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Effect of nutrient and stress factors on polysaccharides synthesis in *Proteus mirabilis* biofilm*

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The extracellular matrix in biofilm consists of water, proteins, polysaccharides, nucleic acids and phospholipids. Synthesis of these components is influenced by many factors, e.g. environment conditions or carbon source. The aim of the study was to analyse polysaccharides levels in *Proteus mirabilis* biofilms after exposure to stress and nutritional conditions. Biofilms of 22 *P. mirabilis* strains were cultivated for 24, 48, 72 hours, 1 and 2 weeks in tryptone soya broth or in modified media containing an additional amount of nutrients (glucose, albumin) or stress factors (cefotaxime, pH 4, nutrient depletion). Proteins and total polysaccharides levels were studied by Lowry and the phenol-sulphuric acid methods, respectively. Glycoproteins levels were calculated by ELLA with the use of selected lectins (WGA and HPA). For CLSM analysis dual fluorescent staining was applied with SYTO 13 and WGA-TRITC. In optimal conditions the levels of polysaccharides were from 0 to 442 μg/mg of protein and differed depending on the strains and cultivation time. The agents used in this study had a significant impact on the polysaccharides synthesis in the *P. mirabilis* biofilm. Among all studied components (depending on tested methods), glucose and cefotaxime stimulated the greatest production of polysaccharides by *P. mirabilis* strains (more than a twofold increase). For most tested strains the highest amounts of sugars were detected after one week of incubation. CLSM analysis confirmed the overproduction of N-acetyloglucosamine in biofilms after cultivation in nutrient and stress conditions, with the level 111–1134%, which varied depending on the *P. mirabilis* strain and the test factor.

Key words: *Proteus mirabilis*, exopolysaccharides, biofilm, nutrient, stress factors

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INTRODUCTION

Proteus mirabilis is an opportunistic pathogen, which can be found in natural environments, e.g. soil, water, and sewage, as well as in animal intestinal tracts. These bacteria cause many different types of diseases — most frequently urinary tract infections (UTIs), especially in patients with urinary catheters (CAUTIs) (Jacobsen *et al.*, 2008). They form biofilm on a catheter surface, detected in 65–85% of CAUTIs cases (Muzzi-Bjornson & Macera 2011). *P. mirabilis* synthesize a lot of virulence factors, e.g. urease. The activity of this enzyme leads to biofilm encrustation with apatite and struvite crystals, which could cause blockade of the catheter lumen and make infection persistent and difficult to treat (Stickler & Feneley 2011). Encrusted biofilms are more resistant to antimicrobial agents, host defenses and environmental stress conditions.

The extracellular matrix (ECM) is a very important biofilm component. It consists of water (97%) and exopolymers, which are a mixture of: polysaccharides (EPS), proteins, nucleic acids, glicoproteins and phospholipids. The average thickness of a biopolymer layer is 0.2–1.0 μm (Czaczyk & Myszka 2007). Carbohydrates found in the matrix are homo- or heteropolysaccharides composed of 2–8 types of sugars. The majority of microorganisms synthesize neutral sugars (e.g hexoses), possessing an anionic charge (uronic acids, pyruvic acids) or containing phosphate or sulphate groups. The molecular weight of the sugars usually ranges between 0.5–2 × 10^6 kDa (Sutherland 2001, Czaczyk & Myszka 2007). Electrostatic and hydrogen bonds are often involved in polysaccharides linking to bacterial cells, another posibility is binding by lipids and glycerol.

Bacterial EPS play an important role in biofilm development, e.g. they participate in the adhesion process, where they intensify the cells binding to solid surfaces. EPS are also neccessary in biofilm matriculation, they increase the distance between cells, which stabilises the biofilm structure. EPS participate also in transport of the nutrients to the bacterial cells (Sutherland 2001, Nwodo *et al.,* 2012). EPS functions include also regulation of biofilm dispersal processes and contribution to bacterial communication (quorum sensing). However, the most important is their protective role. They constitute a mechanical barrier which protects bacterial cells against the harmful environmental influence of e.g antimicrobials, UV radiation or macroorganism immune system agents (phagocytosis, opsonization). EPS compounds can also react with antimicrobial agents, bind them and prevent their atachment to receptors on bacterial cells. EPS is also a virulence factor — strains which produce EPS are

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Abbreviations: ABTS 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), CAUTI catheter associated urinary tract infection, CLSM confocal laser scanning microscope, CTX cefotaxime, DMSO dimethyl sulfoxide, ECM extracellular matrix, ELLA enzyme linked lectinosorbent assay, EPS extracellular polysaccharides, GalNAc N-acetylgalactosamine, GlcNAc N-acetylglucosamine, HPA *Helix pomatia* agglutinin, PBS phosphate buffered saline, TRITC tetramethylrhodamine-5-(and-6)-isothiocyanate, TSB tryptone soya broth, UTI urinary tract infection, WGA wheat germ agglutinin

more pathogenic than EPS non producing strains (Starkey *et al.,* 2004).

