New Microbiologica, 45, 4, 227-236, 2022, ISSN 1121-7138

REVIEW

Biofilm Development and Approaches to Biofilm Inhibition by Exopolysaccharides

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SUMMARY

Bacteria biofilm consists of microorganisms, accounting for 5-35% of the biofilm volume, and of the extracellular matrix (65-95%), made of water (97%), proteins (2%), polysaccharides (1-2%) and nucleic acids (DNA/RNA, both <1%). The physiology of bacteria in the biofilms entails adaptive changes with expression of genes which are different from those translated in the planktonic state. While most of our applied knowledge on bacterial biology stems from the study in the planktonic state, an increasing interest is currently paid to bacterial behaviour as biofilm generators, as it is estimated that 65% of all bacterial infections are associated with bacterial biofilms. Infections of both upper and lower airways, bacterial endocarditis, chronic otitis media, urinary tract infections, periodontitis, ocular infections and chronic wound infections (including diabetic foot ulcer) are all associated with biofilm formation. The role of biofilm is also relevant in case of infections taking place on abiotic surfaces, as in the case of infections occurring on prostheses and several other medical devices. Here, we review current knowledge on biofilm formation and its impact on human infections, discussing recent means for its inhibition, with particular emphasis on an interesting anti-biofilm activity exerted by exopolysaccharides derived from marine strains of *Bacillus licheniformis*.

Received August 10, 2022 *Accepted* September 22, 2022

WHAT BIOFILM IS

Although most of the medical knowledge on bacteria stems from studies carried out on planktonic organisms, a variety of diseases are actually due to or directly originate from bacteria in a sessile form, as part of a sort of resident community named biofilm (Costerton *et al*., 1994). Biofilm can be simply defined as bacteria aggregates living in a matrix mainly composed of their own secretions, where bacteria replication is limited and nutrients circulate through a three-dimensional network of internal channels. Bacteria account for 5-35% of the biofilm volume, while 65-95% consists of the extracellular matrix. The latter is made of 97% of water, 2% proteins. DNA and RNA (both <1%) and 1-2% of polysaccharides (*Figure 1*) (Brandas *et al*., 2005).

By focusing on medical microbiology, biofilms can be described as a particular form of colonisation of human cavities and surfaces, where myriad microbial species are present according to metagenomic studies (Rodrigo Carvalho *et al*., 2017). In more updated language we may define biofilm as part of the microbiome. As in the case of other microorganisms of medical interest, biofilm can be considered a socially organized, "slow" form of bacterial life within an environment conferring protection from the action of local and systemic immunity, shear stress forces, as well as against antimicrobial drugs (de Beer *et al*., 1997; Walters *et al*., 2003). As compared to the same organisms in the planktonic form, the formation of biofilm entails the transcription of specific gene sets, including the ones leading to reciprocal bacterial signalling known as "quorum sensing", which coordinates bacterial behaviour as a group and actually limits bacterial growth inside the biofilm matrix (Sauer *et al*., 2002). From a medical viewpoint, biofilm resembles the biological translation of the concept of "Trojan horse" in human infectious diseases, such as the permanence of living pathogenic organisms in the human body in a relatively dormant state; when the surrounding microenvironment switches toward more permissive conditions, the microorganisms are ready to resume their planktonic profile, including the ability to cause disease. The formation of biofilm can thus be defined as a microbial survival strategy where bacteria live in a dynamic equilibrium and may undergo detachment with subsequent dissemination to new surfaces and/

Key words:
Bacterial biofilm, Exopolysaccharides, Bacillus licheniformis.

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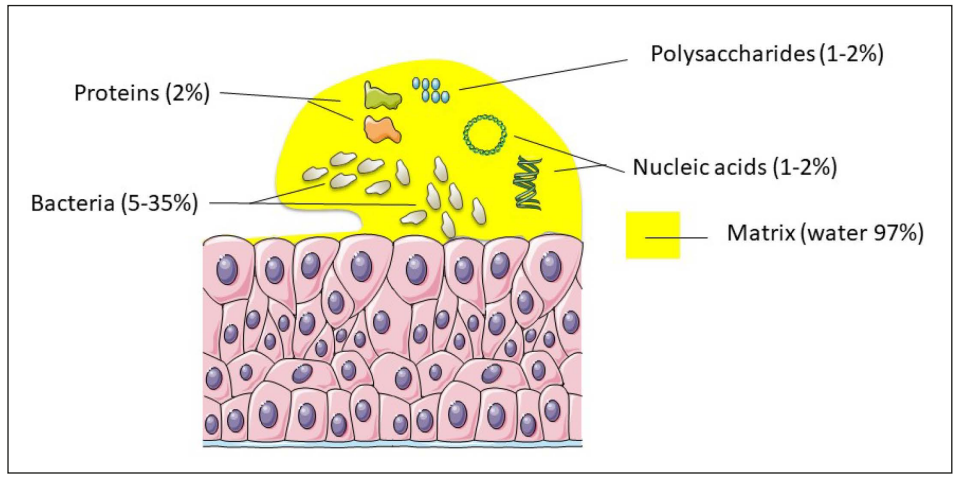

Figure 1 - *Schematic representation of the major components of biofilm.*

or initiation of a "*sensu stricto*" infectious process of clinical relevance (Sufya *et al.*, 2003; Spoering *et al.*, 2001).

