Expert Review of Anti-infective Therapy

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/ierz20

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To cite this article: Rakesh Sikdar & Mikael Elias (2020): Quorum quenching enzymes and their effects on virulence, biofilm, and microbiomes: a review of recent advances, Expert Review of Anti-infective Therapy, DOI: 10.1080/14787210.2020.1794815

To link to this article: https://doi.org/10.1080/14787210.2020.1794815

EXPERT REVIEW OF ANTI-INFECTIVE THERAPY
https://doi.org/10.1080/14787210.2020.1794815

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REVIEW

Quorum quenching enzymes and their effects on virulence, biofilm, and microbiomes: a review of recent advances

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ABSTRACT

Introduction: Numerous bacterial behaviors are regulated by a cell-density dependent mechanism known as Quorum Sensing (QS). QS relies on communication between bacterial cells using diffusible signaling molecules known as autoinducers. QS regulates physiological processes such as metabolism, virulence, and biofilm formation. Quorum Quenching (QQ) is the inhibition of QS using chemical or enzymatic means to counteract behaviors regulated by QS.

Areas covered: We examine the main, diverse QS mechanisms present in bacterial species, with a special emphasis on AHL-mediated QS. We also discuss key *in vitro* and *in vivo* systems in which interference in QS was investigated. Additionally, we highlight promising developments, such as the substrate preference of the used enzymatic quencher, in the application of interference in QS to counter bacterial virulence.

Expert opinion: Enabled *via* the recent isolation of highly stable quorum quenching enzymes and/or molecular engineering efforts, the effects of the interference in QS were recently evaluated outside of the traditional model of single species culture. Signal disruption in complex microbial communities was shown to result in the disruption of complex microbial behaviors, and changes in population structures. These new findings, and future studies, may result in significant changes in the traditional views about QS.

ARTICLE HISTORY
Received 12 February 2020
Accepted 9 July 2020

KEYWORDS
Quorum sensing (QS); Quorum quenching (QQ); signaling; lactonase; microbiome

1. Introduction

Quorum sensing (QS) was discovered in the bioluminescent marine bacteria *Vibrio fischeri* inhabiting the light organ of Hawaiian bobtail squid *Euprymna scolopes* [1]. Bioluminescence produced by the bacteria correlated with its cell density and production of small diffusible molecules called autoinducers (AI) [2]. With increasing bacterial population during growth, the AI concentration reaches a threshold and activates population-wide bioluminescence [3–5]. This phenomenon, by which AIs act as chemical signals, and how, depending on the bacterial biomass, can modulate their individual and group behaviors was termed as QS [6]. Thereafter, QS systems were found to be present in numerous bacterial species. All QS systems demonstrate a common mode of action comprising the following three steps – (i) Intracellular production and extracellular secretion of AIs by bacteria (ii) extracellular AIs reentry into the bacterial cells either actively (via membrane-bound transporter proteins) or passively (via diffusion across the cell membrane) and binding to specific membrane or cytoplasmic receptors and (iii) signal transduction by AI receptors activates downstream signaling pathways which eventually regulate the expression of genes associated with physiological processes such as metabolism, virulence, sporulation, and biofilm formation [7–9]. Regulation and disruption of QS, therefore, present an attractive opportunity to mitigate undesirable bacterial traits controlled by signaling such as virulence and biofilm formation associated with infectious diseases [10]. Interference in QS, known as Quorum Quenching (QQ) [11], inhibits bacterial communication using chemical or enzymatic means and subsequently reduces all behaviors regulated by QS [12]. In this review, we discuss the main QS mechanisms present in bacteria and several key methods and promising developments utilizing enzymes for the disruption of QS.

2. Diversity of QS signals – it's all about autoinducers (AIs)

Bacterial QS systems rely on AIs as specialized signaling molecules. Three main classes of AIs are shown in Figure 1 – *N*-acyl Homoserine Lactones (AHLs) or Autoinducer-1, Autoinducing Peptides (AIPs), and Autoinducer-2 (AI-2) [7]. While the structural diversity of known signaling molecules is already large, it is very likely that more, new signaling molecules involved in QS or similar signaling systems are yet to be discovered. Known AI molecules can passively or actively diffuse across the cell membrane, bind to their cognate receptors with high specificity and trigger a cascade of signaling pathways eventually resulting in transcriptional regulation of target genes. While it is not the focus of this review, we note that signaling using the

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Supplemental data can be accessed here.

Article highlights

- Quorum quenching strategies can interfere with microbial signaling both *in vivo* and *in vitro*.
- Enzymatic quenchers show low diversity in substrate preference, yet the latter property is key in interference strategies.
- Highly stable and active enzymatic quenchers allow investigating signaling in complex communities.
- Interference strategies in complex communities lead to global changes in population structures and behaviors.

above-mentioned molecules is not limited to bacteria, but extend to eukaryotes including plants and animals [13–20].