The composition and quantity of exopolymers depend on microorganisms species, the stage of biofilm matriculation, as well as environmental conditions such as carbon source and balance between the following elements: nitrogen, potassium and phosphorus. The temperature and pH also influence the EPS synthesis and structure (Vu *et al.,* 2009).

The knowledge of the EPS structure could be extended by developing new biofilm eradication methods, based on matrix destruction. Therefore, it is extremely important to characterize the *P. mirabilis* EPS. Chemical structures of polysaccharides in the *P. mirabilis* matrix have not been discovered, but generally these compounds could be similar to O-specific chains of the lipopolysaccharide (Różalski *et al.*, 2007). The purpose of our study was the analysis of differences in the levels of polysaccharides in *P. mirabilis* biofilms after exposure to stress and nutritional conditions. The first step was the determination of the polysaccharides and proteins levels in *P. mirabilis* biofilm in optimal medium (TSB). The second stage involved a study of the effect of nutrient deficiencies, acidic pH, and the presence of an antibiotic (cefotaxime), albumin or an additional amount of glucose in culture medium on polysaccharides synthesis.

MATERIAL AND METHODS

Bacterial strains. 22 *P. mirabilis* strains were obtained from biofilms on urinary catheters collected from long-term catheterized patients. Catheterization lasted from a few months to a few years, however, each catheter was replaced every two weeks. The strains were stored at -80°C in tryptone soya broth (TSB, BTL Corp.) and in 10% dimethyl sulfoxide (DMSO), and cultivated on tryptone soya agar (TSA, BTL Corp.). Bacteria were incubated for 24 h at 37°C. For experiments bacteria were grown in TSB broth for 24 h, 37°C.

Biofilm cultivation. *P. mirabilis* biofilms were cultivated in flat-bottomed polystyrene 96-well plates in TSB or media modified with the addition of glucose (500 mM), albumin (1.54 mg/ml), cefotaxime — CTX (a subinhibitory concentration — 32 μg/ml), TSB tenfold diluted with distilled water, TSB pH 4. 100 μl volumes of particular media, inoculated with *P. mirabilis* (10^7 cells per ml) were transferred into polystyrene plates. The plates were incubated for 24 h, 48 h, 72 h, one or two weeks at 37°C (fresh media were supplied every 72 h). After cultivation biofilms were washed twice with distilled water to remove unbound bacterial cells. Next, levels of polysaccharides and proteins were determined.

Proteins and polysaccharides determination. Concentrations of obtained proteins were measured using the method described by Lowry *et al.* (1951) with bovine serum albumin as a standard. The amount of total carbohydrate was determined using the phenol-sulphuric acid method described by Dubois *et al.* (1956) with glucose as a standard.

An enzyme-linked lectinosorbent assay (ELLA) was used to determine the glycoproteins levels in *P. mirabilis* biofilms. Wheat germ agglutinin (WGA) was applied to detect β(1→4)-N-acetyl-D-glucosamine residues and *Helix pomatia* agglutinin (HPA) to detect α-N-acetylgalactosamine residues were applied. 100 μl of peroxidase conjugated lectins at the concentration of 1.25 μg/ml in PBS were overlaid on plates. After 1 h incubation, biofilms were five times rinsed with PBS with 0.05% Tween 20. Next, ABTS 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (Sigma) was added for 15 min. and absorbance was measured at 595 nm, on microtiter plate reader (Multiscan EX, Labsystems).

CLSM analysis. *P. mirabilis* biofilms were cultivated in transmission flow cells (BioSurface Technologies) for 48–72 h with a flow 0.5 ml/min of medium or on glass slides. After incubation, biofilms were washed with distilled water to remove planktonic bacteria. Nucleic acids were fluorescently stained with SYTO 13 (Molecular Probes) at a concentration of 50 μM. Biofilms were incubated for 15 min., next the dye was washed and extracellular polysaccharides in the matrix were stained with WGA-TRITC lectin — 50 μg/ml (Sigma). After 30 min. incubation, stained biofilms were rinsed to remove unbound lectins. The imaging was performed using a Pascal (Zeiss) confocal laser scanning microscope equipped with a 40× (0.75 numerical aperture) objective lens. The excitation wavelength was 488 nm and emission was 514 nm for SYTO 13, while for WGA-TRITC they were 543 nm and 620 nm, respectively. The images analysis was performed using the AxioVision software, which allowed the quantification of EPS and bacterial cells.