The escape or detachment and dispersal of bacteria from biofilms should be seen in a wide natural epidemiological perspective, as it may ensure the persistence of bacteria in nature by migration into new ecologic niches, the diffusion of bacteria from environmental sources to humans, as well as the colonization of new surfaces of the same host, where an infectious disease may eventually take place (Stoodley *et al.*, 1999; Stoodley *et al.*, 2001a; Stoodley *et al.*, 2001b). While biofilms in nature are evenly distributed, being present on plants and many other different living or inanimate surfaces, the increasing medical interest in biofilm results from its presence on surfaces of many human body districts as a sort of protected form of colonization (Hall-Stoodley *et al.*, 2004).

It is as yet unclear whether the detachment of bacteria from biofilms is a passive process (resulting from increased mechanical and/or physical forces) or the expression of an evolutionary behavioural strategy to proactively look for new nutrients when the original niche approaches saturation and exhaustion (Hall-Stoodley and Stoodley, 2005). Whatever the case, detachment and the ensuing dispersal of bacteria is the final step of biofilm biology, but the entire process should be regarded as a continuous one, as new biofilms are forming when old ones undergo dissolution. From a medical viewpoint it has been estimated that around 65% of bacterial infections are to some extent associated with or related to bacterial biofilms (Lewis *et al.*, 2001). In a wider perspective, since biofilm is one of the forms of bacterial colonization, we may simply consider that all human bacterial infections whose aetiology is attributable to a colonizing organism may actually result from biofilm. In an attempt to classify biofilm-related infections, it is possible to distinguish between biofilms formed on biotic and abiotic surfaces. Among the examples of infectious diseases originating from biofilms on biotic surfaces, bacterial endocarditis is perhaps one of the best known and patho-physiologically characterized. A number of bacteria and fungi are known to cause native valve bacterial endocarditis (Kokare *et al.*, 2009; Donlan *et al.*, 2002). Following some degree of damage to the vascular endothelium, an initially sterile vegetation consisting of platelets and fibrin is formed, which is secondarily infected by circulating microorganisms from either a distant infectious focus or transient bacteraemia originating from skin or mucosal surfaces. Dispersal of bacteria in case of endocarditis is mainly related to the mobile nature of vegetation residing on valvular leaflets. In addition to evolving damage to endocardial tissue and valves, the disease may thus spread to distant districts through metastatic seeding of bacterial aggregates as well as by release of bacteria that have resumed their planktonic status. Other good examples of biofilm-associated infections originating from biotic surfaces are periodontitis (with the resulting plaque-forming process), and osteomyelitis, where bacterial infection may take place from bloodstream seeding or open trauma (including surgery) and an inflammatory reaction leading to local damage promotes bacterial attachment and biofilm formation (Donlan *et al.*, 2001; Percival, 2008). A particular biofilm-associated disease is pulmonary infection by *Pseudomonas aeruginosa* in patients suffering from cystic fibrosis. Patients with cystic fibrosis are prone to experience acute bacterial exacerbations, and biofilm is thought to play the role of reservoir for planktonic bacteria (Goss *et al.*, 2007).

The fast-evolving setting of medical prosthetic devices has generated many clinical circumstances in which abiotic surfaces may be vulnerable to biofilm formation. All medical devices can be considered potential targets for biofilm formation, including contact lenses, central venous catheters, mechanical heart valves, peritoneal dialysis catheters, prosthetic joints, pacemakers, urinary catheters, and voice

Table 1 - *List of human bacterial infections resulting from biofilm formation (Left: infections associated with biofilm formation on natural surfaces; Right: infections that follow biofilm formation on medical devices).*

Non-device-related	*Device-related*
Dental caries	Contact lens
Periodontitis	Sutures
Otitis media	Ventilation-ass.pneumonia
Cystic fibrosis pneumonia	Vascular grafts
Endocarditis	Arteriovenous shunts
Necrotizing fascitis	Endovascular catheter inf.
Osteomyelitis	Peritoneal dyalisis peritonitis
Biliary tract infection	Urinary catheter infection
Infectious kidney stones	IUDs infections
Bacterial prostatitis	Penile prostheses
	Orthopedic prostheses

prostheses (Percival *et al*., 2015). While contamination of the medical device may occur in several ways (including during the procedure required for installation), the surface characteristics may also play a role. Hydrophobic surfaces are deemed to be more prone to biofilm formation, as the repulsion strength between bacteria and the surface can be reduced. As a consequence, bacteria tend to attach more easily to hydrophobic and non-polar materials (Teflon and plastics) than to hydrophilic and polar surfaces like glass and metals (Cerca *et al*., 2005). A comprehensive list of human bacterial infections resulting from biofilm formation is represented in *Table 1*.