2.1. Autoinducing peptides (AIPs)

AIPs (Figure 1(b)) are short cyclical peptides involved in QS found in Gram-positive bacteria [21]. AIPs are synthesized in the cytosol as pro-AIPs which are processed to form mature AIPs either in the intracellular [22] or the extracellular [23] environment depending on the species. Due to their inability to passively diffuse across the cell membrane, AIPs are chaperoned by specialized membrane transporters [24]. AIPs are detected and bound by transmembrane histidine kinases when their extracellular concentration reaches the QS threshold [25]. This binding results in phosphorylation of a downstream response regulator, which regulates the expression of target genes for pathogenic phenotypes such as competence, DNA uptake, sporulation, virulence initiation [26]. AIP-based QS systems are well characterized in *Staphylococcus aureus* [27], *Enterococcus faecalis* [28], *Bacillus subtilis* [29], *Listeria monocytogenes* [30], and *Clostridium perfringens* [31].

2.2. Autoinducer-2 (AI-2)

AI-2 (Figure 1(c)) are synthesized and detected by both Gram-positive [32] and Gram-negative [33] bacteria as well as archaea [34]. AI-2 is a mixture of molecules, including borated furanone derivatives [35], derived from DPD (4,5-dihydroxy-2,3-pentanedione) synthesized by LuxS synthase in the S-adenosyl-methionine (SAM) recycling pathway [36,37]. AI-2 is imported and bound by its cognate receptor leading to a cascade of phosphorylation signaling pathways regulating phenotypes such as virulence and bioluminescence [38]. Examples of well-characterized systems are the LuxPQ system in *Vibrio sp* [39] and the Lsr system in *Salmonella sp*. and *Escherichia coli* [40,41].

2.3. N-acyl homoserine lactones (AHLs)

AHLs (Figure 1(a)) were previously presented to be exclusively synthesized and sensed by Gram-negative bacteria. However, in addition to Gram-negatives [9], there are some examples

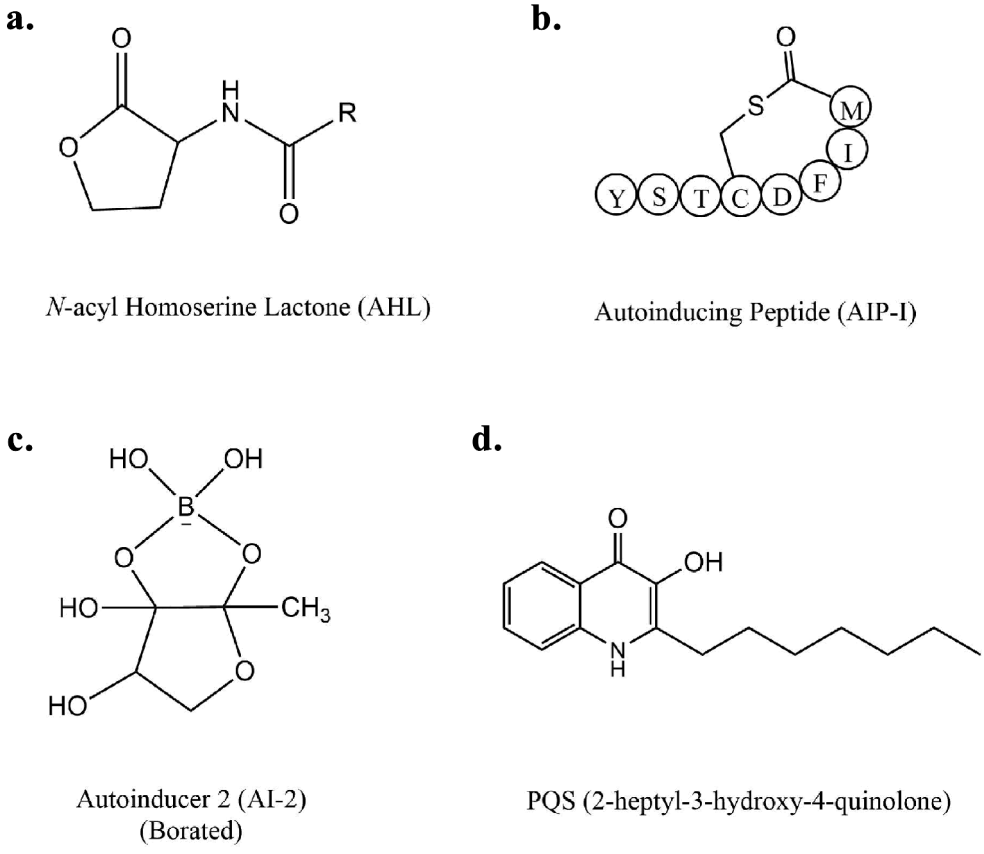

Figure 1. Representative chemical structures of (a) *N*-acyl homoserine lactone, (b) Autoinducing peptide AIP-I from *S. aureus*, (c) Autoinducer 2 (AI-2) from *Vibrio sp*., (d) PQS from *P. aeruginosa*.

