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Regular paper

Opportunistic Gram-negative rods' capability of creating biofilm structures on polivynyl chloride and styrene-acronitrile copolymer surfaces*

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Biofilms are highly organized microbial communities displaying high resistance to disinfectants and other external environmental factors. Medical equipment, such as stents and catheters, can be colonized by a variety of bacteria including opportunistic pathogens circulating in the environment and dangerous to immunocompromised patients. Application of materials resistant to biofilm formation will minimize the risk of patients' infection. Hence, the aim of this research was to determine the biofilm growth of environmental bacteria isolates on polyvinyl chloride and styrene-acronitrile copolymer surfaces. Nine strains (*Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Serratia liquefacies*) isolated from cosmetics, and a reference *P. aeruginosa* strain ATCC 15442, were tested. The ability and dynamics of biofilm formation on intubation catheters (30°C, up to 24 h) in bacterial growth cultures (10^7–10^8 CFU/ml) was investigated, with subsequent sonication and quantification by agar plate count method. The results indicated that all the tested bacteria expressed a strong ability for the polymer surface adhesion, reaching 4.6 to 6.7 log CFU/cm² after 30 minutes. Moreover, for the majority of strains, the level of 24-hour biofilm production was from 6.67-7.61 log CFU/cm². This research indicates that the environmental strains circulating between the cosmetics and patients may pose a threat of biofilm formation on medical equipment surfaces, and presumably in the clinical surroundings as well.

Key words: *Pseudomonas aeruginosa*, biofilm, environmental strains, polymer surfaces

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INTRODUCTION

Materials used in the production of medical devices (catheters, stents, tubes etc.) are perfect niches for microorganisms' colonization. The cells in the biofilm present a high risk factor, involving complications during healing and a prevention process (Hostacka *et al.,* 2010). It is believed that increased resistance can be caused by slow penetration of disinfectants (due to the matrix presence), biofilm heterogeneity, by the presence of "super resistant" cells located in the deeper biofilm layers which are metabolically inactive, and biofilm specific response to stress (bacterial cells are exposed to the disinfectant gradually, they have time to "get used to" the disinfectant and develop defensive strategies) (Coenye, 2010; Van Acker *et al.*, 2013).

Pseudomonas aeruginosa is considered to be one of the most dangerous bacteria causing a range of nosocomial infections in immunocompromised patients. The eradication process of this microorganism is very long and tough because of the complex resistance mechanisms and the ability to acquire insusceptibility to the new antibiotics and disinfectants (Briendstein *et al.,* 2011). It is influenced by many virulence factors which this bacterium aquired. These factors include producing: exopolysaccharide, lipopolysaccharide, exotoxin A, pigments (inter alia pyocyanine), lipase, protease, hemolysin, histamine, exoenzyme S, leucocidin, rhamnolipids, pili (connected with motility) and forming a biofilm structure (Maha *et al.*, 2015; Alhede *et al.*, 2014).

An opportunistic pathogen *Burkholderia cepacia,* besides *P. aeruginosa* and *Staphylococcus aureus,* is the most common cause of infections among cystic fibrosis patients (the so-called cepacia syndrome). For many years this species was classified as *Pseudomonas cepacia.* Nevertheless, in the nineteen nineties it was distinguished from *Pseudomonas* and a new species was created. Due to their metabolic properties, these bacteria are able to colonize abiotic surfaces (glass, plastics) and survive in environments of extremely poor nutrient value (even in distilled water) (Coenye, 2010). What is more, the studies prove that *Burkholderia cepacia* is able to create a biofilm structure alone, or a dual species biofilm together with *P. aeruginosa.* Al-Bakri *et al.* (2004) showed in his research that *P. aeruginosa* can predispose non-colonized parts of surfaces to allow *B. cepacia* to colonize. Furthermore, the treatment of the binary biofilm with commonly used antibiotics, in the infections caused by *P. aeruginosa* and *B. cepacia,* is ineffective in preventing the establishment of the biofilm (Al-Bakri *et al.*, 2005).

Serratia liquefaciens is a Gram-negative rod, a well-known foodborne pathogen. However, bacteria belonging to *Serratia liquefaciens* complex were also reported as a cause of nosocomial infections (sepsis, urinary tract infections, bloodstream infections), linked to contaminated clinical equipment and blood components (Stock *et al.*, 2003). *S. liquefaciens* is capable of producing a range of extracellular enzymes. Givskov & Molin (1992) showed in their research the presence of an extremely thermore-

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Abbreviations: TSB, trypticase soya broth; PBS, phosphate buffered saline; TSA, trypticase soya agar; CFU, colony forming units; ATTC, American type culture collection; ISO, international organization for standardization

sistantant phospholipase A, having a highly specific activity and stability, resistant to some redox agents, detergents and organic solvents. Presumably, the presence of such enzymes can play a vital role in the biofilm formation and the bacterial resistance.