It is clear from the wide spectrum of biofilm-associated bacterial infections that renewed interest in biofilm is fully warranted. Thanks to revived research on biofilm, we now know that encasement of bacteria looking for a slow-motion but protected life is not restricted to a few organisms, but rather seems to be a universal property among bacteria, and polymicrobial biofilms have also been described (Wimpenny *et al*., 2000). Nevertheless, distinct metabolic pathways leading to biofilm formation might be species-specific, and ongoing research is shedding light on biological steps possibly amenable to inhibition and control. In an era when bacterial resistance to antimicrobials keeps increasing, the attempt to interfere with biofilm formation appears to be a possibly fruitful preventive and/or therapeutic option.

INHIBITION OF BIOFILM FORMATION/ INDUCTION OF BIOFILM DISSOLUTION

In the last decades an increasing number of studies have provided *"in vitro"* evidence of anti-biofilm activity by many substances and paved the way for preventive and/or therapeutic interventions aimed at interfering with this form of bacterial colonization (Algburi *et al*., 2017). The basic working hypothesis is to inhibit biofilm by non-toxic substances whose mechanism/s of action is/are different from that of antibiotics so that no selection of antibiotic-resistant bacteria would follow. Indeed, most of the anti-biofilm molecules recently characterized as possibly having a role in this setting were found not to inhibit bacterial growth or survival. Such distance from true antibiotic action, in addition to defining a separate setting for anti-biofilm molecules, is of relevance for at least three good reasons. First, diffusion of antibiotics into the biofilm is limited, thus leading to bacterial exposure to suboptimal drug concentration, a condition favouring the selection of antibiotic-resistant mutants (Stewart and Costerton, 2001). Second, as most antibiotics work by inhibiting replicating steps in the bacterial life cycle and bacterial multiplication inside the biofilm occurs much less frequently as compared to the same organisms in the free-living state, the efficacy of antibiotics would not be expected to be of the required level (Pamp *et al*., 2008). Third, the current global situation concerning antibiotic resistance is such that a more restricted use is universally advocated in order to reduced antibiotic pressure [26].

Although a potential anti-biofilm medication might either inhibit its formation or promote its dispersion (or both), the distinction between these two different actions does not need to be too strict, as the dynamics of biofilm looks like a continuous process where new biofilm is being formed while older aggregates undergo dissolution (Chatterjee *et al*., 2018; Purevdorj-Gage *et al*., 2005). While many substances have so far been challenged for their anti-biofilm properties, their mechanism/s of action has/have been detailed for a limited number of compounds.

Antimicrobial Peptides – Antimicrobial peptides (AMPs) are small molecules (usually up to 30 amino acids) whose targets reside on bacterial membranes. AMPs are produced as mediators of the bacterial innate defence system (Rossi *et al*., 2008). The mechanism/s of action attributed to AMPs is/are to form pores in the cell membrane and/or act as modifiers of membrane functions (Wimley and Hristova, 2011). Additional mechanisms of action may include the down-regulation of genes involved in the synthesis of proteins involved in biofilm formation, as it is the case of *Pseudomonas aeruginosa* when exposed to the peptide LL-37; type IV pili, rhamnolipid synthesis, quorum sensing and assembly of flagella were found to be significantly affected (Overhage *et al*., 2008). Some studies have also successfully combined AMPs with antibiotics in *in vitro* and in experimental infections on vascular catheters, as was the case with tobramycin, piperacillin-tazobactam, or ciprofloxacin in *Pseudomonas aeruginosa* infection (Eckert *et*

al., 2006; Hirakura *et al*., 2002; Hermann *et al*., 2010) and daptomycin, ciprofloxacin, quinupristin-dalfopristin, linezolid or vancomycin with *Staphylococcus aureus* (Mataraci and Dosler, 2012; Dosler and Mataraci, 2013).