Statistical analysis. Experiments were repeated at least three times. Data are presented as the mean ± standard deviation. All statistical analyses were performed with the Statistica 10 PL software. A Mann-Whitney U test was used to compare the means (p value ≤ 0.05 was taken as statistically significant and $p \leq 0.01$ as highly statistically significant).

RESULTS

The amounts of polysaccharides obtained in optimal conditions.

P. mirabilis biofilms were cultivated in TSB medium for 24 h, 48 h, 72 h, one or two weeks. The phenol-sulphuric acid method and the Lowry method were used to examine the amounts of polysaccharides per 1 mg of protein. The polysaccharides amounts obtained in optimal conditions ranged between 0 — and almost 442 μg/mg of protein and differed depending on the strains and cultivation times (Table 1). The highest levels were measured for *P. mirabilis* strains no. 12, 34, 44, 77, 84 and the lowest for strains number 6 and 9. For most of the tested *P. mirabilis* strains the polysaccharides levels did not rise with an increasing incubation time. The highest levels of total carbohydrates were noticed after one week incubation and averaged 106.1 μg/mg of proteins for all tested strains.

Polysaccharides levels after biofilm incubation in stress conditions

Polysaccharides levels in control medium and after biofilm cultivation in different media supplemented with tested factors were compared. The percentage of polysaccharides overproduction in relation to control medium was calculated. The results obtained using both phenol-sulphuric acid and ELLA methods are presented in five similar diagrams — one for each factor (Fig. 1). The figures show the average results obtained after 168 h of cultivation. Value 100% indicates the amounts of polysaccharides equal to the controls, above this level the overproduction of these compounds could be observed.

Enrichment of the growth medium with additional nutrients led to increased synthesis of polysaccharides in

Table 1. Amount of polysaccharides in *Proteus mirabilis* biofilm after cultivation in control medium [µg/mg of proteins]

P. mirabilis strains	Incubation time				
	24 h	48 h	72 h	1 week	2 weeks
5	36.9±7.4	12.2±3.1	16.3±5.4	10.1±3.3	83.1±41.5
6	0	0	4.3±0.4	0	57.3±27.1
7	0	169.4±71.6	0	0	164±54.4
8	0	65.7±22.8	37±15.6	21.9±7.1	53.4±30.2
9	0	0	23.6±5.8	0	62.6±35.9
11	30.8±8.1	40.6±2.6	27.9±9.1	0	28.7±5.2
12	19.9±1.3	107.1±10.8	78.2±30.9	100±22.6	193.9±15.9
15	114.3±47.5	46.4±11.6	30.5±6.7	0	99.7±18.2
20	22.2±5.9	10.9±2.7	29.9±5.4	229.7±110.9	142.0±18.6
24	35.7±10.5	31.1±10.1	50.2±19	99±38.4	132.5±29.0
31	54.4±11.6	20.3±2.6	74.3±12	48.5±15	127.9±34.6
32	0	47.6±14.8	0	94.7±35.1	55.6±21.8
33	9.5±2.2	94.9±5.9	0	301.7±97.1	27.3±1.9
34	5.9±0.7	441.8±184.5	0	0	150.2±50.2
41	0	97.3±35	111.9±26.9	135.4±63.6	0
44	27.3±11.7	17.3±4.7	39±12	93.3±11.6	403.4±179.6
46	13.1±6.0	145.1±46.1	32.8±7.1	159.5±37.5	48.7±16.9
57	16.6±4.1	38.3±5.8	126.6±32.4	48.1±9.3	37.9±4.0
70	74±6.5	39.3±2.8	0	204.7±20.6	119.2±17.6
71	0	28.2±6.2	52.2±12.1	236.7±28.1	18.8±2.8
77	0	355.2±62.2	78.9±18	287±51.2	246.1±47.1
84	113.2±18.8	10.5±1	121.4±7.6	279.5±39	74.9±40.3

