Quenching bacterial communication: Innovative strategies for biofilm inhibition

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1. Abstract

The majority of bacteria are able to grow as surface-associated consortia in biofilms, which have been recognized as a common life style for microbial growth on surfaces in natural, clinical and industrial environments. In contrast to their planktonic growing counterparts, bacteria within biofilms show increased resistance to many classical antimicrobial agents, and thus represent a major challenge in medicine and industry. One of the underlying mechanisms crucial for biofilm formation, pathogenicity and virulence is cell-cell communication (quorum sensing, QS). Thus, QS is an attractive and most likely effective target for novel anti-biofilm drug design in medical, agricultural and industrial applications. In principle, interference with bacterial cell-cell communication (quorum quenching, QQ) can be achieved by degradation or modification of the respective signaling molecules or by antagonistic small molecules. Alternatively, the synthesis, recognition or transport of the signaling molecules can be targeted. It has been demonstrated that QS-interfering compounds have been evolved in bacteria as well as eukaryotes. In addition to those naturally occurring QQ biomolecules, synthetic inhibitors have been designed on the knowledge of the natural inhibitors. In order to screen for such QS inhibitors, several reporter strains have been developed, often based on reporter fusion to a QS-controlled promoter. In this review, we summarize the current knowledge and recent improvements of QS inhibitors as well as their detection using biosensors. Identified QQ mechanisms and their effects on biofilm formation, virulence and pathogenicity are described to speculate about their potential clinical and biotechnological applications.

2. Introduction

It has become evident that most bacteria predominantly exist within biofilms, either in nature or in industrial and clinical environments due to the nutritional and protective advantages associated with the biofilm life style [1]. A biofilm represents an assemblage of microbial cells that is associated with a surface enclosed in a matrix of self-produced polymeric substances (extracellular matrix) [2]. Biofilms form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or drinking water system piping or natural aquatic systems. When biofilms are formed on engineered surfaces or in a medical context, the presence of the biofilm is detrimental, because biofilms can cause material degradation, fouling, contamination, or infections [3]. Bacteria associated within a biofilm are up to 1,000 times more resistant to antibiotic therapies in comparison to their planktonic counterparts and are unresponsive to the host immune system [4]. The National Institute of Health has proposed that up to 80 % of bacterial infections in humans are caused by biofilms [5]; and thus declared biofilms as the most pressing clinical impediment of this century [2]. The biofilm development has been shown to generally include several steps: i) initial attachment, ii) irreversible attachment, iii) formation of microcolonies, iv) biofilm maturation, and v) biofilm dispersal (see Fig. 1). QS has been demonstrated to play a crucial role in cell attachment and detachment from biofilms [6], initiation of biofilm formation to rescue crowded planktonic bacterial populations from stress [5], and to control the population size in a biofilm by promoting dispersion or dissolution of subpopulations [7]. In addition, it impacts gene expression in cells within established biofilms altering the course of biofilm development by inducing or repressing group activities such as bacterial motility [8]. Meanwhile, QS has been shown to be important during biofilm formation for a wide variety of medically relevant species, e.g. *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* or *Staphylococcus aureus* (for review see [9]). Bacterial infections are traditionally combated with antibiotics, which inhibit their growth or even kill the bacteria [10]. This is accompanied by an enormous selection pressure, which promotes the development of resistances, sooner or later resulting in the loss of the antibiotic effect. As antibiotics are losing their effectiveness due to the development of resistance, new antimicrobial strategies have to be considered and developed aiming at suppressing virulence behavior rather than directly targeting the viability of a pathogen, and thus allowing to select for resistance. Since QS appears to be a key player in regulation of virulence and the formation of biofilms [11], interference with QS systems appears to be an innovative strategy for future antimicrobial applications.

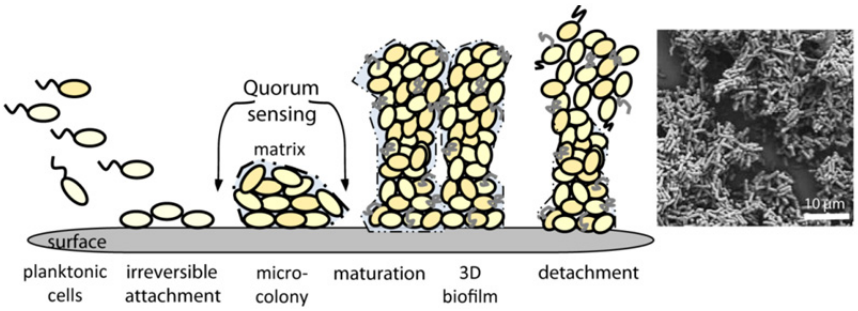


Fig. 1: Biofilm development. Free-moving bacteria initially attach to a solid surface. During maturation micro-colonies encased in a matrix develop. 3D biofilms permanently undergo composition/decomposition. QS is involved in different regulation steps of biofilm development. Right panel: Scanning electron microscopy image (SEM S- 4800, Hitachi) of *Klebsiella oxytoca* M5aI biofilm.

