Searching the next remedies within the bacterial arsenal

Charalampia Korea
Pharmassist Ltd, Contract Research Organisation, Holargos, Athens, Greece
DOI: https://doi.org/10.5281/zenodo.10021168

Summary

Biofilms are bacterial communities, often multi-species, attached on abiotic or biotic surfaces and enclosed in an extracellular matrix. The matrix is composed of extracellular polymeric substances (EPS) such as proteins, polysaccharides and bacterial DNA. Biofilm formation occurs in four steps: first contact, attachment, maturation, and dispersion. After initial adhesion, if the environmental conditions are favorable, this reversible adhesion can lead to irreversible attachment and extracellular matrix production, thus to micro-colony formation and biofilm maturation. Several bacterial adhesins (Type 1 pili, P pili, curli) and biofilm-related molecules, eg. EPS or Quorum sensing molecules are closely involved in all steps of biofilm formation and can be responsible for biofilm persistence.
In the medical sector the particularly resistant profile of biofilms poses serious problems both in the diagnosis of the biofilm-associated infections (BAI) as well as in their eradication. Indeed, biofilm infections exhibit 10 to 1000 times higher resistance to the antibiotic therapies used in clinical practice. This recalcitrance seems to be due to the i) reduced penetration of antibiotics into the EPS, ii) the arise of persisters, ie of cells with a particularly resistant phenotype and iii) the presence of bacteria that have activated stress responses after sensing the induced chemical stress provoked by antibiotics within the matrix.

Up to date it is known that biofilms of medical relevance can be responsible for a series of chronic infections, such as endocarditis, cystic fibrosis, chronic bronchopneumonia, persistent otitis media, chronic rhinosinusitis, chronic osteomyelitis. Further to this biofilms can be also responsible for nosocomial infections related to foreign-body medical devices e.g., catheters, prosthetic heart valves, pacemakers and stents.
The present review presents the current experimental and clinical approaches related to the development of prevention strategies and therapies against biofilm-associated infections. The most recent advances include compounds that can either prevent the bacterial adhesion itself or inhibit a series of biofilm-related structures and proteins. Moreover the progress on the development of vaccines using biofilm-related virulence factors is briefly presented.
Their understanding can pave the way for the discovery and development of diagnostic tools and targeted therapies for an efficient clinical management of the biofilm associated infections.

Corresponding author
Charalampia Korea, PhD, PMP,
Senior Scientific Advisor,
PHARMASSIST Ltd,
CONTRACT RESEARCH ORGANISATION
262 Messogion A., 155 62 Holargos,
Athens – Hellas
Tel.: +30 210 6560700, ext: 116
Fax: +30 210 6512210
e-mail: korea@pharmassist.gr

Key words
biofilms, therapies, anti-adhesive compounds, quorum sensing inhibitors (QSI)

Introduction

Bacteria can live either as free-floating organisms (planktonic cells) or as surface-bound (sessile) cells that form communities. These bacterial communities, attached on abiotic (inert materials) or biotic surfaces and enclosed in an extracellular matrix, are described as biofilms.[1-6] The bacterial consortium can consist of one or more species living in a sociomicrobiological way, while the matrix is composed of extracellular polymeric substances (EPS) such as proteins, polysaccharides, and bacterial DNA.

Biofilms are omnipresent not only in natural environments (lakes, mines, rocks, animals etc) but also in many human activities, especially in the industrial and the medical domain. For this reason, they are of major ecological importance, playing either a positive role (water bioremediation, biolixivation, probiotics) or a negative one (antibiotic resistance, nosocomial infections, biofouling/corrosion etc.).[7-13]

Biofilm formation occurs in four steps: first contact, attachment, maturation, and dispersion. After initial adhesion, if the environmental conditions are favorable, this reversible adhesion can lead to irreversible attachment and extracellular matrix production, thus to a micro-colony formation and biofilm maturation[14-15] (Figure 1). The successful establishment and growth of a biofilm are very much dependent on bacterial adhesins, a category of bacterial virulence factors expressed in all steps of biofilm formation. Indeed, they are often implicated in initial bacterial adhesion, either by modulating the properties of the surface, where bacterial attachment may occur, or by mediating adhesion to different substrates. Additionally, they can also play a role in the most advanced steps in biofilm formation, specifically by mediating interactions between bacteria, either intra- or inter-species. The most important and well-known adhesion factors are pili (Type 1, P pili), curli, Type III Secretion factors, outer membrane proteins (eg. Antigen Ag43), while Extracellular Matrix, or Extracellular Polymeric Substance (EPS) and quorum sensing molecules are actively involved in the most advanced forms of biofilms.

The unique properties of the bacteria living in biofilms drew the attention of the microbiologists very quickly. Indeed, it has been shown that biofilms protect bacteria from several environmental challenges including desiccation, access to nutrients, access to

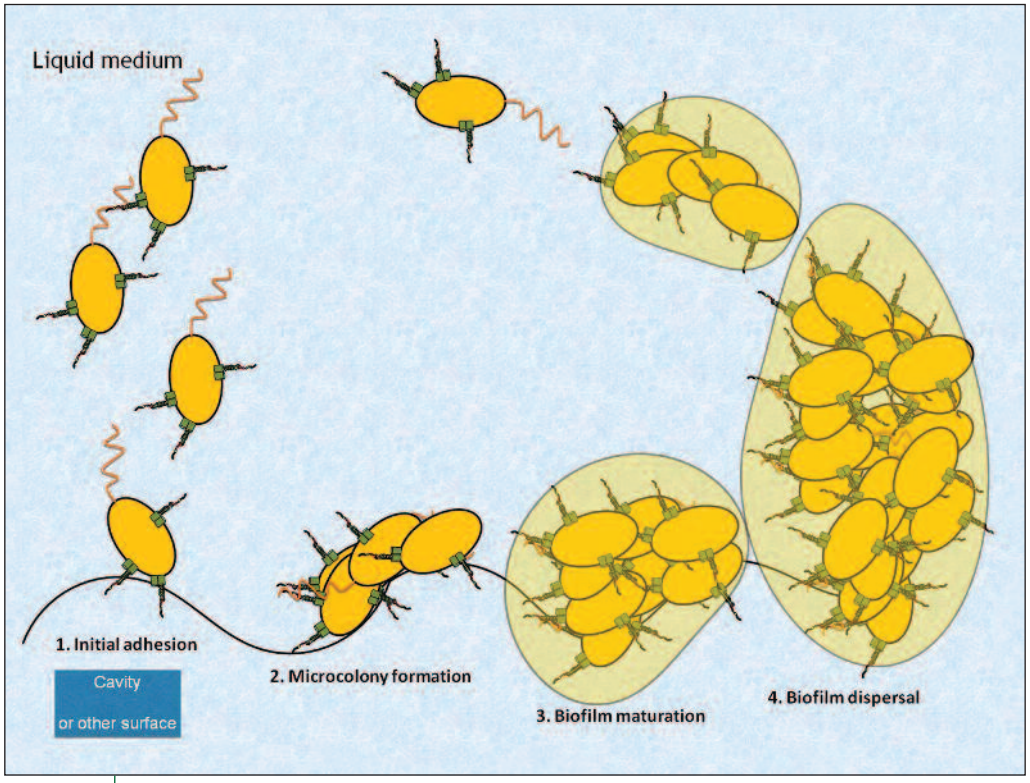

Figure 1 The four steps of biofilm formation. **Stage 1**: Initial adhesion, **Stage 2**: formation of microcolonies, **Stage 3**: maturation and structuring, **Stage 4**: detachment and return to the planktonic growth model. Various adhesion factors – such as flagella and pili depicted here as protrusions on the bacterial cell surface- are employed in the different steps of biofilm growth.

oxygen/CO_2, predators (bacteriophages, amoebae) and biocides used in industry and in hospitals.[2]

