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Book Chapter Bacterial Biofilm (Review)

Natural Compounds Inhibiting *Pseudomonas aeruginosa* Biofilm Formation by Targeting Quorum Sensing Circuitry

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Abstract

The biofilm lifestyle mode certainly represents one of the most successful behaviors to facilitate bacterial survival in diverse inhospitable environments. Conversely, the ability of bacteria to develop effective biofilms represents one of the major obstacles in the fight against bacterial infections. In *Pseudomonas aeruginosa*, the biofilm formation is intimately connected to the quorum sensing (QS) mechanisms, a mode of cell-to-cell communication that allows many bacteria to detect their population density in order to coordinate common actions. In this chapter, we propose an overview *(i)* on *P. aeruginosa* QS mechanisms and their implication in biofilm formation; and *(ii)* on natural products that are known to interfere with these QS mechanisms, subsequently disrupting biofilm formation. The concluding remarks focus on perspectives of these compounds as possible antibiotherapy adjuvants.

Keywords: Biofilm, *las,* Natural products, PQS, *Pseudomonas*, Quorum sensing *rhl*

1. Introduction

Bacterial infections are mainly related to the ability of bacteria to invade and disseminate through their hosts by using different types of motility, by releasing a myriad of virulence factors, by building structured biofilm which lead to host cell and tissue damage but also allow bacteria to evade the immune system and conventional antimicrobial agents [1]. For decades, antibiotics, although less effective in biofilm-growing bacteria [2], have represented our best weapon against bacterial diseases. However, the on-going emergence and worldwide spreading of resistant bacteria is considerably reducing the antibiotic pallet available for the treatment of bacterial infections [3]. This alarming situation forces researchers to consider other strategies to combat bacterial infections, notably the use of phages [4] or the use of alternative agents, such as essential oils [5], silver nanoparticles [6], bacteriocins [7] and antimicrobial peptides [8]. Some interesting strategies propose original compounds that disrupt biofilm formation without affecting the

viability of invading bacteria; this strategy is expected *(i)* to reduce the bacterial aptitude to build protective barriers, but without exerting a selective pressure *per se* [4]; *(ii)* to allow sufficient time for the immune defenses to effectively destroy invaders; and *(iii)* to minimize the use of effective antibiotics.

In most bacteria, the expressions of virulence factors are coordinated by quorum sensing (QS) mechanisms, a cell-to-cell communication which allows bacteria to detect their population density by producing and perceiving diffusible signal molecules to synchronize common actions [9]. This cell-to-cell communication has been largely investigated in *Pseudomonas aeruginosa,* an opportunistic pathogen which mainly affects people who are severely immunocompromised, in part due to its ability to evade from both innate and acquired immune defenses through adhesion, colonization and biofilm forming, and to produce various virulence factors that cause significant tissue damage [10-11]. In this bacterium, QS regulates virulence factors production, motilities and, in particular, biofilm formation for which QS is one of the relevant key actors. Interestingly, within the two past decades, study papers reporting natural and synthetic compounds that interfere with QS and/or biofilm formation are regularly published; QS circuitry and biofilm formation control mechanisms indeed constitute promising targets to struggle against *P. aeruginosa* infection with potential huge clinical interests [12]. The present chapter covers the scope of natural compounds from both prokaryote and eukaryote organisms that have been identified to disrupt the biofilm lifestyle cycle in *P. aeruginosa via* modulation of QS mechanisms. An overview of the entanglement between QS circuitry and biofilm formation is reported as a prerequisite for a better understanding of the mechanisms of action proposed for some of the identified compounds. The concluding remarks focus on the perspectives of these compounds as possible antibiotherapy adjuvants for possible eradication of resistant infections caused by *P. aeruginosa*.

2. *P. aeruginosa* Biofilm lifestyle

Like most bacteria, *P. aeruginosa* can develop two distinct lifestyles, planktonic and sessile cells. The planktonic state is encountered when *P. aeruginosa* evolves freely in a liquid suspension whereas, on natural or synthetic surfaces, *P. aeruginosa* can form sticky clusters in permanent rearrangements characterized by the secretion of an adhesive and protective matrix [13]. Defined as "biofilm", this set of bacterial community adherent to a surface appears as an adaptive response to an environment more or less unsuited to growth in planktonic form [14].

The biofilm formation can be delimited in 5 main stages (Figure 1). A first reversible phase corresponds to the initial adhesion of bacteria to surfaces (image B); this adhesion becomes irreversible in the second stage (image B). Then, thanks to a proliferation period corresponding to the third stage, microcolonies are built concomitantly with the production of extracellular matrix (image C), leading to the fourth stage of biofilm structuration and organization in which the growth of three dimensional communities is observed with amplified extracellular matrix production (image D). This biofilm cycle is completed by a dispersion step (image E) [12].

