MINIREVIEW

Pseudomonas biofilms: possibilities of their control

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Abstract

Genus *Pseudomonas* includes a large number of species that can be encountered in biotechnological processes as well as in the role of serious human or plant pathogens. Pseudomonads easily form biofilms on various types of surfaces. The biofilm phenotype is characterized by an increased resistance to environmental influences including resistance to antibiotics and other disinfectants, causing a number of problems in health care, food industry, and other areas. Considerable attention is therefore paid to the possibilities of eradication/destruction of pseudomonads biofilms both in terms of understanding the mechanisms of biofilm formation and at the level of finding suitable antibiofilm tools applicable in practice. The first part of this review is devoted to an overview of the regulatory mechanisms that are directly or indirectly involved in the formation of biofilm. The most effective approaches to suppressing the formation of biofilm that do not cause the development of resistance are based on the application of substances that interfere with the regulatory molecules or block the appropriate regulatory mechanisms involved in biofilm development by the cells. Pseudomonads biofilm formation is, similar to other microorganisms, a sophisticated process with many regulatory elements. The suppression of this process therefore also requires multiple antibiofilm tools.

Introduction

Biofilm is a structured microbial community and a quite natural form of growth of most microorganisms. Adhesion of microbial cells, subsequent biofilm formation, and maturation of its structure are connected with changes in cell phenotype, which is usually manifested by new metabolic pathways, enhanced resistance to toxic compounds and, in the case of pathogens, increased virulence (Watnick & Kolter, 2000). Biofilm formation is often a very rapid process, which has always several phases that include conditioning of carrier surface, reversible binding of the cells (capture of cells by nonspecific physicochemical forces emerging between the surface of the cells and the surface of the carrier), irreversible binding (firm anchor of cells on the surface with the participation of binding molecules – adhesins, e.g. protein LapA in the case of *Pseudomonas fluorescens*), development of microcolonies, and formation and maturation of biofilm architecture (Nikolaev & Plakunov, 2007). Active participation of microbial cells is essential for the adhesion process. Production of extracellular polymeric substances (EPS) is of considerable importance for the construction of biofilm architecture (Kreft & Wimpenny, 2001), and surface structures such as pili and flagella also come into play (Mandlik *et al.*, 2008). All of these phenomena require a significant change in the physiology of cells leading to biofilm phenotype. The essence of control mechanisms of these changes is chemical signaling, which in the case of biofilm formation is usually realized on the quorum-sensing principle. The best-studied *Pseudomonas aeruginosa* possesses three, mutually connected, QS systems. The two use acyl homoserine lactones and the third uses a quinolone signaling molecule (Jakobsen *et al.*, 2013). Detachment of individual cells or entire blocks of biofilm can be caused by environmental conditions (shear, chemical agents, etc.) or is subject to control of microbial populations alone.

Many of the numerous species of genus *Pseudomonas* are used in biotechnology. With regard to the ability to form biofilms, most attention has long been given to *P. aeruginosa*, an opportunistic human pathogen. For instance, biofilms formed by mucoid strains, wherein the polysaccharide

alginate is an important component of the biofilm matrix (see below), may be fatal for patients with cystic fibrosis. Also, other *Pseudomonas* species take advantage of biofilm growth, which generally increases their resistance to various negative environmental influences and enables them to inhabit a broad range of niches and colonize the soil matrix, plant tissue, etc.

Control mechanisms of the cells involved in the formation of *Pseudomonas* biofilms

A number of regulatory mechanisms involved in the formation of *Pseudomonas* biofilms are currently known. It has been shown that two-component regulatory systems, quorum sensing, cyclic di-GMP signaling, and sigma factors can participate in the control of biofilm processes (Heydorn *et al.*, 2000; de Kievit, 2009; Mikkelsen *et al.*, 2011). Two-component systems allow organisms to constantly evaluate environmental conditions and induce responses which can, for example, start biofilm growth. These systems generally consist of a sensor kinase and a response regulator. Rodrigue *et al.* (2000) found that the genome of *P. aeruginosa* PAO1 contains genes for 127 two-component systems. Quorum sensing, which is responsible for cell-to-cell communication, is also a highly important mechanism involved in cell adhesion and biofilm formation. *Pseudomonas aeruginosa* possesses two quorum-sensing systems based on acyl homoserine lactone (AHL) molecules (de Kievit, 2009). AHL synthase LasI synthesizes N-(3-oxododecanoyl)-L-homoserine lactone (Gambello & Iglewski, 1991), and RhlI AHL synthase synthesizes N-butyryl-L-homoserine lactone (Ochsner *et al.*, 1994). The former compound emerged as a key factor in the maturation and differentiation of biofilm (Davies *et al.*, 1998), and the latter is responsible for the production of biosurfactants – rhamnolipids (Pearson *et al.*, 1997). Rhamnolipids are necessary for maintaining an open channel system in *Pseudomonas* biofilm (Davey *et al.*, 2003). Pesci *et al.* (1999) found another signaling molecule, 2-heptyl-3-hydroxy-4-(1H)-quinolone, referred to as *Pseudomonas* quinolone signal, which participates in cell-to-cell communication of *P. aeruginosa*. Jakobsen *et al.* (2013) in their comprehensive review summarized the current knowledge about the function of quorum sensing in *P. aeruginosa* and state that, depending on growth conditions, 4–6% of genes are subject to control by quorum sensing. These genes are responsible for the synthesis of virulence factors (proteases, elastase, siderophores, rhamnolipids) and are involved in swarming motility and iron metabolism.