The bacteria described above, posing a threat to the human health, are circulating in the environment. They are present on human skin and are transferred to food and cosmetics. The purpose of this study was to find out whether environmental strains of *P. aeruginosa*, *B. cepacia* and *S. liquefaciens* can colonize the polyvinyl chloride and styrene-acronitrile copolymer catheter's surface efficiently and what the pace of adhesion to the suction catheters is.

MATERIALS AND METHODS

Microorganisms. Nine environmental strains: six *P. aeruginosa* (DH1, DH2, DH4, CFII, CFIVa, CFV) two *S. liquefaciens* (CFIV, CFVII) and one *B. cepacia* (DH3), used in the experiment were isolated from cosmetics (CF), the cosmetic's raw material-Dehyquart (DH) and were previously identified. *P. aeruginosa* ATCC 15442 strain (originated from American Type Culture Collection) was used for a comparison. Bacteria were activated in trypticase soy broth — TSB (Merck, Darmstadt Germany) and incubated at 30°C for 24 h.

Material and biofilm cultivation. Suction catheters (vacutip type) made of polyvinyl chloride and styrene-acronitrile copolymer, were cut into 3 cm pieces, ensuring their sterility (by means of a sterile surgical scalpel, on a sterile Petri dish in a box with sterile airflow), put into TSB liquid medium inoculated with a monoculture of one of the tested bacteria (10^7–10^8 CFU/ml) and incubated at 30°C up to 24 hours. For each bacterial strain, three repetitions were made.

Sonication and bacteria enumeration. The catheters and the liquid samples were collected after 0.5, 4, 8, 12 and 24 hours. Each catheter was rinsed three times by subsequent transferring into the test tube with 3ml of 10μM PBS buffer (pH 7.4) and gently shaking for 1 minute; for removing the loosely adhered cells. The edges of the catheter were removed by careful cutting by sterile surgical scissors, leaving 1cm samples, to avoid biofilm lesions produced at the places where the catheter was gripped during the rinsing operations, and put into a sterile test tube with saline solution. Afterwards, the tubes were sonicated for 30 sec (60 Hz) to disperse the adhered cells (Paduch & Niedzielski, 2005). Colony forming units (planktonic cells from the liquid samples and bacteria cells considered as biofilm-creating) were obtained by the plate count method on TSA medium (Merck, Darmstadt Germany). The results were presented as log CFU/1cm^2. For all calculations, the surface of the catheter was the sum of the inner and outer surface of the 1cm long sample.

Safranin staining. The catheters were washed three times with 3ml saline solution to remove non adherent cells. The bacterial cells that had adhered to the catheters were stained in 0.1% safranin (Merck, Darmstadt Germany) for 10 minutes (Balaban *et al.*, 2003).

Statistical analysis. Each sample was tested in triplicate for each bacterial strain and the standard deviation was calculated using Origin 6.1. Differences between strains were statistically evaluated by ANOVA test with a level of significance of 0.05.

RESULTS AND DISCUSSION

Cosmetics applied on the skin can be contaminated during their use and can serve as a source of opportunistic pathogens (Osungunna *et al.*, 2010). There are also some reports (Lundov & Zachariae, 2008; Budecka & Kunicka-Styczyńska, 2014) on microbiologically contaminated cosmetic products due to raw materials' low quality or improper hygiene of the production environment. The tested *P. aeruginosa*, *B. cepacia* and *S. liquefaciens* strains were previously isolated from cosmetics and cosmetic raw materials commonly used in cosmetic production. Their ability of biofilm formation on medical equipment may be a factor predisposing them to the settlement in clinical environments. All the tested strains were able to adhere to the catheter's surface even after a short period of time that leads to the formation of biofilm. The values of colony forming units which were considered as biofilm-creating cells fluctuated from 4.6 to 6.7 log CFU/cm^2 after 30 minute incubation, depending on the strain. The most adhesive strains, three *P. aeruginosa* (CFII, CFIVa, CFV) and two *S. liquefaciens* (CFIV, CFVII) strains, were isolated from the cosmetic products and the number of bacteria which adhered after 30 minutes was higher than 6 log CFU/cm^2 (Fig. 1E–G; I, J).

