of phages. Bacteriophages were isolated from urban wastewater from the Group Sewage Treatment Plant in Lodz and the Station of Water Supply and Sewage in Zgierz, by the modified enrichment technique (Ackermann & Nguyen, 1983). Specimens of wastewater were collected for a few days and pooled. After centrifugation at $9000 \times g$, at 4°C for 30 min., the wastewater was filtered through a paper filter (Schleicher and Carl Chull) and subsequently through a sterile filter with a pore diameter of 0.2 μm (Sartorius). Ten milliliters of purified wastewater were mixed with 10 ml of 2× concentrated tryptone soya broth – TSB (Emapol) and 1 ml of an 18 hour bacterial culture on TSB. After 3 h incubation at 37°C, with shaking at 150 rpm/min., about 1 ml of the suspension was applied on a phage nutrient agar plate (Ślopek *et al.,* 1972). For *P. mirabilis* strains which exhibited swarming growth, a medium containing 0.1% phenol was used. The plates were incubated for 24 h at 37°C. Then, individual plaques were cut out using a Pasteur pipette, transferred into 8 ml of nutrient broth, pH 7.1, containing 50 μl of 18 h culture of a suitable bacterial strain. After 24 h incubation at 37°C, the cultures were centrifuged at $4000 \times g$, at 4°C for 30 min. and the lysates were filtered through a filter with a pore diameter of 0.2 μm. The procedure was repeated 5 times to eliminate bactericidal activity of some chemical compounds present in the wastewater samples and to obtain pure phage lysate. The phage samples were stored at 4°C. Phages were annotated by giving a number and abbreviation coming from the host strain name. If more than one bacteriophage was isolated using the same host strain, after the number there is a successive letter. For example, phage 36PmC20 is the first phage isolated for the *P. mirabilis* C20 strain. Phage 36APmC20 is the second plaque isolated for this strain. In this paper in some cases we used symbols e.g. 36 or 36A. The bacteriophage filtrates' titer is expressed as PFU/ml and plaque morphology was assessed by the double-layer method (Kropinski *et al*., 2009).

Determination of the phage host range (Kutter, 2009). To determine bacterial susceptibility to a phage, a 200 μl of 18 h bacterial culture grown on nutrient broth, pH 7.1, was added to molten nutrient agar, mixed and immediately poured on the phage nutrient agar plates (Ślopek *et al.,* 1972). After drying, 10 μl of the phage suspension (10^8 PFU/ml) was put on the top agar. After 18 h incubation at 37°C, the plates were checked to examine and classify the spots. The following spot evaluation system was used: no clearing – no bacterial lysis in the spot, sp – a few single plaques in the spot, turbid spot – very weak bacterial lysis in the spot, medium turbid spot – weak bacterial lysis in the spot, almost clear spot – very weak bacterial growth in the spot, completely clear spot – complete bacterial lysis in the spot.

Phage DNA isolation. Isolation of genetic material was carried out using a modified technique by Su *et al.* (1998). For DNA isolation, the phages were initially propagated in nutrient broth pH 7.1 or LB-LS medium (1% tryptone (Difco), 0.5% yeast extract (Emapol), 0.5% NaCl, 10 mM $MgSO_4$, pH 7.0). To 1 ml of phage lysate (6.5×10^7–9.0×10^{10} PFU/ml), 2 μl of DNase I (Fermen-

Table 1. Strains of *P. mirabilis* and *P. vulgaris* used in this study (strains cultured on plates with added 0.1% phenol are listed in bold).

Bacteria	Strain number	Source
P. mirabilis	C5, **C6**, **C7**, C8, **C9**, C11, C12, C15, **C20**, C24, **C31**, **C32**, **C33**, C34, C41, C44, **C46**, **C57**, C70, **C71**, **C77**, **C84**	biofilm formed on urological catheters obtained from long-term catheterized patients treated in two outpatient clinics in Lodz (Moryl *et al*., 2013)
	MM, **332**, **484**, **512**, **677**, 687, **942**, 977, **1090**, **1220**, **1281**, **1579**, 1683, **1984**, **2337**, **2733**, **2833**, **2867**, **3059**, **3907**, **4107**, **4490**, **4955**, **5211**, **5932**, **6042**, **5628**, **8709**	urine of patients treated in the Wards of Neurosurgery, Nephrology, Neurology Rehabilitation, The Children's Memorial Health Institute in Warsaw
P. vulgaris	1595	

Table 2. Grouping of bacteriophages by RFLP used in this study.

