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Regular paper

Antimicrobial, antiadhesive and antibiofilm potential of lipopeptides synthesised by *Bacillus subtilis*, on uropathogenic bacteria*

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The aim of this study was to investigate the antimicrobial effect of lipopeptide biosurfactants from surfactin, iturin and fengycin families, synthesised by the *Bacillus subtilis* I'1a strain, on uropathogenic bacteria, including the effects on planktonic growth, processes of biofilm formation and dislodging. Antimicrobial activity was tested against 32 uropathogenic strains belonging to 12 different species of Gram-negative and Gram-positive bacteria. The sensitivity of 25 tested bacterial strains to the *B. subtilis* I'1a filtrate was confirmed by an agar diffusion assay. None of the strains seemed to be sensitive to pure surfactin at concentrations ranging from 0.1 mg×ml^{-1} to 0.4 mg ml^{-1}. After the treatment of uropathogens with *B. subtilis* lipopeptides, the metabolic activity of planktonic cells was inhibited by 88.05±3.96% in the case of 21 studied uropathogens, the process of biofilm formation was reduced by 88.15±4.77% in the case of 24 uropathogens and mature biofilms of 18 strains were dislodged by about 81.20±4.72%. Ten strains of uropathogenic bacteria were selected to study the antimicrobial activity of surfactin (concentrations 0.1, 0.2 and 0.4 mg×ml^{-1}). Surfactin had no influence on the metabolic activity of planktonic forms of uropathogens, however, biofilms of 5 tested strains were reduced by 64.77±9.05% in the presence of this biosurfactant at the concentration 0.1 mg×ml^{-1}. The negative effect of the compound on the biofilm formation process was observed at all concentrations used. The above-described results were fully confirmed by CLSM. It could suggest that synergistic application of biosurfactants could be efficient in uropathogen eradication.

Key words: uropathogens; biofilm; lipopeptides

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INTRODUCTION

Urinary tract infections are a very common disease in humans. Bacteria attach to urinary tract epithelium or in catheterised patients to the outer and inner surfaces of the indwelling catheter and form a biofilm. The process of biofilm formation is complicated and multi-staged. The first step is reversible adhesion, characterised by weak binding of bacterial cells to the surface. The second one involves the formation of specific bonds between the colonised surface and microbial adhesins. Next, the bacterial cells proliferate, differentiate and produce large amounts of extracellular polymers. As a consequence of these processes, a mature biofilm is formed. Cells from peripheral parts of biofilm can detach from this structure, migrate and colonise new niches (Donlan, 2002; Woźniak-Kosek, 2013). Due to the high complexity of the biofilm structure, sessile bacteria express lower sensitivity to antimicrobial agents which makes the biofilm infections very difficult to treat (Chen & Wen, 2011). The doses of drugs needed to eradicate microorganisms in a biofilm often exceed the allowed therapeutic norms. Therefore, there is a need to develop new methods that would be effective in biofilm destruction.

Bacillus strains secrete various secondary metabolites among which a great potential is exhibited by cyclic lipopeptide (LP) biosurfactants belonging mainly to the surfactin, iturin and fengycin families. Similarly to other surface active agents, these compounds reduce surface/interfacial tension, have self-assembly (micellization) properties, may stabilise emulsions, dispersions and foams, act as wetting agents and facilitate sorption or desorption processes (Ongena & Jacques, 2008; Hamley, 2015; Wang *et al.,* 2015).

Most properties of *Bacillus* LPs are a result of their amphiphilic molecular structure containing a hydrophilic cyclic peptide headgroup (built of L- as well as D-amino acids) attached to a hydrophobic fatty acid chain. In detail, surfactin is a heptapeptide linked to a β-hydroxy fatty acid chain by a lactone ring. Iturin also consists of a heptapeptide part but the hydrophobic tail is built of a β-amino fatty acid chain linked to a cyclic peptide by an amide bond. Fengycins are decapeptides (with eight amino acids participating in the peptide ring formation *via* lactone linkage) with a hydrophobic part of a β-hydroxy fatty acid chain (Mnif & Ghribi, 2015; Meena & Kanwar, 2015). It is worth mentioning that most often particular LPs are synthetised by *Bacillus* strains as a mixture of structurally similar variants, distinguished as isoforms (differing slightly in the amino acid sequence of the peptide part) and homologues (varying in the length of the fatty acid chain) (Pecci *et al.,* 2010).

The chemical structure of LPs (i.e. the orientation of hydrophilic and hydrophobic groups, the presence of amino acid residues and the length of fatty acid chain)

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Abbreviations: *B. subtilis, Bacillus subtilis*

may strongly influence their surface and biological activity (Das *et al.,* 2009; Singh & Cameotra, 2014).

Surfactin, iturin and fengycins are considered to be antibiotics due to their broad antimicrobial activities. Bioactive properties result mostly from the LPs capability to disturb the structure and functions of biological membranes, leading to the increase of membrane permeability (Ostroumova *et al.,* 2010; Deleu *et al.,* 2012). These compounds modify bacterial surface hydrophobicity and affect the development of flagella, which could be the source of their anti-adhesive properties. They are also known to have a stimulating effect on the biofilm dispersion process (Paraszkiewicz & Długoński, 2007; Rivardo *et al.,* 2009; Janek *et al.,* 2012).