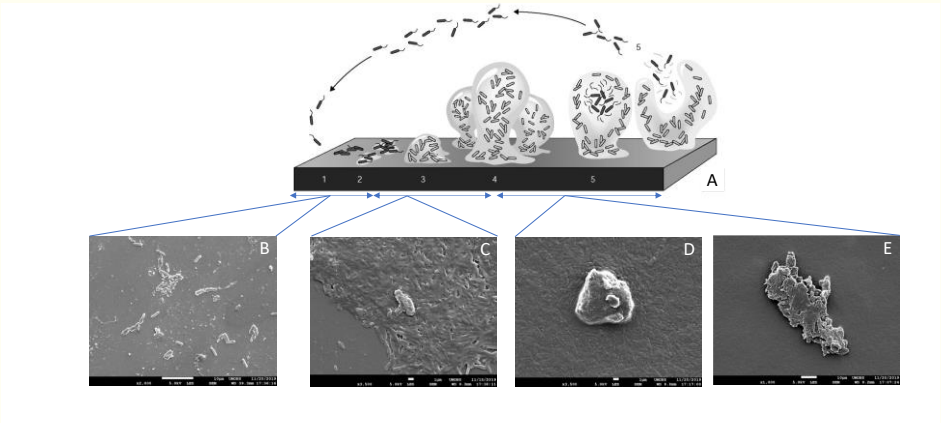

Figure 1. Sketch of the different steps of a biofilm development (A) [15]. Several representative scanning electron microscopy (SEM- JEOL JSM-7200F) images of the *P. aeruginosa* biofilm at different step of development and with different magnifications (B=reversible and irreversible stages at 8h growth, C=microcolonies stage at 12h growth, D=mature biofilm stage at 24h growth and E=dispersion stage at 30h growth). *P. aeruginosa* PAO1 colonies were grown at 37°C with Centers for Disease Control and Prevention (CDC) biofilm reactor (Biosurface Technologies, MT) on tryptone soy broth (TSB).

The secreted extracellular matrix mainly consists of proteins, nucleic acids, lipids and exopolysaccharides (EPS). These account for 50-90 % of total organic matter [16]. *P. aeruginosa* produces at least three types of EPS that are required for biofilm formation and architecture [17] *(i)* Alginate a linear polysaccharide composed of L-guluronic and D-mannuronic acids linked by β-1,4 bonds [18], *(ii)* Pel polysaccharide, a glucose-rich matrix material, with unclarified composition, and *(iii)* Psl polysaccharide, a repeating pentasaccharide consisting of D-mannose, L-rhamnose, and D-glucose. In mucoid strains, EPS are predominantly characterized by the presence of alginate. The alginate participates in the structuring of the biofilm [19], but its real importance is still controversial since some authors claim that it is not essential; indeed architecture and antibiotic resistance profiles of wild-type and alginate-deficient biofilms are identical [20-21]. Nevertheless, the overexpression of alginate was shown to protect *P. aeruginosa* from phagocytosis and host responses [22]. In "non-mucoid" *P. aeruginosa* strains, such as the PAO1 strain isolated from an infected wound [23], alginate is even considered poorly produced at the expense of exopolysaccharides rich in glucose and mannose [24], Pel and Psl, which have been described as being more important in the formation and maintenance of the biofilm [25].

Extracellular DNA (eDNA) is an important component of *P. aeruginosa* biofilm matrix which particularly intervenes in the establishment, maintenance and perpetuation of structured biofilms [26]. Its importance has been demonstrated since *P. aeruginosa* biofilm formation is prevented by exposition to DNase I [27] and biofilms that are deficient in eDNA have been shown to be more sensitive to the detergent sodium dodecyl sulfate [28]. It has been established that eDNA plays roles in bacterial adhesion and in the structural stability of biofilms by maintaining coherent cell alignments [29]; interestingly, its contribution to antimicrobial resistance has also been proposed as eDNA, a highly anionic polymer, is believed to bind cationic antibiotics, such as aminoglycosides and antimicrobial peptides [30].

3. QS mechanisms and their implication in biofilm formation

The complex regulation of biofilm formation involves multiple bacterial machineries including the QS systems. In *P. aeruginosa*, this mechanism is involved in the development of various common bacterial behaviors, including virulence factors expression and biofilm formation, which are mostly implicated in infection success. Three QS systems have been clearly characterized: *(i)* the *las* system and the *rhl* system, two LuxI/R type systems using the signal molecules of the family of acyl-homoserine lactones (AHLs); and *(ii)* the PQS (Pseudomonas Quinolone Signal) system based on molecules of the 2-alkyl-4-quinolone class [10, 31]. The mechanisms of QS in *P. aeruginosa* are summarized in Figure 2 while the main functions regulated by QS systems and involved in the pathogenesis of *P. aeruginosa* are presented in Figure 3.