Phage	Plaque morphology		PFU/ml	EcoRV-RFLP type
	diameter [mm]/shape/clarity	halo [mm]		
39A PmC32	3/ir/cl	5	1.60×10^{10}	I
46 PmC70	4/ir/cl	2	9.07×10^{9}	
28 PmC5	0.5/cir/cl	-	2.08×10^{9}	II
31 PmC8	0.5/cir/cl	-	1.92×10^{8}	
35 PmC15	1/cir/cl	-	6.35×10^{9}	
38 PmC31	1/cir/cl	-	6.37×10^{9}	
56 Pm942	2/cir/cl	3	8.90×10^{8}	III
65A Pm2833	1/cir/cl	-	3.30×10^{10}	IV
66 Pm2867	1/ir/cl	-	3.69×10^{10}	
70 Pm4490	3/ir/cl	3	2.45×10^{10}	V
34 PmC12	0.5/cir/cl	-	2.08×10^{10}	VI
37A PmC24	0.5/cir/cl	-	6.70×10^{9}	
45 PmC57	1/cir/cl	-	1.14×10^{9}	
72A Pm5211	4/ir/cl	3	5.00×10^{10}	VII
72B Pm5211	3/ir/cl	-	8.37×10^{9}	VIII
62 Pm1984	2/ir/cl	1	2.77×10^{10}	IX
65 Pm2833	2/cir/cl	< 1	1.71×10^{10}	X
75 Pm1090	1/cir/cl	< 1	2.62×10^{10}	
61 Pm1683	0.5/cir/cl	-	7.00×10^{9}	XI
65B Pm2833	1/cir/tu	-	9.40×10^{8}	
71A Pm4955	2/ir/cl	2	7.37×10^{9}	XII
81A Pv1595	2/ir/cl	< 1	1.50×10^{10}	XIII
81B Pv1595	2/ir/cl	< 1	1.55×10^{10}	XIV
66A Pm2867	3/ir/cl	-	9.00×10^{10}	XV
70A Pm4490	2/ir/cl	< 1	6.50×10^{7}	
36D PmC20	2/cir/cl	< 1	5.80×10^{9}	XVI
49 PmC84	1/cir/cl	-	1.12×10^{9}	XVII
36 PmC20	4/cir/cl	< 1	1.60×10^{10}	XVIII
36A PmC20	4/cir/cl	< 1	1.35×10^{10}	
36C PmC20	2/cir/cl	< 1	1.22×10^{10}	
29 PmC6	3/cir/cl	< 1	6.17×10^{9}	XIX
30 PmC7	3/cir/cl	< 1	8.05×10^{9}	
36B PmC20	5/cir/cl	1	1.85×10^{10}	XX
44 PmC46	0.5/cir/cl	-	1.93×10^{10}	XXI
49A PmC84	8/cir/cl	2	7.50×10^{10}	XXII
32 PmC9	1/cir/cl	-	1.52×10^{9}	XXIII
81 Pv1595	4/ir/cl	2	5.27×10^{9}	XXIV
58B Pm1220	6/cir/cl	-	3.47×10^{9}	XXV
58C Pm1220	5/cir/cl	-	4.30×10^{9}	
58 Pm1220	3/cir/cl	-	2.72×10^{8}	XXVI
68 Pm3907	3/cir/cl	-	3.43×10^{9}	XXVII
71 Pm4955	5/ir/cl	-	5.00×10^{8}	
72 Pm5211	5/cir/cl	1	1.92×10^{9}	
68B Pm3907	4/ir/tu	< 1	9.08×10^{9}	XXVIII
40A PmC33	5/cir/cl	< 1	1.95×10^{10}	XXIX
54A Pm677	3/cir/cl	-	3.02×10^{9}	XXX
56A Pm942	4/cir/cl	< 1	1.30×10^{9}	
52 Pm484	2/cir/cl	< 1	2.15×10^{8}	XXXI
53A Pm512	0.5/cir/cl	1	1.50×10^{9}	XXXII
64 Pm2733	2/cir/cl	< 1	6.87×10^{9}	XXXIII
58A Pm1220	6/cir/cl	1	7.52×10^{9}	XXXIV

ir, irregular shape; cir, circular shape; cl, clear plaque; tu, turbid plaque; PFU/ml, plaque forming unit per millilitre; EcoRV-RFLP type, the studied phages displayed 34 unique restriction profiles obtained after their DNA digestion with EcoRV. The profiles were numbered using Roman numerals from I to XXXIV.