3. Quorum sensing –how bacteria "talk to each other"

Quorum sensing (QS), the cell-cell communication of bacteria, has attracted increasing research interest since its initial discovery in the luminescent bacterium *Vibrio fischeri* forty years ago [12]. Searching in the National Center for Biotechnology Information (NCBI) for the term "quorum sensing" clearly shows the interest in this research area by publications (currently 10,780 full-text journal articles and 62,527 nucleotide sequences). QS is based on the synthesis and perception of low molecular weight molecules so-called autoinducer (AI), which either diffuse over the cytoplasmic membrane or are actively transported and are specifically detected by a relevant receptor. When the AI binds its corresponding receptor, the subsequent signal transduction is activating the transcription of target genes, often including those encoding the respective AI synthase (autoregulation) [13]. When only a few bacteria are in the vicinity, diffusion reduces the concentration of the autoinducer in the surrounding medium, as a consequence the bacteria produce little autoinducer. However, as the population density increases, the concentration of the signaling molecule is passing a threshold ("quorum"), thus causing more autoinducer to be synthesized via induction of AI synthase. This forms a positive feedback loop, and the receptor becomes fully activated. Activation of the receptor changes the regulation of target genes, in principle leading to synchronized transcription in the population [14]. In the meantime, QS signals are regarded as multifunctional signals, which go beyond the perception of a population density [15]. QS functions are classified in four categories comprising (i) cell maintenance and proliferation (e.g. exoenzyme, siderophore synthesis), (ii) cell behavior (e.g. motility, biofilm formation), (iii) horizontal gene transfer (e.g. conjugation, competence), and (iv) bacteria-host interactions (e.g. bioluminescence, antibiotics, colonization factors) [15]. QS systems have been found in both, Gram-negative and Gram-positive bacteria; and inter-kingdom signaling mediating symbiotic and pathogenic relationships between bacteria and hosts [16].

In Gram-negative bacteria two components, LuxI and LuxR homologs, impact expression of certain target genes, e.g. the *lux* operon (*luxICDABE*) responsible for bioluminescence in *V. fischeri* [17] (see Fig. 2A). A large number of Gram-negative bacteria contain the LuxI/R-system and communicate via AHL signals [18] (see Tab. 1). Gram-positive bacteria communicate using modified oligopeptides and two-component regulatory systems. Signaling is mediated by phosphorylation transfer that impacts the activity of the sensor itself (autophosphorylation) and subsequently the response regulator (see Fig. 2B). Examples of peptide-based QS systems are the ComD/ComE system of *Streptococcus pneumoniae* that controls competence development, the ComP/ComA system of *Bacillus subtilis* that controls competence and sporulation, and the AgrC/AgrA system of *S. aureus* controlling pathogenesis (for review see [19]) (see Tab. 1).

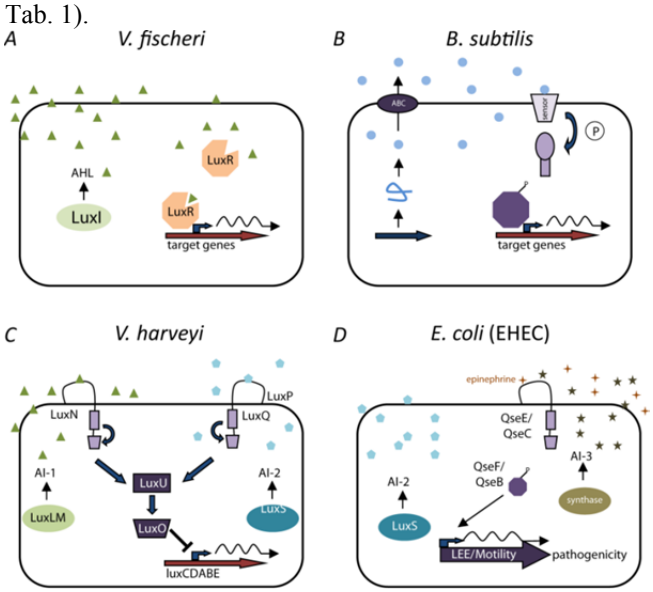
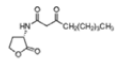
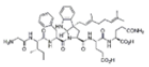
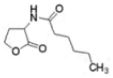
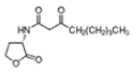
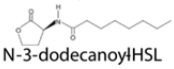
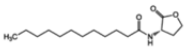
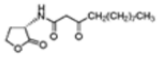
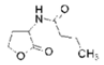
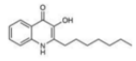
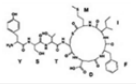
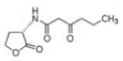
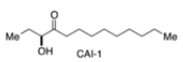


Fig. 2: General signal transduction systems. (*A*) In Gram-negative bacteria LuxI synthesizes diffusible autoinducers (AHL, triangles). The increasing concentration of external autoinducers is measured and represents the density of the population. Inside the cell AHL binds to its cognate receptor LuxR. This complex binds at target gene promoters and activates or reduces their transcription with behavioural consequences. (*B*) A precursor peptide is generated and modified (circles) by Gram-positive bacteria and then exported via an ABC transporter. It is detected by a membrane two-component regulatory system. (*C*) The QS system of *V. harveyi* combines elements of Gram-negative and Gram-positive bacterial QS systems, in which an acyl-HSL (AI-1, triangles) is synthesized by LuxLM and a second autoinducer (AI-2, pentagons) is synthesized by the enzyme LuxS. Accumulated autoinducers are detected by two-component systems. (*D*) Additionally to AI-2, enterohemorragic bacteria produce a third autoinducer (AI-3, stars), which is essential in the pathogenesis of enterohemorrhagic *E. coli* infections and shigellosis.

Tab. 1: Diversity of quorum sensing systems. Selected Gram-negative and Gram-positive bacteria are listed with their respective quorum sensing systems. Further, information on both, intra-species and inter-species systems with corresponding autoinducers and resulting behaviors are given.