that in rare cases AHLs are also produced by Gram-positive bacteria [42] and archaea [43]. AHLs are small diffusible molecules composed of a lactone ring linked to an acyl chain of varying lengths (C4-C14) and C3 modifications [6,9]. AHLs are synthesized by LuxI synthases and detected by their cognate cytoplasmic LuxR receptors [44] (Figure 2(a)). The specificity of AHLs binding to their receptors is determined by the structure of their acyl side chains [9]. When the AHL concentration reaches the QS threshold, typically reported to be in the nanomolar range [45–48], AHL-bound LuxR receptors act as transcriptional modulators of target genes (Figure 2(b)). Many pathogenic behaviors of Gram-negative bacteria such as host adhesion, sporulation, exoenzyme production, toxin secretion, biofilm formation, siderophores, and pigment production are regulated by AHL-mediated QS [9,49]. A non-exhaustive list of examples of well-characterized AHL QS systems is as follows: LuxIR in *Vibrio sp* [50]., TraIR in *Agrobacterium tumefaciens* [51], the hierarchical QS systems LasIR and RhlIR in *Pseudomonas aeruginosa* [52], CviIR in *Chromobacterium violaceum* [53], ExpIR in *Erwinia carotovora* [54], SmaIR in *Serratia sp* [55]., AhyIR and AsaIR in *Aeromonas sp* [56,57]. Putative luxIR homologs have also been detected in the genomes of Gram-positive bacteria and archaea which were shown to produce AHLs [42,43].

It is interesting that LuxI homologs in certain species of α-proteobacteria can produce homoserine lactones (HSLs) with aromatic acid or branched amino acid side chains (instead of the straight fatty acyl side chains of AHLs). Notable examples include *p*-coumaroyl-HSL from *Rhodopseudomonas palustris* (RpaIR) [58], phenylacetyl-HSL from *Prosthecomicrobium hirschii* (HirIR) [59], isovaleryl-HSL from *Bradyrhizobium japonicum* (BjaIR) [60] and cinnamoyl-HSL from *Bradyrhizobium* strain ORS278 (BraIR) [61].

2.4. Other signaling molecules

Several other molecules were found to exhibit autoinducing properties and play distinct roles in QS. For example, Alkylquinolones, underlying the *Pseudomonas* Quinolone Signal (PQS) QS pathway present in *P. aeruginosa*. Alkylquinolones (For e.g.: 2-heptyl-3-hydroxy-4-quinolone; Figure 1(d)) are synthesized by the PqsABCDH system and detected by PqsR [62]. PQS responds to disparate environmental signals such as low iron conditions and response to oxidative stress [63,64] and integrates these signals into the main LasIR and RhlIR signaling systems to fine tune virulence and biofilm formation [65]. In addition to altering the global transcriptional profile of genes via the PqsR dependent pathway, PQS can also function independent of PqsR by binding to hundreds of different cellular receptors [66,67], leading to modulation of host immune responses, cytotoxicity, and key virulence pathways [68].

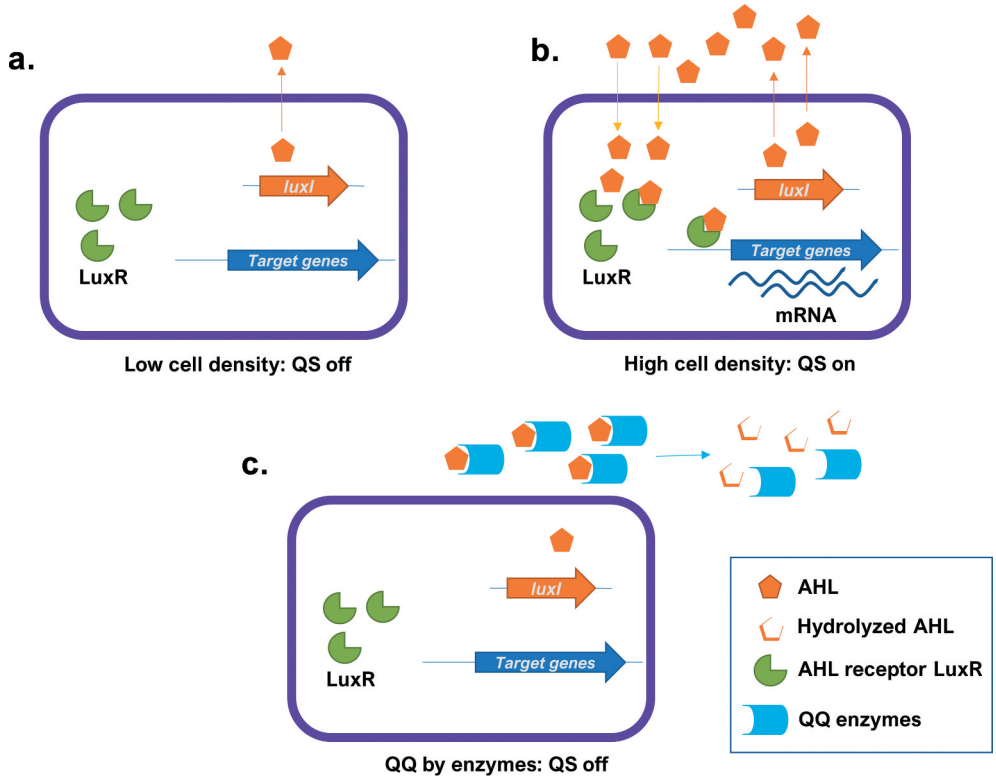

Figure 2. Representation of AHL-mediated Quorum Sensing (QS) (A and B) and enzymatic Quorum Quenching (QQ) (C). (**a**) Bacteria encode a LuxI synthase producing *N*-acyl homoserine lactones (AHLs) in the cytoplasm, which can diffuse out of the cell. At low cell density, the extracellular concentration of AHLs remain low and QS is off. (**b**) When bacterial density increases, so does the extracellular AHL concentration. Upon reaching a threshold level, AHLs bind to their cognate cytoplasmic LuxR receptors. The AHL bonded LuxR acts as a transcription factor and can triggers the expression of target genes, including the production of more AHLs, and resulting in population-wide changes in gene expression profiles and bacterial behavior. (**c**) QQ enzymes degrade extracellular AHLs, reduce the AHL concentration, and prevents the signaling molecule to reach the concentration threshold that triggers QS.