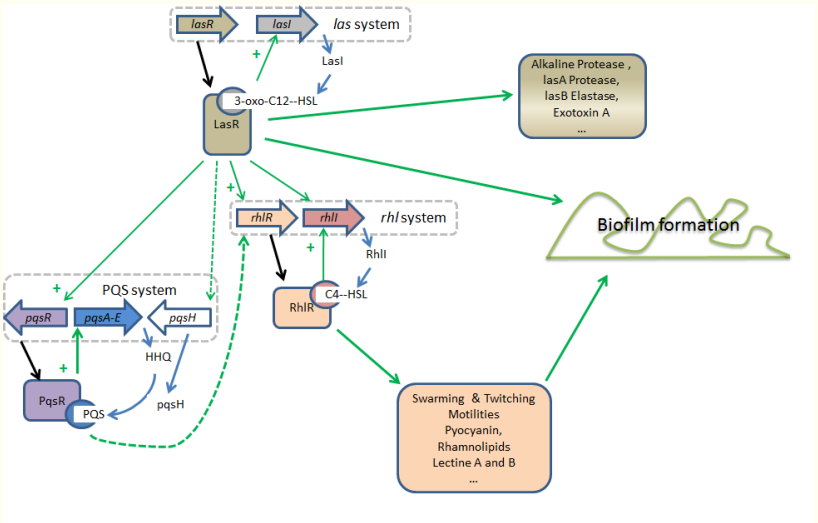

Figure 2. Systems involved in *P. aeruginosa* QS circuitry. The main QS systems in *P. aeruginosa* are the *las, rhl* and PQS systems. The *las* system consists of a *lasR* regulatory gene coding for the LasR protein, a *lasI* gene coding for a LasI synthase involved in the synthesis of a signal molecule of the acyl-homoserine lactone (AHL) family, the 3-oxo-C12-HSL. The LasR/3-oxo-C12-HSL complex is a transcriptional activator of virulence genes (protease, elastase, exotoxine) and *lasI* gene. According to the same model, the *rhl* system consists of *rhlR, rhlI* genes and another AHL, the C4-HSL. This system activates genes in common with the *las* system and also specific genes, such as those coding for the synthesis of rhamnolipids, pyocyanin and swarming/twitching motilities. The *las* system controls the *rhl* system. The third PQS system is interposed between the two main systems. The PqsABCDE operon produces the precursor 2-heptyl-4-quinolone (HHQ), and PqsH catalyzes conversion of HHQ to 2-heptyl-3-hydroxy-4-quinolone (PQS), detected by the receptor PqsR. [10, 31].

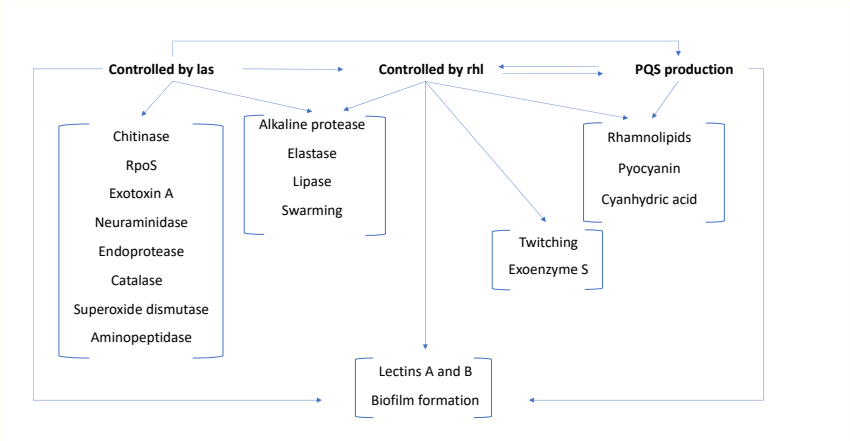

Figure 3. Functions positively regulated by QS in *P. aeruginosa* [10, 31].

Evidence that the *las* system is implicated in biofilm formation has been firstly established when Davies et al. (1998) [32] demonstrated that the biofilm formed by *lasI* mutant appears flat, undifferentiated, and quickly dispersed from the surface upon exposure to sodium dodecyl sulfate, compared to wild type biofilms.

Furthermore, Gilbert et al. [33] observed the binding of the QS regulator LasR to the promoter region of the *psl* operon, suggesting that the *psl* expression may be regulated by the QS. Considering that the *psl* operon is implied in biofilm modulation, the QS then plays a role in the biofilm formation and architecture. The transcription of the *pel* operon seems to be reduced in *rhlI* mutant, suggesting that the *rhl* system plays a biofilm formation role in *P. aeruginosa* by modulating the biosynthesis of the Pel polysaccharide [34]. The *pqsA* mutant produces a biofilm with less eDNA than the wild type biofilm, suggesting that the PQS system also plays a role in biofilm formation, more particularly in the eDNA releasing [34].

Notably, the production of rhamnolipids and lectins is under QS control, indicating a further indirect link between biofilm formation/degradation and QS. Indeed, the *rhl* system controls the production of rhamnolipids [35], that play multiple roles in *P. aeruginosa* biofilm formation: *(i)* as biosurfactant and virulence factor [36]; *(ii)* in the formation of microcolonies [37]; *(iii)* in the maintenance of open channel structures necessary for nutrient circulation [38]; *(iv)* in the development of biofilm mushroom-shaped structures [37]; *(v)* and in cell dispersion from the biofilm [39]. Indeed, an hyper-detaching property has been observed in the *P. aeruginosa* mutants that produce more rhamnolipids compared to wild type strains [40]. Moreover, the *rhl* system also controls the expression of the cytotoxic virulence factors LecA and LecB. Data obtained on mutant strains indicate that these galactophilic lectins probably contribute to the biofilm development [41-42]. Similarly, two types of *P. aeruginosa* motilities implicated in biofilm formation are also QS-regulated. The first movement, swarming motility, accomplishes an organized surface translocation, dependent on cell-to-cell contacts and extensive flagellation [43]; this has been observed during the first stage of *P. aeruginosa* biofilm development and seems to be regulated by the *rhl* system [44]. Flat and uniform biofilms are formed when the strains grow under conditions promoting swarming motility, *e.g.* a growth medium with glutamate or succinate as carbon sources; by contrast, a biofilm without confluent cell aggregates is formed by strains with limited swarming motility [45]. The second movement, a flagella-independent form of translocation, is described as a successive extension and retraction of polar type IV pili [46]. This kind of movement, regulated by the *rhl* system on a Fe-limited minimal medium [47], is necessary to assemble bacteria in monolayers that form microcolonies [38].