tas) was added and incubated at 37°C for 30 min. Next, 2M $ZnCl_2$ (1:50 v/v) was added and incubated at 37°C for 5 min., then centrifuged at 18400×*g*, at 22°C for 2 min. The supernatant was decanted, the pellet dried and resuspended in 700 µl of TENS buffer (0.05 M Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0, 0.1 M NaCl, 0.3% SDS). 2 µl of Proteinase K (Sigma) was added and the sample was incubated at 65°C for 10 min. An equal volume of phenol: chloroform: isoamyl alcohol (BioShop) was added to the suspension, shaken and centrifuged at 18400× g, at 22°C for 10 min. The aqueous phase was transferred to a new tube, and phenol: chloroform: isoamyl alcohol was added, mixed and centrifuged. Again, the aqueous phase was transferred to another tube and 500 µl of isopropanol, and 20 µl of 5M potassium acetate was added, vortexed and incubated at 22°C for 15 min, then centrifuged as above. The supernatant was decanted, the precipitate was dried and washed with 70% ethanol, and centrifuged like before. The supernatant was decanted, the precipitate was dried at 55°C for 10 min., and then suspended in 100 µl of sterile Nuclease Free Water (Promega). 5 µl of RNase I (Fermentas) was added to DNA solution and incubated at 37°C for 30 min. The isolated DNA samples were stored at –20°C. DNA was separated by electrophoresis in a 1% agarose gel, and the bands were visualized under UV light after ethidium bromide staining.

Dendrogram preparation based on endonuclease DNA digestion. Endonuclease for the digestion of the studied bacteriophages DNA was chosen from enzymes which differed in the site of DNA cutting: EcoRV, BamHI, HindIII, XbaI, purchased from Fermentas. 20 µl of the reaction mixture consisted of approx. 1 µg isolated DNA, 1 µl of the restriction enzyme, 2 µl of the Green Buffer (FastDigest) and nuclease-free water. After the enzymatic digestion, the DNA fragments were separated by electrophoresis in a 1.2% agarose gel in the TAE buffer (40 mM Tris, 50 mM EDTA pH 8.0, 20 mM acetic acid), in a Wide Mini-Sub® Cell GT (BioRad) at 5V/cm. GeneRuler™ 1kb DNA Ladder (Fermentas) was used as a size marker. The bands were visualized under UV light after ethidium bromide staining. A band-based dendrogram was generated with BioNumerics software (Applied Maths, Belgium), using the Dice similarity coefficient and clustering by an unweighted pair group method with an arithmetic mean (UPGMA), with 1% tolerance in band position differences. A cluster was defined as all isolates sharing the same pattern. For the discriminatory power of the RFLP method evaluation, the Hunter-Gaston discriminatory index (HGDI) was calculated as described (Hunter & Gaston, 1988).