P. mirabilis biofilm. After biofilm cultivation with a high concentration of glucose, more than a twofold increase (234%) in polysaccharide levels was detected in the case of 14 to 16 strains (depending on the tested method) (Fig. 1A). The largest amounts of polysaccharides were detected for *P. mirabilis* strains no.: 5, 12, 34, 41, 70, 77 (an increase by 364–1775% in relation to the control, $p \leq 0.01$). In the case of strains no. 12, 15, 24, 31, 41, 57, 70, overproduction of polysaccharides was confirmed using all studied methods. A reduction in polysaccharides levels, after the biofilm growth in medium supplemented with glucose, was observed for three strains: 6, 9 and 20 (about 39, 61, 64%, respectively). The addition of the second nutrient factor — albumin to culture medium also resulted in increased synthesis of polysaccharides in *P. mirabilis* biofilm. Total carbohydrate levels in the case of 20 strains were more than threefold higher (335%) compared to those obtained after biofilm cultivation in TSB (Fig. 1B). N-acetyloglucosamine and N-acetylogalactosamine amounts were similar to those detected in control medium, 150% overproduction for 13 strains and 110% for 8 strains were detected, respectively. In the case of 14 *P. mirabilis* strains the N-acetylogalactosamine residues levels were lower (by about 44%) compared to the control. A bacterial culture in adverse conditions, after the application of stress factors, led to the stimulation of the sugars synthesis for most of the tested strains. After biofilm cultivation in medium with the reduced nutrient content, an increase in the amounts of polysaccharides was observed (depending on the method) in the case of 10 to 18 *P. mirabilis* strains (Fig. 1C). The average level of polysaccharides was about 1.25 times higher compared to the control medium. A considerable increase in the N-acetyloglucosamine synthesis was noticed for strains no. 44 and 71 (about 232 fold and 15 fold, respectively; $p \leq 0.01$). Interestingly, this factor inhibited the synthesis of polysaccharides in the case of some strains, e.g. N-acetylogalactosamine levels were reduced by approximately 48% for 12 *P. mirabilis* strains. Total carbohydrate levels were lower (at about 100–5%) for 10 strains. The N-acetyloglucosamine levels were reduced by an average 37% in the case of only 4 strains. Acidic pH also influenced the polysacharide synthesis of studied strains. 15 to 17 *P. mirabilis* strains exposed to this agent produced higher amounts of glicoproteins and total saccharides. In these conditions a twofold increase in the polysacharides levels was confirmed using all tested methods (Fig. 1D). The addition of subminimal doses of cefotaxime to growth medium stimulated polysaccharides synhtesis (depending on the method) in the case of 12 to 15 strains — about 1.5–2.7 higher levels compared to those obtained in control media were detected (Fig. 1E).

Considering all cultivation times and all bacterial strains, it was concluded that the studied factors had a significant influence on polysaccharide synthesis. Slightly different results were obtained depending on the method used (Fig. 2). The N-actetylglucosamine level in biofilms was higher after a shorter incubation period and it significantly decreased after 72 h of incubation — values statistically significant at $p \leq 0.01$ (Fig. 2A). The overproduction of GlcNAc residues was the most effective in