4. Other mechanisms implied in biofilm formation

The QS systems are not the sole key actors intervening in biofilm formation by *P. aeruginosa*. Indeed, the complex regulation of biofilm formation involves multiple bacterial machineries that also include the membrane-bound sensor kinase GacS, the transcriptional response regulator GacA (GacS/GacA two-component regulatory system) and the intracellular second messenger bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP). Briefly, the GacS/GacA system acts as a super-regulator of the *las* and *rhl* systems [48] whereas c-di-GMP is important for the biosynthesis of alginate and Pel polysaccharides and for the switch from planktonic to biofilm lifestyle [49].

5. Natural products that affect QS and biofilm formation by *Pseudomonas aeruginosa*

5.1. From Prokaryotes

✓ ***Enzymes***

Microorganisms known to have the ability to produce anti-QS enzymes are still limited to a few bacteria from the families of *(i) Actinobacteria (Rhodococcus* and *Streptomyces); (ii) Firmicutes-Arthrobacter (Bacillus* and *Oceanobacillus); (iii)) Cyanobacteria (Anabaena); (iv) Bacteroidetes (Tenacibaculum) (v) Proteobacteria (Acinetobacter, Agrobacterium tumefaciens, Alteromonas, Comomonas, Halomonas, Hyphomonas, Klebsiella pneumoniae, P. aeruginosa, Ralstonia, Stappia* and *Variovorax paradaoxus*) [50-56].

Four types of enzymes are known to degrade AHLs [57, 58], a phenomenon sometimes described as "quorum quenching" (QQ) [59]; these include AHL-lactonases and decarboxylases that attack the lactone ring (*Bacillus indicus, B. pumilus* and *B.* sp. SS4 cause significant inhibition of QS-dependent activities in Gram-negative bacteria such as *P. aeruginosa* PAO1, *Serratia marcescens* and *Vibrio*), AHL-acylases that cleave the acyl side chain (*B. pumilus* S8-07 degrades 3-oxo-C12-HSL into the corresponding lauric acid [60]), and deaminases that separate the lactone ring from the acyl side chain. Recently, lactonases and acylases were identified in *Erythrobacter, Labrenzia* and *Bacterioplanes* found in Red Sea sediments; these both degrade AHLs of different acyl chain lengths, particularly the 3-oxo-C12-HSL, and inhibit the formation *P. aeruginosa* PAO1 biofilm [59].

Mycobacteroides abscessus subspecies, emerging pathogens, are capable of degrading both PQS and HHQ. *M. abscessus* subsp. *abscessus*, in coculture with *P. aeruginosa* PAO1, reduced PQS levels through a PQS dioxygenase (encoded by the *aqdC* gene, *M. abscessus* subsp. *massiliense*, a recombinant strain overexpressing the *aqdC* gene, reduces the level of the virulence factors pyocyanin, pyoverdine, and rhamnolipids, suggesting that AqdC is a QQ enzyme [61]. Its impact on biofilm formation would have been interesting to investigate as another dioxygenase, the 2-alkyl-3-hydroxy-4(1H)-quinolone 2,4-dioxygenase (HodC), was described to cleave PQS, attenuate the production of virulence factors but conversely increase the viable biomass, in both newly formed and established biofilms, by increasing iron availability [62].

✓ ***Organic acids***

The acetic and phenyl lactic acids, found in the supernatant of probiotic strains *Lactobacillus paracasei* subsp. *paracasei* CMGB isolated from newborn faeces, were shown to inhibit, at non-bacteriostatic/bactericide levels, the expression of QS genes in *P. aeruginosa*, preventing adherence of bacteria to an inert substratum [63, 64]. Similarly, the lactic acid produced by a potential probiotic *Pediococcus acidilactici* M7 strain, also isolated from newborn faeces, inhibits the production of *P. aeruginosa* short-chain AHLs, elastase, protease, pyocyanin, and biofilm as well as the swarming-swimming-twitching motilities [65].

5.2. From Fungi

✓ ***Antibiotics and mycotoxins***

Penicillin produced by *Penicillium* spp. has been shown to be effective in controlling a bacterial infection. Recently, about 33 *Penicillium* spp. have been recognized as producers of QS inhibitors such as the small lactone mycotoxins patulin and penicillic acid. The use of patulin can significantly reduce lung infection caused by *P. aeruginosa* on a mouse model. Interestingly, a synergy has been shown *in vitro* between patulin and tobramycin towards *P. aeruginosa* PAO1 biofilms whereas patulin alone does not affects the development of biofilm [66]. Although the anti-infective property of patulin has been demonstrated, its genotoxicity and potential carcinogenic properties [67] probably preclude clinical applications.