Other classes of QS signals are (a) fatty-acid derivatives – for e.g. 3-hydroxypalmitate methyl ester (3-OH-PAME) produced by *Ralstonia sp* [69] and cis-11-methyl-2-dodecenoic acid (DSF, Diffusible Signal Factor) from *Xanthomonas campestris* [70] and (b) amino acid derivatives – for e.g. diketopiperazines produced by proteobacteria [71] and archaea [72].

3. Enzymatic quorum quenching – inactivating the messenger

The process of inhibition of QS is referred to as QQ [11]. Although QQ can be achieved by various means, in this review, we will focus on the basics, progress and potential of enzyme-mediated QQ.

Disruption of AHL-mediated QS by enzymes can be achieved by degrading AHLs (Figure 2(c)). As illustrated in Figure 3, three different classes of enzymes, based on their mechanism of action, can inactivate AHLs – (i) Lactonases, that break open the lactone ring [73,74], (ii) Amidases (or acylases), that hydrolyzes the amide bond of the AHLs and breaks it down into the corresponding fatty acid and homoserine lactone [75], (iii) Oxidoreductases, that either oxidize the acyl chain of AHLs or reduces 3-oxo-AHLs to their corresponding 3-hydroxy-AHL counterparts [76–78].

AHL degrading enzymes exist naturally and are produced by bacteria [79], archaea [74], and eukaryotes [80]. Some bacteria like *Pseudomonas* and *Agrobacterium* encode genes for AHL degrading enzymes – the corresponding enzymes, PvdQ and BlcC, respectively, degrade their own AHL signals and are hypothesized to prevent their concentration from reaching toxic levels by AHL recycling [81,82].

A comprehensive list of QQ enzymes is listed in Table S1 along with their substrate specificities and description of their characteristics. Different classes of QQ enzymes demonstrate disparate substrate specificities. For example, while known acylases preferentially hydrolyze AHLs with long acyl chains, lactonases can demonstrate significantly higher activity against a broader (and sometimes complete) spectrum of AHL substrates. It is intriguing to note that most of the well-characterized AHL degrading enzymes fall into two categories: (i) those with very broad substrate specificities and (ii) those with a marked preference for longer acyl chain AHLs.

3.1. Oxidoreductases

Some oxidoreductases were characterized for their ability to modify the QS signal molecule rather than degrading it. They either oxidize the ω1, ω2, or ω3 carbon of the acyl chain or reduce the C3 carboxyl group of AHLs into a hydroxyl group (Figure 3(c)). This process results in alteration of the AHL signal and subsequently its capacity to bind to its cognate receptor, thereby modulating the QS response. For example, BpiB09