Recently *B. subtilis* I'1a strain has been recognised as a surfactin, iturin and fengycin co-producer (Plaza *et al.,* 2015). Simultaneous synthesis of those three biosurfactants is a unique feature due to the *Bacillus* LPs synergic mode of action.

The aim of this study was to investigate the antimicrobial effect of compounds secreted by the *Bacillus subtilis* strain IETU I'1a (surfactin, iturin and fengycin) on uropathogenic bacteria. We investigated their influence on uropathogen planktonic growth, biofilm formation and eradication processes. To investigate the surfactin effect on the studied bacteria, the activities of commercial surfactin and *B. sublilis* I'1a LPs were compared.

MATERIALS AND METHODS

Microorganisms. The *B. subtilis* I'1a strain, kindly supplied by the Institute for Ecology of Industrial Areas (Katowice, Poland), was an isolate from the sludge of a 100-year-old oil refinery in Czechowice-Dziedzice (Poland). The taxonomic identification of the strain as well as its capability to produce LP biosurfactant has been described previously (Berry *et al.,* 2006; Plaza *et al.,* 2006; Plaza *et al.,* 2010; Plaza *et al.,* 2011; Plaza *et al.,* 2015).

32 uropathogenic strains belonging to 12 different species of Gram-negative and Gram-positive bacteria (*Escherichia coli*, *Enterobacter cloacae*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Proteus mirabilis*, *Providencia stuartii*, *Morganella morganii*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*), owned by the Department of Immunobiology of Bacteria, University of Lodz, were used in these studies. The microorganisms were isolated from encrusted biofilms formed on urinary catheters. The catheters were obtained from long term catheterised patients who were treated in two outpatient clinics in Łódź.

Bacterial strains were stored at (–70°C) as stocks of 24-h-old cultures using Luria–Bertani (LB) medium (Fluka, Germany) pH 7.0, supplemented with 20% (v/v) glycerol or with 10% (v/v) dimethyl sulfoxide (DMSO).

***B. subtilis* culture conditions**. A seed culture prepared in LB medium was maintained for 24 h under agitation conditions (140 rpm) at 28°C. Afterwards, it was diluted in LB medium to OD_{600}=0.8 and 3 ml were used to inoculate 97 ml of a fresh LB medium. The second step of the culture was performed in a 300 ml Erlenmeyer flask for 48 h under the conditions described above. A culture supernatant obtained after centrifugation (10 000 × *g*, 10 min) was divided into two parts. The first part was used for surfactin content analysis by liquid chromatography–mass spectrometry (LC-MS/MS). The second part of the supernatant was sterilised through a 0.2 μm filter and used to investigate its antimicrobial properties. To avoid the influence of highly alkaline pH on the tested bacteria, the filtrate was neutralised with hydrochloric acid.

Lipopeptide isolation and analysis by LC-MS/MS. The methods described by Plaza *et al.* (2015) were used for preparation of LP extracts and the sample analysis by an Agilent 1200 HPLC (Santa Clara CA, USA) system and a 3200 Q Trap mass spectrometer (AB Sciex, Framingham, MA, USA) equipped with an electrospray ionization (ESI) source. The mobile phase consisted of water (A) and methanol (B) supplemented with 2 mM ammonium formate and 0.2% formic acid. The flow rate was 600 ml × min^{-1}. The samples (5 μl) were injected onto an Allure® PFP Propyl column (50 mm × 2.1 mm, 5 μm particle size; Restek, Bellefonte, PA, USA) and maintained at 40°C.

The MS/MS data were processed using the Analyst™ v1.5.1 software (AB Sciex, Framingham, MA, USA). The surfactin standard (Sigma-Aldrich) was used for quantitative analysis. The electrospray source was operated at 600°C and 5500 V. For the same chromatographic conditions, an information-dependent acquisition (IDA) method, enhanced MS (EMS)/enhanced product ion (EPI), was used to identify iturin and fengycin homologues. The IDA method was used with the exclusion of the list of surfactin homologues (m/z 1030, 1044 and 1058) to avoid the unnecessary determination of surfactin (surfactin was determined quantitatively). In the EPI mode, the spectra were obtained in the range from m/z 200 to 1550. The EPI scan rate was 4000 amu × s^{-1}.

The antimicrobial assays. The sensitivity of planktonic forms of bacteria to surfactin and *B. subtilis* filtrate was tested using two different methods: agar diffusion and microdilution tests. For all assays uropathogenic bacteria were cultivated overnight at 37°C in tryptone soya broth (TSB). Next, the bacteria were diluted in TSB to yield a bacterial concentration of 10^7 CFU × ml^{-1}.

Three concentrations of commercial surfactin (Sigma-Aldrich): 0.1, 0.2, and 0.4 mg × ml^{-1} in PBS were used in this study.