Erythromycin, a macrolide antibiotic isolated from *Saccharopolyspora erythraea*, has been recently demonstrated to reduce virulence factors in *P. aeruginosa* PAO1, including various motilities, biofilm formation, and production of rhamnolipids, total protease, elastase, and pyocyanin at non-microbicidal level (1.6 µg/mL) [68]. Comparably, the erythromycin derivate, azithromycin, shows a strong *P. aeruginosa* QS and biofilm inhibitory effect [69-71] with inhibition of alginate synthesis [69], a reduction of each type of bacteria movement [72] and a diminution of *gacA* gene expression [73]. At weak antibiotic concentration (2 µg/mL), a biofilm inhibition is observed, probably explained by a lower production of both AHL signal molecules, C4-HSL and 3-oxo-C12-HSL, and of virulence factors [74-76].

✓ ***Alkylcyclopentanone***

Recently, Kim et al. [77] indicated that the alkylcyclopentanone terrein, isolated from *Aspergillus terreus*, reduced virulence factors (elastase, pyocyanin, and rhamnolipids) and biofilm formation *via* antagonizing QS receptors without affecting *P. aeruginosa* cell growth. Beyond a negative impact on the production of QS signaling molecules and expression of QS-related genes, terrein also reduced c-di-GMP levels, an important secondary messenger for the switch from planktonic to biofilm lifestyle mode, by decreasing the activity of a diguanylate cyclase required for c-di-GMP biosynthesis [78].

5.3. From Plants

✓ ***Derivates of shikimic acid, phenols and polyphenols***

Many phenolic compounds and derivatives with anti-QS and antibiofilm activities have been isolated from plants [79]. Cinnamaldehyde (the dominant compound of certain essential oils, in particular *Cinnamomum camphora* (L.) J. Presl) and its derivatives modulate a wide range of anti-QS and antibiofilm activities of *P. aeruginosa* [80-82]. *Curcuma longa* L. produces curcumin, which inhibits the expression of virulence genes of *P. aeruginosa* PA01 [83].

Ellagic acid derivatives from *Terminalia chebula* Retz. downregulate *lasIR* and *rhlIR* genes expression and decrease AHLs production, leading to an attenuation of virulence factor production and to an enhanced sensitivity of biofilm facing a tobramycin treatment [84].

Flavonoids have been investigated for their roles as QS modulating compounds. From these, naringenin and taxifolin reduced the expression of several QS-controlled genes (i.e. *lasI, lasR, rhlI, rhlR, lasA, lasB, phzA1* and *rhlA*) in *P. aeruginosa* PAO1. Similarly, the flavan-3-ol catechin, extracted from the bark of *Combretum albiflorum* (Tul.) Jongkind, reduces the production of QS-dependent virulence factors, such as pyocyanin, elastase, and the formation of biofilm by *P. aeruginosa* PAO1 [85]. Interestingly, Baicalin, an active natural compound extracted from the traditional Chinese medicinal *Scutellaria baicalensis*, has been demonstrated to inhibit the formation of *P. aeruginosa* biofilms and enhance the bactericidal effects of antibiotics such as amikacin.

Moreover, at sub-minimal inhibitory concentration (256 µg/mL), this flavonoid has been shown to reduce LasA protease, LasB elastase, pyocyanin, rhamnolipids, and exotoxin A production and to downregulate the three QS-regulatory genes, including *lasI, lasR, rhlI, rhlR, pqsR* and *pqsA* [86]. Consistently, *in vivo* experiments indicated that baicalin treatment reduces *P. aeruginosa* pathogenicity in *Caenorhabditis elegans* and enhances the clearance of *P. aeruginosa* from the peritoneal implants of infected mice.

Furocoumarins from grapefruit can inhibit the QS signaling (AHLs and AI-2) of *V. harveyi* BB886 and BB170 strains as well as biofilm formation in pathogens such as *E. coli* O157: H7, *Salmonella typhimurium* and *P. aeruginosa* [87]. These purified furocoumarins (dihydroxybergamottin and bergamottin), tested at the concentration of 1 µg/mL, cause 94 % inhibition of autoinducers (AHLs) without affecting bacterial viability. Biofilm inhibition was up to 58.3 and 72%, respectively for *E. coli* O157: H7 but modest for *P. aeruginosa* (27.3 and 18.1%, respectively).

Malabaricone C, a diarylnonanoid isolated from the bark of *Myristica cinnamomea* King inhibited the QS-regulated pyocyanin production and biofilm formation in *P. aeruginosa* PAO1 [88].

A screening of various herbs revealed that a clove extract (*Syzygium aromaticum* (L.) Merr. Et Perry) inhibits QS-controlled gene expression (*las* and PQS systems) in *P. aeruginosa* with eugenol as major active constituent [89]. Recently, the effects of eugenol and its nanoemulsion on *P. aeruginosa* QS–mediated virulence factors and biofilm formation have been identified by Lou et al., [90] at a 0.2 mg/mL concentration. Similarly, the anthraquinone emodin from *Rheum palmatum* L,. a traditional Chinese medicinal plant, was found to inhibit the *P. aeruginosa* biofilm formation at 20 µM, increasing the antibiotic activity of ampicillin [91]. Finally, the 6-gingerol, isolated from fresh ginger oil, reduces the production of several virulence factors, decreasing the mortality induced in mice by *P. aeruginosa*. A DNA microarray analysis revealed that the application of the 6-gingerol on biofilm-encapsulated cells down-regulates several QS-related genes, notably those involved in the production of rhamnolipids, elastase, pyocyanin, all of which are involved in biofilm formation [92].