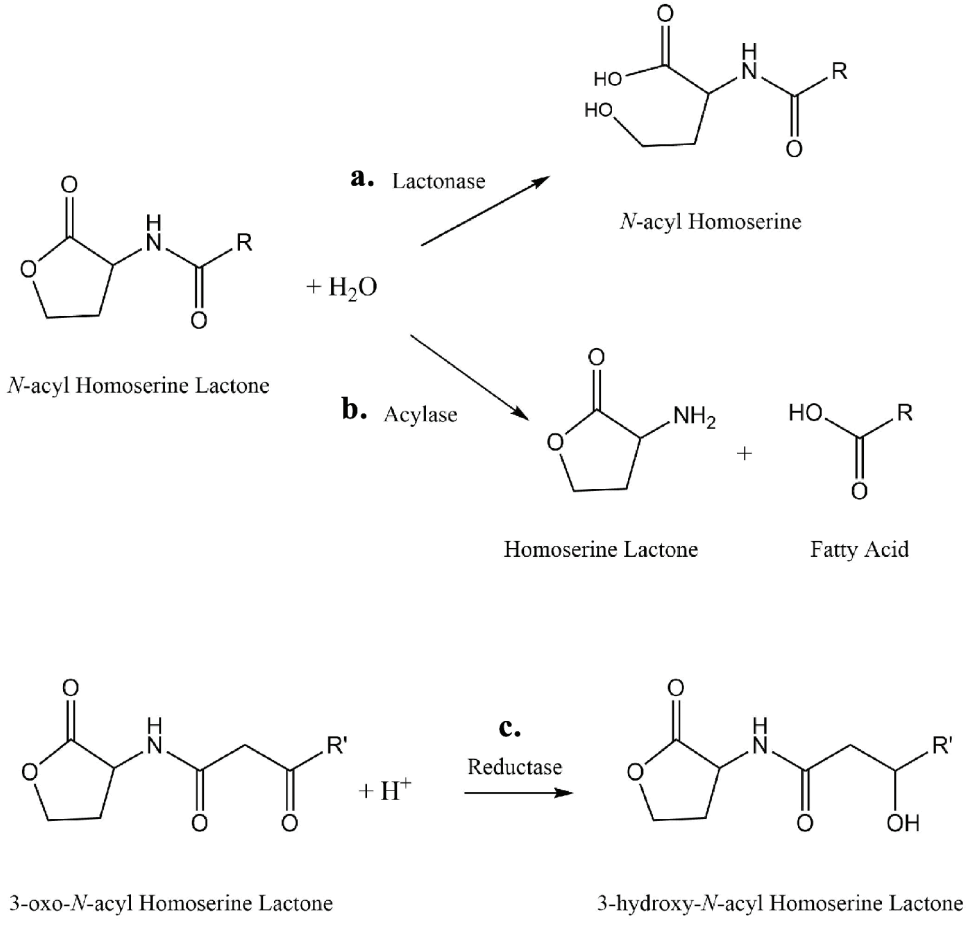

Figure 3. Chemical mechanisms of hydrolysis of AHLs by (**a**) lactonases and (**b**) acylases or (**c**) reduction of 3-oxo-AHLs by reductases into 3-hydroxy-AHLs.

reductase (Figure 4(a)) from *Acidobacterium sp*. reduces 3-oxo-AHLs to 3-hydroxy-AHLs and can alter the QS response of *P. aeruginosa* [76].

3.2. AHL Acylases

Acylases hydrolyze the amide bond of AHLs (Figure 3(b)) [83]. They belong to the superfamily of N-terminal nucleophile hydrolases (Ntn-hydrolases) with a characteristic αβ/βα fold. Their active sites show a large hydrophobic binding pocket to accommodate long acyl chains of AHL substrates [84–86]. A well-characterized QQ acylase is PvdQ (Figure 4(b)) from *P. aeruginosa* [81]. PvdQ preferentially cleaves AHLs with acyl chain lengths exceeding 10 carbons [87] and plays a key role in the production of pyoverdine [88].

3.3. AHL Lactonases

Lactonases degrade AHLs by hydrolyzing their lactone ring (Figure 3(a)) [73]. They can show preference for certain types of AHLs and other lactone substrates such as δ-, ε- and γ-lactones, and some representative from extremophiles are active under a variety of environmental conditions such as high temperature [74,89–91]. Intriguingly, different classes of lactonases were identified in a variety of protein superfamilies and folds (**Table S1**), including:

(i) **The Phosphotriesterase-like lactonases (PLLs)** – Found in bacteria and archaea, they exhibit a $(\alpha/\beta)_8$ fold (TIM barrel). They prefer AHLs with long acyl chains. SsoPox (Figure 4(c)) is a well-characterized PLL representative from *Saccharolobus solfataricus* [91,92]. PLLs are metalloenzymes and numerous characterized PLL representatives are thermostable enzymes, a desirable property for biotechnological applications e.g. SacPox [93], VmoLac [94], SisLac [95], and GkL [96]. PLLs are proficient lactonases that typically show a substrate preference for long acyl chain AHLs.

(ii) **The Metallo-β-lactamase-like lactonases (MLLs)** – Found in bacteria, archaea, and eukaryotes, their main structural highlight is the presence of a conserved metal-binding motif HXHXDH, exhibiting a characteristic αβ/βα fold bonded with two metal cations. These lactonases are very proficient enzymes and are found to exhibit a broad AHL substrate preference [74,89,97,98]. AiiA from *Bacillus sp*. is a well-characterized example [99] (Figure 4(d)).

(iii) **The α/β hydrolase fold lactonases** – Isolated from bacteria, these possess a characteristic α/β hydrolase