For the agar diffusion assay, 1 ml of bacterial suspensions were spread on Mueller-Hinton agar. Next, 50 μl of surfactin at three concentrations (described above) and 50 μl of the *B. subtilis* filtrate were deposited onto the surface of the agar. The plates were incubated at 37°C for 24 h. Then, the transparency of halo zones was determined and the diameter of microbial growth inhibition was measured in millimeters by using a ruler.

For the microdilution method, 50 μl of the *B. subtilis* filtrate or surfactin solutions were placed in a polystyrene plate F. Next, 50 μl of each bacterial solution (density 10^7 CFU × ml^{-1}) was added. Additionally, the bacterial growth and filtrate sterility controls were prepared. The plate was incubated at 37°C for 24 hours in a humid chamber. Next, the plate was vortexed and 70 μl of suspensions were transferred to new wells and the absorbance at a wavelength of 595 nm was measured (Ultrospec 2000, Pharmacia Biotech). The absorbance of bacterial growth control and cultures exposed to surfactin or the filtrate were compared and the percentage reductions in the absorbances were calculated.

In all colorimetric methods applied, the results were considered as significant if the reduction in the absorbance was higher than 50%.

To study the effect of surfactin and the *B. subtilis* I'1a filtrate on the biofilm formation process**,** bacteria and the tested antimicrobial agents were mixed in a polystyrene plate in the same way as described above. After 24 h incubation, the biofilms in the wells were washed with 0.85% NaCl to remove planktonic cells. Next, the bio-

films were visualised using an MTT assay. 100 µl of TSB medium and 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in concentration 5 mg×ml^{-1} were added to each well. The plate was incubated at 37°C for 3 hours in a humid chamber. Then, 100 µl of DMSO and 25 µl of glycine buffer were added to each well to dissolve the formazan crystals. The plate content was mixed and the absorbance was measured at a wavelength of 550 nm (Ultrospec 2000, Pharmacia Biotech). The results were calculated and presented as a percentage of the reduction in the absorbance of cultures after the incubation with surfactin or the *B. subtilis* filtrate, relatively to a biofilm control sample.

To investigate the influence of surfactin and filtrate on the biofilm dislodging process, 100 µl of each bacterial culture with a density of 10^7 CFU×ml^{-1} was added to a multi-well plate. After 24 h incubation at 37°C, the mature biofilms were formed on the surface of the wells. Next, the plate was washed with 0.85% NaCl to remove unbound cells and biofilms were treated with 50 µl of surfactin or the *B. subtilis* filtrate. The biofilm growth and antimicrobial sterility controls were prepared. The plate was incubated at 37°C for 24 hours in a humid chamber. Next, the MTT assay was performed and the results were calculated as described above.

Confocal laser scanning microscopy (CLSM). For CLSM analysis, biofilms of uropathogens were cultivated in glass-bottomed dishes (Greiner Bio One) for 24 h. Next, the biofilms were washed with distilled water to remove planktonic bacteria, and exposed to surfactin (0.1, 0.2 and 0.4 mg×ml^{-1}) or the *B. subtilis* I'1a cell free supernatant. The biofilm formation process in the presence of tested antimicrobials was also analysed by CLSM. In this case, the LPs and uropathogen cultures were added simultaneously into the plate in a volume of 200 µl. After 24 h incubation, biofilms were washed with distilled water and fluorescently stained with SYTO 13 (Molecular Probes) at a concentration of 50 mM. The imaging was performed using a Pascal (Zeiss) confocal laser scanning microscope equipped with a 40× (0.75 numerical aperture) objective lens. For SYTO 13, the excitation/emission maxima were 488/514 nm. The image analysis was performed using the AxioVision software.

Kinetics of bacterial growth and biofilm formation in the presence of LPs. To study the planktonic growth kinetics in the presence of antimicrobials (surfactin or the *B. subtilis* I'1a filtrate), two uropathogenic strains: *S. marcescens* 23 and *E. coli* 84 were selected. Bacteria were inoculated into the TSB medium and incubated at 37°C for 24 h. Then, the bacterial cultures were diluted, using TSB medium, to obtain a density of 10^5 CFU×ml^{-1}. Next, equal volumes of the filtrate (or surfactin at concentration 0.1 or 0.4 mg×ml^{-1}) and the bacterial cultures were mixed (0.5 ml). The control of bacterial growth was also performed. After the incubation times of: 0, 1, 2, 4, 6 and 24 h, at 37°C and 150 rpm, the bacterial cells were counted to determine the colony forming units (CFU) per ml.

Kinetics of biofilm formation were assessed by growing the biofilms of selected uropathogens on urological catheters. 1 cm pieces of a silicone Foley catheter were placed into test tubes. Next, the bacterial cultures (diluted in TSB medium to a density of 10^5 CFU×ml^{-1}) and the surfactin solution or the filtrate, were added in equal volumes. The controls of biofilm growth were also prepared. After the incubation for 1, 2, 4, 6 and 24 h at 37°C, the catheters were rinsed to remove planktonic cells and biofilms were sonicated for 5 min (Sonic 6, Polsonic) to detach the settled cells from the catheter surface. The bacterial cells were counted by obtaining the number of CFU cm^{-1} of catheter.