Organism	Intra-species system	Autoinducer identity	Function	Inter-species system	Function
Agrobacterium tumefaciens	TraI/TraR	N-(3-Oxooctanoyl)-HSL 	Conjugation	Unknown	-
Bacillus subtilis	Com CSF	Pentapeptide (ERGMT) 	Competence Sporulation	LuxS/ LsrB	Biofilm formation
Chromobacterium violaceum	CviI/CviR	N-Hexanoyl-HSL 	Antimicrobial Violacein, exoprotease, chinolytic enzymes	Unknown	-
Escherichia coli	SdiA	N-(3-Oxooctanoyl)-HSL 	Cell division	LuxS/LsrB 	Biofilm formation and motility
Klebsiella pneumoniae	Unknown	N-octanoyl-HSL  N-3-dodecanoyl-HSL 	Unknown	LuxS/LsrB	Biofilm formation
Pseudomonas aeruginosa	LasI/LasR RhlI/RhlR PqsABCD_H/ PqsR QscR	N-(3-Oxododecnoyl)-HSL  N-Butyryl -HSL  PQS 	Biofilm formation Rhamnolipid Virulence	Unknown	Virulence factor production
Staphylococcus aureus	Agr	AIPs 	Virulence	Unknown	Capsular polysaccharide production
Vibrio fischeri	LuxI/LuxR	N-(3-Oxohexanoyl)-HSL 	Luminescence	LuxS/LuxP	Luminescence
Vibrio harveyi	LuxLM/ LuxN CqsA /CqsS	N-(3-Oxobutanoyl)-HSL  CAI -1 	Luminescence	LuxS/LuxP 	Luminescence, colony morphology, biofilm formation

QS processes allow bacteria not only to communicate within, but also between species. This assumption arose with the discovery and study of the autoinducer AI-2, which is one of the signal molecules used by *V. harveyi* [20] (see Fig. 2C). AI-2 is synthesized by the LuxS enzyme [21]. The AI-2 synthesis pathway exists in many bacteria; in 2012, LuxS homologues were identified in 537 of 1,402 bacterial genomes sequenced at the time [20]. However, it has to be taken into account that besides AI-2 synthesis, LuxS plays a general role in the methionine metabolism. AI-2 is involved in a wide range of bacterial phenotypes, namely biofilm formation, cell motility; conjugation and virulence factor production [9] (see Tab. 1). Today, it is known that even microorganisms and their hosts communicate with each other through an array of hormonal signals that are produced by eukaryotic hosts and hormone-like chemicals that are produced by microorganisms [22]. Bacterial QS compounds can be important modulators of microbe-host interactions.

Thus, it is not surprising that some pathogenic species have adapted to these signaling systems to promote disease. The AI-3/epinephrine/norepinephrine signaling system (see Fig. 2D) is the prime example. The enteric pathogen *E. coli* generally senses AI-3 produced by the microbial gastrointestinal (GI) community to activate the expression of virulence genes resulting in colon lesions. Eukaryotic hormones epinephrine and norepinephrine present in the GI tract also activate expression of the virulence genes in enterohemorrhagic *E. coli* due to the adaptation or modulation of the bacterial signal system [23].

Besides the presented classical ones, further autoinducers have been identified in recent years, for example, CAI-1 controlling virulence factor production and biofilm development in *Vibrio cholerae*. Further, the diffusible signal factor DSF from *Xanthomonas campestris* and 3-OH-PAME from *Ralstonia solanacearum* are known as well as diketopiperazines from *Pseudomonas*, *Citrobacter* and *Enterobacter,* and diketopiperazine cyclo-(L-prolyl-L-Val) from the halophilic archaeon *Haloterrigena hispanica*. Other known autoinducers are butyrolactones in *Streptomyces spp*. and quinolones in *Pseudomonas* (for review see [15]). These examples illustrate the diversity of QS signals in different bacterial and even archaeal clades and within a certain species, e.g. *Pseudomonas* [24].

4. Interference with QS

Since many bacterial pathogens use QS to control the expression of virulence factors, the interference with this cell-cell communication mechanism constitutes a novel and promising strategy to control bacterial biofilm formation and infectious diseases [25]. The mechanisms causing the inactivation of QS systems are generally known as "quorum sensing interference" (QSI) or "quorum quenching" (QQ) [26]. QQ alone or in combination with antibiotics represents an attractive novel strategy for the treatment of infectious diseases by resistant pathogens. QSI does not kill the bacteria or limit their growth, but it affects the expression of specific QS-controlled functions. QQ strategies exert less selective pressure for microbial survival than biocide treatments with antibiotics. In the present context of increasing antibiotic resistance, QQ strategies represent a beneficial tool for developing novel biocontrol or therapeutic procedures. The interference with bacterial cell-cell communication can be in general achieved by inhibition of signaling molecule **synthesis**, inhibition of signal **transport,** inhibition of signaling **molecule/receptor interaction**, and modification or degradation of **signaling molecules** or by antagonistic small molecules (see Fig. 3).