Proteolytic Enzymes – The exopolysaccharide matrix (EPS) can be the target of biofilm-degrading enzymes. DNAase I, α-amylase from *Bacillus subtilis* and Dispersin (DspB) were proven to exert, although to a different extent, a reducing activity on *Pseudomonas aeruginosa* and Methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms. DNAase I is thought to act by modifying the matrix structure, which in turn make both *Pseudomonas aeruginosa* and *Staphylococcus aureus* more susceptible to antibiotic action (Dosler and Mataraci, 2013; Izano *et al*., 2007; Eckart *et al*., 2007; Whitchurch *et al*., 2002; Kalpana *et al*., 2012). A concerted strategic action between degrading enzymes and antibiotics is also suggested by the experimental studies carried out with DspB (from *Actinomyces actinomycetemcomitans*) (Donelli *et al*., 2007) and Lysostaphin (from *Staphylococcus simulans*), which also included the clearance of *Staphylococcus aureus* biofilm from medical devices in experimental infections (Wu *et al*., 2003; Aguinaga *et al*., 2011). According to available knowledge, these enzymes, whose production might be costlier than other candidate anti-biofilm molecules, can actually be developed as synergizers of antibiotics in the treatment of chronic infections.

Quorum Sensing Inhibitors – In the process of biofilm formation, when bacterial density has reached optimal level, small molecules known as auto-inducers (AIs) are secreted by bacteria to reciprocally reduce their own replication. The inter-bacterial communication network named Quorum System modulates bacterial behaviour in accordance with environmental variables and intervenes in the expression of genes concerned with virulence, toxin production, motility, chemotaxis and biofilm production (Uroz *et al*., 2009). Auto-inducers can be classified in three main categories, such as autoinducing oligopeptides (AIPs, for Gram+ve bacteria), acyl-homoserine lactones (AHLs, for Gram-ve bacteria), and universal (for both Gram+ and Gram-ve bacteria) autoinducer-2 (AI-2). Enzymes able to degrade AIs, such as lactonase, acylase, oxidoreductase and paraoxanase, were found to exert a promising effect in quorum-sensing inhibition. Since Ais, following penetration into bacterial cells, work by regulating gene expression, quorum-sensing inhibitors (QSIs) might inhibit biofilm formation without killing planktonic bacteria (Kiran *et al*., 2011). Likewise, in case of quorum-sensing inhibitors, a strategy including the concurrent use of antibiotics can be envisaged, as the action of many QSIs was found to make bacteria more sensitive to the antibiotic effect (Brackman *et al*., 2011), as mostly seen in case of biofilm produced by *Pseudomonas aeruginosa*.

Essential Oils – Essential oils are easy to extract, non-toxic in tissue culture and fully degradable in water, all such suitable characteristics suggesting promising development for clinical use (Fabian *et al*., 2006; Warnake *et al*., 2006). Several mechanisms account for the antimicrobial properties of essential oils. Production of ATP and ATPase activities have been associated with exposure of biofilm to essential oils (Algburi *et al*., 2017). The latter may also work by increasing bacterial membrane permeability, which leads to the loss of ions and metabolites. By analogy with QSIs, essential oils may also down-regulate the quorum sensing genes and inhibit the transcription of virulence factors (Isman, 2000). Several essential oils were also found to inhibit bacterial efflux pumps, an effect leading to increased antibiotic activity (Songa *et al*., 2018).

Nanoparticles – Anti-biofilm activity of nanoparticles has been proven both for biofilm and planktonic bacteria. The mechanism of action seems to be related to disruption of ATP-associated metabolism and increase in membrane permeability (Rabin *et al*., 2015). The anti-biofilm action of nanoparticles was found to fully synergize with antibiotic use, particularly in case of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Some uncertainties remain regarding the choice of the best size of nanoparticles and their safety in clinical use.

Polysaccharides – Although exopolysaccharides are natural constituents of the biofilm matrix and have been associated with pathogenicity, promotion of adherence to surfaces and biofilm formation, several polysaccharides have been found to be able to both inhibit biofilm formation and promote dispersal of pre-formed biofilm (Jiang *et al*., 2011; Joseph and Wright 2004; Davey and Duncan 2006; *Valle et al*., 2006; Qin *et al*., 2009). A notable exopolysaccharide, EPS273, produced by the marine bacterium *Pseudomonas stutzeri 273*, was found to exert anti-biofilm action through various mechanisms (Das and Manefield, 2012). EPS273 interferes with the production/release of several virulence factors such as pyocyanin, exoprotease, and rhamnose. Reduction of pyocyanin production is associated with decreased H_2O_2 release and this confers potent antioxidant activity to EPS273. Such property is also associated with a decreased release of *eDNA* (environmental DNA), which is required for formation of stable biofilm. Its molecular weight is 190kd and consists of glucosamine (35.4%), rhamnose (28.6%), glucose (27.2%) and mannose (8.7%) (Wu *et al*., 2015). Many other polysaccharides have been investigated and found to exert anti-biofilm activities. In addition to bacterial sources, polysaccharides with anti-biofilm properties have been obtained from plants, animals, and algae. While most of these polysaccharides (particularly those of bacterial origin) display broad-spectrum anti-biofilm activities, only a few are