Statistical analysis. The results were expressed as mean ± standard deviation. Statistical analyses were performed with the Statistica 12 PL software and the means were compared using the Mann-Whitney U test.

RESULTS AND DISCUSSION

Surfactin, iturin and fengycin production in the *B. subtilis* I'1a culture

The results obtained by LC-MS/MS analysis revealed that after 48 h of cultivation, *B. subtilis* I'1a produced 8.02±0.54 mg l^{-1} of surfactin, present as four surfactin homologues with the acyl chain length ranging from C13 to C16. Two surfactin homologues (C14 and C15) strongly dominated in the analysed sample and comprised about 89% of the whole surfactin content. In the analysed samples, various compounds from the iturin and fengycin families were also detected. Literature data confirm that surfactin is synthesised mostly as a mixture of three or four homologues. For example, *B. licheniformis* V9T14 was reported to produce C13, C14 and C15 surfactin homologues (Pecci *et al.,* 2010; Li *et al.,* 2010). On the other hand, Bacon *et al.* (2012) observed that some *B. mojavensis* strains were able to synthesise as many as seven surfactin homologues with the acyl chain length ranging from C11 to C17. It was revealed that the majority of *B. mojavensis* isolates secreted from 0.7 to 35.9 mg×l^{-1} of surfactin. In the context of these data, *B. subtilis* I'1a produces surfactin with medium intensity. It is also worth mentioning that the number of reported *Bacillus* strains capable of simultaneous surfactin, iturin and fengycin production is limited (Chen *et al.*, 2008; Kim *et al.,* 2010; Plaza *et al.,* 2015). Therefore, *Bacillus* strains with such properties seem to be interesting both as research models and biocontrol agents.

Antimicrobial activity of *B. subtilis* LPs

The agar diffusion method was used to study the effect of surfactin and the *B. subtilis* I'1a filtrate on planktonic forms of uropathogens (on solid medium). The results are summarised in Table 1. In an agar diffusion assay, the sensitivity of 25 tested bacterial strains to the *B. subtilis* I'1a cell free supernatant was indicated based on the transparency of halo zones and their size, which ranged from 10 to 17 mm. None of the strains tested seemed to be sensitive to pure surfactin at any concentration tested (data not shown). The surfactin activity was too weak to visualise changes in the microorganisms' density. In contrast, the *B. subtilis* I'1a cell free supernatant was active against bacterial planktonic forms probably due to the synergistic effect of the filtrate components. The mixture of extracellular bacterial substances secreted by *B. subtilis* had a greater impact on uropathogens growth than purified compounds. Similarly, Compaoré *et al.* (2013) in their research on the *B. subtilis* cell free supernatant, containing, among others: surfactin, subtilosin and subtilin, used the diffusion method to demonstrate its strong influence on the inhibition of 36 Gram-positive and Gram-negative bacteria and yeasts.

However, the effect of biosurfactants on the planktonic bacteria is still unclear and there are many studies which describe ineffectiveness of the compounds in bacterial growth inhibition. Rivardo *et al.* (2009) demonstrated that biosurfactants V9T14 and V19T21 had no

Table 1. Influence of LPs, present in the *B. subtilis* I'1a filtrate, on uropathogens' growth assessed by the agar diffusion method

No.	Bacterial strain	*B. subtilis* I'1a filtrate	
		Zone transparency*	Zone size (mm)
1	*E. coli* C9	+	12
2	*E. coli* C56	++	15
3	*E. coli* C84	+	13
4	*P. aeruginosa* C11	-	0
5	*P. aeruginosa* C53	-	0
6	*P. aeruginosa* C56	-	0
7	*S. marcescens* C19	++	17
8	*S. marcescens* C23	++	16
9	*M. morganii* C1	+/-	12
10	*M. morganii* C41	+/-	11
11	*M. morganii* C67	+/-	12
12	*E. cloacae* C30	++	15
13	*E. cloacae* C64	+	14
14	*E. cloacae* C72	++	16
15	*P. stuartii* C11	+/-	10
16	*P. stuartii* C53	+	12
17	*P. stuartii* C56	-	0
18	*P. mirabilis* C11	++	14
19	*P. mirabilis* C41	+	12
20	*P. mirabilis* C70	++	15
21	*C. freundii* C16	+/-	11
22	*C. freundii* C61	++	15
23	*C. freundii* C79	++	16
24	*K. pneumoniae* C46	+/-	10
25	*K. pneumoniae* C56	-	0
26	*K. pneumoniae* C71	+/-	11
27	*S. aureus* C65	+/-	10
28	*S. aureus* C85	+/-	12
29	*S. epidermidis* C35	+	13
30	*E. faecalis* C9	-	0
31	*E. faecalis* C46	-	0
32	*E. faecalis* C84	+	12

*++ zone with full transparency; + zone with incomplete transparency; +/- zone with weak transparency; – lack of transparency

influence on planktonic survivability at every concentration tested.