The adhesion process of bacteria to different surfaces depends on a large number of factors, such as hydrophobicity, cell-surface, charge, electron acceptor, donor properties, properties of attachment surfaces (roughness, physico-chemical stability, resistance to corrosion) and environmental conditions — pH, osmolarity, O_2, temperature, nutrient composition, presence of other bacteria (Briandet *et al.*, 1999; Giaouris *et al.*, 2014). As bacteria are negatively charged, their adherence should not be possible due to electrostatic repulsive force. Nevertheless, the presence of fimbriae, flagella and lipopolysaccharides make bacterial cells hydrophobic which reduces the negative interaction between surfaces (Shi & Zhu, 2009).

B. cepacia was considered as the weakest biofilm producer, and the mature biofilm (6.5 log CFU/cm^2) could be observed after 12 hours, which was confirmed by the decrease of the number of cells considered as biofilm-creating, and the increase of planktonic cells (Fig. 1H). A similar behaviour was noticed for *P. aeruginosa* ATCC 15442 strain. However, the number of bacteria in a biofilm structure was higher and reached 7.1 log CFU/cm^2 (Fig. 1A).

Release of the cells from the structure signifies that the biofilm reached a mature stage when the colonization is followed by disaggregation of the biofilm matrix and detachment of single and clustered cells (Francolini *et al.*, 2010). The greatest biofilm producers among the tested *P. aeruginosa* strains were DH1 and CFII, CFIVa and CFV capable of creating a biofilm at the level of 7 log CFU/cm^2 (Fig. 1B, E–G). What is more, the research showed a weaker ability to create biofilm by *P. aeruginosa* DH4 (6.7 log CFU/cm^2), Fig. 1D. The study proved that both *S. liquefaciens* strains also express the ability to colonize the polyvinyl chloride and styrene-acronitrile copolymer surfaces as efficiently as *P. aeruginosa*. The rate of attachment and colonization of these bacteria came up to 7.2 and 7.4 CFU/cm^2, for CFIV and CFVII strains, respectively, after 8-hours incubation, which characterizes these bacteria as strongly adherent microorganisms (Fig. 1I, J). Furthermore, the results indicated that *S. liquefaciens* did not create a mature biofilm after 24 hour incubation, as the disaggregation process was not observed.