able to promote dispersal of biofilm in the early formation phases (Rendueles *et al*., 2013). One example is PslG, an endoglycosidase polysaccharide produced by *Pseudomonas aeruginosa*; Psl is a constituent of the biofilm matrix and PslG was found to determine both the dispersal of preformed mature biofilm and to inhibit biofilm formation. In *ex-vivo* studies, exposure to PslG was also associated with higher biofilm vulnerability to the action of antibiotics and the host immune system (Yu *et al*., 2015).

In addition to the above-mentioned EPS273 produced by the marine bacterial species *Pseudomonas stutzeri*, other marine bacteria were found to synthesize exopolysaccharides of potential medical interest as anti-biofilm agents. *Bacillus licheniformis* has been studied by two groups and found to produce two seemingly distinct exopolysaccharides displaying anti-biofilm properties. An 1800 kDa polysaccharide was identified from the supernatant of a *Bacillus licheniformis* strain associated with the marine sponge *Spongia officinalis*. This polysaccharide was found to reduce the initial adhesion and biofilm development by *Escherichia coli* and *Pseudomonas fluorescens* on abiotic surfaces. Its action was devoid of any effect on bacterial growth and independent from quorum sensing (Sayem *et al*., 2011).

From another marine strain of the same bacterial species, *Bacillus licheniformis*, another group identified an exopolysaccharide of 1000 kDa able to inhibit biofilm formation on abiotic surfaces by several multidrug-resistant bacterial species, including *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus* (Spanò *et al*., 2013; Spanò *et al*., 2016).

EXOPOLYSACCHARIDES (EPS) WITH ANTI-BIOFILM ACTIVITY FROM MARINE STRAINS OF *BACILLUS LICHENIFORMIS*

The interest in bacterial polysaccharides in the context of biofilm currently regards two aspects, as these extracellular substances secreted by bacteria may be both the constituents of the biofilm extracellular matrix as well as bacterial products able to inhibit biofilm formation ((Jiang *et al*., 2011; Joseph and Wright, 2004; Davey and Duncan, 2006; Valle *et al*., 2006; Qin *et al*., 2009). While exopolysaccharides (EPS) may be produced by various sources, those extracted from bacteria have been shown to display properties not found in polysaccharides from algae or plants (Rendueles *et al*., 2013). In the last decade, two apparently distinct EPS of 1800 and 1000 kd, respectively, were found to be secreted by marine strains of *B.licheniformis* (Sayem *et al*., 2011; Spanò *et al*., 2013; Spanò *et al*., 2016). Both extracts displayed *in vitro* activity against biofilm formation by various human pathogens, such as *E.coli, P.aeruginosa, Klebsiella sp*, and *S.aureus*. *Bacillus licheniformis* is a gram-positive bacterium with mesophilic and thermophilic variants (Warth, 1978; Sadiq *et al*., 2017). The mesophilic strains are commonly found in soil, and their optimal temperatures for growth and enzyme secretion are around 50°C and 37°C, respectively (Warth, 1978). Mesophilic strains of *B.licheniformis* are already well-known to industry, as their submerged fermentation gave rise to the production of a protease-rich product suitable for use at high temperature. This product is used in detergent formulations to remove protein-based stains and is the original alkaline protease contained in biological washing powders since 1960 (Kumar *et al.*, 2008).

Bacteria from marine sources have the uncommon ability to grow in extreme environmental conditions, such as high temperature, salt water, high concentration of hydrogen sulphide and heavy metals, and the EPS released by marine species were found to have unique physical properties and molecular structure (Fitter *et al*., 2001). Microorganisms like *Bacillus horikoshii*, actinomycetes, marine *Vibrio spp*. and *Pseudoalteromonas* were found to synthesize and release exoproducts able to inhibit biofilm formation by a variety of human bacterial pathogens (Jiang *et al*., 2011; Nithyanand *et al*., 2010; Dheilly *et al*., 2010).