This particular transcriptional and functional profile poses serious problems during bacterial infections, as it renders challenging both the diagnosis of biofilms in infection as well as their eradication. A major obstacle in the efficient treatment of biofilm infections is their recalcitrance to conventional antibiotic therapy. This reduced susceptibility to antibiotics, 10 to 1000 times less than in planktonic cells,[16] is caused by a combination of different factors, such as the i) reduced penetration of antibiotics into the EPS, ii) the arise of persisters, i.e. of cells with a particularly resistant phenotype, iii) the presence of bacteria that have activated stress responses after sensing the induced chemical stress provoked by antibiotics within the biofilm matrix and iv) the pre-existing presence of antibiotic resistance genes expressed by the bacteria.[17-19]

Additionally, biofilms activate a series of protective mechanisms to evade antibiotics effect, e.g. they may produce enzymes that degrade antibiotics, express efflux pumps or upregulate porin proteins and display antibiotic targets of low affinity.[8,20-23] This enzymatic defense, coupled with a higher mutation rate and increased horizontal gene transmission, leads to resistance against several antimicrobial agents, i.e. beta-lactam antibiotics, aminoglycosides and fluoroquinolone.[19] Characteristic example is the case of *Pseudomonas aeruginosa*, where studies demonstrated that subinhibitory concentrations of antibiotics, eg. aminoglycosides, tetracycline and cephadrine may induce biofilm formation as a reaction to the presence of antibiotics.[24]

Up to date it is known that biofilms of medical relevance grow on many natural and inert surfaces of the human body such as teeth, heart valves, lungs, middle ear and can be responsible for a series of chronic infections, such as endocarditis, cystic fibrosis, chronic bronchopneumonia, persistent otitis media, chronic rhinosinusitis, chronic osteomyelitis. Further to this biofilms can be also responsible for nosocomial infections related to foreign-body medical devices e.g., pacemakers, catheters, prosthetic heart valves and stents medical implants.[25-26] Some of the bacterial species known to form biofilms leading to persistent and recurrent infections are *Micrococcus* spp., *Enterococcus* spp., group B streptococci, beta-haemolytic streptococci, *Proteus mirabilis, Bacteroides* spp., *P. ae-*

ruginosa, Escherichia coli, Staphylococcus aureus and *S. epidermitis* (Table 1).

Below we provide with a concise review of the experimental and clinical approaches related to the development of prevention strategies and therapies against Biofilm-Associated Infections (BAI). The most recent advances on agents aiming at the prevention of either the bacterial adhesion itself or at the inhibition of a series of biofilm-related structures and proteins such as pili, curli, Type III Secretion System, the extracellular matrix, quorum sensing are reviewed. Moreover, the progress on the development of vaccines using biofilm-related virulence factors is briefly presented.

Given the extent of consequences bacterial biofilms can have in medical practice, the study of clinically relevant biofilms in the context of specific anatomic sites or host tissues is of high importance.[26] Their understanding can pave the way for the discovery and development of both diagnostic tools and guidelines as well as of targeted therapies for an efficient clinical management of the biofilm infections.

A. Inhibiting adhesion

Initial adhesion is the first, and consequently the most important, step in bacterial colonization. For this reason, scientists have focused their efforts on finding ways to block this initial interaction, principally by inhibiting the interaction between the bacterial adhesins (or pili) and the host cell. Several artificial or natural carbohydrate derivatives have been proposed as substitutes of the natural host receptors of the adhesins, thus indirectly blocking bacterial attachment on the cell. The anti-adhesion approach has been first proposed as a therapeutic strategy 30 years ago and it is always in development. Latest advances are regularly reviewed.[27]

1. Anti-adhesive compounds

The most extensive studies on anti-adhesives have been done on adhesins of pathogenic *E. coli* strains related to urinary tract infections (UTI). During infection, the uropathogenic *E. coli* (UPEC) express mainly type 1 pili, which are key role players in pathogenesis.[28] The tip adhesin of type 1 fimbriae, FimH, can recognize the mannose residues, which are exposed on uroplakin Ia (UPIa), a membrane glycoprotein, found on the epithelial cells of the urinary tract. Of great interest, type 1 pili mediate the invasion of the epithelial cells in an effort to evade the innate host immune response, which is activated after attachment. When inside the eukaryotic cells, bacteria replicate rapidly and develop intracellular bacterial communities (IBCs) with characteristics similar to biofilms. IBCs are used as a source of re-infection, since bacteria can disperse from there and invade neighbouring cells in the bladder lumen. The persistence of these bacteria in the bladder can lead to the emergence of the recurrent urinary tract infections (rUTI).[28]

Given the extended roles of FimH, the anti-adhesive compounds can act on various levels, i.e. not only by blocking bacterial adhesion on uroepithelial cells, but also by antagonizing invasion and the subsequent IBCs formation. In nature, cranberries are known to contain 2 compounds that prevent fimbriated *E. coli* from adhering to uroepithelial cells in the urinary tract. Results of clinical studies conducted in several target populations have suggested a positive clinical benefit of cranberry juice for the UTI prevention, especially for sexually active women with UTI history. Especially for these women, the clinical studies have demonstrated a 50% reduction in disease-related morbidity.[29]

Further to this, intensive efforts have been done for the design and creation of synthetic compounds that can bind FimH and therefore inhibit bacterial adhesion. The first experiments started more than 30 years ago; in 1985 Abraham and colleagues[30] reported that the use of antibodies targeting mannose residues present on host mucosal surfaces or on bacterial adhesin can protect against the colonization of certain mucosal surfaces by pathogenic bacteria.[30] In this first study, it is also shown the first effort to block FimH-mediated adhesion by using mannoside derivatives. This has found a fertile ground for the development of the antiadhesives with regard to the UTI infections.

The main important consideration in order to ensure the efficacy of any anti-adhesive compound is the design of the compound so that it mimicks very accurately the structure of the natural epitopes, eg. these of a-D-mannose. As a general scheme, such inhibitors bear the a-D-mannopyranoside of high-mannose N-glycans as a basis, being also the ones recognized by the tip adhesion FimH. A hydrophobic aglycon is then linked to the mannose by the O atom, which is subsequently substituted by a carbon. This change renders the compound even more stable, because the created C-glycosidic bond cannot be degraded by enzymes.

The created molecules can antagonize FimH at the level of mannose epitopes present on the host surface.[31] Studies in mouse models have demonstrated that these mannose compounds can be highly efficient in the clinical treatment of the UTI and Catheter-Associated UTI (CAUTI). In a characteristic recent experiment, mice treated with a FimH inhibitor prevented the *in vivo* acute infection and the treatment

Table 1 Biofilm-associated infections and involved microorganisms[25-26]