The control of *Pseudomonas* biofilm formation often involves a second messenger, cyclic di-GMP. High intracellular levels of cyclic di-GMP evoke the processes leading to the production of biofilm matrix, while a decrease in its level causes an increase in cell motility and transition into the planktonic forms of growth. Borlee *et al.* (2010) confirmed the earlier findings that *P. aeruginosa* uses cyclic di-GMP for positive regulation of the production of polysaccharides Pel and Psl, which play an irreplaceable role in the structure of the biofilm. They also found that the cyclic di-GMP is involved in the synthesis of *P. aeruginosa* adhesin CdrA. Extracellular CdrA could act as a cross-linker of Psl strands within the matrix. Alternatively, cell-associated CdrA may serve to anchor the cells to Psl in the matrix. Alg44 is an important protein involved in the synthesis of alginate, a polysaccharide produced by mucoid *P. aeruginosa*, which requires for its activity binding to cyclic di-GMP (Merighi *et al.*, 2007). Ueda & Wood (2009) showed that *P. aeruginosa* tyrosine phosphatase TpbA forms the connection between the quorum-sensing regulatory mechanism and intracellular level of cyclic di-GMP, which consequently affects the production of Pel polysaccharides and biofilm formation.

Sigma factors are important regulatory molecules that engage in a general stress response. There is indirect evidence that sigma factor RpoS is included in biofilm growth control of *P. aeruginosa* because a *rpoS* mutant formed significantly thicker biofilms than the wild-type strain (Heydorn *et al.*, 2000). RpoS is to a greater extent produced in the stationary phase of planktonic *P. aeruginosa* growth, which leads to increased resistance to certain stress factors. On the other hand, the *rpoS* mutant induced the twitching-motility phenotype, which indicates that type IV fimbriae were affected (Suh *et al.*, 1999). Xu *et al.* (2001) compared the level of RpoS in a stationary-phase planktonic population of *P. aeruginosa* to that of a continuously fed biofilm population and they found similar levels of the sigma factor. Growing biofilm thus exhibited some characteristics of the stationary phase, which could be related to the high resistance of the *Pseudomonas* biofilm.

The mechanisms involved in the regulation of adhesion and biofilm formation can be specifically affected by the modification of corresponding genes. Genetically modified strains are often used in explaining the effects of various tools (antibiotics, compounds interfering with signal molecules, etc.) that show the ability to influence the process of biofilm formation (Friedman & Kolter, 2004; Vallet *et al.*, 2004; Sadovskaya *et al.*, 2010).

The following text is devoted to various approaches used to regulate/suppress *Pseudomonas* biofilm formation. As described above, biofilm formation is a targeted process driven by sophisticated regulatory mechanisms, both at the single-cell level and at the level of cell populations. Biofilm can be affected (eradicated) without intervention in the system of regulatory mechanisms of its formation,

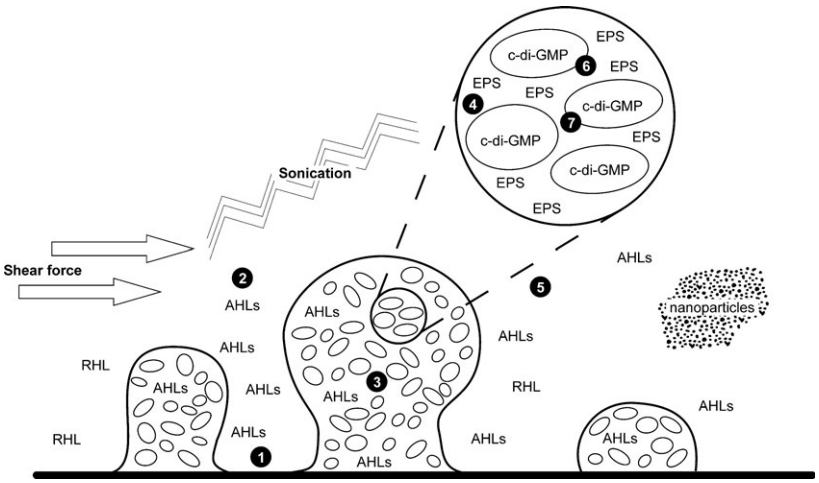

Fig. 1. The main targets and some potential tools (in parentheses) to modify (eradicate) *Pseudomonas* biofilm. ❶ – surface properties of biofilm carrier (modification of roughness, hydrophobicity, charge, cold plasma application, surface conditioning); ❷ – mechanical stability of biofilm (liquid flow rate, shear force); ❸ – biofilm architecture (nutrient composition, modification of rhamnolipid concentration, modification of alginate concentration, application of ultrasound, surfactants, or nanoparticles); ❹ – EPS (application of hydrolytic enzymes, surface active compounds, natural products); ❺ – quorum sensing (application of compounds interfering with the synthesis and activities of AHLs and other regulation molecules); ❻ – cyclic di-GMP (application of compounds interfering with its biosynthesis and activity) ❼ cell viability (application of antibiotics, disinfectants).

that is, by direct disruption of existing bonds and structures in the biofilm (mechanical destruction, modification of the carrier surface, physicochemical properties of the surrounding environment), as well as by influencing the regulatory mechanisms by substances with appropriate biological activity, that is, systemically (Fig. 1).

Biologically nonsystemic eradication of *Pseudomonas* biofilm

There are relatively effective procedures to influence microbial biofilms, regardless of the control mechanisms that microorganisms use for their formation. In this context, attention is being directed to the properties of potential biofilm carriers, on the use of tools or physical processes for mechanical removal of an existing biofilm, or on application of chemicals which more or less nonspecifically prevent the biofilm formation or cause its eradication. Biofilms are relatively very resistant to mechanical destruction. They also often show surprising resistance to aggressive chemicals. Simple scraping of biofilm from a carrier is therefore ineffective (Bjerkan *et al.*, 2009).