- ✓ ***Alkaloids***

Recently, caffeine (a purine alkaloid) has been shown to inhibit AHLs production and swarming mobility in *P. aeruginosa* PAO1 without causing AHLs degradation [93].

- ✓ ***Terpenoids and Triterpenoids***

The pentacyclic triterpenoids ursolic acid was identified as an inhibitor of biofilm formation from *Diospyros dendo* Welw, the tree used for ebony from Gabon, Africa [94]. Tested at a dose of 10 µg/mL, ursolic acid reduces biofilm formation by 79 % in *E. coli* and 57-95 % in *V. harveyi* and *P. aeruginosa* PAO1. Similarly, oleanolic acid inhibits the *in vitro* biofilm formation by *S. aureus* and *P. aeruginosa* [95]. However, these triterpenoids showed no inhibitory effect on QS mechanisms contrarily to triterpenoid coumarate esters isolated from *Dalbergia trichocarpa*, a tropical legume from Madagascar. Indeed, oleanolic aldehyde coumarate at 200 µM inhibits the formation/maintenance of *P. aeruginosa* PAO1 biofilm and the expression of the *las* and *rhl* QS systems as well as *gacA* gene [96]. Consequently, the production of QS-controlled virulence factors, including, rhamnolipids, pyocyanin, elastase and extracellular polysaccharides, as well as twitching and swarming motilities is reduced. Other African plants harbor terpenoids and triterpenoids with antivirulence properties. Indeed, cassipourol and β-sitosterol (both at 100 µM), isolated from *Platostoma rotundifolium* (Briq.) A. J. Paton, a Burundian medicinal plant, inhibit quorum sensing-regulated and -regulatory genes expression in *las* and *rhl* systems. These triterpenoids can still disrupt the formation of biofilms at concentrations down to 12.5 and 50 µM [97].

- ✓ ***Isothiocyanates and organosulphur compounds***

Isothiocyanates produced by many plants are also QS inhibitors in *P. aeruginosa* PAO1. For example, iberin, isolated from horseradish (*Armoracia rusticana* G. Gaertn et al.), specifically blocks the expression of QS-regulated genes in *P. aeruginosa* PAO1 at the concentration of 100 µM; its impact on biofilm formation has not been investigated [98]. Sulforaphane and erucine, two isothiocyanates isolated from broccoli, inhibit the *P. aeruginosa* PAO1 *las* and *rhl* system as well as biofilm formation at concentrations of 50 and 100 µM, respectively [99].

A further compound known to affect the QS-regulated genes in *P. aeruginosa*, including the rhamnolipids production, is ajoene, an allyl sulfide isolated from *Allium sativum* L.. Ajoene, at the concentration of 100 µg/mL and combined with the antibiotic tobramycin, leads to killing of biofilm-encapsulated *P. aeruginosa*. In a mouse model of pulmonary infection, this synergy improves the clearance of *P. aeruginosa* from lungs [100]. The S-phenyl-L-cysteine sulfoxide and its derivatives, notably diphenyl disulfide, have shown a significant impact on the biofilm formation by *P. aeruginosa* [101]; the sulfoxide derivative seems to interfere with both *las* and *rhl* systems whereas the diphenyl sulfide only disturbs the *las* system.

4. From Marine Organisms

✓ ***Furanones***

A series of studies have indicated that marine organisms are a potential source of anti-QS [102-104]. The halogenated furanones produced by the red alga *Delisea pulchra* inhibit QS-induced activities in bacteria by competing with AHL signals related to their receptor site (LuxR) [104].This protein-ligand binding is destabilized, causing rapid receptor recycling [102]. Inspired from natural compounds, the halogenated furanones C-30 and C-56 have been demonstrated to exhibit biofilm reduction and target the *las* and *rhl* systems in *P. aeruginosa* [105].

✓ ***Terpenoids***

Following a screening of 284 extracts from the marine sponge *Luffariella variabilis,* 36 extracts were revealed as inhibitors of *P. aeruginosa* QS, targeting the *las* system [103]; from these, the sesterterpenoid manoalide displays antibiofilm activities. Note that this molecule doesn't generate bactericidal effects on *P. aeruginosa* [103], but presents an antibiotic activity against gram-positive bacteria [106].

5.5. From Animals and Human

✓ ***Enzymes***

Type I porcine kidney acylase inactivates QS signals such as C6-HSL and 3-oxo-C12-HSL but not C4-HSL [50]. This type I acylase moderately reduces biofilm formation in *Aeromonas hydrophila*, *P. putida* and probably *P. aeruginosa* [107]. This degradation is dependent on the length of the acyl chain, since only C6-HSL and 3-oxo-C12-HSL are degraded [108].

Mammalian cells release enzymes called paraoxonases 1 (extracted from human and murine sera) that have lactonase activity; degrading *P. aeruginosa* AHLs. They prevent, in an indirect way, QS and biofilm formation [109]. Similarly, Human epithelial cells and particularly human respiratory epithelia have the capacity to inactivate a *P. aeruginosa* QS signal by inactivating AHLs (3-oxo-C12HSL) produced by *P. aeruginosa* [108, 110]. However, the enzyme or enzyme-like compound involved in acyl-homoserine lactone inactivation have not been identified and characterized yet. Recently, Losa et al. [111], demonstrated that polarized airway epithelial monolayers, in contrast to nonpolarized cells, are also able to degrade 3-oxo-C12-HSL using membrane-associated paraoxonase 2 that catalyzes the opening of the lactone ring.