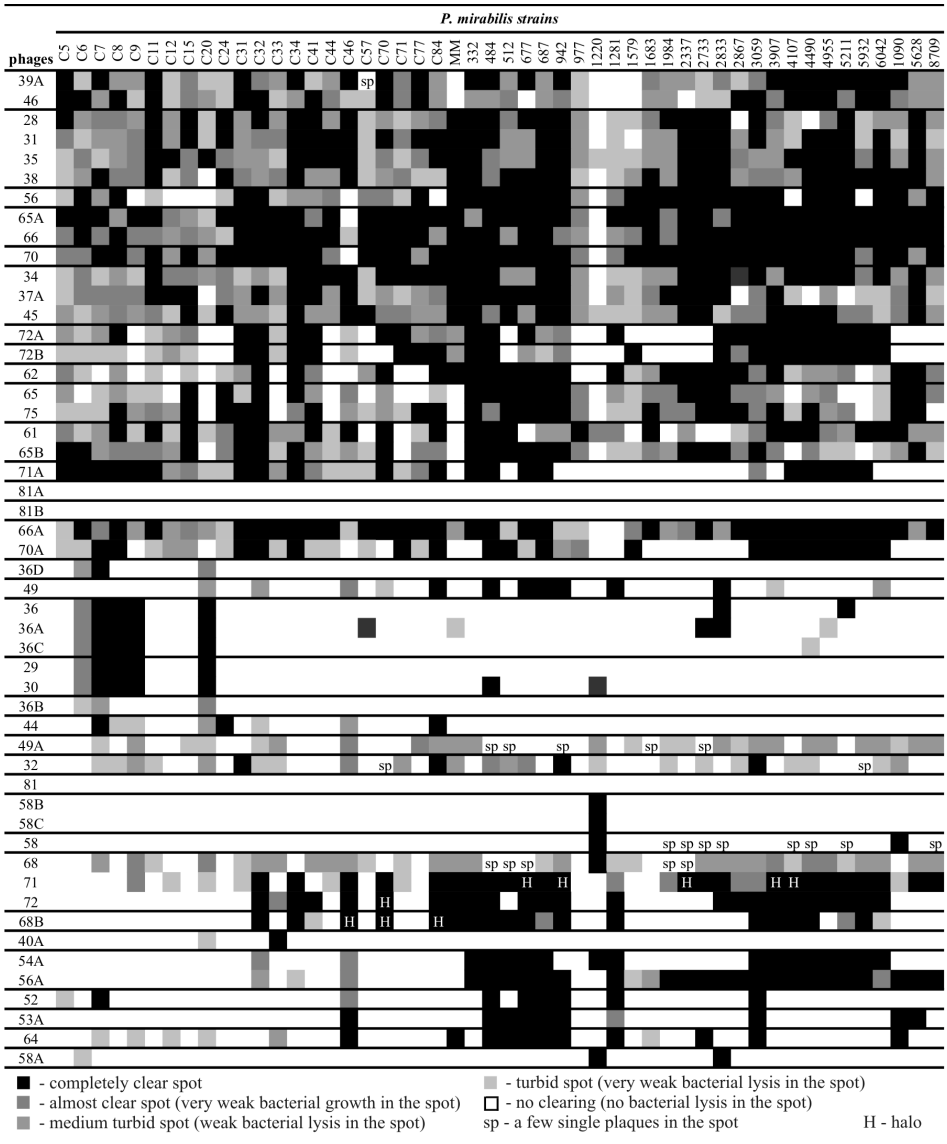
RESULTS

Phage isolation and host range determination

As a result of the isolation process, 48 phage isolates specific to 31 *P. mirabilis* strains and 3 isolates for one *P. vulgaris* were obtained (Table 2). At least one phage isolate was obtained for 32 of all tested strains, while for 11 strains two to five phages were isolated. Phage plaque parameters such as size, turbid or clear appearance, presence of a halo around plaques were assessed for all isolates. Some of them had clearly different, while others identical, plaque morphology suggesting that these could be the same phages. The diameter of plaques formed by the isolated phages ranged from 0.5 mm to 8 mm, however, 69% of them were above 1 mm in size. All except two phages, 65B and 68B, which produced turbid plaques, the remaining phages formed clear plaques. The plaques of 29 bacteriophages were surrounded by a hazy zone — a halo, which suggested production of polysaccharide depolymerases by the viruses. As shown in Table 2, RFLP analysis performed for each isolate allowed to group phages by assigning them into one of the unique RFLP patterns observed. In a few cases, the plaque morphology of each virus belonging to the same restriction profile was different. For example, profile XI consisted of two phages which produced markedly different plaques: phage 61 – clear plaque, 0.5 mm in diameter, and phage 65B – 1 mm turbid plaque. *P. mirabilis* phage titers ranged from 6.50×10^7 PFU/ml for phage 70A, to 7.50×10^{10} PFU/ml for phage 49A. A few phages with the same restriction profile propagated in different bacterial hosts had various titers, for comparison phage 31 – 1.92×10^8 PFU/ml and phages 28, 35, 38 – from 2.08 to 6.37×10^9 PFU/ml or phage 66A – 9.00×10^{10} PFU/ml and phage 70A – 6.50×10^7 PFU/ml.