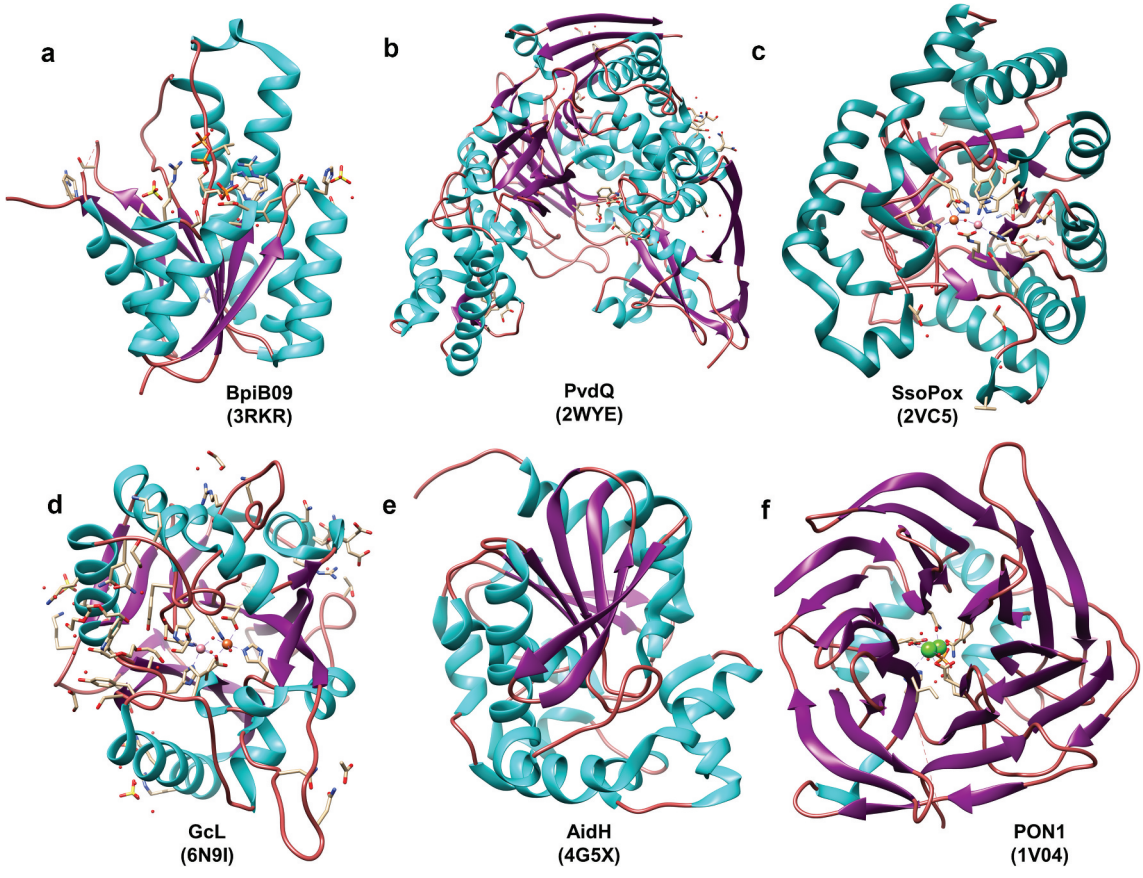

Figure 4. Crystal structures of representatives from different QQ enzyme families – (**a**) AHL reductase BpiB09 from an uncultured bacterium Bio5, (**b**) AHL acylase PvdQ from *Pseudomonas aeruginosa*, (**c**) phosphotriesterase-like lactonase SsoPox from *Saccharolobus solfataricus*, (**d**) metallo-β-lactamase-like lactonase GcL from *Parageobacillus caldoxylosilyticus*, (**e**) α/β hydrolase fold lactonase AidH from *Ochrobactrum sp*. T63. and (**f**) serum paraoxonase PON1 from *Oryctolagus cuniculus*. RCSB Protein Data Bank ID for each structure is provided in brackets.

fold but lack the conserved HXHXDH motif of MLLs [100]. These lactonases also demonstrate preference for a broad spectrum of AHL substrates [100,101,102,103,104null–null]. One well-characterized example is AidH (Figure 4(e)) produced by the soil bacterium *Ochrobactrum sp* [100,101].

(iv) **The Paraoxonases (PONs)** – They derive their name from their ability to cleave paraoxon [105], a derivative of the organophosphate insecticide parathion. PONs possess a characteristic six-bladed β-propeller fold (Figure 4(f)) [106]. PONs are found in both mammals and bacteria [107]. PONs hydrolyze a wide variety of substrates which includes long acyl chain AHLs (weak activity against short chain AHLs, preference for longer acyl chains AHLs), arylesters, organophosphates, fatty acids, δ-lactones and γ-lactones [107,108].

It is interesting to note that an evolutionary convergence in the structures and catalytic mechanisms of PLLs, MLLs, and PONs was observed [109]. Their active site regions and the usage of a metal cation to bind to the carbonyl oxygen of the lactone substrate leading to its subsequent hydrolysis are common to these classes of lactonases [107]. It is also intriguing to observe that most lactonases that have been identified and characterized exhibit a low variety of substrate preferences (**Table S1**). PLLs, specifically PLL-A [94], appear to be the most specific lactonases, with a marked preference for long-chain AHLs.

3.4. QQ enzymes against AI-2 and other QS signaling molecules

Proficient enzymes capable of neutralizing AI-2 remain elusive. In a known interference mechanism, AI-2 can be extracellularly phosphorylated by the kinase LsrK, which destabilizes it and prevents intracellular uptake by its cognate receptor [38,40,41,110]. Recently, oxidoreductase enzymes degrading AI-2 were discovered using metagenomic approaches [111,112] and may represent a promising way to interfere with AI-2 based QS.

The quinolone-based QS signals of *P. aeruginosa* (PQS) and *Burkholderia sp*. (AQ) can be degraded by a class of enzymes known as quinolone dioxygenases (see Table S1 for details). These enzymes open the PQS ring to form N-octanyl-anthranilic acid and carbon monoxide [113,114]. The first identified member of this class of enzymes is Hod (1H-3-hydroxy-4-oxoquinaldine 2,4-dioxogenase) from *Arthrobacter sp*. Rue61a [113]. Other QS signals such as 3-OH-PAME can be degraded by recently identified microbial esterases [115,116] and diketopiperazines were shown to be biologically degraded by yet unidentified enzymes in a cell-free extract of *Streptomyces albulus* [117].