The two marine strains of *B.licheniformis* concerned here were isolated, respectively, from the marine sponge *Spongia officinalis* and from shallow hydrothermal vents in the sea surrounding the island of Panarea (Aeolian Islands, Italy) (Spanò *et al*., 2013; Spanò *et al*., 2016). Although both *B.licheniformis* strains were of marine origin, it seems likely that, due to substantial differences between the habitats where the two strains of *B.licheniformis* were recovered, their biological properties, including the characteristics of the polysaccharides secreted, are also dissimilar. The marine sponge *Spongia officinalis*, with which the first strain of *B.licheniformis* was associated, is reported to grow at a median temperature of around 25°C (Kaschner *et al*., 2016), while the T° in the submarine environment (hydrothermal vent) where the second strain was isolated is around 50°C. Bacteria from salty and hot water are expected to have unique physiological profiles and express thermostable biomolecules, such as enzymes, exopolysaccharides, and lipids.

Among the *B.licheniformis* strains recovered from *Spongia officinalis*, a specific isolate, termed SP1, was studied for its anti-biofilm activities. The supernatant form SP1 (ESP-SP1) was first tested in the inhibition of biofilm formation by *E.coli* and *P.fluorescens*, and its activity was found to be concentration-dependent with no effect on bacterial growth. It is worth noting that the activity of ESP-SP1 against biofilm formation by *Staphylococcus aureus* approached 90%. The inhibitory properties of ESP-SP1 were also found to be T°-dependent, with optimal activity around 50°C

and decreasing effects with increasing temperatures. In order to explore the mechanism of action of ESP-SP1 in the inhibition of biofilm formation, its activity was also tested with and without the concomitant exposure of *E.coli* to quorum sensing signals. No additive effects were shown, which implies that the action of SP1 is not attributable to interference with QS mechanisms. ESP-SP1 was also evaluated in a test of adherence of target cells to surfaces (*E.coli* and *P.fluorescens*) and a significant effect in reducing hydrophobicity was measured, particularly for *E.coli*. Likewise, the EPS containing supernatant produced by the SP1 strain was tested for its effects on attachment of biofilm-forming bacteria to an abiotic surface. Both the pre-coated and uncoated wells of microtiter polystyrene plates were challenged with a strain of *E.coli*, and biofilm formation was found to be inhibited by 75% in the uncoated wells following the addition of the SP1 supernatant, while the magnitude of biofilm inhibition rose to 92.5% in the case of pre-coated wells, in which ESP-SP1 was added before *E.coli*. In accordance with these results showing better inhibition when the SP1 supernatant is added to the wells before exposure to biofilm-forming *E.coli*, it was found that the exposure of mature biofilm to SP1 supernatant produced a much lower effect, thus suggesting that the supernatant works by modifying the target surface in a manner that actually prevents the initial steps of biofilm formation, with gradually decreasing effects along the process of biofilm development (Sayem *et al*., 2011).

The second strain of *B.licheniformis* investigated for the properties of its EPS in the inhibition of biofilm formation was recovered from the extreme environmental habitat (at the sampling site the water T° was 50°C and the pH was 5.42) of the marine surfaces surrounding a hydrothermal vent near the island of Panarea, part of the Aeolian Archipelago. The latter is of volcanic origin and the recovery site of *B.licheniformis* is in a wide ancient submerged crater where a variety of submarine volcanic activities can be found in shallow water. The strain was termed T14 and the exopolysaccharide obtained from its supernatant (EPS1-T14) was investigated for its ability to inhibit biofilm formation by several medically relevant bacteria species isolated from patients, such as *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The experiments were conducted on 96-well polystyrene microtiter plates and biofilm formation with or without EPS1-T14 was measured spectrophotometrically. The growth of the bacterial isolates tested here was not modified by EPS1-T14, even at the highest concentrations, which implies that no antibiotic activity *sensu stricto* is attributable to the compound. EPS1-T14 was found to exert a concentration-dependent anti-biofilm activity. All bacteria here challenged with the highest ESP1-T14 concentration (400 μg ml^{-1}) were found to be significantly inhibited in their ability to form biofilm (*E. coli* 74%, *K. pneumoniae* 56%, *P. aeruginosa* 54%, and *S. aureus* 60%) (Spanò *et al*., 2013). The activity by *K.pneumoniae* against biofilm formation is notable, as in addition to EPS1-T14, among polysaccharides such property was only shown by PAM galactan (Bendaoud *et al*., 2011). This also applies for the wide spectrum shown by EPS1-T14, including both Gram-positive and Gram-negative biofilm-forming bacteria, since other EPS were found to display their inhibitory activity only against Gram-positive organisms (Ec300p, Pel and PI80 EPS) (Rendueles *et al*., 2011; Kanmani *et al*., 2011) or had activity only at much higher concentrations (EPS from *Lactobacillus acidophilus* A4) (Kim *et al*., 2009) or exerted a wide-spectrum inhibitory activity of much lesser magnitude (EPS from *Lactobacillus plantarum* YW32) (Wang *et al*., 2015). Among the properties of EPS1-T14, its activity as surfactant should not be disregarded, as this might