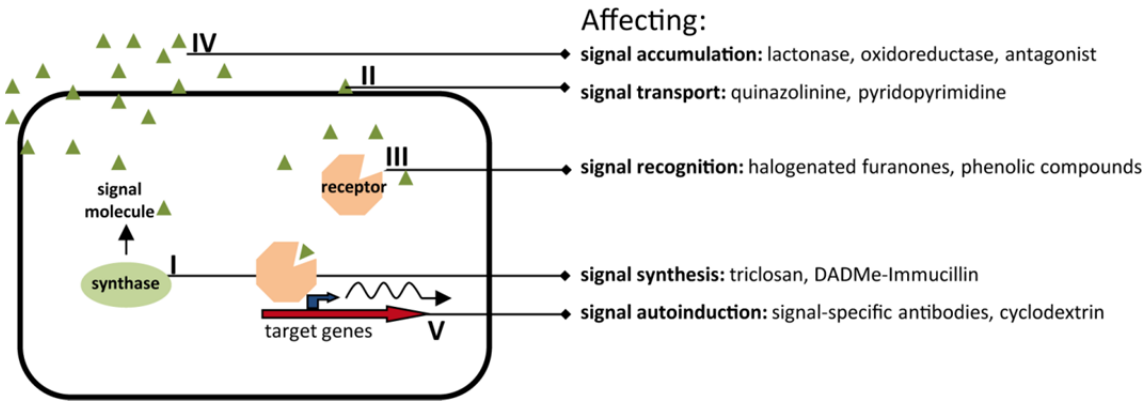


Fig. 3: Blocking cell-cell communication. Possible interventions in bacterial communication by QQ mechanisms are shown using the example of Gram-negative quorum sensing system via AHLs. The critical communication processes that could be targeted by quenching approaches are indicated with selected research examples.

4.1 Inhibition of autoinducer synthesis

To date, only a few substances are known to inhibit autoinducer synthesis (see Fig. 4). In addition to the signaling molecule synthases, also the synthesis of chemical precursors is a possible target. In the case of AHLs, which are synthesized from the two precursors S-adenosylmethionine (SAM) and acyl-acyl carrier protein (acyl-ACP), the Enoyl-ACP reductase FabI catalyzes the last step of the fatty acid elongation of acyl-homoserine lactones. This enzyme can be inhibited by triclosan, which forms a stable complex with the enzyme resulting in inhibition of AHL synthesis, e.g. in *P. aeruginosa* [27]. In addition, several SAM analogs (e.g. butyryl-SAM and sinefugin) inhibit AHL synthesis by antagonistic completion with this Co-factor [15]. However, since SAM is also essential for various metabolic pathways in Eukarya and Bacteria, therapeutic suitability is insufficient.

AI-2 is synthesized from S-adenosyl-homocysteine (SAH) by two enzymatic steps involving the enzymes 5′-methylthioadenosine/S-adenosylhomo-cycteine nucleosidase (MTAN) and LuxS [20]. Based on the importance of S-ribosyl-homocysteine (SRH) in the synthesis of the AI-2 precursor (S)-4,5-Dihydroxy-2,3-pentandione (DPD), several research groups focused on finding analogues of SRH as potential inhibitors that target AI-2 synthesis. For two SRH

analogues, S-anhydroribosyl-L-homocysteine and S-homoribosyl-L-cysteine, exhibited inhibitory activities against LuxS were demonstrated [28]. 5-methylthioadenosine nucleosidase (MTAN) encoded by the *pfs* gene is also an important enzyme during the synthesis process of SRH catalyzing the hydrolytic deadenylation of its substrates to form adenine and SRH. According to the mechanism of the reaction catalyzed by MTAN, several transition state analogues, e.g. 5'-butylthio-DADMe-Immucillin-A were designed and synthesized inhibiting AI-2 synthesis [29].

Furthermore, halogenated anthranilic acids were shown to inhibit the synthesis of PQS quinolone signals of *P. aeruginosa* by competitive inhibition [30]. Structural analogs as well as the fungal secondary metabolite ambuic acid inhibit the synthesis of signal peptides of various Gram-positive bacteria, such as *S. aureus* and *Listeria innocua* [31].

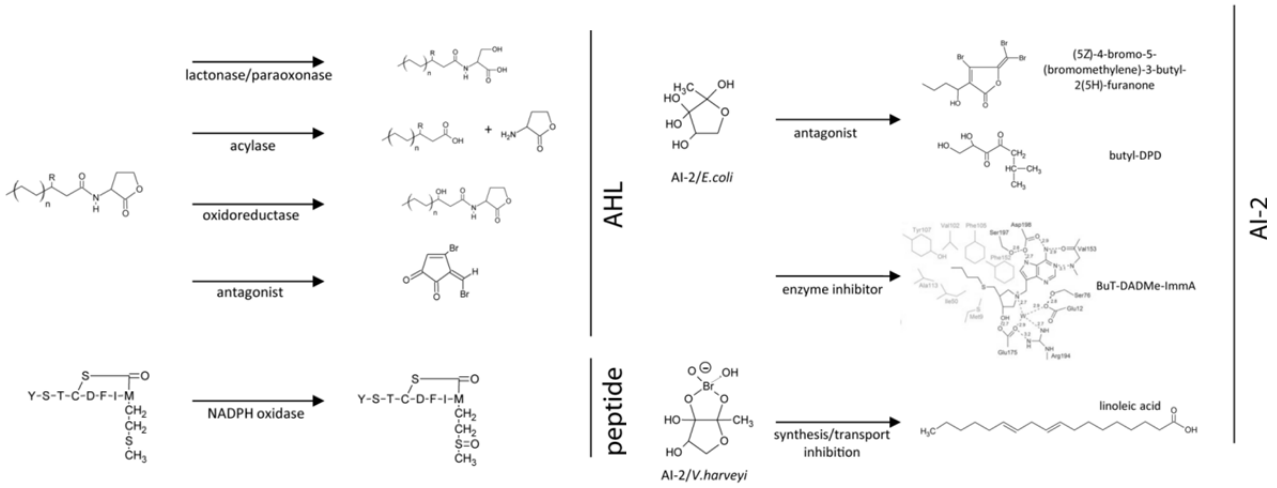


Fig. 4: Quorum quenching mechanisms. The inhibition of bacterial cell-cell communication can be achieved by degradation, modification or antagonistic processes. Several identified quenching pathways for Gram-negative, Gram-positive or Inter-species signal systems are represented schematically.