4. Quorum quenching enzymes and biotechnology

Because QS modulates bacterial behaviors, the use of QQ enzymes is fundamentally different from the use of antimicrobials: the enzymes show no toxicity and little to no effects on growth [118–120]. Rather than entering cells or binding to a receptor, lactonases hydrolyze signaling molecules secreted in the media to affect bacteria's behavior [121]. It is noteworthy that while QQ can also be achieved by various small molecule chemicals known as Quorum Sensing Inhibitors (QSIs) such as 5-Fluorouracil (5-FU) and halogenated furanones [122], QQ enzymes are significantly more potent than QSIs while lacking the cytotoxicity of the latter [103,123]. Resistances to these strategies have been observed. Yet, contrary to the resistance to QSIs that can be achieved by the overproduction of efflux pumps, resistance to QQ enzymes is possibly limited to cells that either lost the ability to respond to QS (e.g. social cheaters) or to cells producing a hypothetical enzyme inhibitor [124,119,125].

4.1. AHL Acylases and Lactonases as anti-virulence agents

The effect of QQ enzymes has been extensively investigated with the Gram-negative pathogenic bacterium *P. aeruginosa*. This pathogen is associated with 8–10% of nosocomial infections in health care as it frequently infects immunocompromised patients and is listed among the top priority pathogens by the WHO for immediate R&D of new antimicrobials [126,127]. *P. aeruginosa* possesses three overlapping QS circuits – LasIR, RhlIR and the PQS systems following a strict signaling hierarchy (LasIR at the top), interwoven signaling pathways, and overlapping signaling targets [65]. This sophisticated QS circuitry enables this bacterium to be a versatile and opportunistic pathogen that can adapt to a variety of environmental conditions in the host tissue and forms a robust biofilm [128]. While all these three QS systems contribute to virulence, the QS signals 3-oxo-C12 AHL (sensed by LasR), C4-AHL (sensed by RhlR) and PQS (sensed by PqsR) have been successfully targeted by QQ enzymes.

A multitude of QQ enzymes (AiiA, AiiM, SsoPox, PvdQ, MomL, BpiB09, and HodC) was shown to reduce the production of virulence factors, motility and biofilm formation of *P. aeruginosa via* interference in QS *in vitro* [76,85,98,113,103,123,129,130], as well as *in vivo* in various models, including *Caenorhabditis elegans, Drosophila melanogaster* and rodents [130–133].

As previously noted in **Table S1**, AHL degrading enzymes are found to exhibit two main substrate preferences: broad spectrum or preference for long acyl chain AHLs. This distinct property of QQ enzymes was recently used to specifically inhibit the different QS circuits in *P. aeruginosa*, i.e. systems based on C4-AHL and 3-oxo-C12-AHL. With clinical isolates from lung-adapted strains, the use of two lactonases with distinct specificity resulted in different inhibitions of virulence factors, the broader spectrum enzyme being a more potent inhibitor [134] Using the same enzymes, the use of *in vivo* amoeba models unexpectedly showed that only the most specific enzyme could protect amoebas from infections. Analysis, including proteomics, in fact, revealed large variations in protein levels involved in antibiotic resistance, biofilm formation, virulence as a function of lactonase specificity [135]. These results suggest that the specificity of the interference in signaling may be a key parameter in improving the potency of quorum quenching approaches.

In addition, numerous animal studies were performed. For example, the lactonase SsoPox-I was administered directly in the trachea of rats, immediately after infecting them with *P. aeruginosa* [132]. The treated rats showed a significantly reduced mortality rate compared to untreated rats, during a 50 h observation period. A similar work was performed with the acylase PvdQ [133] that was administered to mice *via* the nasal route after initiating a lethal *P. aeruginosa* infection. Subsequently, the lung bacterial load of PvdQ treated mice was reduced fivefold and less morbidity, reduced tissue inflammation, and prolonged survival were observed compared to untreated control mice. Combinations of QQ were also tested *in vivo*: Combining the *Rhodococcal* lactonase QsdA and *Mycobacterial* dioxygenase AqdC resulted in enhanced QQ and reduction of virulence of *P. aeruginosa* in *C. elegans* and epithelial lung cell infection models [136]. Another study found that *Deinococcus radiodurans* produces a lactonase and an acylase, which demonstrated a more robust QQ of *P. aeruginosa* and a major reduction of virulence in a *C. elegans* infection model [137]. These studies demonstrate the underlying potential of QQ enzymes to inhibit infections *in vivo*, and that QQ strategies can putatively be improved by combining enzymes and possibly targeting multiple QS systems.

Additionally, lactonases were also shown to inhibit the virulence of numerous other pathogens in infection models, including *Aeromonas hydrophila* and subsequent mortality in crucian carp by AiiA [138], reduction of virulence of marine pathogenic *Vibrio sp*. in brine shrimp and manila clam [139] and *Erwinia carotovora* in plants [140]. The acylase PvdQ was also shown to be effective in an infection model. An engineered PvdQ variant hydrolyzed C8 AHL in addition to its natural long chain AHL substrates such as 3-oxo-C12 AHL, with high efficiency. Since C8 AHL is used by *Burkholderia cenocepacia* for QS, application of this engineered PvdQ acylase was shown to reduce the virulence of *B. cenocepacia* and subsequently reduced mortality in a *Galleria mellonella* infection model [87].