Type of infection	Biofilm producing microorganism	Review reference
Tissue-associated		
Dental Caries	*Actinomyces* spp., *Tannerella forsythia, Fusobacterium nucleatum, Spirochaetes, Synergistetes, Candida albicans*	26
Periodontitis	*Streptococcus* spp., *Fusobacterium* spp.	26
Cystic fibrosis lung infections	*P. aeruginosa*	26
Chronic otitis media	*H. influenzae, P. influenzae*	26
Chronic Rhinosinusitis	*S. aureus, P. aeruginosa, H. influenzae*	26
Chronic tonsillitis	Gram positive and Gram negative (*Klebsiella, Pesudeomonas, Staphylococcus, E. coli* etc)	26
Chronic wounds	*Prevotella, Clostridium,* and *Porphorymonas*, *Staphylococcolus, Pseudomonas*	26
Musculoskeletal infections		
Osteomyelitis	*Peptostreptococcus productus, Peptostreptococcus anaerobius, Bacteroides fragilis, Enterococcus, Pseudomonas aeruginosa*, *Serratia marcescens, Staphylococcus aureus, Staphylococcus epidermidis,* gamma *Streptococcus*	26
Endocarditis	*Bartonella quintana, Tropheryma whipplei*	26
UTI	*Klebsiella* spp., *Escherichia coli, Pseudomonas* spp., *Enterobacter* spp., *Staphylococcus, Enterococcus, Citrobacter* spp., *Acinetobacter* spp., *Proteus* spp.	26
Infectious kidney stones/biliary tract infections	*E. coli, Enterococcus* spp., *Veillonella* spp, *Fusobacterium* spp, *Bifidobacterium* spp, *Streptococcus anginosus*	26
Implant/medical devices associated		
Sutures/surgical meshes	*Corynebacterium, Streptococcus milleri, Staphylococcus*	26
Stents	*Enterococcus faecalis , Enterococcus faecium*	26
Vascular grafts	*Streptococcus viridans*	26
Artificial voice prostheses	*Candida albicans, Streptococcus mitis, Streptococcus salivarius, Rothia dentocariosa, Candida tropicalis, Streptococcus sobrinus, Staphylococcus epidermidis, Stomatococcus mucilaginous*	25
Artificial hip prosthesis Replacement joints	Coagulase-negative Staphylococci, b-hemolytic Streptococci, enterococci, *Proteus mirabilis, Bacteroides species, Staphylococcus aureus, Streptococcus, Escherichia coli,Pseudomonas aeruginosa* *S. aureus, S. epidermidis*	25 25
Prosthetic heart valves	*Streptococcus viridans,* coagulase-negative Staphylococci, *enterococci, S. aureus*	25, 26
Cardiac pace makers	*S. aureus*	25, 26
CSF shunts	*S. aureus, S. epidermidis, Enterococcus*	25
Endotracheal tubes	*S. aureus, S. epidermidis, C. albicans, P. aeruginosa*	25
Urinary catheters	*S. epidermidis, Klebsiella pneumoniae, Enterococcus faecalis, P. mirabilis*	25, 26
Peritoneal dialysis catheters	Streptococci, Staphylococci	25
Central venous catheters	*S. epidermidis, S. aureus, E. faecalis, K. pneumoniae, P. aeruginosa, C. albicans*	25
Contact lenses	*P. aeruginosa* and *Gram-positive cocci*	25, 26
Dental implants	Acidogenic Gram-positive cocci (e.g. *Streptococcus)*, Gram-negative	25, 26
Implanted prosthetic devices for erectile dysfunction	*S. aureus* and *S. epidermidis*	25
Intrauterine contraceptive devices	*Micrococcus sp., Enterococcus sp., C. albicans*, Group B Streptococci	25
Orthopaedic implants	Hemolytic streptococci, *Enterococci, P. mirabilis, Bacteroides* spp., *P. aeruginosa, E. coli*	25, 26
Breast implants	*S. aureus, Enterococcus* spp., *S. epidermidis*	25

of chronic cystitis caused by a multi-resistant *E. coli* UPEC strain *E.coli* O25b:H4-ST131 by lowering the bacterial burden more than 1000 times.[27,32]

Other examples of synthetically created anti-adhesive compounds include heptyl a-D-mannose, whose use prevented type 1-piliated *E. coli* from binding to the human bladder cell line 5637, as well as it decreased both adhesion and invasion of the *E. coli* UTI89 isolate. Finally, similar studies are being conducted for the P pili, e.g. the development of a compound mimicking the receptor for PapG.[33]

Apart pathogenic *E. coli*, the idea of anti-adhesive compounds has been also tested in other bacterial species. Polysaccharides derived from plant extracts have been tested against a series of bacteria, eg. *Helicobacter pylori, Campylobacter jejuni, Porphyromonas gingivalis* and the obtained results showed very promising anti-adhesive effects. More specifically, compounds from immature okra fruits (*Abelmoschus esculentus*), enriched with glucuronic acid, polysaccharides from *Glycyrrhizia glabra*, also enriched with glucuronic acid, and *Pelargonium sidoides* extracted proanthocyanidins blocked successfully the adhesion of *H. pylori* in *vitro,* the latter in a dose-dependent manner. *A. esculentus* was efficient for *C. jejuni* too, while *G. glabra* polysaccharides showed strong anti-adhesive properties against *P. gingivalis*.[34] Finally tests with salvianolic acid B (SA-B), a polyphenol from the radix *Salviae miltiorrhizae* showed that it may prevent meningococcoal infections by inhibiting meningococcal binding to host cells.[35]

However, the tropism and the redundancy of adhesins employed during bacterial infections make difficult a quick development of an efficient mainstream use of anti-adhesion therapies. This can be compensated with the use of a combination of inhibitors that are either directed against multiple adhesins or they are used in conjunction with the mainstream antibiotic therapy.[27] Once more the efficacy and accuracy of these inhibitors are dependent on the precise design of these molecules.[27] An encouraging result is the demonstration that the use of a small molecular weight FimH inhibitor 4'-(a-D-mannopyranosyloxy)-N,3'- dimethylbiphenyl-3-carboxamide can result in reduced colonization of the bladder in murine models of urinary tract infections (UTI) provoked by a multi-drug resistant UPEC strain *E.coli* O25b:H4-ST131.[27]

Overall the development of anti-adhesive compounds constitutes a very promising approach for the treatment of bacterial infections. Both the encouraging results on mannosides so far, as well as the future studies at preclinical and clinical level can provide us with more insight and eventually set the ground for the introduction of the anti-adhesive compounds as a novel antibiotic therapy. Their contribution as a therapy adjacent to the traditional treatments can be even more important in the case of persistent infections, e.g. UTI, where the anti-adhesive compounds can act at two levels, i.e. by blocking both bacterial adhesion and invasion, and therefore by preventing eventual recurrence of the infection.

2. Anti-adhesive Nanoparticles

Nanotechnology is another field, which has become increasingly popular thanks to the development of nanoparticles (NP), or nanodiamonds, (ND) with antimicrobial potential and even anti-biofilm effects.

This new paradigm for the effective treatment of infectious diseases represents a new type of antimicrobial drugs, called nanoantibiotics, which possesses many advantages over other antimicrobial agents, including increasing effectiveness against drug-resistant species, lack of adverse effects and overcoming resistance development interfering with multiple biological pathways. These nanoantibiotics either show antimicrobial activity by themselves or elevate the effectiveness and safety of conventional antibiotics administration creating high local concentrations. Among the mechanisms of action by which these NPs kill bacteria is the disturbance of the bacterial cell wall membrane following their conjugation with carbohydrates that can block bacterial interaction with the surface and therefore the subsequent adhesion.[36]

NPs have unique physicochemical characteristics, since their surface to volume ratio is high, they are easily modifiable and they can be delivered through various platforms, eg. liposomes, solid lipid NPs, polymeric NPs and dendrimers. These features facilitate the delivery of the antimicrobial molecules to the infection site and allow easier access to the bacterial and host cells.[36]

In a recent work, NPs (or NDs) were conjugated to trimeric thiomannosides in an effort to check whether the high mannose density obtained with NPs can lead to an effective inhibition of *E. coli* type 1 fimbriae. The study by Khanal *et al*.[37] confirmed that these triads of mannosides conjugated with NPs act as potent inhibitors of type 1 fimbriae-mediated *E. coli* adhesion to yeast and T24 bladder cells, as well as of the respective biofilm formation. Similar tests with NPs and *S. aureus* biofilms showed a similar effect, although modest.[36-37]

B. Inhibiting bacterial secretion systems

1. Pilicides

A second strategy for the inhibition of the pili-mediated adhesion is the development of molecules that

prevent pili biogenesis by blocking the respective secretion system responsible for their formation. The chaperone-usher pathway (CUP) is the most characteristic example, pathway employed for the generation of pili produced by several Gram-negative bacteria eg Type 1 pili, P fimbriae, etc.