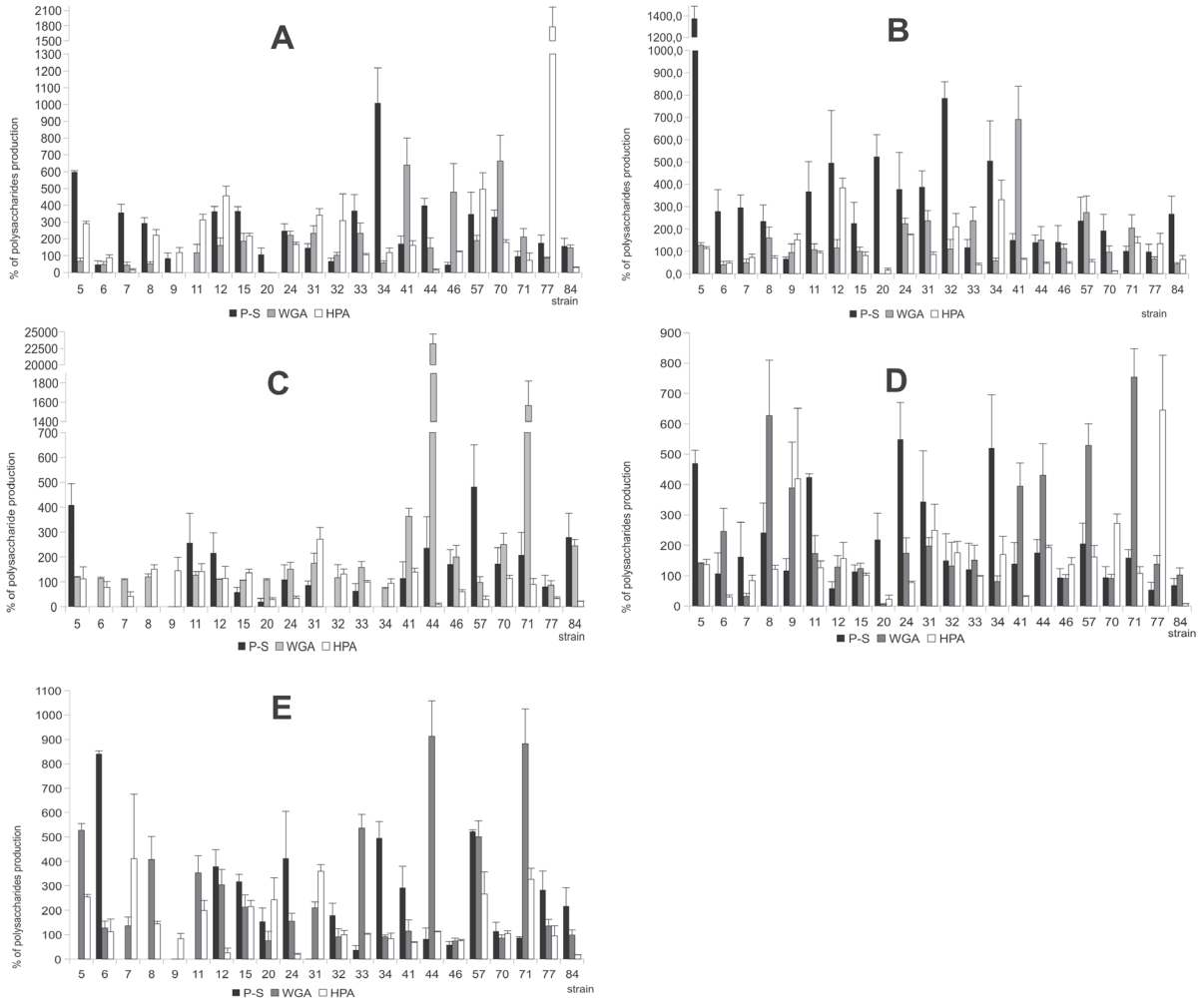

Figure 1. Effects of tested factors (A — glucose — 500 mM; B — albumin — 1.54 mg/ml; C — nutrient depletion, D — pH 4, E — cefotaxime — 32 µg/ml) on polysaccharides synthesis in 22 *P. mirabilis* biofilms cultivated for 168 h.
Results were obtained using three methods: P-S — phenol-sulphuric acid, WGA — ELLA with WGA lectin, HPA — ELLA with HPA lectin and presented as a percentage of the saccharides amount in relation to the control (100%). Results above 200% were statistically significant p≤0.05; results above 300% were highly statistically significant p≤0.01.

media containing cefotaxime. The lowest polysaccharides production (similar to the control) was noticed in media with albumin above 48 h of cultivation.

N-acetylgalactosamine levels rose with an increasing incubation time (especially to 168 h) (Fig. 2B). The overproduction of GalNAc residues was observed above 72 h of incubation. A similar trend was detected for all studied factors.

Total carbohydrates levels were similar in all cultivation times and they were about 2–3-fold higher compared to the control conditions (Fig. 2C). The lowest levels of saccharides were observed after biofilm cultivation in media with nutrient depletion, the values of these compounds were slighty higher than those obtained in optimal medium.

Table 2. Polysaccharides production analyzed by CLSM (% of polysaccharide overproduction in relation to control — 100%)

Factor		Polysaccharide production (%)	
		P. mirabilis 41	*P. mirabilis* 70
	Control TSB medium	100	100
Nutrients factors	Glucose	474.2±17.7*	204.9±18.6*
	Albumin	451.5±25.7*	110.6±36.5
Stress factors	Nutrient depletion	469.3±5.8*	1134.5±156.2*
	pH4	586.7±23.6*	226.2±31.0*
	CTX	422.2±29.9*	438.7±214.2*