In the next step, the host range was examined using 50 *P. mirabilis* strains (Table 3). The phages listed in Table 3 were arranged on the basis of RFLP similarities. All bacteriophages (except for 58B and 58C) that presented the same restriction profiles had similar but not identical host range patterns. Lytic activity of phages differed in the number of lysed strains and the bacterial lawn lysis degree. For phages 36B and 36D, contrary to others, the lytic activity differed slightly in the bacterial lawn lysis degree, but not in host range profiles. Among 51 exam-

Table 3. The host range of *Proteus* bacteriophages determined by spot test.



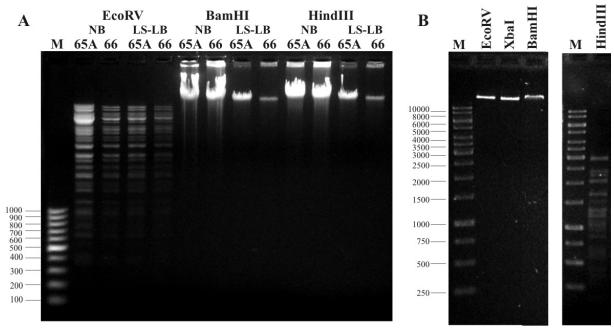


Figure 1. (A) Electrophoresis in 1% agarose gel of EcoRV, BamHI and HindIII digested DNA of phages 65APm2833 and 66Pm2867. (B) Electrophoresis in 1.2% agarose gel of 58A Pm1220 bacteriophage DNA digested with different restriction enzymes.
NB — digested DNA, isolated from phage propagated in nutrient broth, pH 7.1, LS-LB — digested DNA, isolated from phages propagated in the LS-LB medium; numbers 65A and 66 are the symbols of 65APm2833 and 66Pm2867 phages, respectively. M — marker; the sizes of the molecular size markers are shown in bp on the left side of the figure.