Briefly, during pilus formation, individual pilus subunits (pilins) are exported through the inner membrane and to the periplasm by the Sec pathway and guided to the usher via a chaperone, e.g. FimC/PapD, in the case of Type 1 and P fimbriae, respectively. The chaperone protein facilitates the appropriate folding of each pilus subunit and acts as a checkpoint prior to pilin transfer to the pilus.[33,38]

Insertion of the pilin into the pilus is realized through a process called Donor-Strand Exchange (DSE) reaction.[33,38] Each pilin's Ig structure is incomplete, namely, there is one strand missing and for this reason an external G1 strand is provided by either the chaperone or the preceding subunit of the pilus in order to rectify its structure. Small molecules that can disrupt these chaperone:subunit complexes or the interaction between the usher, they can actually prevent pilus polymerization and hence act indirectly as adhesion and biofilm inhibitors. Of importance, the CUP can be found not only in *Escherichia* species but also in *Salmonella, Klebsiella, Yersinia* and *Pseudomonas*, meaning that CUP inhibitors, or pilicides, may have a broad spectrum of activity.[32,36,39-41]

So far several pilicides have been rationally designed with encouraging results regarding type 1 or P piliation inhibition. In the case of *E. coli*, these compounds target the DSE reaction between FimH and FimG/FimF, the subunits that connect the tip adhesin to the pilus rod. These pilicides are derivatives of ring-fused 2-pyridones. A very potent inhibitor of type 1 piliation is the pilicide ec240. When tested in the cystic isolate UTI89, ec240 was shown to mediate a strong biofilm formation inhibition, with a 50% inhibitory concentration of 7 μM.[42] Based on the same principle a PapD inhibitor has been also created.[39,43] The effectiveness of these candidate pilicides awaits future studies in animal models.

Finally, more recently new compounds that can prevent the formation of pili have been studied. Among the ones finally selected after an *in silico* screening, a molecule called AL1 was shown not only to prevent the formation of type 1 pili, but also to take away pili already present on the bacterial surface. Its mechanism of action would be possibly the destabilization of the basal subunit:chaperone complex leading to dissociation of the pili. AL1 represents the first small molecule to successfully target DSE, providing an exciting basis for further design and development of antivirulence drugs.[39]

Pilicides can be of great pharmaceutical potential, as stated above; first, their interference with the CUP can inhibit the formation of all these surface structures, whose formation is dictated by the Chaperone-Usher Pathway. Indeed, studies initially conducted on pilicides against type 1 fimbriae revealed that the disrupting effect of these molecules was also effective against at least other three pili types formed through the CUP, namely the P-, S-, and Dr- pili. For example, co-incubation of 3.5 mM pilicides with Dr+ bacteria decreased bacterial adherence to CHO cells expressing Dr fimbrial DAF receptor protein by 75 to 87%.[44] The mechanism of action on these other pili was different and independent of the one targeting type 1 pili.[42,45] Given that CUP is employed by important bacterial pathogens in a similar way- secretion systems are highly conserved-, we can hypothesize that well-designed pilicides can likely have a very broad-spectrum of activity across numerous bacterial species. This property, along with their low molecular weight and peptidic nature makes them excellent candidates for an effective treatment in the near future.[42,45]

2. Curlicides

Curli are functional extracellular amyloid fibers and the main protein component of the extracellular matrix in *E. coli* pellicle biofilms, as well as other bacterial cells, that contribute to immune system activation, host colonization and cell invasion. Curli are produced by uropathogenic *Escherichia coli* (UPEC) and other *Enterobacteriaceae* (Figure 2). Compounds that can affect the polymerisation of the main curli subunit formation of these amyloids, named curlicides, can also be used as effective treatments against biofilms.

Like pilicides, curlicides are made of ring-fused 2-pyridones and seem to be able to interfere with both curli and type 1 pili formation. In a characteristic example, two curlicides named FN075 and BibC6 were shown to block curli formation by preventing the polymerization of the CsgA subunit protein of curli.[39,46] Additionally, when tested in a mouse model of UTI, FN075 was able to block UPEC virulence by blocking the polymerization of both curli and type 1 pili.[46] Once more, the fact that small protein molecules can target two distinct virulence factors targeting two different mechanisms of pili formation underlines their valuable therapeutic potential. Given the particular properties of these adhesins, prevention of their formation can protect the host both from the bacterial adhesion and from the biofilms mediated by these organelles. This discovery can also show a new landscape for the study and better understanding of curli virulence, as set by their amyloid characteristics.

Of importance, a range of cyclic-2-pyridones has

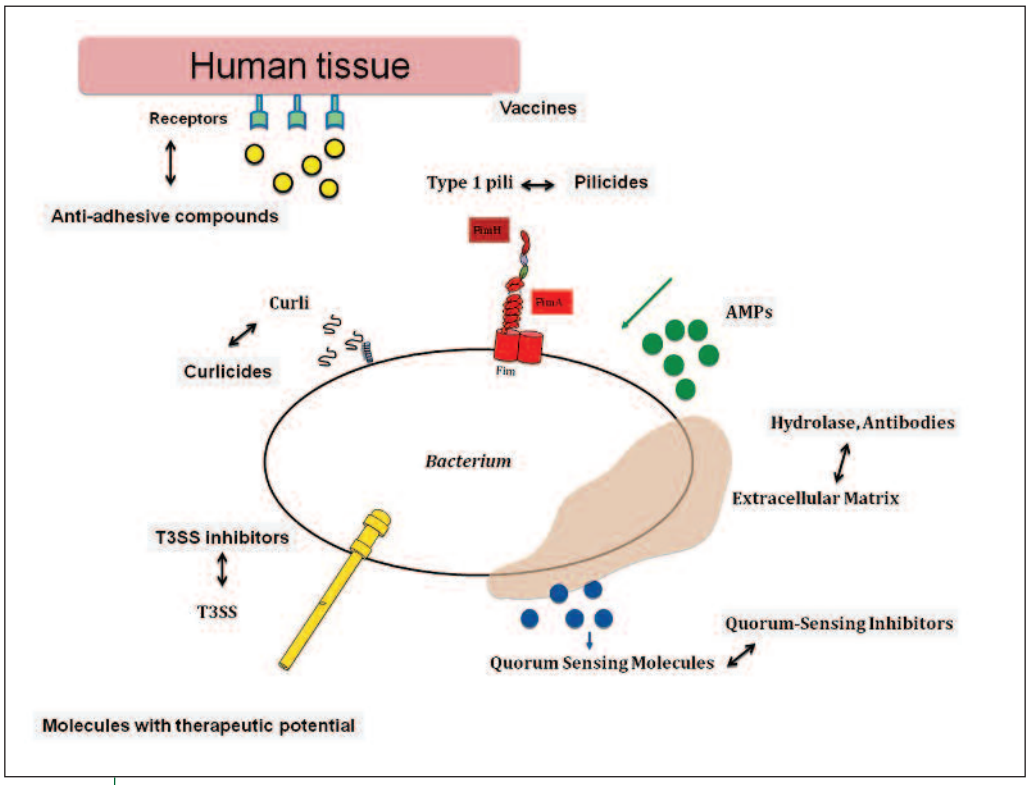

Figure 2 Overview of bacterial structures and molecules of therapeutic potential. Schematic view of some of the different adhesion factors and biofilm elements that can be found on the surface of Gram-negative bacteria: 1) chaperone–usher pili (e.g. Type 1 pili), 2) curli, 3) Type III secretion system[38], 4) Quorum Sensing molecules, 5) Extracellular Matrix (EPS). Anti-adhesive compounds and well-tailored inhibitors can target either one or several bacterial adhesion factors in parallel, leading to biofilm decrease. Antimicrobial peptides (AMPs) have been found to disrupt biofilms too.

been found to have an enhancing or inhibitory effect on amyloid formation, making them potential drug candidates for the treatment of Alzheimer's disease too[39].