The antimicrobial, anti-adhesive and antibiofilm activities of LPs produced by *B. subtilis* I'1a were also analysed using colorimetric methods. To determine the effect of surfactin on the selected uropathogenic bacteria, the commercial surfactin was used at the concentrations of 0.1, 0.2 and 0.4 mg×ml^{-1}.

The results were calculated on the basis of the observed reduction in the absorbance of cultures exposed to the *B. subtilis* I'1a filtrate in relation to the bacterial growth control and are presented in Fig. 1 as the growth inhibition percentages. In the case of 21 studied uropathogens, the metabolic activity of planktonic cells was inhibited by 88.05±3.96% after the treatment with the *B. subtilis* cell free supernatant. *B. subtilis* products also affected the process of biofilm formation - an average reduction of 88.15±4.77% was observed in the case of 24 studied uropathogenic strains. It was also shown that the cell free supernatant was active against mature biofilms, in 18 tested strains the reduction of about 81.20±4.72% in biofilm biomass was noted. The inhibitory activity of *B. subtilis* extracellular products was lower in the case of several species: *Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis* and *Enterococcus faecalis*.

The tested compounds had the greatest influence on the process of biofilm formation, probably due to the strong anti-adhesive properties of the mixture of *B. subtilis* I'1a metabolic products. This effect could be related to biosurfactants' influence on the reduction of bacterial cell hydrophobic properties or on the repulsion between bacteria and abiotic surfaces (Zezzi do Valle Gomez & Nitschke, 2012). Similar results were observed by Rivardo *et al.* (2009), who demonstrated that biosurfactants produced by *B. subtilis,* at a proper concentration could decrease the biofilm formation process. Anti-adhesive properties were also found for pseudofactin II secreted by *Pseudomonas fluorescens* BD5. This compound significantly decreased the adhesion of tested bacteria and yeast to abiotic surfaces and had lower activity in a biofilm dislodging process (Janek *et al.,* 2012). A similar effect was demonstrated by Biniarz *et al.* (2015), who studied the pseudofactin and surfactin activity on *Candida albicans*. The authors observed a decrease in the adhesion of all tested *C. albicans* strains, and determined the synergistic interactions between the two tested lipopeptides. When the plates were pre-treated with surfactin and pseudofactin simultaneously, the process of *C. albicans* adhesion was more strongly inhibited when compared to the experiments in which only one compound was used.

10 strains of uropathogenic bacteria were selected to study the antimicrobial activity of surfactin. The LP at all concentrations tested had no influence on the metabolic activity of planktonic forms of uropathogens (Fig. 2A). Our results are in contradiction with the studies of Sabate & Audisio (2013), who demonstrated the listericide effect of surfactin. The compounds synthesised by various *B. subtilis* strains inhibited the pathogen at concentrations ranging from 0.125 mg×ml^{-1} to 1 mg×ml^{-1}. A similar observation was made by Loiseau *et al.* (2015), who found surfactin was active against all tested *Legionella* strains in contrast to other bacterial strains studied, which seemed to be resistant to surfactin, even at a concentration as high as 265 μg×ml^{-1}.

A very small effect of surfactin on mature uropathogenic biofilm was observed (Fig. 2C). The biofilms of 5 tested strains were reduced by 64.77±9.05% in the presence of the biosurfactant at the concentration of 0.1 mg×ml^{-1}. However, surfactin exhibited an anti-adhesive properties and exerted a negative effect on the biofilm formation process at each concentration used (Fig 2B). Surprisingly, lower concentrations of surfactin had greater impact on the biofilm formation process. The adhe-