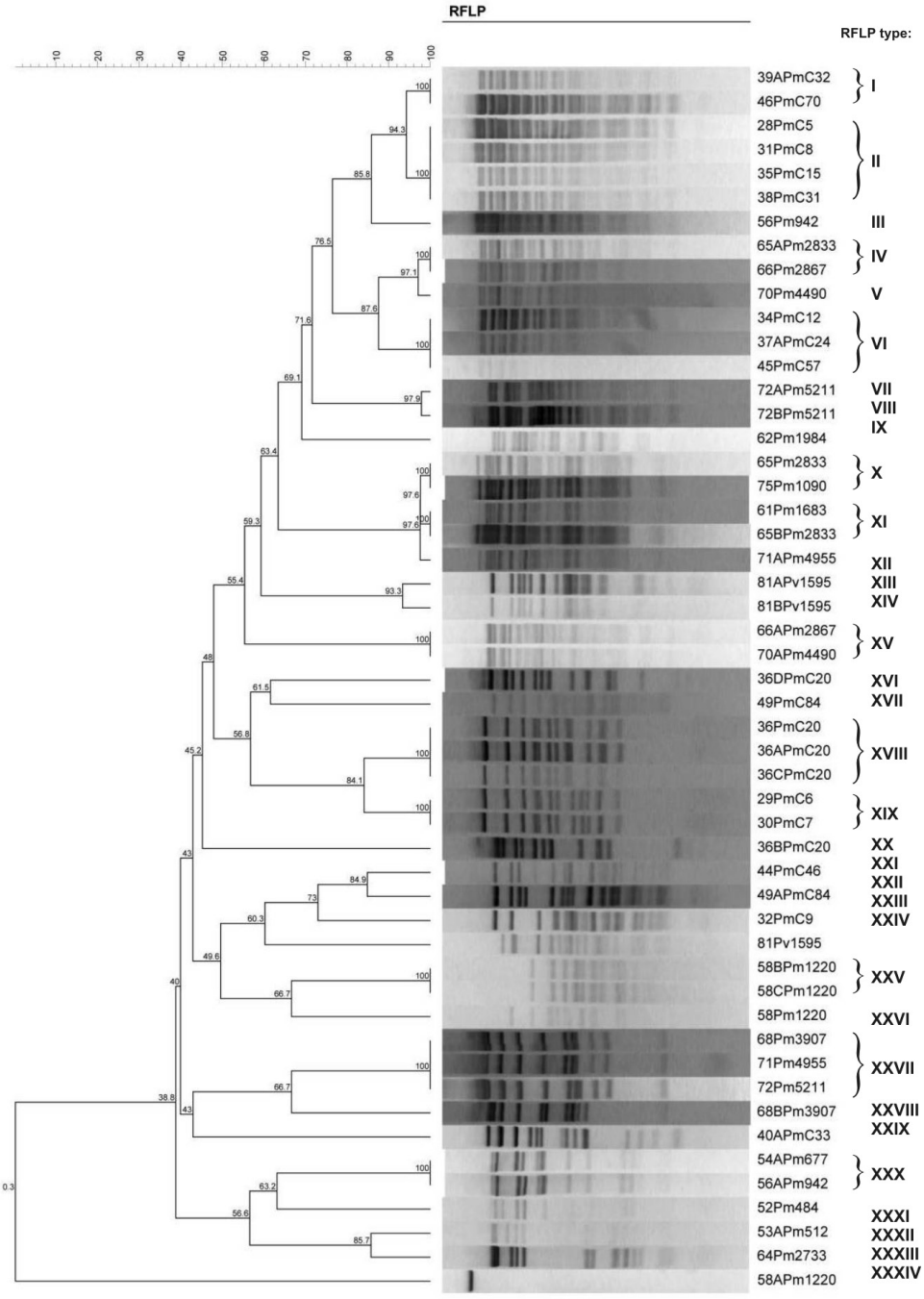


Figure 2. EcoRV-RFLP based dendrogram showing relationships among *Proteus* phages.
The EcoRV-RFLP analysis classified 51 phages into 34 unique EcoRV-RFLP types numbered from I to XXXIV. The tree diagram presents the Dice similarity index (%).

ined phages, 12 lysed to a different degree above 45 tested strains of *P. mirabilis*, while phages 35 and 45 lysed all of them (Table 3). 19 phages caused complete lysis of 20 or more *P. mirabilis* strains. Four of them led to complete lysis of 30 or more strains: 65A (39 strains, 78%), 70 (37 strains, 74%), 66 (33 strains, 66%) and 66A (31 strains, 62%). Moreover, for phages 68B, 71 and 72, a halo zone around the spot on the lawn of certain bacterial strains was observed (Table 3). It suggests that these bacteria produce exopolysaccharides which are substrates for the polysaccharide depolymerases produced by these phages. Another thirteen phages had a very narrow host range, they lysed between 1 and 9 strains. All 3 bacteriophages specific to *P. vulgaris* (81, 81A, 81B) failed to lyse *P. mirabilis* isolates.

Phage DNA restriction fragment length polymorphism

Differentiation of the studied phages was also carried out by determining the restriction profile of phage DNA. For the preliminary evaluation of experimental conditions, two phages: 65APm2833 and 66 Pm2867 were used. These two viruses were randomly selected from the group of viruses representing a broad range of hosts among *P. mirabilis* strains. Bacteria were cultivated in two media: LB-LS recommended by Su *et al.* (1998), and in nutrient broth, pH 7.1, used for phages amplification in our laboratory. Genomic DNA of bacteriophages 65A and 66 was digested by restriction enzymes: EcoRV, BamHI and HindIII, and separated in an agarose gel in the presence of a mass marker (Fig. 1A). Restriction profiles were obtained only when endonuclease EcoRV was used. The type of the medium used did not affect the results of the experiment. As a result, for the RFLP analysis, phages were propagated on nutrient broth, pH 7.1, and phage DNAs were digested with EcoRV. The obtained RFLP profiles were used for the construction of a dendrogram showing the relationships between the phages (Fig. 2). 33 EcoRV-RFLP profiles consisting of about 9 to over 20 bands, and one consisting of 1 band (phage 58APm1220 insensitive to EcoRV), were obtained for the 51 studied bacteriophages. EcoRV-RFLP displayed high differentiating power of *Proteus* specific bacteriophage genomes reaching a discriminatory index (HGDI) of 0.982. EcoRV resistant phage 58A DNA was treated with other endonucleases: BamHI, XbaI and HindIII and showed to be sensitive only to the HindIII enzyme (Fig. 1B). The obtained EcoRV-RFLP profiles were numbered using Roman numerals from I to XXXIV (Fig. 2). Twenty two subtypes were unique and displayed by singular phages. The most common presented cleavage pattern was number II observed in the case of four viruses. Restriction profiles XI, XVIII and XXVII were displayed by three phages. Eight profiles consisted of two phages.