3. Type III secretion system (TTSS).

The secretion system of the Gram-negative bacteria attracting the most attention is the type III secretion system (TTSS). TTSS is a multiprotein appendage in form of syringe, through which bacteria can "inject" effector proteins and toxins into the host cell, which in their turn modulate the cell response to the bacterial infection (Figure 2). The TTSS machinery exists in many pathogens, eg. *Escherichia, Shigella, Salmonella, Pseudomonas, Chlamydia* and *Yersinia* spp.[32] Although TTSS was not linked to biofilm infections so far, a recent study showed that *P. aeruginosa* TTSS translocon dependent biofilm formation can occur during infection of the epithelial barrier.[47-48]

Many diverse classes of compounds have been investigated as inhibitors of Type III secretion system, but the lack of a clear mode of action for these compounds has prevented any major developments. So far phenoxyacetamides and benzimidazoles have been shown to inhibit the TTSSs from *P. aeruginosa* and *Yersinia pseudotuberculosis*, respectively, while thiazolidinones have been shown to block TTSS from *Salmonella typhimurium* and *Yersinia enterocolitica*, reduce the virulence of *Pseudomonas syringae*. In addition to TTSS inhibition, thiazolidinones have been shown to interfere with other secretion systems such as the type II in *Pseudomonas* spp. and the type IV in *Francisella*.[49]

Further to this, high-throughput screening has identified as potential TTSS inhibitors diverse classes of compounds from natural sources, e.g. a series of salicylidene acylhydrazides with effect against both intracellular (*Chlamydia trachomatis*) and extracellular pathogens (*Yersinia* spp.).[50] Of interest, when tested in mouse models, some of these molecules demonstrated protective activity against the sexually transmitted pathogen *C. trachomatis*.[32] However, the mechanisms by which these compounds act have yet to be elucidated, making difficult an immediate use of them in the near future.[39]

C. Inhibiting biofilm-related factors

1. Extracellular matrix/polysaccharides (EPS)

The extracellular polymeric substance (EPS) is the main constituent of the biofilm matrix, the physical barrier of the biofilm from the host immune response and the external environmental threats. EPS is made of various proteins and polysaccharides, including among them extracellular DNA, DNABII proteins, pili, flagella, and outer membrane vesicles.[51] Apparently, these components are heavily involved in biofilm protection: EPS likely limits engulfment by host cells, as well as the penetration of immune components. The extracellular DNA (eDNA) and polysaccharides can bind and sequester immune components, particularly those with a charge differential such as AMPs and matrix eDNA. Opsonization by complement and immunoglobulins are also negatively affected by EPS. The bacteria within the biofilm respond by producing factors that limit the oxidative and non-oxidative capabilities of phagocytic cells, aiding bacterial survival.[51] Altogether these elements can facilitate biofilm survival and, consequently, can favor the establishment of persistent or recurrent infections.

Apart their immunoprotective role, extracellular DNA (eDNA) and the DNABII family of proteins are also crucial for the structural stability of the intracellular DNA and the overall integrity of the biofilm matrix[52]. In fact, when antisera were used against an *E. coli* DNABII family member in an *in vitro* study using EPS from various human pathogens, the interaction led to biofilm disruption[52]. Moreover, the study demonstrated that the production of antibodies against a common family of bacterial nucleoid-associated proteins, such as IHF, led to EPS disruption and consequently to biofilm dissolution.[52] This methodology can be used in order to indirectly expose bacterial biofilms to more conventional therapies, such as antibiotics.[52]

Another approach for the disruption of biofilms formed by *P. aeruginosa* has involved the use of glycoside hydrolases to target exopolysaccharides present within the EPS, such as the enzymes PelAh, PslGh and Sph3h shown to disrupt existing *P. aeruginosa* biofilms *in vitro*.[51,53]

For *Salmonella*, cellulase has been used *in vitro* to target cellulose, which often (depending on growth conditions) has a dramatic negative effect on biofilm formation. DNase is also highly effective at disrupting eDNA-rich biofilms formed by *Salmonella*.[51]

Likewise, the enzyme Dispersin B in combination with an AMP showed synergistic antibiofilm/antibacterial activity in a chronic wound model of *P. aeruginosa* infection.[51]

Overall, EPS disruption molecules, enzymes or antibodies, have the potential to become a common therapeutic approach for recurrent diseases, either as first-line therapy or as a complementary one.[52]

2. Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are oligopeptides with a varying number (from five to over a hundred) of amino acids used by the host for his defense. AMPs target the lipopolysaccharide layer of the cell membrane, which is ubiquitous in microorganisms, but not in eukaryotic cells. AMPs can target a broad spectrum of organisms ranging from viruses to parasites and as such, they can be also used as an alternative therapy to antibiotic resistant bacteria. In line with this, several studies have reported that various types of AMPs (naturally occurring or synthetic) can act upon bacterial biofilm.[54] For example Wang *et al*.[55] demonstrated that the use of a cationic antimicrobial peptide named chrysophsin-1 could be employed as an alternative treatment, or even as a prevention technique, against pathogenic *S. mutans* biofilms, who predominate in dental caries. In another study, a natural AMP called LL-37, and some of its synthetic fragments, managed to successfully inhibit biofilm formation of *P. aeruginosa* PAO1 strain.[36] Finally, when bovine AMPs were tested at subinhibitory concentration, they still managed to inhibit biofilm formation, although they did not kill bacteria. Further to this finding, these peptides were shown to be efficient agents to eradicate pre-formed biofilm and for the treatment of cystic fibrosis diseases provoked by *P. aeruginosa, S. maltophilia* and *S. aureus*.[36]

3. Quorum-sensing inhibitors as anti-biofilm agents (QS)

One alternative approach is targeting the bacterial communication system (quorum sensing, QS). QS is a process by which bacteria produce and detect signal molecules and thereby coordinate their community-wide behaviours in a cell-density-dependent manner, such as formation, virulence, conjugation, sporulation, and swarming motility. QS is important in many human and plant pathogens such as *P. aeruginosa, S. aureus, E. coli, S. typhimurium, Erwinia*, and *A. tumefaciens*. The mechanism of cell-to-cell communication that is based on the production, secretion, and detection of small signalling molecules, called autoinducers (AIs).

Three main QS systems can be distinguished: 1) the acylhomoserine lactone (AHL) QS system in Gram-negative bacteria, 2) the autoinducing peptide (AIP) QS system in Gram-positive bacteria and 3) the autoinducer-2 (AI-2) QS system in both Gram-negative and -positive bacteria.[32,36]

QS networks can be inhibited or modulated at three levels: (i) through inhibition of signal generation

e.g. by blocking synthesis of AHL using AHL analogues, (ii) by degrading the signal molecule with the means of enzymes, antibodies or by altering the pH and (iii) by blocking the QS signal molecule/receptor interaction. This latter approach is the one mostly used. So far there are several natural and synthetics molecules already discovered exhibiting a role as antagonists of many bacterial "sensors" , eg from enterobacteria, *Pseudomonas, Staphylococcus*. Moreover, some of them have been tested in animal models of infection with positive results.[32, 56-57]

Targeting QS systems in Gram-negative and Gram-positive bacteria constitutes a novel pharmacological approach to control bacteria virulence and biofilm formation. In recent years, the development of new anti-quorum sensing drugs has been gaining ground since they have the advantage to affect bacterial behaviours, without killing or inhibiting their growth[36,58]. Although much remains to be learned about the involvement of QS in biofilm formation, maintenance, and dispersal, QS inhibitors (QSI) seem to be promising agents against biofilm-associated infections.[59-60].

D. Applications in vaccines

Immunization using adhesion-based vaccines may be an effective strategy to ward off other diseases such as otitis media, pneumonia, meningitis, pyelonephritis, and gonorrhea. Organisms responsible for these diseases, i.e. *Haemophilus influenzae, K. pneumoniae, Neisseria meningitidis*, pyelonephritic *E. coli*, and *Neisseria gonorrhoeae*, respectively, express pilus-associated adhesins. Vaccination with these bacterial adhesins may elicit antibodies that will effectively block microbial colonization of mucosal surfaces and be an effective means to treat or prevent these infections as well.[61] Furthermore, binding of anti-adhesin immunoglobulins on the bacterial surface tends to promote the opsonization of the pathogen.