4.2 Inhibition of signal transport

In general, autoinducers either diffuse freely across the cytoplasmic membrane or are actively transported out of the cell, where they are recognized by membrane bound two-component regulatory systems or imported by ABC transporters [32]. In Gram-positive bacteria, Apolipoprotein B sequestered the autoinducing peptide (AIP) 1 signal in *S. aureus,* and thus prevented binding to its receptor AgrC [33]. In addition, antibody AP4–24H11 sequestered AIP4 in *S. aureus* [34], whereas antibody RS2–1G9 quenched against 3-oxo-C12-HSL in *P. aeruginosa* [35]. Those antibody-based QQ efforts are nowadays traded as promising novel vaccines to inhibit pathogenic biofilms [36].

4.3 Inhibition of signal perception - and response

Several studies on QQ described the identification of small molecules, which are either agonist implying a function like the native signal molecule based on mimicking the structure, or an antagonist blocking the receptor binding site and preventing binding of the QS signal and/or modifying the normal conformation of the receptor-signal complex (Fig. 4) [37]. For instance, an AHL agonist for *P. aeruginosa* was identified, which shows no obvious structural connection to the native 3-oxo-C12-HSL, but was predicted by *in silico* analysis to efficiently bind in the same protein pocket of the receptor protein as the respective AHL [38]. Various natural as well as synthetic analogs of AHLs act as QSIs, such as thiolactones [39], lactams [40], the Solenopsin A alkaloid from *Solenopsis invicta* [41], the isothiocyanate iberin from horseradish [42], anthocyanins from fruits [43], ajoen from garlic [44], curcumin from turmeric and eugenol from cloves block [45]. Brominated furanones of the red alga *Delisea pulchra* bind to various receptors, including LuxR, thus preventing QS-controlled behavior as swarming in *Serratia liquefaciens* or bioluminescence in *V. fischeri* and *V. harveyi* [15]. The flavonoid quercetin also binds to AHL receptor homologues, and thus prevents the biofilm formation of *Pseudomonas spp*., *Salmonella spp*., *Campylobacter jejuni* and *Yersinia enterocolitica* [46].

Several synthetic agonist ligands have been reported for *V. harveyi* receptor protein LuxP, most of which are DPD or AI-2 (S-THMF-borate) analogues competing for binding to LuxP with natural AI-2 [47]. In addition, nucleoside analogs have been shown to interfere with AI-2 mediated QS and, in some cases, affect biofilm formation [48]. Bentley and coworkers have described novel synthetic AI-2 analogs that were capable of inhibiting maturation of *E. coli* biofilms *in vitro*, and when combined with antibiotics near minimum inhibitory concentrations; almost completely cleared pre-formed *E. coli* biofilms in a microfluidic device [49]. By screening a large number of samples from plants, ursolic acid and 7-hydroxyindole were found as inhibitors for enterohemorrhagic *E. coli* biofilm formation by blocking

the AI-2 pathway [50]. Further screens using reporter strain *V. harveyi* BB170 showed that certain food components inhibit AI-2 signaling [32]. AI-2 QS inhibitors linoleic acid, oleic acid, palmitic acid, and stearic acid contained in poultry meat wash samples showed AI-2 inhibition ranging from approximately 25 % to 99 % [51].

Regarding the inhibition of peptide-type signals in Gram-positive bacteria, the most studied system is the AgrC/AgrA system of *S. aureus*, and its AIP signals, due to the enormous clinical relevance of this pathogen. Cross-inhibition of AIP mediated signaling in *S. aureus* represents an example of a QQ mechanism because each of the four AIPs present in *S. aureus* specifically inhibits QS in competing *S. aureus* groups [52]. Cyclic peptides comprising about six to twelve amino acids in length and including sequences corresponding to the native peptide from staphylococci bacterium were isolated and purified to act as inhibitor peptides [53]. Solonamide A and B from a marine γ-Proteobacterium inhibit the AgrC receptor competitively [54]. Hamamelitannin from the American witch-hazel competitively inhibits the signal transduction protein TRAP, which is involved in the QS cascade, and finally leads to a reduced cell wall thickness ultimately improving effects of antibiotics [55]. In addition, the novel QSI savirin has been identified from a high throughput screen. Savirin blocks the transcriptional activity of the response regulator AgrA, and thus inhibits the QS pathway in *S. aureus* consequently reducing tissue damage in mice [56]. The QS signaling molecule farnesol of the yeast *C. albicans* inhibits the PQS-mediated communication of *P. aeruginosa* by conformational alteration of the PQS receptor to protect them against the toxic effect of *P. aeruginosa* AHLs [15].

4.4 Enzymatic interference with signal molecules

Another possibility to interfere with QS is signal inactivation by enzymatic degradation or modification (Fig. 4). AHL-lactonases hydrolyze the ester bond of the homoserine lactone ring of AHL molecules [57]. The first reported natural AHL-lactonase encoded by the *aiiA* gene was characterized from *Bacillus* isolate 240B1 [58]. Homologues have been identified in a range of bacteria including Gram-positive and Gram-negative species. AHL-lactonases can be grouped into two clusters based on their sequence homologies. The first one is the AiiA cluster with representatives from *Bacillus* [59]. The second one is the AttM cluster with Gram-negative members, e.g. *Agrobacterium tumefaciens* and *K. pneumoniae* [60, 61]. AHL-lactonases are by far the most specific AHL-degradation enzymes among known QQ enzymes. They hydrolyze both short- and long-chain AHLs, but show no residue activity to other small molecules [62]. Paraoxonases (PON) also capable of hydrolyzing the homoserine lactone ring of AHLs have been identified in mammals, other vertebrates and invertebrates [63, 64]. PONs appear to be most active with long-chain AHL molecules, often typical for pathogens, e.g. *P. aeruginosa* [53]. In contrast to AHL-lactonases, PONs are known for their broad substrate spectrum, and are thus less substrate specific [65]. AHL-acylases inactivate AHL signals by cleaving the amide bond of AHL producing the corresponding fatty acids and homoserine lactone [66]. These enzymes are widely conserved in several bacteria, including *Variovorax*, *Ralstonia* and *P. aeruginosa* [67]. There are notable differences in the substrate specificities among AHL-acylases, which are manifested in the effectiveness of degrading long-chain AHLs [68]. AHL-oxidoreductases modify the 3-oxo group of AHLs to generate corresponding 3-hydroxy derivates [69, 70]. Depending on the specificity of the AHL receptor, the modification may or may not affect the signaling activity of the respective AHL [71].