✓ ***Alkaloids***

The *P. aeruginosa* pyocyanin production is inhibited by a molecule found and isolated from the ant *Solenopsis invicta*, the piperidine alkaloid Solenopsin A alkaloid. The biofilm formation is also reduced in a dose-dependent manner. This molecule probably disrupts the signals from the *rhl* system [112].

6. Concluding remarks

This review presents natural compounds reported to exhibit anti-QS and antibiofilm properties against *P. aeruginosa* (summarized in Table 1); these highlight the great potentiality of living organisms as reservoir of compounds susceptible to modulate virulence mechanisms without affecting bacterial viability. Overall, it appears that prokaryotes as well as animals and humans are sources for enzymes that degrade or antagonize AHLs whereas plants harbor larger panels of anti-QS and anti-biofilm compounds with very diverse chemical structures, including alkaloids, organosulfurs, phenolics and terpenoids. Contrarily to animals and humans, plants are not able to deploy elaborate defense through humoral and cell-mediated immunity (antibodies and phagocytes) to struggle against bacterial invasions [113]. Plants immune defenses rely on the secretion of antibacterial compounds (bactericide and/or bacteriostatic agents [114]), including resistance modulating compounds [115] (*e.g.* inhibitors of efflux pumps [116]), and mostly on their ability to recognize molecules released from pathogens through plant cell surface receptors. This recognition triggers specific signaling cascades, activating series of defense responses, including the synthesis of antimicrobial lytic proteins, enzymes, phytoalexins and other secondary metabolites. Some of these exert non-microbicidal anti-virulence properties [117-118]. Finally, marine organisms and fungi produce also bioactive secondary metabolites (halogenated furanones and antibiotics, respectively) and other original and promising compounds, such as terrein which was identified as the first dual inhibitor of QS and c-di-GMP signaling at 30 µM.

The increasing presence of antibiotic-resistant bacteria certainly pushes scientists to reorient the strategy of fight against bacterial infections to defer entry into a post-antibiotic erea where major antibiotics would not be effective even for banal infections. Anti-virulence approaches and anti-virulence drugs are being increasingly considered as potential therapeutic alternatives and/or adjuvants to currently failing antibiotics. For example, oleanolic aldehyde coumarate and cassipourol, anti-QS compounds, exert interesting antibiofilm properties, restoring the effectiveness of the antibiotic tobramycin in the clearance of biofilm-encapsulated *P. aeruginosa* (Figure 4); also the association between biofilm formation and antimicrobial resistance has been highlighted in carbapenem-resistant *P. aeruginosa* [119]. Such non-microbicidal drugs inhibit virulence factors essential for establishing infection and pathogenesis through targeting non-essential metabolic pathways which should not lead to activation of bacterial evasion mechanisms. This approach should reduce the selective pressure and consequently could slow down the development of resistance. Compounds that target QS may be particularly interesting as they impact planktonic and biofilm lifestyles, by reducing at the same time the production of virulence factors and the generation of biofilms. This should lead to less severe infections at levels that can be cleared by the host's immune defense and with increased activity of antibiotics.

Table 1. Natural compounds inhibiting *P. aeruginosa* QS and biofilm formation

	Origin	**Compounds (Class)**	**Target (QS)**	**Synergy with antibiotic**
Prokaryotes	*B. indicus, B. pumilus*	AHL-acylase (Enzyme)	AHL degradation	NC

	B. sp. [60]; *Erythrobacter, Labrenzia, Bacterioplanes* [59]	AHL-lactonase (Enzyme)		NC
	Lactobacillus paracasei subsp. Paracasei [64]; *Pediococcus acidilactici* M7 [65]	Acetic acid, lactic acid, phenyl lactic acid	AHL antagonist	NC
Fungi	*Penicillium* species [66]	Penicillic acid (Furanone)	LasR and RhlR	NC
		Patulin (Furopyrannone)	LasR and RhlR‡	+(1)
	Saccharopolyspora erythraea [68]	Erythromycin (Macrolide)	*rhl system* and *GacA*	NC
	Aspergillus terreus [77]	Terrein (alkylcyclopentanone)	LasR and RhlR antagonist; c-di-GMP	NC
marine organisms	*Delisea pulchra* [102, 104]	halogenated furanones and derivative	AHL antagonist	+(1)
	Luffariella variabilis (Polejaeff, 1884) [103]	Manoalide (Sesterterpenoid)	*las* system	NC
Plants	*Platostoma rotundifolium* (Briq,) A, J, Paton [97]	Cassipourol (terpenoid), β-sitosterol (terpenoid)	*las* and *rhl* systems	+(1)
	Combretum albiflorum (Tul.) Jongkind [85]	Catechin (Flavonoid)	*las* and *rhl* systems	NC
	Dalbergia. trichocarpa Baker. [96]	Oleanolic aldehyde Coumarate (Phenolic compound)	*las* and *rhl* systems	+(1)
	Allium sativum L. [100]	Ajoene (Organosulfur)	*las* and *rhl* systems	+(1)
	Armoracia rusticana G. Gaertn et al. [98]	Iberin (Isothiocyanate)	*las* and *rhl* systems	NC
	Terminalia chebula Retz. [84]	Ellagic acid derivatives (Phenolic compound)	*las* and *rhl* systems	+(1)
	Syzygium aromaticum (L.) Merr. Et Perry [89, 90]	Eugenol (Phenylpropanoid)	*las* and PQS systems	NC
	Curcuma longa L. [83]	Curcumin (Phenolic compound)	AHLs inhibition	NC
	Citrus paradisi Macfad. (Rio Red and Marsh White grapefruits) [87]	Bergamottin and dihydroxybergamottin (Furocoumarins)	AHLs inhibition	NC
	Rheum palmatum L. [91]	Emodin (Anthraquinone)	docking traR*	+(2)
	Scutellaria baicalensis Georgi. [86]	Baicalin (Flavonoid)	*las*, *rhl* and PQS systems	+ (1)
	Zingiber officinale Rosc. [92]	6-gingerol (Phenolic compound)	docking lasR	NC