Currently, lots of patents for bacterial antigens proposed as potential vaccine targets have been submitted, namely for adhesins that contribute to the virulence of various pathogens, such as *Y. pseudotuberculosis, Staphylococcus* spp (like *S. aureus; S. epidermidis*), *Streptococcus* spp., *N. meningitidis, N. gonorrhoeae, H. influenzae, Helicobacter pylori, E. coli, Burkholderia pseudomallei, Burkholderia mallei, Bartonella* spp., *Haemophilus, Actinobacillus, Shigella, Brucella, Ralstonia, Sinorhizobium, Bradorhizobium, Burkholderia*.[62] Even trimeric autotransporters (TAA), such as BCAM0224 of *Acinetobacter baumannii* have been proposed as vaccine candidates against infections.[63]

Genomics has enabled large-scale identification of potential targets through *in silico* comparisons of pathogenic and nonpathogenic strains and mining of existing microbial metabolic databases in order to discover targets with therapeutic potential.[64]

The FimCH vaccine is a characteristic example of a subunit vaccine targeting UTI[61]: Extensive preclinical testing has been done so far, all with positive results: as expected, sera from vaccinated mice and cynomolgus inhibited uropathogenic *E. coli* from binding to human bladder cells *in vitro*. Similarly, passive systemic immunization with FimH abolished almost completely the *in vivo* UPEC colonization of the bladder mucosa[61], presumably due to the FimH-specific antibodies produced from the mice, also detected in urinary samples. In line with the above finding, polyclonal antibodies have been also shown to block FimH along with a general stimulation of the immune response (phagocytes, complement etc) in order to clear the infection.[61]

In line with the approach of Type 1 pili, more vaccine candidates have been designed targeting other CUP pilus types, eg. P pili and Dr fimbriae. A vaccine against the P pili can be of great utility against pyelonephritis and for the prevention of complicated advanced UTIs. In a preclinical setting, administration of the subunit PapDG vaccine intraperitoneally protected mice from infection and elicited a specific IgG antibody response in cynomolgus monkeys.[45,65].On the other hand, vaccination with purified Dr. fimbriae, although it gave high titers of serum antibodies, it did not decrease the colonization on the bladder or kidney host cells.[61]

Finally, it is important to consider that the correct choice of the subunit will define the efficacy of the vaccine candidate. In a recent experiment, for example, it was shown that vaccination of mice EbpA, the tip adhesin of Ebp pilus of *E. faecalis*, resulted in significant protection against *E. faecalis* CAUTI, but not when the mice got vaccinated with other Ebp pilus protein subunits.[66] In fact, it has been demonstrated that the N-terminal portion of EbpA, which contains the fibrinogen-binding domain, would be sufficient for vaccinating and thus protecting mice from subsequent infection, and it could be even more protective than vaccinating with the entire adhesion.[45]

Reverse vaccinology, a strategy developed for the identification of antigens as potential vaccine targets, has also given promising results forvaccines against e.g. various serotypes of *N. meningitidis. Neisseria meningitides* is a commensal bacterium of the human nasopharynx which, under certain conditions, can be fatal to susceptible subjects, such as children and young adults. In fact, *N. meningitidis* can cross the mucosal and the blood-brain barrier, multiply in the bloodstream or in the cerebrospinal fluid and lead to se-

pticemia or meningitis, respectively. The developed vaccines are conjugate ones, meaning that a combination of the capsular polysaccharides from the five most known serotypes, i.e. A, B, C, Y and W13 have been used as antigens[67-68]. In these vaccines the adhesin NadA from *Neisseria meningitidis* was shown to bind to monocytes derived from dendritic cells, acting as an immunopotentiator, leading to cytokines expression and T lymphocyte proliferation and differentiation.[62,68]

Similarly, reverse vaccinology has revealed 19 putative antigenic proteins as potential vaccine candidates for *Corynebacterium urealyticum*. *C. urealyticum* is an opportunistic pathogen that under normal conditions lives on the skin and mucous membranes, but which, in immunocompromised patients, can cause acute or encrusted cystitis. Among the discovered proposed proteins is also the spaDEF operon, which encodes pili forming proteins, suggesting that spaDEF may be closely involved in the adhesion of the pathogen to the host tissue.[69] Similar studies with pili of Gram-positive bacteria have been conducted with equally promising results, e.g. for *S. pneumonia, S. agalactiae*[70-72].

Adhesins can be used not only as vaccine antigens, but also as specific immunogens in order to generate antibodies for the treatment of the related disease.[62] A notable example of a patent, in which the use of both the adhesin and antibodies is described, is found in the US0150943 (US 20100150943 A1). This patent describes a new adhesin island found in *Streptococcus* bacteria, which is composed of genes that encode surface proteins (mainly adhesins and sortases) that are important for bacterial virulence. The proteins encoded by this genomic island are associated with the pilus formation, and are believed to be involved in the bacterium initial adherence mechanism. Thus, as a consequence, an immune response against these proteins will tend to slow or prevent the infection. Besides, these proteins seem to be involved in biofilm formation, which can improve bacteria resistance to the host immune response and also to antibiotics. Associated with the fact that these genomic islands can occur in many Gram-positive bacteria (*Staphylococcus, Streptococcus, Enterococcus, Clostridium, Listeria* and *Corynebacterium*), these particular adhesins may represent promising antigens to compose diverse future vaccines.[62]

Conclusions

Biofilm-associated infections (BAI) are based on the communication of the pathogen with the host as well as of the microbes that reside within biofilms. A very good understanding of the bacterial arsenal and of the underlying mechanisms of interaction will allow scientists to explore all these therapeutic strategies that can make use of the bacterial tools, i.e. pili, curli, proteins, polysaccharides in favor of the patients. The present review gives a glimpse of the progress done so far on this field and the current status of the developed strategies. Drugs that may block bacterial adhesion, as well as vaccines against bacterial adhesins, seem to be the most promising therapies for the time being; already there are preclinical tests performed in mice, as well as clinical trials in various phases of development.

A more systematic discussion on biofilm-associated infections (BAI) including physicians, medical microbiologists, and biofilm researchers can set the ground for better diagnostic tools and treatments of BAI.

Funding

There is no specific funding received for this work.

Transparency declarations

The author has worked in the past in the field of vaccines with European funds. There is no conflict of interest to declare.

Transparency and authorship declaration

Charalampia G. Korea, hereby confirm that both figures included in the review have been designed by me and have not been reproduced by another source.

Περίληψη

Ψάχνοντας τι επόμενες θεραπείες μέσα στο οπλοστάσιο των βακτηρίων

Χαραλαμπία Κορέα
Pharmassist Ltd, Contract Research Organisation, Χολαργός, Αθήνα, Ελλάδα

Οι βιομεμβράνες είναι κοινότητες βακτηρίων, συχνά πολυμικροβιακές, προσκολλημένες σε αβιοτικές ή βιοτικές επιφάνειες και εγκλωβισμένες σε εξωκυτταρικό πολυμερές (EPS). Το πολυμερές περιέχει πολυσακχαρίτες, πρωτεΐνες και DNA μικροβιακής προέλευσης.
Η δημιουργία βιομεμβρανών πραγματοποιείται σε τέσσερα στάδια: πρώτη επαφή, προσκόλληση, ωρίμανση και διασπορά. Σε ευνοϊκό περιβάλλον, η αρχικά αναστρέψιμη προσκόλληση οδηγεί στη δημιουργία μικρο-αποικιών και στην παραγωγή εξωκυτταρικού υλικού και ως εκ τούτου στην ωρίμανση της βιομεμβράνης. Διάφορες βακτηριακές προσκολλητίνες (πχ, Ivίδια τύπου 1, P, curli), το εξωκυτταρικό πολυμερές ή τα μόρια για τη διακυτταρική επικοινωνία μεταξύ βακτηρίων (Quorum-Sensing) εμπλέκονται στενά σε όλα τα στάδια δημιουργίας των βιομεμβρανών και ευθύνονται για την αντοχή τους.
Στον ιατρικό τομέα το ιδιαίτερα ανθεκτικό προφίλ των βιομεμβρανών θέτει σοβαρά προβλήματα τόσο στη διάγνωση των λοιμώξεων που σχετίζονται με τις βιομεμβράνες (Biofilm-Associated Infection, BAI), όσο και στην καταπολέμησή τους. Το μεγαλύτερο εμπόδιο στην αποτελεσματική αντιμετώπιση των BAI είναι η μη ανταπόκριση στη συμβατική αντιβιοτική θεραπεία. Αυτή η μειωμένη ευαισθησία στα αντιβιοτικά προκαλείται από ένα συνδυασμό διαφορετικών παραγόντων, όπως 1) η ελλιπής διείσδυση της αντιβίωσης στο εξωκυτταρικό πολυμερές, 2) η παρουσία εμμένοντων κυττάρων και 3) η παρουσία είτε στάσιμων κυττάρων είτε κυττάρων που έχουν ενεργοποιήσει απαντήσεις ενάντια στο χημικό στρες μέσα στο πολυμερές της βιομεμβράνης.
Μέχρι σήμερα είναι γνωστό ότι οι βιομεμβράνες ευθύνονται για μια σειρά χρόνιων λοιμώξεων όπως η ενδοκαρδίτιδα, η κυστική ίνωση, η χρόνια βρογχοπνευμονία, η χρόνια εμμένουσα ωτίτιδα κτλ. Επιπρόσθετα οι βιομεμβράνες ευθύνονται συχνά για τις νοσοκομειακές λοιμώξεις που σχετίζονται με τις βιοατρικές συσκευές, πχ τους βηματοδότες, τους καθετήρες, τις προσθετικές καρδιακές βαλβίδες, κτλ.
Η παρούσα ανασκόπηση παρουσιάζει τις τρέχουσες πειραματικές και κλινικές προσεγγίσεις για την ανάπτυξη στρατηγικών πρόληψης και θεραπειών ενάντια στις BAI. Οι πιο πρόσφατες ανακαλύψεις περιλαμβάνουν ουσίες που μπορούν είτε να εμποδίσουν τη βακτηριακή προσκόλληση ή να αναστείλουν μια σειρά από δομές και πρωτεΐνες που σχετίζονται με τις βιομεμβράνες. Επιπρόσθετα παρουσιάζεται εν συντομία η πρόοδος που έχει γίνει για την ανάπτυξη εμβολίων χρησιμοποιώντας τους σχετικούς παράγοντες μολυσματικότητας ως αντιγόνα.
Η κατανόησή τους μπορεί να ανοίξει το δρόμο για τη δημιουργία και την ανάπτυξη διαγνωστικών εργαλείων και στοχευμένων θεραπειών για μια αποτελεσματική αντιμετώπιση των λοιμώξεων που σχετίζονται με τις βιομεμβράνες.