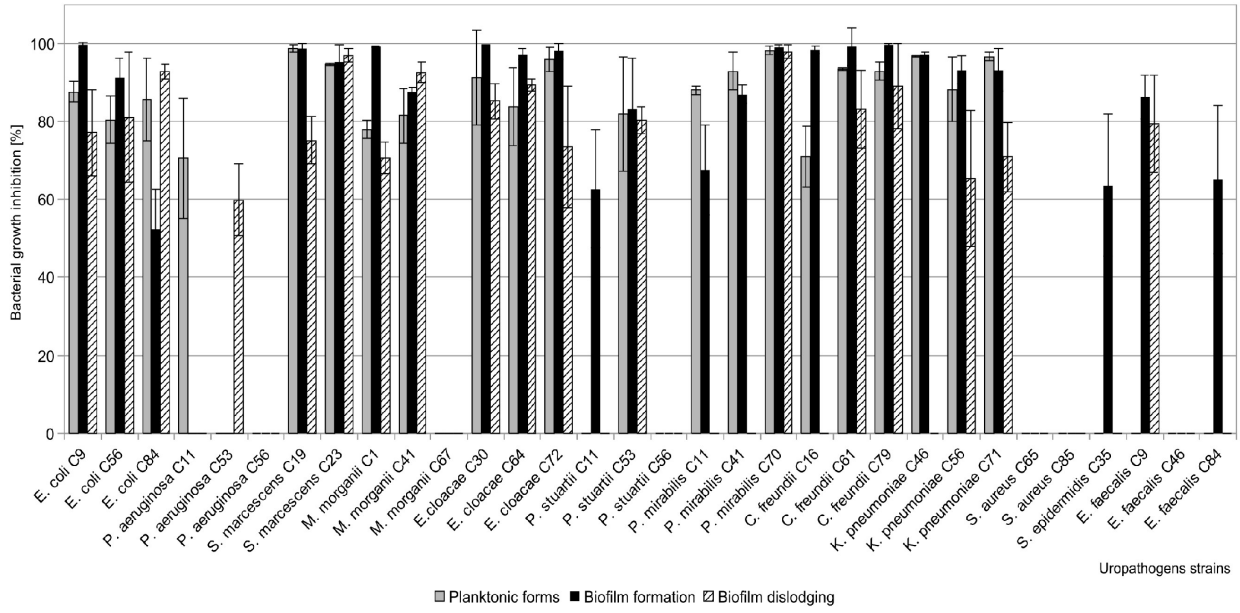
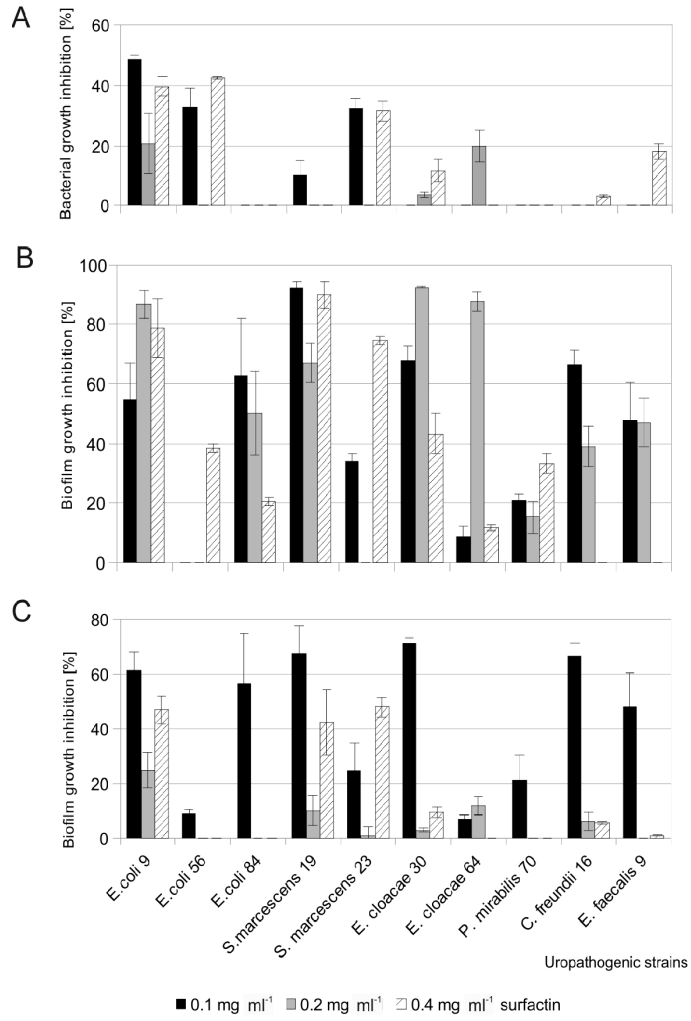


Figure 1. Influence of LPs, present in the *B. subtilis* I'1a filtrate, on uropathogenic bacteria assessed by colorimetric methods.
The results are presented as a percentage of the reduction in the absorbance of cultures after incubation with the *B. subtilis* filtrate, relatively to a growth control sample.



sion of 5 strains was decreased in the presence of surfactin at the concentrations of 0.1 and 0.2 mg×ml^{-1}, by 68.94±8.63% or 76.86±5.76%, respectively, whereas the presence of 0.4 mg×ml^{-1} caused a 81.02±5.22% reduction in the adhesion of only 3 strains. Biniarz *et al.* (2015) found that pre-treatment of a polystyrene plate with surfactin was more effective when the concentrations of this compound were higher than critical micelle concentration (0.1 mg×ml^{-1}). A concentration-dependent effect was also observed for pseudofactin II — the higher the concentration was used, the greater influence of the biosurfactant on microbial adhesion was observed (Janek *et al.,* 2012). These discrepancies could be explained by the use of the biosurfactant in different isoforms, application of different research methods or experimental conditions, use of different species and strains of bacteria. Rivardo *et al.* (2009) observed that the anti-adhesion activity of biosurfactants was correlated with increasing concentration for one tested strain — *E. coli*. The results obtained for *S. aureus* showed that lower concentrations of biosurfactants had a greater inhibition effect on the biofilm formation process.

Two selected strains: *E. coli* 84 and *S. marcescens* 23 were chosen for CLSM. The analysis fully confirmed the results obtained by the colorimetric methods. In the control, where bacterial cultures without surfactin or the

Figure 2. Influence of surfactin (0.1, 0.2, 0.4 mg ml^{-1}) on uropathogens' planktonic growth (A), biofilm formation (B) and biofilm dislodging (C).
The results are presented as a percentage of the reduction in the absorbance of cultures after incubation with surfactin, relatively to a growth control sample.