Animals and Human	Porcine kidney [50, 107]	Type I acylase	AHL degradation	NC
	Human and murine sera [109-110]	Paraoxonases 1 Enzyme (lactonase)	AHL degradation	NC
	Solenopsis invicta (insect; ant) [112]	Solenopsin A (Alkaloid)	*rhl* system	NC

+, yes; -, no; NC, not communicated
ǂ, patulin alone does not affect the development of biofilm
*, LuxR-type transcription factor of *Agrobacterium tumefaciens*
(1) Aminoglycosides, (2) Ampicillin

Despite these important prospects however, the big breakthrough in antibacterial strategies is still out of reach. This is probably due to a very complex entanglement between different QS systems, to the ability of *Pseudomonas* to compensate deficient systems and to the intervention of key actors involved in biofilm formation, outside of QS circuitry [12]. Millenia of coevolution between plants and bacteria have led to complex defense strategies, with plants producing cocktails of bioactive compounds with multiple targets [114] and/or compounds such as terrein that impact dual inhibitory targets. In the current state of research, much remains to be done in understanding these mechanisms and the real impact of such combinations before arriving at a commercial use. Nevertheless, following a combined approach for "adjuvant antibiotherapy" and "combined antibiotherapy" will undeniably lead to a renewed concept of "complex drugs for complex diseases", a well-known presupposed in traditional medicines [120].

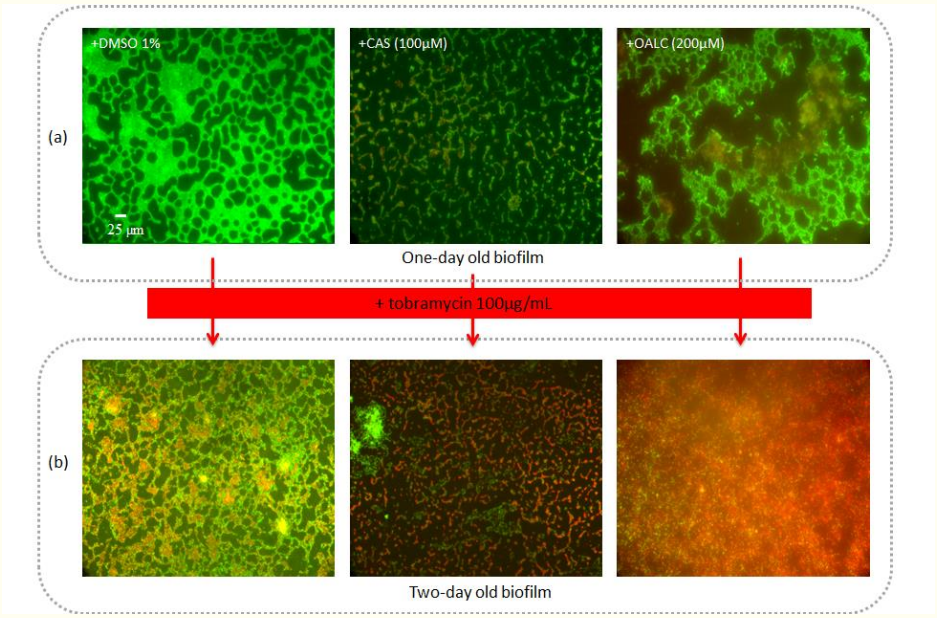

Figure 4: *P. aeruginosa* biofilm phenotypes and effectiveness of tobramycin treatment in presence of DMSO 1 % or, cassipourol (CAS: 100µM) or oleanolic aldehyde coumarate (OALC: 200µM). (a) After 1 day of incubation, *P. aeruginosa* fails to form structured confluent aggregate in presence of CAS or OALC as compared to DMSO treatment. (b) CAS and OALC considerably increase the susceptibility of *P. aeruginosa* to tobramycin (100 µg/mL), as shown by the increased proportion of dead cells compared with DMSO. Similar results are observed when tobramycin is added simultaneously with CAS or OALC to one-day old untreated biofilms. The bacterial viability was assessed by staining the cells with SYTO-9 (green areas zones—live living bacteria) and propidium iodide (red areas zones—dead bacteria) furnished in the LIVE/DEAD BacLight kit. Cells were visualized using a

LeicaDMIRE2 inverted fluorescence microscope using equipped with a 40x objective lens and colored images were assembled using Adobe Photoshop.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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