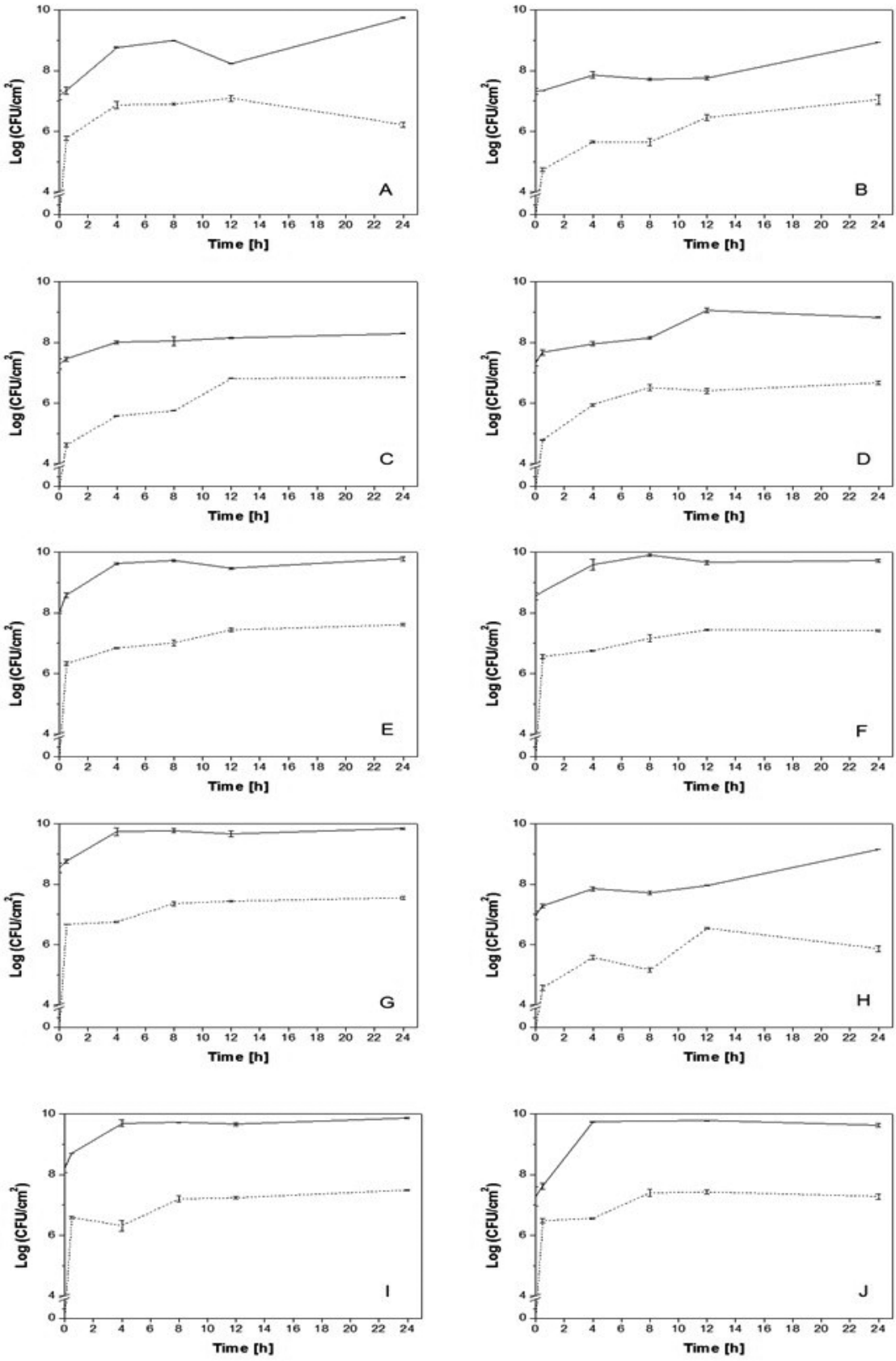

Figure 1. Colonization of the polyvinyl chloride and styrene-acronitrile copolymer suction catheter surface:
(**A**) *P. aeruginosa* ATC15442; (**B–G**) *P. aeruginosa* DH1, DH2, DH4, CFII, CFIVa, CFV; (**H**) *B. cepacia* DH3; (**I, J**) *S. liquefaciens* CFIV, CFVII; solid line — planktonic cells, dotted line — biofilm.

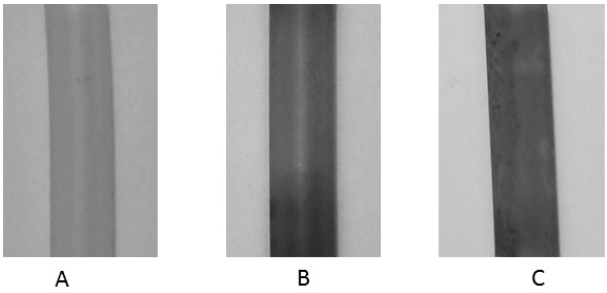

Figure 2. Biofilm formed on the polyvinyl chloride and styrene-acronitrile copolymer suction catheter's surface (safranin staining) after 30 minute incubation:
(**A**) the unstained catheter, (**B**) *P. aeruginosa* ATCC15442, (**C**) *P. aeruginosa* CFII

Analysis performed for the set of the tested strains showed statistically significant differences ($p < 0.05$) in biofilm formation between all the strains, up to 8 hours of incubation, and for planktonic cells in a course of the experiment (up to 24 hours). For the strains isolated from cosmetics (CF), no statistically significant differences in biofilm level, from 12 to 24 hours of contact with the catheter, were observed.

Taking into account the bacteria's ability of catheter surface colonization within the period of 30 min – 8 hours, the quantitative scale was proposed: fast and strong biofilm formers: 6–7.5 log CFU/cm^2, moderate biofilm formers: 5–7 log CFU/cm^2, slow and weak biofilm formers: 4.5–6.5 log CFU/cm^2, respectively. According to this scale, the tested bacteria can be classified as follow: fast and strong biofilm formers — *P. aeruginosa* CFII, CFIVa, CFV and *S. liquefaciens* CFIV, CFVII; moderate biofilm former — *P. aeruginosa* ATCC 15442; slow and weak biofilm formers — *B. cepacia* DH3, *P. aeruginosa* DH1, DH2, DH4. The differences in the dynamics of biofilm formation by *Pseudomonas* and Pseudomonads may be attributed to both strains' abilities and the environmental conditions (Rasamiravaka *et al.*, 2015). According to the literature data, the important determinants for the stability of the biofilm structure are polysaccharides in the biofilm matrix (Qing & Luyan, 2013; Mann & Wozniak, 2012), and bacterial motility (Harmsen *et al.*, 2010; Klausen *et al.*, 2003). Bacterial strains may substantially differ in EPS composition. Especially the wild-type biofilms are characterized by dynamic changes due to extensive cell motility, competition and selection occurring during biofilm development (Klausen *et al.*, 2003).