In general, the universal signal molecule AI-2 is intracellularly subsequently phosphorylated by LsrK to activate the QS cascade in bacteria with Lsr signaling system, such as *E. coli*. When LsrK is exogenously added to the culture medium of *E. coli*, *S. typhimurium* and *V. harveyi,* phosphorylating extracellular AI-2, the negative charge of phospho-AI-2 prevents its transport into the cell. Moreover, phospho-AI-2 is unstable and decays over time to 2-phosphoglycolic acid [72]. Recently, the first metagenome-derived AI-2 quenching enzyme QQ-2 was identified, which was highly effective in inhibition of *Klebsiella spp*. biofilm formation due to an observed oxidoreductase activity already known for AHL modification [73].

5. Strategies to control biofilm formation

Aggregated bacterial cells that are attached to and growing on a surface produce extracellular polymeric substances (EPS), which likely provide the biofilm with increased resistance to antimicrobial agents, biocides and disinfectants. Within a given system, the biofilm mass often varies with location, and is typically composed of many species of microorganisms, including bacteria, fungi, algae and protozoa and phages [74]. Once initial adhesion occurs, a biofilm is difficult to remove. Even small numbers of persisting cells can regrow introducing the risk of an inflammation. Additionally, biofilms can contain disease-causing microorganisms which are easier inactivated in their planktonic forms [75]. In general, medicine and industry would benefit from a novel promising and feasible method for controlling biofilm formation.

5.1 Chemical and physical strategies

Chemical agents and physical approaches are routinely used to control biofilms on surfaces and the problems they cause. Most effective strategies for biofilm treatment include prevention of bacterial cell adhesion to the substratum, reduction of polysaccharide production, and the highly innovative approach to disrupt cell-cell communication -

involved in biofilm formation, through physical, chemical and biological applications. Chemical treatment includes a wide variety of antimicrobial agents, such as biocides and antibiotics as well as chemical cleaners that assist in removing biotic or abiotic constituents of the biofilm. Mechanical cleaning of biofilms can be accomplished by scraping, brushing, or hydraulic flushing [10]. Another strategy comprises the change of physical surfaces properties with defined nanostructure to prevent initial attachment and adhesion of bacteria to surfaces [76]. In order to control biofilm formation, especially in the medical sector, it is recommended that not only preventive strategies are used but also frequently changing control strategies be implemented such as hygienic layout, design of equipment, choice and coating of materials, correct use and selection of detergents and disinfectants [5]. Many of the strategies for the inhibition of biofilms are complicated by the fact that a biofilm is characterized by its resistance to biocides, antibiotics and interference with host defense mechanisms [77]. Bacteria evolve antibiotic resistance through either acquired or intrinsic mechanisms. Biofilm cells show significant reduced metabolic activity than planktonic cells leading to significantly slower growth and subsequently to reduction of antimicrobial susceptibility [78]. Furthermore, biofilm cells express genes distinct to planktonic cells potentially resulting in stress resistance response [79]. Another difficulty are persister cells, which are responsible for biofilm re-growth after antibiotic treatment often causing more resistant re-formed biofilms [80]. Thus, treatments with traditional biocides or antibiotics are mostly ineffective in controlling biofilm populations. In order to control unfavorable biofilm formation on a variety of surfaces important to medical practices and industry, novel preventive strategies and methods for biofilm control are urgently required.

5.2 Novel strategies

Bacterial biofilms are a major threat to human health and enormously costly in industry as they become inherently resistant to "traditional" clearance [81]. Finding treatments that can alter the phenotype of the bacteria without a strong selection for viability of the bacteria that ultimately might lead to resistance, is the key in combating bacterial biofilms. When paired with conventional biofilm control strategies, e.g. use of antibiotics; such strategies have the potential to prevent and degrade biofilms.

One approach for clearance of established biofilms is to destroy the integrity of the biofilm matrix, typically by enzymatic degradation of components of the EPS leading to subsequent detachment of cells from the biofilm. Glycosidases, proteases, and DNases degrade various components of the EPS. DNase thermonuclease produced by *S. aureus*, the glycoside hydrolase dispersin B produced by *Aggregatibacter actinomycetemcomitans*; and alginate lyase produced by *P. aeruginosa* as well as several proteases are used to modulate biofilms by degradation of biofilm matrix components [82].