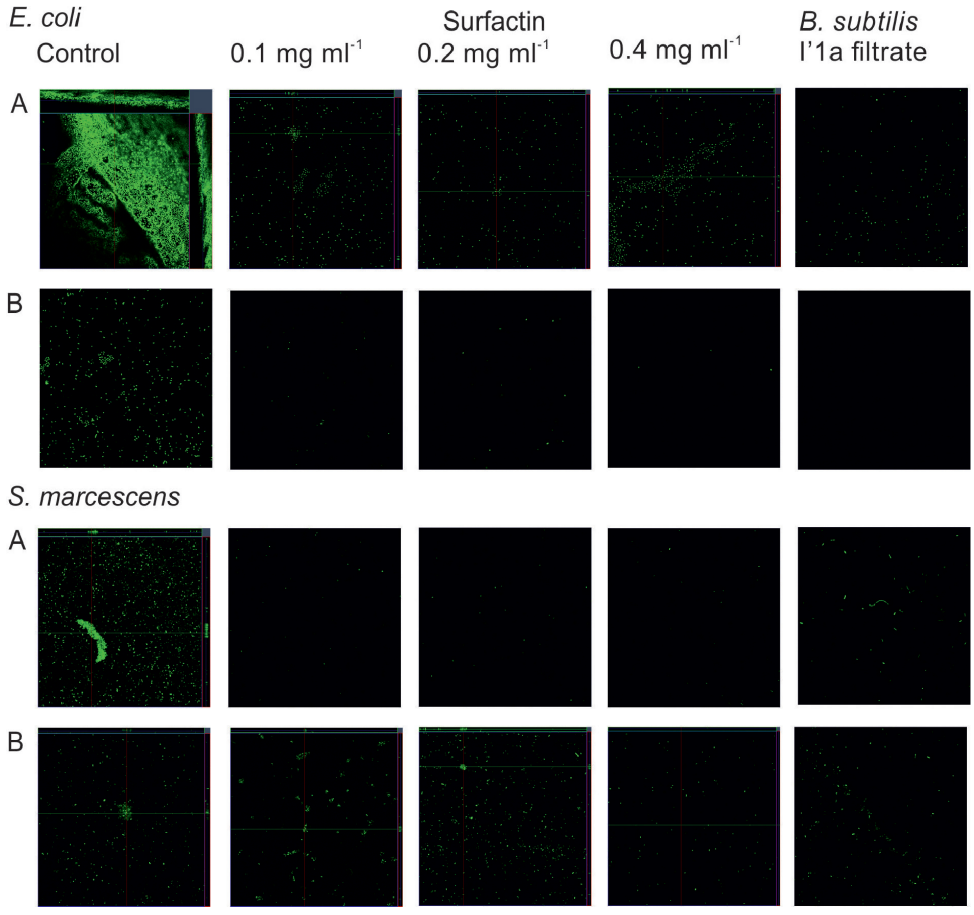


Figure 3. Influence of surfactin (0.1, 0.2, 0.4 mg ml^{-1}) and the *B. subtilis* l'1a filtrate on *E. coli* and *S. marcescens* biofilm dislodging (A) and biofilm formation (B) processes.