Lieleg *et al.* (2011) conducted an extensive study of mechanical resistance of *Pseudomonas* biofilms. They found that different species have the ability to effectively repair *Pseudomonas* biofilms after mechanical damage. The viscoelasticity of these biofilms has not been changed over a wide range of mechanical factors or by treatment with various chemical compounds. Only trivalent ions and citric acid significantly affected the biofilm elasticity, the first of which also altered the texture of the biofilm material.

Chen & Stewart (2000) examined biofilm removal by chemical agents, including the surfactants SDS, Tween, and Triton. The biofilm protein removal and cell viability reduction were not found to be very efficient when pure agents were used; the best results were obtained by combining SDS with the antimicrobial agent chloramphenicol (91% protein removal and 97% cell viability reduction).

Oxygen glow discharge plasma treatment is one of the possible applications of plasma-based methods employed to modify polymer surfaces to prevent cell attachment (Øiseth *et al.*, 2002). Triandafillu *et al.* (2003) reported an *in vitro* study of adhesion of *P. aeruginosa* clinical isolates onto native and oxygen glow discharge plasma-treated poly(vinyl chloride) material from endotracheal intubation devices. Oxygen plasma treatment of the poly (vinyl chloride) yielded a hydrophilic surface and reduced the number of adhering bacteria by as much as 70%. The authors, however, report that this reduction is unlikely to be sufficient to prevent *P. aeruginosa* colonization of endotracheal intubation devices (Triandafillu *et al.*, 2003).

The application of ultrasound to disrupt *Pseudomonas* biofilm was studied on sessile bacteria in biofilms of *P. aeruginosa* on a polyethylene substrate (Qian *et al.*, 1997). In this study, ultrasound effect at frequencies of 70 kHz, 500 kHz, 2.25 MHz, and 10 MHz was evaluated and biofilm viability was measured. The results indicate that although ultrasound by itself did not have any effect in reducing bacterial viability within the biofilm, a

significantly greater fraction of the cells was inhibited when they were subjected to ultrasound after exposure to 12 μg mL^{-1} gentamicin sulfate. Interestingly, lower frequencies were found to be significantly more effective than higher frequencies.

Photodynamic antimicrobial chemotherapy is a method designed to target sessile growing cells. The principle of this method is the action of reactive oxygen species generated by the photoactivation of a photosensitizer by a light source. Donnelly *et al.* (2007) studied biofilm eradication in *P. aeruginosa* clinical isolates by two photosensitizers (toluidine blue and meso-tetra-N-methyl-4-pyridyl-porphine-tetratosylate) in combination with red light (635 nm). The results of this study show that this method is capable of achieving high levels of kill of clinical *P. aeruginosa* isolates grown both planktonically and in biofilm. Although drug and light diffusion through mucus were impaired, sufficient light and concentrations of both drugs crossed a relatively thick mucus barrier to yield photodynamically effective concentrations in the receiver compartment. Photodynamic antimicrobial chemotherapy may therefore be a suitable option for the treatment of *P. aeruginosa* infection, either alone or in combination with conventional antibiotics or other antimicrobial agents (Donnelly *et al.*, 2007).

As the mature biofilm removal strategies were found to be insufficient or are inapplicable in implant treatments, other strategies for the control of microbial biofilm are being developed. These involve surface modification, for example incorporation of antimicrobial agents to produce an intrinsically bactericidal surface or the design of antimicrobials that are targeted at biofilm cells (high-diffusion–reaction molecules, agents affecting nongrowing cells).

Wood *et al.* (1998) reported a surface catalysis approach that involves catalytic generation of biocidal species from a less active treatment agent due to the presence of catalysts incorporated into the surface on which the biofilm is formed. The strategy was reported to overcome the barrier that biofilm matrix poses for the access of antimicrobial, commonly associated with resistance of biofilms to cleaning strategies. The catalysts – transition metals – incorporated into polymers enabled the reaction of persulfates yielding reactive oxygen species. When *P. aeruginosa* biofilms were exposed to dilute solutions of potassium monopersulfate, significant reduction in cell viability was observed in catalyst-containing surfaces over that of controls. Laser confocal microscopy fluorescence probes and viability staining provided evidence that reactive species were generated at the biofilm–material boundary and the cell viability reduction was a direct result of the generation of free radicals in this region (Wood *et al.*, 1998).

Development of the production of nanomaterials and a continually growing understanding of their characteristics show their considerable potential also in the modulation of biofilm formation and stability. Nanomaterials tend to have quite different properties than particles of chemically the same material, but with dimensions differing by several orders of magnitude. The actual dimensions of the nanoparticles as well as their chemical activity allow them to enter into extensive interactions with individual microbial cells and biofilm matrix.

Here are a few examples of various types of nanoparticles which make it possible to suppress *Pseudomonas* biofilm. Frequently used materials are nanoparticles based on silver. Lungu *et al.* (2013) have prepared nearly spherical nanocomposite particles 15 and 30 nm in size, consisting of TiO_2 nanoparticles with different concentrations of deposited silver nanoparticles. A higher proportion of silver provides the material with increased antimicrobial activity. High antibiofilm activity was confirmed for a number of Gram-positive and Gram-negative microorganisms including *P. aeruginosa*. Biofilm of *P. aeruginosa* NCIM 2948 was suppressed by another type of nanoparticles containing silver, namely Ag-nanoparticles embedded in orthorhombic nanotubes of lithium vanadium oxides (LiV_2O_5/Ag) 40–50 nm in diameter and a length of 5–10 μm (Diggikar *et al.*, 2013). Similarly, Naik & Kowshik (2014) prepared sol-gel coatings AgCl-TiO_2, which effectively prevented the initial adhesion of microorganisms (including *P. aeruginosa*) for 10 days through a gradual release of silver particles. Tseng *et al.* (2013) created nanohybrids of silver particles on nanoscale silicate platelets. They used the fact that silicate platelets have an enormous surface area and high anionic charge, which increases their ability to attract polar organic substances and, because of the composition of cell surfaces, also microorganisms. This nanomaterial released the cells and the extracellular matrix from the surface layer of biofilm and prevented the subsequent re-colonization of the surfaces. Antibiofilm activity against *Pseudomonas* has also been demonstrated *in vivo* on mouse corneas. de Faria *et al.* (2014) decided to capture silver on graphene oxide sheets. This method allowed them to obtain Ag particles with a size of 7.5 nm on the surface of graphene oxide. The material so obtained had a strong biocidal activity and was able to inhibit the growth of adherent cells of *P. aeruginosa* and thus prevent the process of biofilm formation.