As outlined above it is now well established that bacterial cells communicate through the transfer and uptake of small signaling molecules [32]. Since QS plays such a substantial role in biofilm formation one strategy considered for preventing biofilm formation is to coat or embed surfaces with compounds capable of interfering with related signaling mechanisms [83]. Over the last twenty years, a range of QQ enzymes and inhibitors have been identified from different sources, including both prokaryotic and eukaryotic organisms mainly interfering with the AHL-QS system of Gram-negative bacteria [26]. These novel enzymes and inhibitors are the key molecules for establishing the concept of QQ as novel anti-biofilm strategy [83, 84]. Blocking one of the QS pathways might prevent harmful biofilms, a strategy that led to the foundation of numerous biotechnology companies. These companies are mainly screening for synthetic compounds to prevent and disrupt industrial or medical biofilms. A couple of Gram-positive, Gram-negative as well as broad spectrum QS inhibiting compounds were identified and are subjected to clinical test series [85]. A search in the US patent database (04/21/2017) revealed a total of 50 applications related to "QSI strategies". The following sections summarize and gain insight into the identification of novel QQ compounds using biosensors and provide examples for effective QQ molecules and their applications for biofilm inhibition.

5.2.1 Biosensors for screening novel quorum quenching compounds

In order to screen extracts or synthetic libraries for QQ compounds, several strains have been developed for detecting QS activators and inhibitors, based on fusing a QS-controlled promoter to a reporter gene [86]. These strains often lack the ability to produce native QS signals; however, they are able to respond to exogenous autoinducers, often with a clearly detectable phenotype, such as violacein pigment production in *Chromobacterium violaceum* CV026 [87] and bioluminescence production in *V. harveyi* [88] or *A. tumefaciens* A136 [89]. All of these reporter strains were initially designed to identify new signaling molecules. The QS-promoter is induced by signal molecules possibly present in the environment, leading to the expression of the respective phenotype. By simultaneous addition of defined amounts of promoter-inducing autoinducers in the assay, these biosensors can also be used in order to identify QQ compounds, which interfere with these signal molecules. Remarkably, there are mostly AHL-QS-based reporters published, which allow the identification of AHL-interfering compounds. Using such reporter strains, numerous AHL-degrading [90] or -modifying [91] compounds as well as AHL agonists [38] and antagonists [92] have been already identified. With *V. harveyi*, a reporter for the detection of QQ compounds against Gram-negative and interspecies-specific QS has been developed. The respective autoinducer synthases were mutated resulting in two strains, which were no longer capable of generating either the autoinducer A1-1 or AI-2, whose accumulation leads to bioluminescence. By incubating the

respective reporters with a mixture of autoinducer, potential QQ active compounds can be identified by the absence of luminescence [93]. A screen based on this type of system will indicate a QS-interfering compound by the disappearance of the reporter signal. One crucial problem of this procedure is that factors other than QQ compounds can also cause a reduction in the signal, e.g. by reducing cell growth. If the biosensor bacterium is exposed to a toxic substance (such as an antibiotic) in sub-lethal concentrations, growth will be reduced. The underlying interference with protein synthesis, particularly with the reporter synthesis can be misleading in the analysis, such that the test substance is classified as a QQ-positive but actually represents a "false positive". Thus, both, the production of reporter signal and growth must be carefully monitored and the specific activity of the reporter signal should be calculated regarding the degree of QS-specific inhibition. However, it can be difficult to obtain reliable information regarding the specificity of a QS-interfering compound that shows additional pleiotropic effects because the decrease in reporter signal is not necessarily proportional to the decrease in monitored growth rate [94]. To circumvent these problems Rasmussen *et al*. designed another type of screening system termed quorum sensing inhibitor selector (QSIS). The QSIS system is based on *E. coli,* which comprises an AHL-inducible lethal gene encoding a toxic protein. When the strain senses AHLs in the surrounding environment, the lethal gene is expressed and consequently growth will be inhibited. In contrast, the presence of a QS-interfering compound rescues the bacteria, since expression of the lethal gene is not induced and the bacteria are able to grow [95]. Hence, growth of the reporter can only be achieved if non-toxic QQ compounds are present, which not affect bacterial growth. This method of positive selection for growth has proven powerful for isolation of both synthetic compounds and extracts of plants and fungi with AHL-quenching activities [96]. In contrast to AHLs, only a few AI-2-quenching compounds, mostly small antagonistic molecules, have been identified to date that directly or indirectly interfere with the AI-2 QS processes, probably due to the comparatively small knowledge about AI-2 QS systems in various bacteria and the lack of appropriate reporter systems [25]. A few reporter systems for the detection of AI-2 like compounds have been reported that in principle can be used for identification of AI-2 quenching activities. One example is the above mentioned *V. harveyi* based reporter system with a mutated autoinducer synthase (LuxS) that can be used to detect external accumulation of AI-2, leading to bioluminescence [97]. A second reporter system is based on *lacZ* fusion to the *E. coli* AI-2 inducible promoter *lsrA* [98]. Recently, reporter strain AI2-QQ.1 was established based on the innovative strategy of Rasmussen and collaborators, which now allows to identify for novel non-toxic biomolecules interfering AI-2-based QS using positive selection [99]. Bacterial isolates, extracts and even metagenomic libraries containing tens of thousands clones can be rapidly screened for QQ compounds, while toxic substances are excluded based on the positive selection on growth. Several QQ enzymes were identified with those reporters, which are effective in preventing biofilm formation of several pathogens [73, 99].