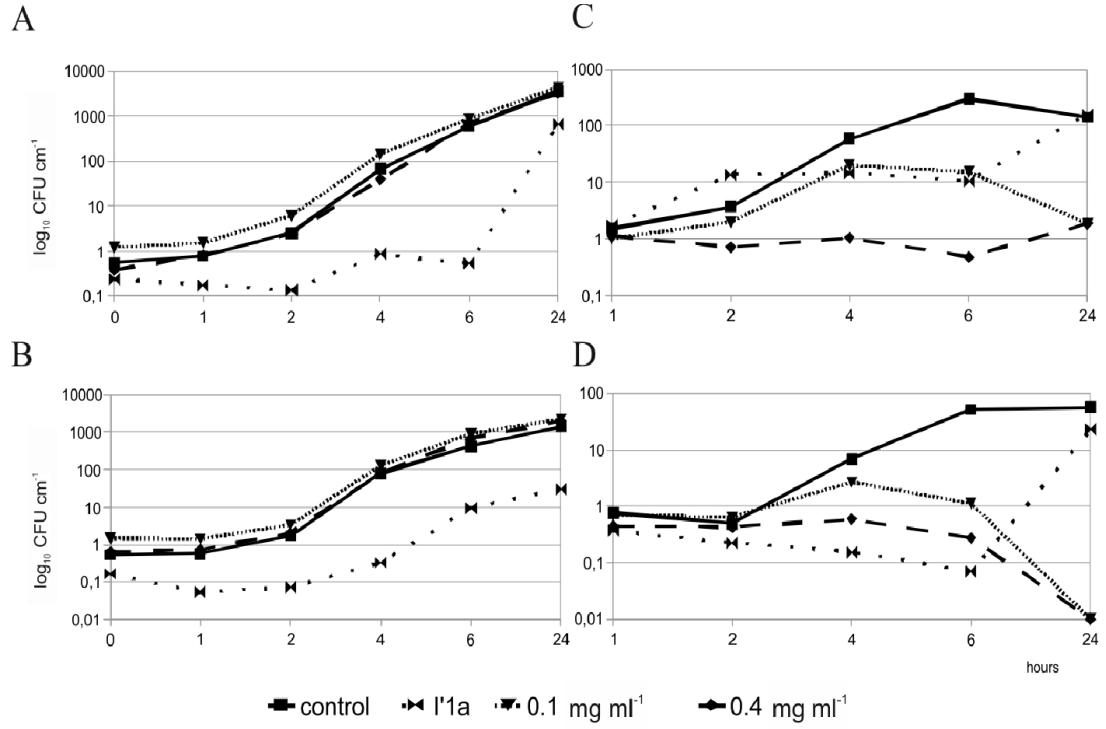


Figure 4. The influence of surfactin (concentrations 0.1 and 0.4 mg ml^{-1}) and LPs present in the *B. subtilis* l'1a filtrate on the: *S. marcescens* planktonic growth kinetics (A); *E. coli* planktonic growth kinetics (B); *S. marcescens* adhesion kinetics (C); *E. coli* adhesion kinetics (D).
Statistical significance in comparison to the controls was shown to be at least $p \leq 0.01$.

B. subtilis I'1a filtrate were cultivated, the aggregates of biofilms with a thickness of 20.40±7.69 μm for *E.coli* and 11.23±3.74 for *S. marcescens* were found. In the wells where the uropathogenic biofilms were treated with the LPs, there was no biofilm and only single microbial cells were observed. Representative images of *E. coli* and *S. marcescens* biofilms, obtained in the control medium and after surfactin or *B. subtilis* I'1a filtrate application, are shown in Fig. 3.

Two strains *E.coli* 84 and *S. marcescens* 23 were chosen to study the microbial cell growth (Fig. 4A, B) and biofilm formation kinetics (Fig. 4C, D) in the presence of surfactin (0.1 or 0.4 mg×ml^{-1}) or the *B. subtilis* I'1a cell free supernatant. In this part of the research, we confirmed that at all concentrations tested surfactin did not inhibit the planktonic growth of bacteria — the growth rate in media containing surfactin was the same as in the control medium (Fig. 4A, B). However, a significant decrease (56 to 99%, $p<0.01$) in the *E. coli* and *S. marcescens* growth rates was observed after incubation with the *B. subtilis* I'1a filtrate from 0 h to 24 h.

The kinetics of biofilm formation by *E. coli* and *S. marcescens* strains were shown to be interfered with by the tested biosurfactants. A significant decrease in the adhesion and biofilm formation by *S. marcescens* 23 was noted after the incubation with surfactin at the concentration of 0.4 mg ml^{-1} from 2 h to 24 h, with the inhibition rate ranging form 26.12% ($p\leq0.01$) to 99.83% ($p\leq0.001$). A smaller effect was shown for surfactin at the concentration of 0.1 mg ml^{-1}, with the highest decrease of about 67.53% ($p\leq0.01$) after 24 h. Biosurfactants produced by *B. subtilis* affected the *S. marcescens* adhesion only after 4 h and 6 h of incubation (74.93% ($p\leq0.01$) and 96.93% ($p\leq0.001$), respectively). A similar observation was made after the *E. coli* 84 exposure to the tested biosurfactants. An interesting phenomenon was detected after 24 h of the bacterial incubation with surfactins (both concentrations used), when no cells were isolated from the surface of the catheter. A high impact of the *B. subtilis* filtrate on the *E. coli* adhesion was observed, with the inhibition rate ranging from 52.29% ($p\leq0.01$) at 1 h to 99.86% ($p\leq0.001$) at 6 h.

Studies of the kinetics of the surfactin action on microorganism growth are very popular and the obtained results are most frequently associated with the tested bacterial species. Araujo *et al.* (2011) examined the adhesion kinetics of *Listeria monocytogenes* in the presence of biosurfactants, for e.g. surfactin, and observed a decrease in the number of bacterial cells by maximum 55% after 6 h incubation. Similar observations were made by Mireles *et al.* (2001), who studied the impact of surfactin on preformed *S. enterica* biofilm. The biosurfactant addition to the growth medium caused an 85% decrease in the total biofilm at 22 h of the experiment.

In conclusion, the *B. subtilis* I'1a filtrate containing a mixture of lipopeptides: surfactin, iturin and fengycin, demonstrated significant anti-adhesive and antibiofilm activities. These compounds also had an influence on the planktonic growth of the tested uropathogens, while pure surfactin at concentrations tested had mainly anti-adhesive properties. The strong influence of the filtrate on uropathogens may be related to a synergistic effect of various compounds. Biosurfactant application in the protection of biomaterials from bacterial colonisation and the removal of bacterial biofilm from surfaces (e.g. urinary catheters) could become a new strategy for biofilm eradication in medicine.

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