Similar to silver, bismuth also exhibits a strong antimicrobial property. Badireddy *et al.* (2013) prepared lipophilic bismuth dithiopropanol nanoparticles having a rhombohedral crystal structure with a size of approximately 18 nm. At the minimum inhibitory concentration of 12.5 μM, the particles can totally inhibit the growth of *P. aeruginosa* for 30 days and also lyse the cells captured in biofilm. The mechanism of action is based on the fact that the lipophilic nanoparticles are transported into the

cell envelope, wherein the nanoparticles are dissolved and bismuth ions react with thiol groups of key respiratory enzymes which they inactivate. Very effective growth inhibitors with antibiofilm properties also belong to another type of lipophilic nanoparticles. These are solid lipid nanoparticles which contain stearic acid, lauric acid, and oleic acid in various proportions. The advantage of these materials is also that they contain only those compounds which are naturally present in human tissues (Taylor *et al.*, 2014).

The modification of surface characteristics to prevent biofilm formation including nanoscale topographical alterations has been studied by Puckett *et al.* (2010). The relationship between the nanostructure of titanium surfaces and bacterial attachment was investigated and certain nanometer-sized titanium topographies were proven to reduce bacterial adhesion. *Pseudomonas aeruginosa* adhesion to conventional titanium, nanorough titanium produced by electron beam evaporation, and nanotubular and nanotextured titanium produced by two different anodization processes was studied. The surface compositions were similar for the conventional and nanorough titanium, therefore ensuring only a difference in surface roughness and configuration. Of all of the nanomodifications, the nanorough surfaces produced by electron beam evaporation decreased the microbial adherence the most.

Substances interfering with control mechanisms of biofilm formation

Targeted interference with the control of cellular mechanisms involved in adhesion and formation of complex biofilm structures inherently allows one to obtain a more efficient control over the biofilm formation than in the case of nonsystem interventions (processes not interfering with regulatory mechanisms – see previous chapter). In this area, a considerable importance was often proved of various groups of natural substances or their synthetically prepared analogues (Table 1). A number of these substances do not have antibiotic activity and microorganisms do not readily create resistance toward them. The antibiofilm effect is then usually long term (Sharma *et al.*, 2014). The fact that these compounds are usually nontoxic to eukaryotic organisms offers a wide range of applications in medicine, food, and other industries. In some cases, the exact target of action of these compounds in controlling biofilm formation is known; sometimes only the final effect is understood.

Pseudomonas biofilm matrix is composed of various polymeric substances, polysaccharides (Pel, Psl, and alginate – in mucoid strains) and proteins forming a significant part. Hydrolytic enzymes can disrupt this structure. Many fungi produce a broad spectrum of hydrolytic enzymes capable of degrading various polysaccharide structures. Enzymes produced by *Trichoderma viride* cultured in a medium with pectin, or enzymes from *Aspergillus niger* grown in a medium with arabic gum, cause effective destruction of *P. fluorescens* biofilm (Orgaz *et al.*, 2006). Alkawash *et al.* (2006) reported that alginate lyase enhances the killing effect of antibiotics against mucoid *P. aeruginosa*. These findings were not confirmed in the more recent work of Lampp & Griswold (2013), who compared the biofilm disruption effect of alginate lyase in combination with either tobramycin or the commodity protein bovine serum albumin or simple amino acids and found the dispersion of *P. aeruginosa* biofilms equivalent in all cases. A novel AF phage infecting *P. putida* has been described in connection with studies on the utilization of phage therapy. This phage has an EPS-degrading activity and can play an important role in emphasizing biofilm degradation (Cornelissen *et al.*, 2012). DNA is another component of the extracellular matrix. Allesen-Holm *et al.* (2006) stated that extracellular DNA functions as a cell-to-cell interconnecting matrix component in *P. aeruginosa* biofilms. With experiments with mutant strains, they confirmed that the extracellular DNA is generated via lysis of a subpopulation of the bacteria. They suggested that the extracellular DNA found in *P. aeruginosa* biofilms is generated via two different pathways. A basal level of extracellular DNA present in biofilms is generated via a pathway which is not linked to quorum sensing. The increased amount of extracellular DNA found in *P. aeruginosa* wild-type biofilms in comparison with lasIrhlI biofilms provides an evidence of a dependence on quorum sensing. Application of DNase-I can reduce biofilm formation in the lungs of patients with cystic fibrosis and thus prevent chronic *P. aeruginosa* infection (Whitchurch *et al.*, 2002).

Iron concentration in the external environment affects the activity of type IV pili and *P. aeruginosa* twitching motility. A decrease in the concentration of free iron, for example, by chelation to lactoferrin, a ubiquitous and abundant constituent of human external secretions, brings stimulation of twitching and suppression of biofilm formation (Singh *et al.*, 2002). Musk *et al.* (2005) found that too high concentrations of iron also resulted in an inhibition of biofilm formation. In correlation with the findings of Singh *et al.* (2002) mentioned above, they indicated that the optimal concentration of iron for the formation of *P. aeruginosa* biofilm is in the range of about 1–100 μM.