5.2.2. Effective QQ compounds – applications for biofilm inhibition

In general, QS interfering compounds should not kill the bacteria or limit their growth, but they have to affect the expression of specific QS-controlled behaviors. AHLs have provided a frame for many potential biofilm inhibitors [100-102]. The synthesis and activity of several unnatural AHLs was reported, significantly reducing biofilm formation in *P. aeruginosa* PA01 [103]. Several inhibitors of LuxS and MTAN, both key enzymes in AI-2 synthesis, have also been described affecting AI-2 production and biofilm formation of e. g. *Edwardsiella tarda* [104]. In the plant pathogen *E. carotovora*, also the expression of an AHL-lactonase significantly reduces its virulence by degrading the synthesized AHL-autoinducers [58, 105]. Transgenic plants expressing the AHL-lactonase can effectively quench AHL-QS signaling and prevent bacterial population density-dependent infections, whereas untransformed control plants develop fatal disease symptoms [58]. In *S. aureus*, toxin production is induced by the protein RNAIII-activating protein (RAP) and by AIPs, and is inhibited by an RNAIII-inhibiting peptide (RIP) and by inhibitory AIPs. RAP has been shown to be a useful vaccine target site, and RIP and inhibitory AIPs therapeutic molecules to prevent and suppress *S. aureus* infections [106]. RIP has been evaluated extensively and has been shown to prevent infections, including those by antibiotic resistant strains, in several animal models [107] without any signs of toxicity or induction of RIP resistance [106]. A notable report on inhibition of bacterial infection was published by Rasko and coworkers using an AI3-QQ agent [108]. Here, the small QQ molecule was able to rescue mice three hours after lethal infections with *S. typhimurium* and *Franciscella tularensis*. This study impressively demonstrated that QS interference might have therapeutic value, but also illustrated that the overall feasibility of QQ strategies might be very pathogen-specific.

It may be premature to discuss the potential implications of QQ agents in the context of clinical applications, as several additional studies have to be performed on the enzyme delivery, stability, efficacy, toxicity and side effects. However, some QQ compounds are per se not suitable for the treatment of microbial biofilms on plants, animals or human due to their toxicity which needs to be taken under consideration. For instance, halogenated furanones produced by the marine red alga *D. pulchra* are capable to inhibit both, AHL and AI-2 QS [109] and affect growth of Gram-positive bacteria [110]. They are effective QQ compounds, which prevent biofilm formation of *P. aeruginosa* and *E. coli* [111, 112]. However, these halogenated furanones without modifications are too reactive, and therefore presumably highly toxic for the treatment of bacterial infections in living organisms [113]. Many known QQ compounds are cytotoxic and the fundamental QQ mechanisms are still poorly understood. In addition, it is still unknown whether these compounds would be stable and effective in humans. All of the previously identified and reported QQ compounds are still under investigation for their potential use in industry and particularly in medicine. No candidate has reached

clinical stage development yet, since transformation of laboratory findings into clinically viable drug development programs has been lacking. Further research is needed to investigate the involvement of QQ in biofilm formation, maintenance, and dispersal. Non-toxic effective QQ agents have to be developed and examined before they can be used in practice. Ones these problems are successfully addressed, QQ might have great implications as antibiotic alternative and as anti-biofilm strategy in the medical, biotechnological and agricultural sector. Particularly, it is a promising novel strategy e.g. to control diseases in aquaculture animals for which use of antibiotics is highly restricted.

6. Resistance to quorum quenching

The finding of new QQ strategies and their effective application in controlling bacterial biofilms and virulence has raised the question about the potential for resistance development against QQ agents. This has become a controversy discussion and an important research field in the last years. The lack of direct effects on the viability of bacteria resulted in the hypothesis that selection for as well as appearance of resistant mutants might be less frequent compared to classic antibiotic treatment. However, Defoirdt *et al*. controverted the assumption that QS disruption is not leading to resistance and suggested that fitness of bacteria can be affected through variability in QS core genes [114]. Additionally, a study has demonstrated that QQ compounds can indeed generate QQ resistance in *P. aeruginosa* [115]. Bacteria were able to easily escape from QQ approaches by altering the expression of core genes in the targeted QS signaling pathway, such as the genes involved in the signal synthesis, detection and transduction. Thus, the hypothesized mechanism of QQ resistance appears very similar to the conventional antibiotics resistance. In fact, there is increasing evidence of QQ treatments leading to resistant bacterial phenotypes. Further studies are necessary to determine the molecular mechanisms of mutations that confer the QQ resistant bacterial phenotypes and to elucidate whether the selection of such mutations is random and would confer advantages in fitness. Nevertheless, future studies in the therapeutic development of anti-virulence strategies should proceed to avoid the undesired consequences currently associated with antibiotic development. In summary, it still appears unclear whether QQ agents tend less to select for resistant mutants, but a number of interesting findings have been published and future work will illustrate whether QQ compounds have the assumed high potential to attenuate microbial virulence. Thus, nowadays it seems that a combined strategy of antibiotics and QQ might be more effective [66].

7. Conclusion

The vast amount of reports that describes the identification of QQ activities present in nature contrasts to the few QQ compounds, which have been characterized in detail at a molecular level. Just a few were experimentally evaluated with respect to their biological role and the underlying QQ mechanisms, or even the use as antibacterial treatments under realistic conditions. For their application as anti-biofilm compounds it is of high importance that, in addition to their anti-biofilm activity, they do not affect host cells and are simple and inexpensive to produce. Overall, QQ strategies might become an effective alternative to combat bacterial biofilms and infections either as single agents or in combination with antibiotics. QQ can be further developed as a tool for disrupting or retarding the ability of a pathogen to sense cell density and reduce or inactivate the capability for biofilm formation and pathogenicity mechanisms. This incapability would ensure that the host has time to eliminate the pathogens through immune system functions, resulting in overcoming the pathogenic infection. A combination of a QQ approach with common treatments, such as antibiotics, to obtain a synergistic effect is a strategy that could potentially increase the susceptibility of bacteria to antibiotic treatment. The possible development of QQ resistance cannot be underestimated; however more studies will provide further insight and guide eventual biotechnological and clinical evaluation of QQ agents.

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