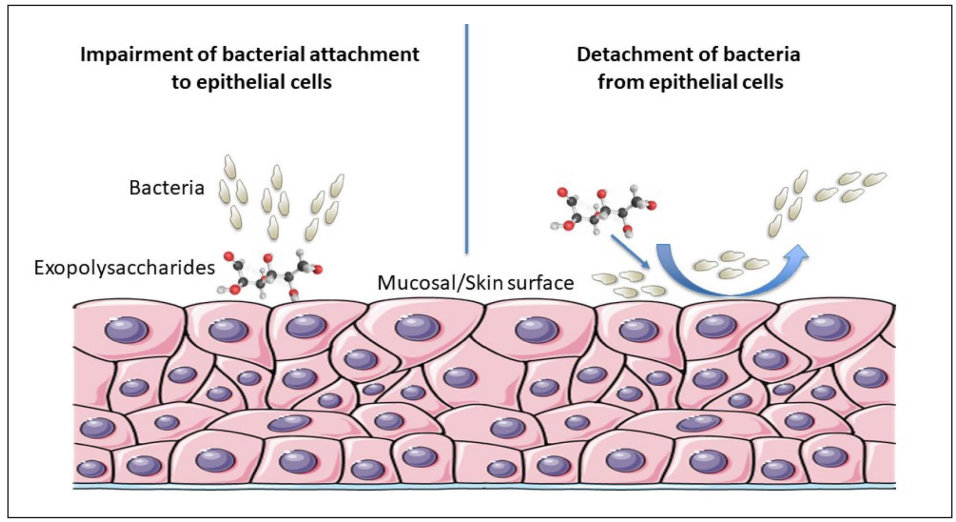

Figure 2 - *Proposed anti-microbial mechanisms of action for exopolysaccharides derived from marine strains of* Bacillus licheniformis T14. *Both chemical/physical properties and surfactant activity can be responsible for either impeding the attachment of microorganisms to epithelial cells or facilitating their removal, respectively.*

be part of its mechanism/s of action; by modifying the hydrophobicity of bacterial cells it might inhibit the initial step of adhesion, which is a prerequisite for biofilm formation, or even be able to detach bacteria from mucosal surfaces in the initial phases of bacteria adherence to the epithelial cells (*Figure 2*). The high fructose and fucose content of EPS1-T14 might also be of relevance, as both such sugars are inhibitors of the formation and activity of bacterial surface lectins (fimbriae or pili), an action that is likely to result in decreased adherence by bacteria (Imberty *et al*., 2004). Significantly, this surfactant activity of EPS1-T14 might also apply to other microorganisms adhering to mucosal epithelial cells, thus facilitating their removal and possibly impairing the infective load. Nevertheless, further investigation will be needed to clarify whether this EPS might be useful in counteracting infections by pathogenic microorganisms other than bacteria, including viruses. Beyond the still undisclosed mechanism of action of EPS1-T14 in the inhibition of biofilm development by the most relevant bacteria of medical interest, some of its general features, like water solubility, the lack of toxic *in vitro* effects, thermostability within a wide temperature range, and its full biodegradability currently suggest possible fruitful development in the human clinical setting (Spanò *et al*., 2013; Spanò *et al*., 2016). Given the recently recognized importance of biofilm as a protected form of bacterial colonization of skin and mucous membranes as well as of abiotic material, new directions in the prevention of bacterial diseases seem to be currently pursuable by taking advantage of the ability of EPS to inhibit biofilm formation. Furthermore, our conventional weapons against offending bacteria (antibiotics) have lost a sizeable part of their potential due to the increasing diffusion of drug-resistant species, and therefore any additional strategy aimed at reducing the risk of bacterial infectious diseases deserves serious consideration.