Microorganisms can produce simple molecules that inhibit the formation or decompose existing biofilm of other microorganisms (including *P. aeruginosa*). Production of a mixture of D-Leu, D-Met, D-Tyr, and D-Trp at nanomolar concentrations by *Bacillus subtilis* causes the

Table 1. Natural substances or their synthetically prepared analogues with antibiofilm or biofilm-promoting activity (arranged alphabetically by author)

Compounds or mixtures of compounds	Action	References
Aqueous extracts *Conocarpus erectus, Chamaesyce hypericifolia, Callistemon viminalis, Bucida buceras, Tetrazygia bicolor*, and *Quercus virginiana*	Inhibition of LasA protease, LasB elastase, pyoverdin production, and biofilm formation	Adonizio *et al.* (2008)
Alginate lyase + gentamicin or ceftazidime	Elimination of mucoid bacteria from biofilms	Alkawash *et al.* (2006)
Azathioprine	Inhibition of 5-aminoimidazole-4-carboxamide ribotide transformylase, cyclic diguanylate	Antoniani *et al.* (2013)
Anacardic acids from *Amphipterygium adstringens*	Inhibition of pyocyanin, rhamnolipid, and violecin production	Castillo-Juárez *et al.* (2013)
Aztreonam, ceftazidime, cefoperazone, cefepim, netilmicin, amikacin, ofloxacin, and ciprofloxacin	Activity against biofilms	Cernohorska & Votava (2008)
ε-Viniferin	Inhibition of biofilm formation	Cho *et al.* (2013)
Water extracts of rhubarb, Fructus Gardeniae, *Andrographis paniculata*	Anti-quorum-sensing activity	Chu *et al.* (2013)
Essential oils (cinnamon, tea tree, palmarosa)	Decrease in biofilm biomass and the number of biofilm-entrapped cells	Coelho & Pereira (2013)
5,11,17,23-Tetrakis-[2(α-L-C-fucosyl)acetyl-glycylamido]-25,26,27,28-tetra-propoxycalix[4]arene	Fucose–carbohydrate (biofilm) interactions	Consoli *et al.* (2011)
Desmethylyangonine-4′-O-(6′′-O-(3-hydroxy-3-methyl)glutaryl)-β-D-glucopyranoside, 7-O-β-D-(6′-O-malonyl)-glucopyranosyl-5-methoxy-1(3H)-isobenzofuranone, etc.	Antibiofilm activity	D'Abrosca *et al.* (2013)
Oligomers of N,N′-(6-(((2S,3R,4R,5R,6R)-3-acetyl-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)amino)-6-oxohexane-1,5-diyl) dipalmitamide, etc.	Promotion and inhibition of biofilm formation	Dane *et al.* (2014)
cis-2-Dodecenoic acid	Decrease in the production of quorum-sensing signals	Deng *et al.* (2013)
Rhein, chrysophanol, nodakenetin, shikonin, emodin, fraxin	Inhibition of biofilm formation	Ding *et al.* (2011)
Triclosan, benzalkonium chloride, chlorhexidine dichloride	Modification of morphology and architecture of biofilm	Dynes *et al.* (2009)
Garlic, resveratrol, erythromycin, azithromycin, clarithromycin, spiramycin	Quorum-sensing inhibition	Fulghesu *et al.* (2007)
(Z)-5-(Bromomethylene)furan-2(5H)-one and similar compounds	Antagonist of N-acyl homoserine lactones	Hentzer *et al.* (2002)
Chlorhexidine digluconate, polyhexamethylene biguanide	Reduction in the total amount of biofilm, the bacterial metabolism in biofilms, count of colony-forming units	Hübner *et al.* (2010)
Clove oil	Reduction in the production of protease, chitinase, pyocyanin, exopolysaccharide	Husain *et al.* (2013)
Natural phenol compounds (e.g. anacardic acid, cardanol, epigallocatechin, tannic acid)	Antibiofouling effect on biofilm formation	Jagani *et al.* (2009)
Secomanoalide, sulforaphane, erucin, ajoene, rosmarinic acid, naringin, morin, baicalin, chlorogenic acid, manoalide, azithromycin, ceftazidime, ciprofloxacin, niclosamide, nifuroxazide, chlorzoxazone, salicylic acid, (Z)-5-(Bromomethylene)furan-2(5H)-one, (Z)-4-bromo-5-(bromomethylene)furan-2(5H)-one, (R)-10-isothiocyanato-3-oxo-N-(2-oxotetrahydrofuran-3-yl)decanamide, (R)-10-isothiocyanato-3-oxo-N-(2-oxotetrahydrofuran-3-yl) decanamide, 4-(((2-nitrophenyl)sulfonyl)methyl)-N-(thiazol-2-yl)benzenesulfonamide, etc.	Quorum-sensing inhibitors	Jakobsen *et al.* (2013)
Pseudofactin II	Lowering of adhesion to surfaces	Janek *et al.* (2012)
Limonene-carvone, citral, α-pinene, β-pinene, cineol, α-zingiberene, pulegone	Inhibition of short-chain acyl homoserine lactones	Jaramillo-Colorado *et al.* (2012)
Bacillus subtilis factor (mixture of D-Leu, D-Met, D-Tyr, D-Tyr)	Prevention of biofilm formation (D-alanine blocks biofilm formation by the replacement of alanine in the peptide side chain)	Kolodkin-Gal *et al.* (2010)
Norspermidine	Direct and specific interaction with exopolysaccharide	Kolodkin-Gal *et al.* (2012)
Norfloxacin	Increase in biofilm formation	Kumar & Ting (2013)