*values statistically significant at p≤0.05

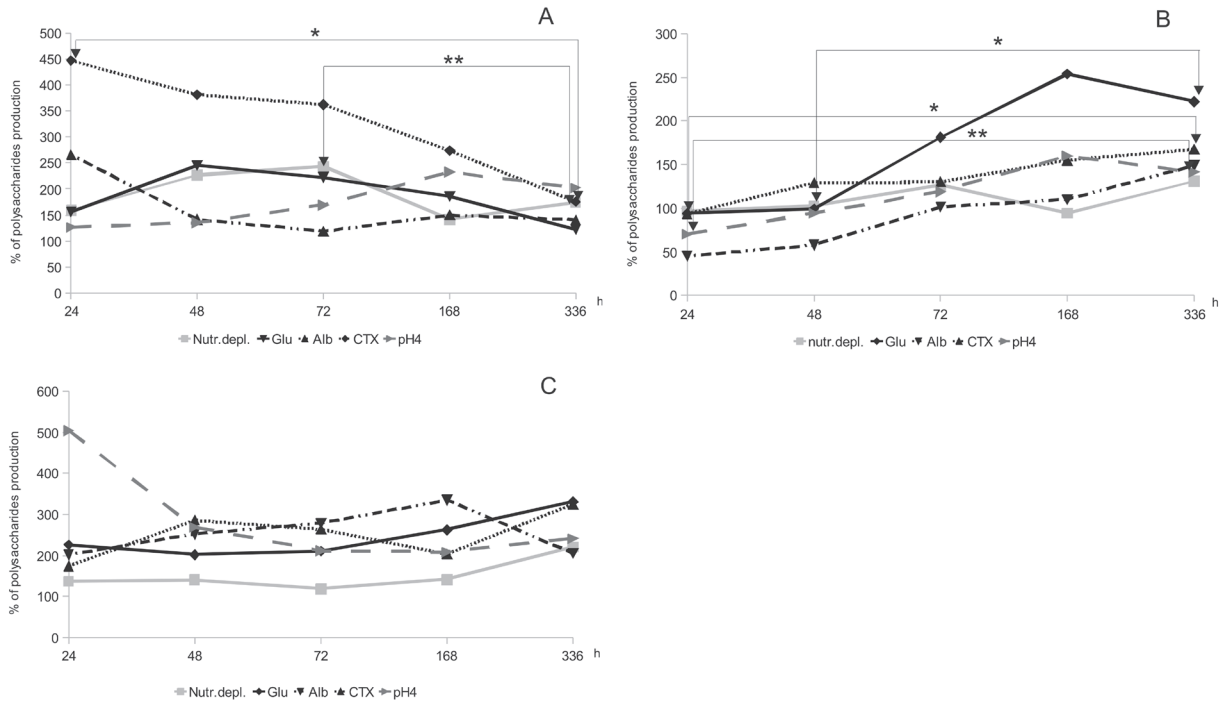

Figure 2. Comparison of polysaccharides levels after *P. mirabilis* biofilm cultivation with the addition of various factors (% of polysaccharides production in relation to the control medium), obtained using three studied methods: A — ELLA-WGA; B — ELLA-HPA; C — phenol-sulphuric acid.
*values statistically significant at p≤0.05; ** values highly statistically significant at p≤0.01.

CLSM analysis

The results obtained by the colorimetric methods were confirmed by biofilm analysis in a confocal laser scanning microscope. They are presented for two selected strains, after 48 h cultivation in media supplemented with tested factors. The bacterial cells and the polysaccharide layers were visualized and the levels of fluorescence: green emitted by bacterial cells (SYTO 13) and red emited by WGA-TRITC were compared. The ratios between those signals were counted and they are presented in Table 2, as percentage values calculated in relation to control conditions. The overproduction of polysaccharides (the red fluorescence dominated) was determined in all cases but sometimes on very low levels (albumin — *P. mirabilis* 70 — about 111%). The overproduction of N-acetyloglucosamine varied between 422–587% for *P. mirabilis* 41 and 111–1134% for *P. mirabilis* 70.

Figure 3 presents samples of images where red fluorescence (WGA-TRITC) and green fluorescence (SYTO 13) are merged. *P. mirabilis* biofilm cultivated under optimal conditions (Fig. 3A, C) was characterized by a small amount of EPS — a balance between the red and the green signals was observed. After merging of images obtained after biofilms cultivation in media supplemented with a high concentration of glucose, the EPS overproduction was revealed and the red fluorescence dominated (Fig. 3B, D). At the same time, additional images were made, which allowed assessing the biofilm structure and thickness (images not shown). After cultivation in optimal condition the biofilm was well organized and its thickness was about 16.1±1.8 mm. The biofilm thickness in media supplemented with glucose reached about 22.3±3.1 mm.

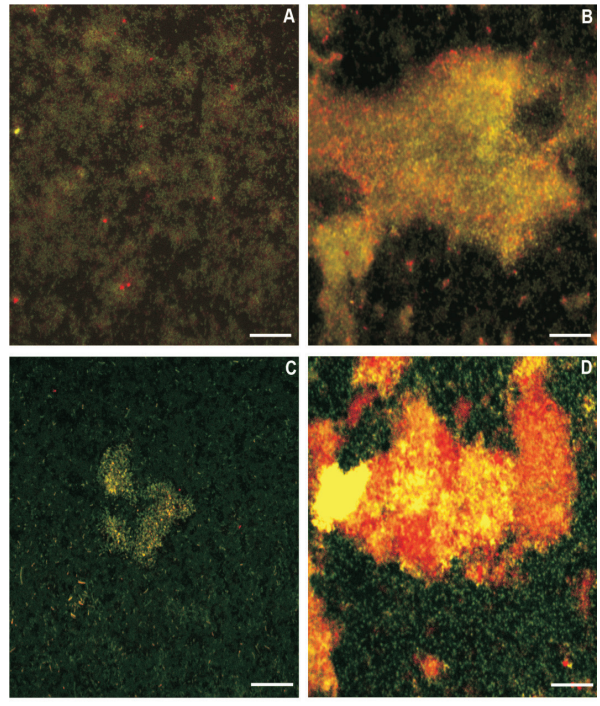

Figure 3. Fluorescence microscopy analysis of *P. mirabilis* biofilm (dual staining: SYTO 13 and WGA-TRITC).
(**A**) *P. mirabilis* 41 biofilm, after cultivation in optimal conditions, (**B**); *P. mirabilis* 41 biofilm, after cultivation in media supplemented with glucose, (**C**) *P. mirabilis* 70 biofilm, after cultivation in optimal conditions, (**D**) *P. mirabilis* 70 biofilm, after cultivation in media supplemented with glucose.