Phages within the profiles containing more than one representative, except for variants representing subtypes XVIII and XXV, were isolated for different bacterial strains. Comparison of DNA cleavage profiles allowed determining the relationships between the studied phages. The 33 RFLP-EcoRV profiles showed a Dice similarity index of 38.8%. The phage variants I and II, IV and V had a 94.3% and 97.1% relationship, respectively. These phages had cleavage profiles comparable to viruses from III and VI restriction types, showing numerous bands and 76.5% similarity. Out of the 13 phages belonging to these subtypes, 9 viruses were isolated for *P. mirabilis* strains obtained from urological catheters and another four were specific to *P. mirabilis* strains isolated from urine. Next three subtypes X, XI and XII, which consisted of phages isolated for *P. mirabilis* strains coming from urine, had a Dice similarity index of 97.6%. Also, phages 72A and 72B showed a high (97.9%) resemblance, and these phages were obtained for one strain. Phages presenting subtypes XXVIII and XXIX, XXXII and XXXIII, XXI and XXII having slightly different band patterns, displayed 84.1%, 85.7% and 84.9% similarity, respectively. Phages, between which the relationship degree was below 80%, had very different restriction profiles. Interestingly, *P. vulgaris* specific phages 81A and 81B, which did not lyse *P. mirabilis* strains, presented 63.4% of RFLP similarities to *P. mirabilis* specific phage types from I to XII.

RFLP analysis allowed classification of the studied 51 phages into 34 unique EcoRV-RFLP types, 6 of which consist of bacteriophages presenting wide host range. Phages classified into 24 restriction profiles might have produced polysaccharide depolymerases, as evidenced by the presence of a halo around their plaques.

DISSCUSION

Antibiotic treatment of UTIs caused by *P. mirabilis* is often ineffective due to the antibiotic resistance of these bacteria and formation of antimicrobial agent resistant biofilm on urological catheters (Jacobsen & Shirtliff, 2011; Moryl *et al.*, 2013). One alternative to antibiotic treatment, especially useful for combating biofilms on biomaterials or medical devices, is application of bacteriophages (Donlan, 2009, Hughes *et al.*, 1998; Tait *et al.*, 2002; Comeau *et al.*, 2007). Because of high phage specificity, the host range of bacteriophages has a great impact on the success in controlling drug-resistant bacteria (Vandamme, 2014; Sillankorva *et al.*, 2010; Zhang *et al.*, 2013). Some of the bacteriophages isolated in our study demonstrated a wide spectrum of specificity against *P. mirabilis* strains. Thus, two phages, namely 39A and 45, infected all *P. mirabilis* strains used, and another 10 phages infected more than 45 strains. Furthermore, 4 of these phages were highly lytic, because they caused complete bacterial lawn lysis in the case of 61% to 76% of *P. mirabilis* strains. Relationships between bacteriophages isolated in our study were determined by the RFLP method (Sekaninova *et al.*, 1994). RFLP analysis confirmed isolation of at least 6 different highly lytic phages with wide spectrum of specificity. Two out of all phages produced plaques surrounded by a halo, suggesting high activity of depolymerases that make them interesting candidates for UTIs treatment, including associated formation of infection biofilm. The rate of bacteriophages representing favorable phenotyping features among all isolates is similar to that observed by Holmfeldt *et al.* (2007) that isolated two phages from among 46 viruses, which infected all except one *Cellulophaga baltica* strains. The EcoRV enzyme was selected experimentally, however, DNA of one phage (58APm1220) proved to be EcoRV, BamHI and XbaI resistant, but sensitive to the Hind III enzyme. The observed insensitivity of this bacteriophage DNA to the used endonucleases may have resulted from the absence of specific cleavage sites or the presence of DNA modifications in the virus genome (Labrie *et al.*, 2010). DNA of 50 other phages was sensitive to the EcoRV enzyme and RFLP analysis revealed 33 different patterns, showing significant diversity (a Dice similarity index of 38.8%, HGDI= 0.982) among tested phages. Our results are comparable to those for 32 Shiga-toxin encoding phages isolated from VTEC