Λέξεις κλειδιά
βιομεμβράνες, θεραπείες, αναστολείς quorum-sensing, αναστολείς προσκόλλησης

References

1. Costerton JW, Irvin RT, Cheng KJ. The role of bacterial surface structures in pathogenesis. *Crit Rev Microbiol.* 1981;8:303-38
2. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science*. 1999;284:1318-22
3. Dunne WM Jr. Bacterial adhesion: seen any good biofilms lately? *Clin Microbiol Rev.* 2002;15:155-66
4. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol*. 2004;2:95-108
5. Hall-Stoodley L, Stoodley P. Evolving concepts in biofilm infections. *Cell Microbiol.* 2009;11:1034-43
6. Jacques M, Aragon V, Tremblay YD. Biofilm formation in bacterial pathogens of veterinary importance. *Anim Health Res Rev.* 2010;11:97-121
7. Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature.* 2000;407:762-4
8. Singh R, Paul D, Jain RK. Biofilms: implications in bioremediation. *Trends Microbiol.* 2006;14:389-97
9. Goodman AL, Kulasekara B, Rietsch A, Boyd D, Smith RS, Lory S. A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in Pseudomonas aeruginosa. *Dev Cell.* 2004;7:745-54
10. Cohen Y. Bioremediation of oil by marine microbial mats. *Int Microbiol.* 2002;5:189-93
11. Costerton JW, Montanaro L, Arciola CR. Biofilm in implant infections: its production and regulation. *Int J Artif Organs.* 2005;28:1062-8
12. Jain A, Gupta Y, Agrawal R, Khare P, Jain SK. Biofilms--a microbial life perspective: a critical review. *Crit Rev Ther Drug Carrier Syst.* 2007;24:393-443
13. Macfarlane S. Microbial biofilm communities in the gastrointestinal tract. J Clin Gastroenterol. 2008;42 Suppl 3 Pt 1:S142-3
14. Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated communities. *Annu Rev Microbiol.* 2002;56:187-209
15. Salwiczek M, Qu Y, Gardiner J, Strugnell RA, Lithgow T, McLean KM, Thissen H. Emerging rules for effective antimicrobial coatings. *Trends Biotechnol.* 2014;32:82-90.
16. Gilbert P, Allison DG, McBain AJ. Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? *J Appl Microbiol.* 2002;92 Suppl:98S-110S
17. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet.* 2001;358(9276):135-8
18. Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol.* 2002; 292(2): 107-13
19. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents.* 2010;35:322-32
20. Abbas HA; Serry FM.; El-Masry, Eman M. Biofilms: the Microbial Castle of Resistance. *Research Journal of Pharmacy and Technology,* [S.l.], p. 1-3, jan. 2013. ISSN 0974-360X.
21. Khameneh B, Diab R, Ghazvini K, Fazly Bazzaz BS. Breakthroughs in bacterial resistance mechanisms and the potential ways to combat them. *Microb Pathog.* 2016;95:32-42.
22. Richards, J.J. and Melander, C. Small Molecule Approaches Toward the Non-Microbicidal Modulation of Bacterial Biofilm Growth and Maintenance. *Anti-Infective Agents in Medicinal Chemistry,* 2009;8:295-314
23. Donlan RM. Role of biofilms in antimicrobial resistance. *ASAIO J.* 2000 Nov-Dec;46(6):S47-52. Erratum in: ASAIO J 2001;47:99
24. Balcázar JL, Subirats J, Borrego CM. The role of biofilms as environmental reservoirs of antibiotic resistance. *Front Microbiol.* 2015;6:1216.
25. Veerachamy S, Yarlagadda T, Manivasagam G, Yarlagadda PK. Bacterial adherence and biofilm formation on medical implants: a review. *Proc Inst Mech Eng H.* 2014;228:1083-99.
26. Hall-Stoodley L, Stoodley P, Kathju S, Høiby N, Moser C, Costerton JW, *et al.* Towards diagnostic guidelines for biofilm-associated infections. FEMS *Immunol Med Microbiol.* 2012;65:127-45
27. Stones DH, Krachler AM. Fatal attraction: how bacterial adhesins affect host signaling and what we can learn from them. *Int J Mol Sci.* 2015;16:2626-40.
28. Wellens A, Garofalo C, Nguyen H, Van Gerven N, Slättegård R, Hernalsteens JP, *et al.* Intervening with urinary tract infections using anti-adhesives based on the crystal structure of the FimH-oligomannose-3 complex. *PLoS One.* 2008;3:e2040. Erratum in: PLoS ONE. 2008;3
29. Raz R, Chazan B, Dan M. [Cranberry juice and urinary tract infection]. *Harefuah.* 2004;143:891-4
30. Abraham SN, Babu JP, Giampapa CS, Hasty DL, Simpson WA, Beachey EH. Protection against *Escherichia coli*-induced urinary tract infections with hybridoma antibodies directed against type 1 fimbriae or complementary D-mannose receptors. *Infect Immun.* 1985;48:625-8
31. de Ruyck J, Lensink MF, Bouckaert J. Structures of C-mannosylated anti-adhesives bound to the type 1 fimbrial FimH adhesin. *IUCrJ.* 2016;3(Pt 3):163-7