Figure 2 illustrates the biofilm formed by *P. aeruginosa* ATCC 15442 and the environmental isolate *P. aeruginosa* CFII.

Colonization of catheters, stents and other medical equipment by opportunistic pathogens is a huge challenge for medicine. The most frequent nosocomial infections are urinary tract and respiratory tract infections, which are closely related with using medical devices. According to the data of European Centre for Disease Prevention and Control, approximately 4.1 mln patients are estimated to acquire a healthcare-associated infection in the EU each year. These infections are believed to contribute to additional 110 000 deaths each year. In relation to the data from 2010, *P. aeruginosa* Gram-negative rods are reported to be responsible for 17.5% of pneumonia cases, 7.4% of bloodstream infections and 14.2% of urinary tract infections (http://www.ecdc.europa.eu).

The suction catheters used in the experiment are intend for oro-, nasopharyngeal, tracheobronchial suctioning and also for observation of mucus (http://www.convatec.com.au/). They are attached to the intubation tube, and therefore their exposure to the human body depends on the intubation time, which sometimes may take ten days. The Intensive Care Unit patients are exposed to the risk of nosocomial infections due to the fact that the intubation devices can be colonized by opportunistic pathogens circulating between the patients and the environment. The endotracheal aspirates examined by Vasanthi *et al.* (2104) contained predominantly Gram-negative rods, and approximately 27% of isolates were identified as *P. aeruginosa.*

Various studies had determined *P. aeruginosa* and *S. liquefaciens* as good biofilm producers. Fu and coworkers (2010) observed in their study the formation of *Pseudomonas aeruginosa* biofilm on the silicone surfaces (Foley catheters), noticing a high value of biofilm cells (6 log CFU/cm^2) after 4 hours of catheters' exposure to the bacteria, which is similar to the results obtained by us. Regarding *S. liquefaciens*, Xu *et al.* (2011) discovered that this bacterium has the strongest ability to adhere to polystyrene, when compared to other examined strains. Ghasemian and coworkers (2015) investigated the development of biofilm on the glass and stainless steel surfaces by *P. aeruginosa*. For both materials the level of biofilm cells increased to 5.7 log CFU/cm^2 after 8 hour incubation. All of these results confirm that opportunistic pathogens, such as *P. aeruginosa* and *S. liquefaciens,* are able to colonize abiotic surfaces. The ability to develop biofilms on polyvinyl chloride and styrene-acronitrile copolymer has never been reported before, in contrast to silicone and polypropylene materials described the most frequently. The researchers are still looking for the best and the most efficient method to prevent the biofilm growth on the medical devices. Generally, the methods can be divided into two categories: (i) a change of material composition, (ii) related to the coating the material with different substances (antibiotics, polymer, plant extracts, human serum albumin, and impregnation) or other biofilm-resistant materials (Wang *et al.*, 2014). Kim and coworkers (2015) proved the effectiveness of cinnamon bark oil and cinnamaldehyde against *P. aeruginosa* by coating PLGA-poly(lactic-co-glycolic acid) with those substances.

Overall, Gram-negative rods *P. aeruginosa*, *B. cepacia* and *S. liquefaciens* express capability of colonizing polyvinyl chloride and styrene-acronitrile copolymer surface even within 30 minutes of contact with this abiotic surface. The strains isolated from the cosmetic products and raw materials, circulating between the patient and the environment, may pose a threat for humans also in the clinical surroundings. It is important to focus on environmental strains, as the danger already occurs during the production process and subsequently in the finished cosmetic products. According to the ISO standard from 2014 (PN-EN ISO 17516: 2014-11), the presence of *P. aeruginosa* (as well as *Candida albicans* and *S. aureus*) is not allowed in 1 g of the cosmetic product. As the research showed, not only *P. aeruginosa* is a threat to the cosmetic users. *B. cepacia* and *S. liquefaciens,* known better from the food environment, were able to form biofilm structures with as high extent as *P. aeruginosa.*

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