Table 1. Continued

Compounds or mixtures of compounds	Action	References
Zingerone	Inhibition and eradication of biofilm	Kumar *et al.* (2013)
3-Indolylacetonitrile	Decrease in the production of 2-heptyl-3-hydroxy-4(1H)-quinolone, pyocyanin, and pyoverdine	Lee *et al.* (2011)
Resveratrol, ε-viniferin, suffruticosol A, B, vitisin A, B	Inhibition of biofilm formation	Lee *et al.* (2013)
Ambroxol	Reduction in the production of guanosine diphospho-D-mannose dehydrogenase (alginate)	Li *et al.* (2008)
Sansanmycin, gentamicin, carbenicillin, polymyxin B, roxithromycin, piperacillin, and tazobactam	Inhibition of the generation of biofilms and proliferation of bacteria	Li *et al.* (2009)
4-Bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone (fimbrolide)	Inhibition of quorum sensing (homoserine lactones)	Liu *et al.* (2010)
Ambroxol	Reduction in alginate production by undefined mechanisms	Lu *et al.* (2010)
(S)-2-([1,1′-Biphenyl]-4-yl)-N-(2-oxotetrahydrofuran-3-yl)acetamide, etc.	Activation and inhibition of quorum-sensing control repressor	Mattmann *et al.* (2011)
Pyocyanin, phenazine methosulfate, phenazine-1-carboxylate, and methylene blue	Prevention of formation of structured colony biofilms by the inhibition of respiration	Morales *et al.* (2013)
Andrographis paniculata methanol extract (e.g. methyl acetyllithofellate, 4-[(E)-3-hydroxyprop-1-enyl]-2-methoxyphenol)	Antibiofilm activity on quorum sensing	Murugan *et al.* (2013)
Ferric ammonium citrate and other iron salts	Inhibition of biofilm production	Musk *et al.* (2005)
Glycosides of soyasapogenol B	Inhibition of diguanylate cyclase	Ohana *et al.* (1998)
Chlorolactone, chloro-thiolactone, meta-chloro-thiolactone, meta-bromo-thiolactone	Inhibition of the production of pyocyanin and biofilm formation	O'Loughlin *et al.* (2013)
Proteinase, cellulase, pectinesterase, pectin lyase, alginate lyase from *Aspergillus niger, Trichoderma viride,* and *Penicillium* spp.	Biofilm removal	Orgaz *et al.* (2006)
Laherradurin	Increase in biofilm production	Parellada *et al.* (2013)
4-Hydroxybenzoic, gallic, cinnamic, sinapic, ferulic, chlorogenic acids	Increase in biofilm formation	Plyuta *et al.* (2013a)
Salicylic, indole-3-acetic, gibberellic, abscisic acids	Stimulation of biofilm formation	Plyuta *et al.* (2013b)
Phenazines	Influence on swarming motility	Ramos *et al.* (2010)
Di-3-N-(4-anilinophenyl)benzamide, di-4(R)-1-(benzylsulfonyl)-3-phenoxy-2-propanol, etc.	Inhibition of diguanylate cyclase and inhibition of biofilm formation	Sambanthamoorthy *et al.* (2012)
N′-((1E)-(4-ethoxy-3-[(8-oxo-1,5,6,8-tetrahydro-2H-1,5-methanopyrido[1,2-a][1,5]diazocin-3(4H)-yl)methyl]phenyl)methylene)-3,4,5-trihydroxybenzohydrazide, etc.	Inhibition of diguanylate cyclase and biofilm formation	Sambanthamoorthy *et al.* (2014)
Carvacrol, thymol	Adherence and biofilm formation	Soumya *et al.* (2011)
Coumarin-4-carboxylate ligands with Ag (e.g. 6-methoxycoumarin-4-carboxylic, 7-methoxycoumarin-4-carboxylic, 8-methoxycoumarin-4-carboxylic acids)	Antibacterial activity against *P. aeruginosa* grown as a biofilm	Sullivan *et al.* (2014)
Solid lipid nanoparticles from free fatty acids (lauric acid, oleic acid)	Multiple mechanisms at the nanoscale	Taylor *et al.* (2014)
Antibiotics (e.g. rifampicin, polymyxin B)	Destruction of biofilm	Toté *et al.* (2009)
Silver nanoparticles	Elimination of biofilms	Tseng *et al.* (2013)
Derivatives of uracil (e.g. 5-fluorouracil)	Repression of biofilm formation	Ueda *et al.* (2009)
Dianthus orientalis, Origanom majorana, Cyclamen coam extracts, essential oil of *Zataria multiflora*	Biofilm formation (the antibiofilm effect)	Varposhti *et al.* (2013)

Table 1. Continued

Compounds or mixtures of compounds	Action	References
Palmitoyl-DL-carnitine	Simulation of motility, inhibition of activity of the Las quorum-sensing system	Wenderska *et al.* (2011)
Ginseng aqueous extract	Formation of biofilm, destruction of mature biofilm	Wu *et al.* (2011)
Extracts from *Hemidesmus indicus, Holarrhena antidysenterica, Mangifera indica, Punica granatum, Psoralea corylifolia*	Inhibition of violacein production and swarming motility	Zahin *et al.* (2010)
Nitrofurazone, furazidin, nitrofurantoin, nifuroxazide	Enlargement of biofilm formation	Zaitseva *et al.* (2009)
Indirubin, ferulic acid, psoralen, esculetin, esculin, ephedrine, protocatechuic acid, baicalein, baicalin	Inhibition of biofilm formation	Zeng *et al.* (2008)

release of amyloid fibers, which connect the cells in the biofilm (Kolodkin-Gal *et al.*, 2010).