O103:H2. The presented 21 RFLP patterns also demonstrated a low Dice similarity index of 44.92% (Karama & Gyles, 2008). The 12 restriction profiles obtained in our work were represented by 2 to 4 phages, other cleavage patterns consisted of single phages. This indicates that the test phage collection consists of at least 34 different viruses. The phages classified into the same restriction type, with the exception of the phages of subtype XXV, had very similar but not identical lytic patterns. For example, phages 61 and 65B having the same restriction profile or X, XI and XII subtypes of phages showing a very high Dice similarity index (97.6%), formed very different plaques. Additionally, the differences were also observed in plaque morphology and phage lysate titer. This may be due to the special host-induced modifications. A similar phenomenon was previously observed in *Salmonella* Enteritidis specific bacteriophages, where lytic efficiency was dependent on the propagation of the host strains. Phages amplified in different *Salmonella* hosts also showed slightly different lytic patterns (Sillankorva *et al.* 2010).

Some of the restriction profiles differed in the presence of one or two bands, which indicates a very close family relationship of these phages, for example phages from cleavage patterns XVIII and XIX. Kunisaki & Tanji (2010) found that small differences within a genome of bacteriophages PP17 and SP22 specific to *E. coli* resulted from an exchange of short DNA fragments between phages, when they coexisted in a host cell (a case of superinfection). The studied *Proteus* specific bacteriophages existed in the same wastewater environment, which may have led to a number of insertions and deletions within their genomes when viruses infected the same host cell. As a result of such coexistence, small differences within the tested phage genomes' cleavage patterns could be observed. On the other hand, the broad-host-range *Salmonella* phage phi PVP-SE1 displayed the same restriction patterns after amplification in its natural host and *E. coli* Bl21 nonpathogenic strain (Santos *et al.*, 2010). It suggests that the examined *P. mirabilis* viruses from XVIII and XIX RFLP-profiles, amplified in the same bacterial strain, could have identical restriction patterns. It is intriguing to note that *P. vulgaris* specific phages 81A and 81B, showing a relatively close relationship (63.4%) to polyvalent phages infecting *P. mirabilis* strains, did not lyse any of the tested strains. They probably do not possess genes encoding ligands recognizing specific cell-surface receptors on *P. mirabilis* strains, which results from the high specificity of phages (Labrie *et al.*, 2010).

The RFLP method was sometimes used to estimate the size of bacteriophage genomes (Karama & Gyles, 2008; Sillankorva *et al.*, 2010; Soykut & Tunail, 2014). This approach, however, has several technical limitations because bands identified under intensive UV light illumination might be composed of a few DNA fragments slightly different in size. However, it is worth to note that slightly different lytic patterns of *P. mirabilis* specific bacteriophages representing restriction profiles I to XV, which had about 20 different bands, seem to have bigger genomes in comparison to other viruses examined in this study.

RFLP proved to be a suitable method for quick and proper assessment of the genetic variety among newly isolated bacteriophages infecting different *P. mirabilis* strains. The obtained genomic fingerprints from different phages infecting *P. mirabilis* strains allowed diversifying the collection, determining the relationship and the number of the studied phages, whereas phage lytic profiles allowed determining the lytic activity of the studied bacteriophages. These two methods allowed selecting phages — classified as restriction types I, II, IV, V, VI and XV — characterized by a wide host range and production of polysaccharide depolymerases. These phages are interesting candidates for combating infections caused by *P. mirabilis*, in particular those associated with biofilm formation. They will be subjected to further research to assess their suitability for phage therapy.

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