RESISTANCE TO ANTIBIOTICS

In the last several years, a series of critical bacterial species, particularly those involved in nosocomial infections, have become increasingly resistant to available antibiotics (European Center for Disease Prevention and Control, 2018). The formerly parallel race between the emergence of new bacterial resistances and the development of new active antibacterials is no longer symmetrical, as some species have developed complex resistance patterns against which the currently available antibiotic armamentarium is far from providing the required coverage. Widespread uncontrolled use of antibiotics (including industrial applications in agriculture), an increased demand for antibiotic use in the growing elderly population, and more severely ill patients with relevant comorbidities are among the main factors accounting for the ongoing crisis in antibiotic resistance, by far the most severe since the beginning of the antibiotic era (Meyera *et al*., 2013). Bacterial species like *Pseudomonas aeruginosa*, Enterobacteriaceae (*K. pneumoniae, E.coli*), *Stenotrophomonas maltophilia*, *Acinetobacter sp*, *Staphylococcus aureus, Enterococcus sp.*, and *Clostridium difficile* have shown a steady tendency toward decreased susceptibility to antibiotics. While for Gram-positive organisms, newer drugs characterized by different mechanisms of action were released to counteract the progressive loss of Vancomycin efficacy and new options have also been developed against *C.difficile*, the worst scenario is represented by Gram-negative infections. The progressive and continuous selection of new β-lactamases by Enterobacteriaceae and *P.aeruginosa* are responsible for a growing number of isolates virtually unresponsive to any single antibiotic. The recent wave of antibiotic development in this specific setting has brought several newer antibacterials able to overcome some of the existing resistance patterns. However, the development of new β-lactamases inhibitors in association with new or old β-lactams is just a small step forward in the progress required here. Since no new mechanisms of action have been introduced, it appears unlikely that these new molecules (belonging to classes that are largely responsible for the increase in drug-resistant organisms) will provide long-lasting relief to the current scenario. Although a series of initiatives aimed at improving the policies of antibiotic prescription at different levels of healthcare have been implemented under the term of "antibiotic stewardship" (Raad *et al*., 2016), it appears unlikely that these measures will significantly impact a trend which currently keeps increasing.

POSSIBLE CLINICAL SCENARIOS FOR EXOPOLYSACCHARIDES (EPS)

According to the properties of EPS in the inhibition of biofilm formation shown in the laboratory, it does actually seem that its most appropriate strategic position is in the prevention of biofilm development rather than in the clearance of a steady, mature biofilm. The best performances of EPS have been recorded when biofilm-forming bacteria were added on EPS-coated surfaces, while effects of lower magnitude were seen when the EPS-containing supernatant was added on a developing biofilm (Sayem *et al*., 2011; Spanò *et al*., 2016). Such findings suggest that pre-exposure of abiotic surfaces (e.g., prosthetic devices) should reasonably be the best option in order to take full advantage of the anti-biofilm properties of EPS. We might envisage how, in addition to existing preventive procedures, the application of EPS throughout the surface of an orthopaedic prosthesis before its final positioning might actually reduce the

risk of bacterial contamination, biofilm formation, and ensuing bacterial infections (Raad *et al*., 2016; Mistry *et al*., 2016; Lemire *et al*., 2013). The search for suitable dispensing devices to be adapted to different prosthetic devices are warranted in order to optimize the surface coverage by EPS, a requirement that appears to be feasible considering the physical and structural molecular characteristics of EPS (Fabbri *et al*., 2016; Urish *et al*., 2014). Other examples of medical devices likely to benefit from an anti-biofilm remedy are vascular (Gominet *et al*., 2017) and urinary catheters (Murugan *et al*., 2016), whose frequency of use is rather high in the medical setting, and are fully recognized as responsible for generating bacterial infections following biofilm formation. The same applies to endotracheal tubes employed for mechanical ventilation in intensive care units, where such practice is associated with the most severe forms of nosocomial pneumonia by multidrug-resistant organisms (Gil-Perotin *et al*., 2012).

But the potential use of EPS as a means to prevent infections is likely not to be limited to abiotic surfaces. Although the anti-biofilm action of EPS seems to be mainly in preventing the formation of biofilm, such formation is however a continuous process, as the life of biofilm is finite and new biofilm is formed when old biofilm disaggregates. As a consequence, the use of EPS on mucous surfaces, albeit of limited activity against the pre-formed biofilm, might actually reduce the formation of new biofilm, and in the long run this might turn into a limited persistence of biofilm in the mucosal district exposed to EPS. This is necessarily a speculative view, as the clinical experience in biofilm formation is still limited and additional work is required to fully elucidate the scope of effects of the application of anti-biofilm solutions (Oliveira *et al*., 2014). Nevertheless, the effects in a medical/biological scenario (e.g., nasal cavities, oropharyngeal mucosa) are worth investigating, as the interference with biofilm formation on a selected mucosal surface might actually determine downstream variations in the different forms of bacterial residency (microbiota) along the mucosal surfaces in continuity with the one exposed to the anti-biofilm measure, i.e., mucosal surfaces beyond the reach of the dispensing devices might also benefit from the anti-biofilm measures. Another possible use of anti-biofilm products is the prevention of biofilm formation in skin areas closely surrounding a surgical or traumatic wound (Edmiston *et al*., 2016). Exposure of such lesions to skin, intestinal, as well as to environmental bacteria represents a serious risk of developing bacterial infections of the skin and soft tissues, whose prevention with ordinary measures might be difficult, especially in vulnerable patients (e.g., diabetics).

Therefore, the potential applications of anti-biofilm interventions actually look quite extensive, and it is time to move quickly to the clinical ground.

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