DISCUSSION

P. mirabilis is the main cause of urinary tract infections in patients with urinary catheters (Jacobsen *et al.*, 2008, Stankowska *et al.*, 2012). These bacteria form an extremely durable biofilm, which is very difficult to eradicate, mainly due to the resistance (in high concentrations) of sessile bacteria to most of recommended antibiotics. In our previous studies we confirmed the high resistance of *P. mirabilis* biofilms to most antibiotics (Moryl *et al.,* 2013). Biofilms are also resistant to many chemical and physical factors, disinfectants and immune system agents. Therefore, it is essential to examine the structure and properties of biofilm. The matrix that surrounds the bacterial cells in biofilm plays a crucial role in sessile bacteria resistance to antibiotics (Flemming *et al.,* 2008). Extracellular polymers are also involved in cells adhesion to a solid surface and in the formation of a three-dimensional structure of biofilm (Wingender *et al.,* 1999). In the future identification of matrix components may allow easier elimination of biofilm from abiotic surfaces.

The aim of the study was the analysis of differences in the levels of polysaccharides in *P. mirabilis* biofilms after cultivation in optimal and stress conditions. It is necessary to identify factor, which might delay or even prevent matrix formation. We investigated nutrient and stress conditions, that could influence the matrix quantity. Different factors affecting biofilm formation (data not shown) were chosen for the study, e.g. albumin at a concentration of 1.54 mg/ml, which corresponds to albuminuria observed in the urine of patients with kidneys disorders (nephritic syndrome) or cefotaxime (CTX) at a subinhibitory concentration of 32 mg/ml. In the previous studies the sensitivity of *P. mirabilis* to recommended antibiotics had been determined. Most of *P. mirabilis* strains in planktonic forms were susceptible to cephalosporins, but in biofilms they occurred to be resistant (Moryl *et al.*, 2013).

Polysaccharides levels in biofilms were determined using different techniques. The well known methods by Dubois *et al.* (1956) and Lowry *et al.* (1951) were applied to study the ratio of the quantity of proteins to polysaccharides in biofilm. The phenol-sulphuric acid colorimetric method allows investigating the total saccharides concentration — neutral sugars in oligosacharides, proteoglycans, glycoproteins and glycolipids. This commonly used 96-well microplate method is very reliable, and requires only 1–150 nM of polysaccharides per well for accurate analysis (Masuko *et al.,* 2005). The well known enzyme linked lectinosorbent assay (ELLA) using lectins, which recognize glicoproteins residues most frequently observed in biofilm matrices, was useful for characterization and quantification of selected sugars in biofilm (Leriche *et al.,* 2000). Two lectins: wheat germ agglutinin (WGA), which binds N-acetyl-D-glucosamine and/or N-acetylneuraminic acid (sialic acid) and *Helix pomatia* aglutinin (HPA) binding N-acetylogalactosamine were used. The CLSM technique was used for quantification of several biofilm parameters, e.g.: biofilm density and distribution on the surface. CLSM combined with fluorescent lectins is a method of choice for the *in situ* investigation of biofilm matrix components — it allows the analysis of composition and structural chemistry of biofilms (Lal *et al.,* 2010).