32. Heras, B, Scanlon, MJ. and Martin, JL. Targeting virulence not viability in the search for future antibacterials. *British Journal of Clinical Pharmacology,* 79:208–215
33. Lillington J, Waksman G. Ordered and ushered; the assembly and translocation of the adhesive type I and p pili. Biology (Basel). 2013;2:841-60
34. Wittschier N, Lengsfeld C, Vorthems S, Stratmann U, Ernst JF, Verspohl EJ, Hensel A. Large molecules as anti-adhesive compounds against pathogens. *J Pharm Pharmacol.* 2007;59:777-86
35. Huttunen S, Toivanen M, Liu C, Tikkanen-Kaukanen C. Novel anti-infective potential of salvianolic acid B against human serious pathogen *Neisseria meningitidis. BMC Res Notes.* 2016;9:25
36. Ivanova K, Fernandes MM and Tzanov T. Current advances on bacterial pathogenesis inhibition and treatment strategies. Chapter from "Microbial pathogens and strategies for combating them: science, technology and education, Publisher: Formatex Research Center, Editors: A. Méndez-Vilas, pp.322-336.
37. Khanal M, Larsonneur F, Raks V, Barras A, Baumann JS, Martin FA, *et al.* Inhibition of type 1 fimbriae-mediated *Escherichia coli* adhesion and biofilm formation by trimeric cluster thiomannosides conjugated to diamond nanoparticles. *Nanoscale.* 2015 ;7:2325-35
38. Fronzes R, Remaut H, Waksman G. Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria. *The EMBO Journal*. 2008;27: 2271-2280
39. Steadman D, Lo A, Waksman G, Remaut H. Bacterial surface appendages as targets for novel antibacterial therapeutics. *Future Microbiol.* 2014;9:887-900
40. Oliver A, Canton R, Campo P, Baquero F, Blazquez J. High frequency of hyper- mutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 2000;288:1251–3.
41. Ciofu O, Riis B, Pressler T, Poulsen HE, Høiby N. Occurrence of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. *Antimicrob Agents Chemother* 2005;49:2276–82.
42. Greene SE, Pinkner JS, Chorell E, Dodson KW, Shaffer CL, Conover MS, *et al.* Pilicide ec240 disrupts virulence circuits in uropathogenic *Escherichia coli. MBio.* 2014;5(6):e02038
43. Pinkner JS, Remaut H, Buelens F, Miller E, Aberg V, Pemberton N, *et al.* Rationally designed small compounds inhibit pilus biogenesis in uropathogenic bacteria. *Proc Natl Acad Sci* U S A. 2006;103:17897-902
44. Piatek R, Zalewska-Piatek B, Dzierzbicka K, Makowiec S, Pilipczuk J, Szemiako K, et al. Pilicides inhibit the FGL chaperone/usher assisted biogenesis of the Dr fimbrial polyadhesin from uropathogenic *Escherichia coli. BMC Microbiol.* 2013;13:131
45. Spaulding CN, Hultgren SJ. Adhesive Pili in UTI Pathogenesis and Drug Development. *Pathogens.* 2016;5. pii: E30. doi: 10.3390/pathogens5010030.
46. Cegelski L, Pinkner JS, Hammer ND, Cusumano CK, Hung CS, Chorell E, *et al.* Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. *Nat Chem Biol.* 2009;5:913-9
47. Du Toit A. Biofilms: The T3SS translocon in biofilm formation. *Nat Rev Microbiol.* 2015;13:2-3
48. Tran CS, Rangel SM, Almblad H, Kierbel A, Givskov M, Tolker-Nielsen T, *et al.* The *Pseudomonas aeruginosa* type III translocon is required for biofilm formation at the epithelial barrier. PLoS Pathog. 2014 Nov 6;10:e1004479
49. Duncan MC, Linington RG, Auerbuch V. Chemical inhibitors of the type three secretion system: disarming bacterial pathogens. *Antimicrob Agents Chemother.* 2012;56:5433-41
50. Pan N, Lee C, Goguen J. High throughput screening for small-molecule inhibitors of type III secretion in *Yersinia pestis. Adv Exp Med Biol.*2007;603:367-75
51. Gunn JS, Bakaletz LO, Wozniak DJ. What's on the Outside Matters: The Role of the Extracellular Polymeric Substance of Gram-negative Biofilms in Evading Host Immunity and as a Target for Therapeutic Intervention. *J Biol Chem.* 2016;291:12538-46
52. Goodman SD, Obergfell KP, Jurcisek JA, Novotny LA, Downey JS, Ayala EA, *et al.* Biofilms can be dispersed by focusing the immune system on a common family of bacterial nucleoid-associated proteins. *Mucosal Immunol.* 2011;4:625-37
53. Yu S, Su T, Wu H, Liu S, Wang D, Zhao T, *et al.* PslG, a self-produced glycosyl hydrolase, triggers biofilm disassembly by disrupting exopolysaccharide matrix. *Cell Res.* 2015;25:1352-67.
54. Bahar AA, Ren D. Antimicrobial peptides. *Pharmaceuticals (Basel).* 2013;6:1543-75
55. Wang W, Tao R, Tong Z, Ding Y, Kuang R, Zhai S, Liu J, Ni L. Effect of a novel antimicrobial peptide chrysophsin-1 on oral pathogens and *Streptococcus mutans* biofilms. *Peptides.* 2012;33:212-9
56. Tay SB, Yew WS. Development of quorum-based anti-virulence therapeutics targeting Gram-negative bacterial pathogens. *Int J Mol Sci.* 2013;14:16570-99
57. Hirakawa H, Tomita H. Interference of bacterial cell-to-cell communication: a new concept of antimicrobial chemotherapy breaks antibiotic resistance. *Front Microbiol.* 2013;4:114
58. Hentzer M, Givskov M. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *J Clin Invest.* 2003;112:1300-7
59. Brackman G, Coenye T. Quorum sensing inhibitors as anti-biofilm agents. *Curr Pharm Des.* 2015;21:5-11
60. Kalia VC. Quorum sensing inhibitors: an overview. *Biotechnol Adv.* 2013;31:224-45
61. Langermann S, Palaszynski S, Barnhart M, Auguste G, Pinkner JS, Burlein J, *et al.* Prevention of mucosal

Escherichia coli infection by FimH-adhesin-based systemic vaccination. *Science.* 1997;276:607-11.
62. Carvalho E, Ching Ching AT, Estima Abreu PA, Ho PL, Barbosa AS. Breaking the bond: recent patents on bacterial adhesins. *Recent Pat DNA Gene Seq.* 2012;6:160-71
63. Mil-Homens D, Leça MI, Fernandes F, Pinto SN, Fialho AM. Characterization of BCAM0224, a multifunctional trimeric autotransporter from the human pathogen *Burkholderia cenocepacia. J Bacteriol.* 2014;196:1968-79
64. Nagaraj NS, Singh OV. Using genomics to develop novel antibacterialtherapeutics. *Crit Rev Microbiol.* 2010;36:340-8
65. Roberts JA, Kaack MB, Baskin G, Chapman MR, Hunstad DA, Pinkner JS, Hultgren SJ. Antibody responses and protection from pyelonephritis following vaccination with purified *Escherichia coli* PapDG protein. *J Urol.* 2004;171:1682-5.
66. Flores-Mireles AL, Pinkner JS, Caparon MG, Hultgren SJ. EbpA vaccine antibodies block binding of *Enterococcus faecalis* to fibrinogen to prevent catheter-associated bladder infection in mice. *Sci Transl Med.* 2014;6:254ra12.
67. Esposito V, Musi V, de Chiara C, Veggi D, Serruto D, Scarselli M, *et al.* Structure of the C-terminal domain of Neisseria heparin binding antigen (NHBA), one of the main antigens of a novel vaccine against *Neisseria meningitidis. J Biol Chem.* 2011;286:41767-75
68. Serruto D, Bottomley MJ, Ram S, Giuliani MM, Rappuoli R. The new multicomponent vaccine against meningococcal serogroup B, 4CMenB: immunological, functional and structural characterization of the antigens. *Vaccine.* 2012;30 Suppl 2:B87-97.
69. Guimarães L, Soares S, Trost E, Blom J, Ramos R, Silva A, et al. Genome informatics and vaccine targets in *Corynebacterium urealyticum* using two whole genomes, comparative genomics, and reverse vaccinology. *BMC Genomics.* 2015;16 Suppl 5:S7
70. Lauer P, Rinaudo CD, Soriani M, Margarit I, Maione D, Rosini R, *et al.* Genome analysis reveals pili in Group B *Streptococcus. Science.* 2005;309:105.
71. Rosini R, Margarit I. Biofilm formation by *Streptococcus agalactiae:* influence of environmental conditions and implicated virulence factors. *Front Cell Infect Microbiol.* 2015;5:6
72. Margarit I, Rinaudo CD, Galeotti CL, Maione D, Ghezzo C, Buttazzoni E, *et al.* Preventing bacterial infections with pilus-based vaccines: the group B streptococcus paradigm. *J Infect Dis.* 2009;199:108-15