Pseudomonas aeruginosa uses two quorum-sensing molecules, N-(3-oxododecanoyl)-L-homoserine lactone and N-butanoyl-L-homoserine lactone, to control the production of extracellular virulence factors and biofilm formation. Substances affecting the functioning of quorum sensing are usually potent inhibitors of *Pseudomonas* biofilm formation. Such substances can be inhibitors of the *Pseudomonas* quorum-sensing receptors LasR and RhlR (O'Loughlin *et al.*, 2013). The first structures with proved interaction with quorum sensing, halogenated furanone compounds, were isolated from the Australian marine alga *Delisea pulchra* (Kjelleberg *et al.*, 1997). Natural furanones, however, do not have a significant antibiofilm activity against *Pseudomonas*. Hentzer *et al.* (2002) and Liu *et al.* (2010) have tested a synthetic brominated derivative of furanone capable of interfering with the AHL-mediated quorum sensing in *P. aeruginosa* and found them to have a significant inhibitory activity on the activation of quorum-sensing signal receptor by 3-oxododecanoyl-homoserine lactone.

Methanol extract of the herb *Andrographis paniculata* contains substances (e.g. the diterpenoid lactone andrographolide) that mimic *P. aeruginosa* quorum sensing and thus suppress the formation of biofilms (Murugan *et al.*, 2013). The newly synthesized derivative 14α-lipoyl andrographolide inhibits biofilm formation and increases the sensitivity of *P. aeruginosa* to many antibiotics (Zeng *et al.*, 2011). Similar effects have also been described for aqueous extracts from *Conocarpus erectus*, *Bucida buceras,* and *Callistemon viminalis*, plants from South Florida (Adonizio *et al.*, 2008), although the extracts themselves have a marginal effect on the growth of bacteria. Similarly, aqueous extracts of common fruits, herbs, and spices (raspberry, blueberry, blackberry, cranberry, grape, oregano, thyme, basil, kale, ginger, turmeric) significantly affect quorum sensing, which was demonstrated in the case of *P. aeruginosa* by the inhibition of swarming motility (Vattem *et al.*, 2007). Methyl eugenol contained in the methanolic extract of *Cuminum cyminum* is the main component interfering with AHL, leading to damage of the biofilm architecture and formation of new *P. aeruginosa* PAO1 biofilm *in vitro* (Sybiya Vasantha Packiavathy *et al.*, 2012). Clove oil even at subinhibitory concentrations reduces las- and rhl-regulated virulence factors, swimming motility, and EPS production by *P. aeruginosa* PAO1. The result is a reduction of biofilm formation (Husain *et al.*, 2013). Citral contained in the essential oil of the Colombian plant *Lippia alba* inhibits the long-chain quorum-sensing system in *P. putida* F117/pKRC12 (Jaramillo-Colorado *et al.*, 2012). The prophylactic role of garlic (*Allium sativum*) was evaluated as an alternative to

conventional antibiotics in the treatment of infections caused by biofilm-forming bacteria. Persson *et al.* (2005) found six sulfur compounds in a garlic extract, which were not toxic to bacteria but had a high activity toward the LuxR quorum-sensing system. Simultaneous application of the garlic extract and the antibiotic tobramycin enabled effective destruction of *P. aeruginosa* biofilm (Bjarnsholt *et al.*, 2005; Rasmussen *et al.*, 2005). Extracts of plants used in traditional medicine are also being carefully examined to see whether some of their components may interfere with quorum sensing and influence biofilm formation. The pharmaceutical plant *Amphipterygium astringents* known as 'cuachalalate' contains anacardic acids in hexane extracts. The mixture of anacardic acids has a strong anti-quorum-sensing activity and in the case of *P. aeruginosa*, besides inhibition of the formation of a virulence factor pyocyanin, it also inhibits the production of rhamnolipids, reduces the elastase activity (Castillo-Juárez *et al.*, 2013), and suppresses biofilm formation (Jagani *et al.*, 2009). Chusri *et al.* (2013) found that one of the plant extracts used in traditional Thai medicine (THR-SK010E) brought about a significant reduction in *P. aeruginosa* biofilm formation. After a 24-h treatment with THR-SK010E at 62.5 mg mL^{-1}, 97.3% of the preformed biofilms were destroyed.

Besides natural substances that interfere with the mechanisms of quorum sensing, synthetic molecules with similar activity are described with increasing frequency. O'Loughlin *et al.* (2013) have tested some 30 compounds and they found meta-bromo-thiolactone as the most effective inhibitor of *P. aeruginosa* quorum-sensing receptors LasR and RhlR, which blocked the expression of virulence factors and biofilm formation. Ambroxol [2-amino-3,5-dibromo-N-(trans-4-hydroxycyclohexyl) benzylamine] is used as an expectorant and a bronchosecretolytic in the treatment of bronchial asthma, chronic bronchitis and is associated with a number of other therapeutic effects (Stetinova *et al.*, 2004; Yamada *et al.*, 2009). Ambroxol has recently been found to have also a strong anti-quorum-sensing activity allowing significant inhibition of biofilm formation. Given that ambroxol is a commonly used substance in medicine, its use can be easily extended to support the treatment of cystic fibrosis and for reducing colonization of indwelling devices (Lu *et al.*, 2010).