The environmental signals had a different influence on bacterial biofilm growth and matrix synthesis. Some bacteria exist as sessile forms only in rich environments, e.g. EPS production by *Cronobacter sakazakii* is enhanced after biofilm cultivation in TSB (Jung *et al.,* 2013). Other species better form biofilms in adverse conditions. For example, in the case of *Piscirickettsia salmonis* nutrient limitation induces stress, which leads to bacterial cells aggregation, stronger biofilm formation and production of large amounts of polysaccharides (Marshall *et al.,* 2012). The same effect was observed after *P. mirabilis* biofilm cultivation in medium containing a tenfold lower amount of nutrients. An about fourfold increase (compared to optimal conditions) in total polysaccharides levels was observed. Zalewska-Piatek *et al.* (2013) studied the effects of nutrient and stress factors on the process of *Escherichia coli* biofilm formation. They revealed that nutritional suplementation with glucose, glucose with iron salts or casamino acids supported biofilm formation by *E. coli* strains. Similar results were obtained by Jung *et al.* (2013), who confirmed that the addition of glucose or sucrose to culture medium stimulated EPS synthesis and biofilm growth of *C. sakazakii*. A high concentration of glucose in growth medium resulted in an increase in polysaccharide levels in *P. mirabilis* biofilms in all cultivation times (24–336 h). We noticed an about fourfold increse in total carbohydrate levels for 80% strains and an about twofold increase in glycoproteins levels for 70% strains. Petry *et al.* (2000) also observed a high level of extracellular polysaccharides after the cultivation of *Lactobacillus delbrueckii* in media containing 10 mg/ml of glucose. The most EPS were produced at the end of growth — in the stationary phase. It suggests that glucose (in the late stationary phase) is used for EPS production. It is well known that the pH of the environment has a huge impact on the growth of bacteria. It influences, e.g. enzyme activity, cell membrane morphology and affects nutrient solubility and its uptake by microorganisms (Bajaj *et al.,* 2009). Most bacteria exhibit the highest growth rate at optimal pH. Razack *et al.* (2013) demonstrated that the maximum productivity of EPS by *Bacillus subtilis* occurred at 35°C and pH 7. Similar observations were made by Lee *et al.* (1997) and Petry *et al.* (2000), who determined the optimal conditions for the synthesis of EPS by *Bacillus polymyxa* and *L. delbrueckii,* respectively. For *B. polymyxa* the highest yield of exopolysaccharides was observed at pH 7–8 in sucrose containing medium and a relatively low concentration of EPS was obtained when pH was 4.5. Growth of *L. delbrueckii* in pH-controlled (pH 6) medium resulted in a 3–4 times higher yield of EPS than that obtained under non-pH-regulated conditions. All these studies confirmed the observation that lowering of culture medium pH leads to a reduction in bacterial growth and the synthesis of extracellular polysaccharides. In contrast to the above-described results, Jung *et al.* (2013) did not observe a reduction in polysaccharides synthesis with decreasing environmental pH (to pH 5) in tested *C. sakazakii* strains but, interestingly, found a significant decrease in the nuber of microorganisms cells. In our studies we confirmed that acidic pH (4) strongly affected the bacterial population (data not shown), but the amounts of polysaccharides were higher compared to those obtained in optimal conditions (pH 7).

We also found (using all tested methods) that subinhibitory doses of cefotaxime stimulated *Proteus* bacteria (80% of tested strains) to greater synthesis of polysaccharides. This phenomenon could confirm the protective role of EPS in the biofilm structure, which in stress conditions is necessary for bacterial survival. On the other hand, many studies concerning the influence of drugs on sessile bacteria growth revealed that subminimal doses of antibiotics inhibited bacteria adhesion and biofilm formation processes. It is probable that chemio-

therapeutic agents have an effect on bacterial molecules (e.g. fimbriae) expressed on the bacterial surface, which consequently leads to changes in bacterial cell surface hydrophobicity (Balague *et al.,* 2011). However, those agents could also influence the EPS layer, e.g. reduce the amount of matrix. Wojnicz *et al.* (2007) found that ciprofloxacin and amikacin decreased the number of *E. coli* strains which had a polysaccharide capsule.

An important role of cephalosporins in the reduction of EPS was also demonstrated (La Tourette Prosser *et al.,* 1997, Balague *et al.,* 2011). Due to the fact that antibiotics have been observed to influence the synthesis of biofilm matrix of different bacteria species in variety of ways, it is very important to be wary of antibiotic therapy implementation.

The application of CLSM combined with fluorescent lectins allowed us to reveal extracellular glycoconjugates distribution in an intact biofilm. Both ELLA and CLSM confirmed that *P. mirabilis* matrix contained different amino sugars like: N-acetylglucosamine and N-acetylgalactoseamine as well as sialic acids. The results obtained by the colorimetric methods have been fully confirmed by CLSM photographs.

Lectins which have the ability to bind to specific glycoproteins, are frequently used to visualize these compounds in EPS. Zippel & Neu (2011) used a large panel of lectins (70) to investigate distribution of EPS glycoconjugates in tufa-associated biofilm. They detected the binding of 40 lectins to biofilm and confirmed the presence of neutral sugars e.g. fucose, galactose, amino sugars and sialic acids in the biofilm matrix. Lal *et al.* (2010) also demonstrated that CLSM coupled with fluorescent stains could be a successful tool for the study of the *Candida albicans* matrix (extracellular material containing glucose and mannose residues). Bridier *et al.* (2013) also observed a highly regular cells organization in *B. subtilis* biofilm, with cells connected by EPS material, composed partly of N-acetylglucosamine residues.

The presented study provides background information essential for our future studies on the analysis of the matrix composition and proper characterisation of sugars in *P. mirabilis* biofilms. The obtained results show that the culture medium and environmental conditions play a crucial role in the biofilm structure formation, which should always be taken into consideration in the treatment of infections caused by these microorganisms.

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