There is relatively little information on the interference of natural substances or other compounds with the activity of the second messenger cyclic di-GMP, which would result in the suppression of adhesion of *P. aeruginosa*. The first substance that affects biofilm formation of Gram-negative bacteria and for which it has been assumed that this effect is due to inhibition of cyclic di-GMP biosynthesis was sulfathiazole (Antoniani *et al.*, 2010). One of the few other synthetic compounds, for which the inhibition of biofilm formation and synthesis of CDG was confirmed in *P. aeruginosa*, is N-(4-anilinophenyl) benzamide (Sambanthamoorthy *et al.*, 2014). Azathioprine immunosuppressant, an inhibitor of 5-aminoimidazole-4-carboxamide ribotide transformylase, is also able to inhibit the WspR protein, a *P. aeruginosa* enzyme synthesizing cyclic di-GMP – diguanylate cyclase (DGC; Antoniani *et al.*, 2013). However, the authors found that azathioprine failed to prevent the formation of *P. aeruginosa* biofilm. Sambanthamoorthy *et al.* (2014) very recently prepared four small molecules that inhibit DGCs and also inhibit biofilm formation of some bacteria including *P. aeruginosa*. In this case, urinary catheters were used as model biofilm carrier. The triterpenoid saponin isolated from the extracts of etiolated pea (*Pisum sativum*) shoots is a natural compound possessing strong inhibitory activity against purified DGC from *Acetobacter xylinum* (Ohana *et al.*, 1998). This compound could be a basis for finding active substances capable of reducing biofilm formation on the level of cyclic di-GMP regulation in other microorganisms.

The resulting effect of substances with antibiofilm activity can be further modified via their mode of application. Recently, processes were described that use nanoparticles as carriers of these compounds to the biofilm. Unlike microparticles, the nanoparticles may easily penetrate into the biofilm and thus exhibit greater efficiency. Cheow *et al.* (2011) engineered polymer–lipid hybrid nanoparticles [e.g. polymer – poly (lactic-co-glycolic acid); lipid – phosphatidylcholine] with encapsulated antibiotic levofloxacin. This material had a high antibacterial activity against 1-day-old biofilms of *P. aeruginosa*. The presence of lipid shell in the hybrid provides a slower release of antibiotics. Hybrid nanoparticles exhibit higher antibacterial activity against biofilm cells compared to liposomes.

It is logical that in addition to compounds with antibiofilm activity, compounds are also found that in turn stimulate the formation of biofilms. Such materials include phenolic compounds such as vanillin, epicatechin, 4-hydroxybenzoic, gallic, cinnamic, sinapic, ferulic, and chlorogenic acids. Plyuta *et al.* (2013a) demonstrated that these substances increased biofilm formation several times, which they attributed to an increase in the production of N-(3-oxododecanoyl)-L-homoserine lactone in *P. aeruginosa* PAO1 and thus interference with the Las-determined quorum sensing. The resulting interaction of certain substances with biofilm varies depending on the concentration. Plant hormones such as salicylic, indole-3-acetic, gibberellic, and abscisic acids at subinhibitory concentrations stimulate the formation of *P. aeruginosa* biofilms. After exceeding concentrations that more significantly inhibit the growth, however, they act as suppressors of biofilm formation (Plyuta *et al.*, 2013b). Also, subinhibitory concentrations of some antimicrobial agents based on nitrofuran, such as nitrofurazone, furazidin and nifuroxazide, nitrofurantoin (Zaitseva *et al.*, 2009), or norfloxacin

(Kumar & Ting, 2013), cause strengthening of *P. aeruginosa* biofilm formation. Linares *et al.* (2006) reported induction of biofilm formation and expression of determinants influencing the virulence by tobramycin, tetracycline, and norfloxacin at subinhibitory concentrations. The biofilm formation enhancement by subinhibitory concentrations of the aminoglycoside antibiotic tobramycin was tentatively linked to alterations in the level of cyclic di-GMP by Hoffman *et al.* (2005).

These findings point to the strategy of some pathogens to survive in the conditions of a gradual increase in the concentration of antibiotics during treatment. *Annona cherimola* seeds contain two annonaceous acetogenins squamocin and laherradurin. Parellada *et al.*, (2012) found that both of these substances stimulate *Pseudomonas plecoglossicida* J26 biofilm. These substances are indirectly involved in the higher production of AHLs because they are acting as stressors to which cells respond by increased production of autoinducer-1 from the group of acyl homoserine lactones (Parellada *et al.*, 2013).

A separate aspect of this issue is the situation in multispecies biofilms. In addition to the interference of biologically active compounds with the cell control mechanisms, an extensive interaction occurs between populations of different species that may result in stimulating the formation or eradicating such biofilms. A large number of original works have been published in this field, *P. aeruginosa* being often included in these multispecies biofilm experiments as an important opportunistic pathogen. This topic is very well summarized, for example, in the review articles of Elias & Banin (2012) or Rendueles & Ghigo (2012).

Conclusion/Outlook

Members of the genus *Pseudomonas* usually readily form biofilms. Except where these microorganisms are used in bioremediation processes and biofilm is a desired technology instrument, the formation of *Pseudomonas* biofilms is a general problem, especially in food industry and medicine. In this regard, the most problematic is certainly the opportunistic pathogen *P. aeruginosa*, especially its mucoid strains, and quite rightly, it has received the most attention in connection with biofilm formation. A considerable amount of knowledge about the possibilities to influence *Pseudomonas* biofilms has been collected, especially in recent years, which is moreover documented in this review. None of the mentioned tools have a universal utilization, and an appropriate approach depends on the site where biofilm is formed. The development of new materials with 'antiadhesive' surfaces appears the most promising. Also, finding natural compounds and understanding the principles by which they interfere with the control mechanisms of biofilm formation, without creating pressure leading to the development of resistant strains, can bring considerable advances in this field. This is a challenging task, given the complexity of often mutually independent mechanisms involved in the formation of biofilm structures. For example, very little is as yet known about the possibilities of affecting the function of cyclic di-GMP in biofilm formation. We believe that a very promising approach could involve a combination of the application of nanomaterials and natural substances, both with antibiofilm activity.

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