It is noteworthy that QQ enzymes and QSIs can also be used in combination to achieve a more robust QQ in pathogenic bacteria. In a recent study with *P. aeruginosa*, it was observed that combining QQ enzyme AiiM and the QSI G1 resulted in almost complete quenching of the Las and Rhl QS systems, which was significantly better than using either AiiM or G1 separately [141].

4.2. AHL Acylases and Lactonases as anti-biofilm agents

Biofilms are complex 3D structures composed of a mono- or multispecies community of bacteria attached to a surface. Bacteria in the biofilm secrete an extracellular matrix composed of exopolysaccharide (EPS), nucleic acids, proteins, and lipids [142], which provide structural support, adaptability to diverse environmental conditions and resistance to a wide variety of antimicrobial compounds including antibiotics [143,144]. In most ecological niches including infected tissues [145], bacteria exist in multispecies biofilms.

The ability of QQ lactonases to inhibit biofilm formation in *P. aeruginosa* was reported in several reports [103,123,146]. Inhibition of biofilm formation of other pathogens was also reported, including *A. baumannii*. Application of QQ lactonases such as GkL [147], AaL [74] and GcL [89] resulted in reduced biofilm formation by the bacterium. In addition, engineered lactonase GkL with increased catalytic efficiency also disrupted existing biofilms of *A. baumannii* [147]. The effect of lactonases in inhibition or dispersal of biofilms is illustrated in Figure 5(a).

Utilization of QQ enzymes from extremophiles and/or engineering for higher stability has allowed us to use these enzymes in formulations to control biofilm formation on

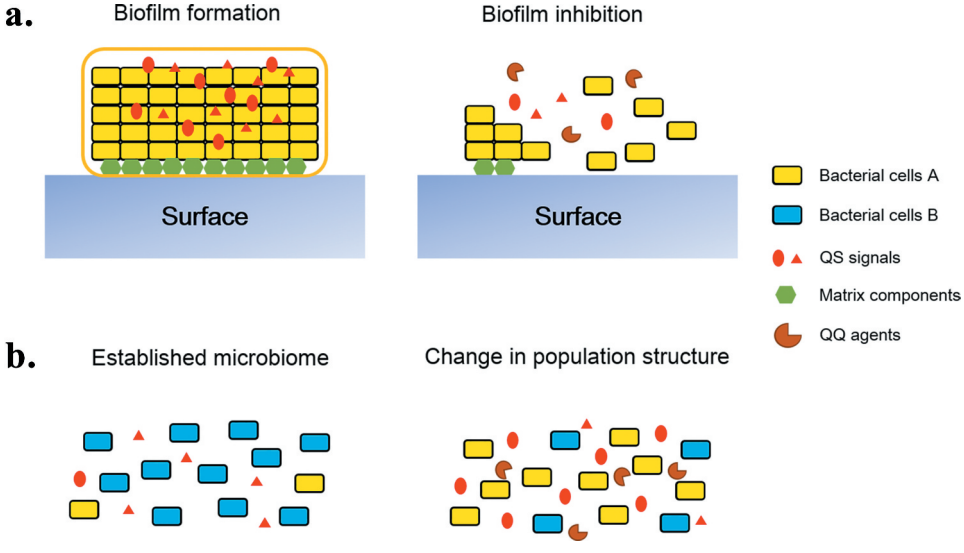

Figure 5. Effect of QQ on – (a) biofilm inhibition and (b) altering the species composition of the mammalian gut microbiome to prevent dysbiosis. (a) *Left*. The formation of stable biofilms relies extensively on QS dependent processes such as production of EPS and other components of the biofilm matrix. *Right*. QQ can prevent the formation of biofilms. (b) *Left*. Microbiome balance results from a complex interaction between several microbial species and therefore dependent on QS. *Right*. Because interference in QS leads to change in microbial population structure, future research will reveal interactions between microbial species and might aid in restoring healthy microbiome.

medically and industrially relevant surfaces. Medically relevant surfaces are foreign inserts into the human body such as prosthetic valves, vascular prosthetics, catheters, endotracheal tubes, cardiac pacemakers, cerebrospinal fluid shunts, ocular prosthesis, orthopedic implants, and intrauterine contraceptive devices [148]. These inserts provide a natural surface for the growth of pathogenic bacteria and often result in the formation of recalcitrant biofilms and subsequently difficult-to-treat infections [148,149]. The relevance of preventing biofilm formation, and thereby prophylactically preventing infection, by using surface-immobilized QQ lactonases becomes more profound in the context of increasing frequency of antibiotic resistance in bacteria.

Recently, the silicone surface of a urinary catheter was coated with a QQ acylase derived from *Aspergillus melleus*. The immobilized enzyme imparted anti-biofilm properties to the catheter surface for a period of 7 days. This treatment caused a reduction of 75% of *P. aeruginosa* biofilm after 24 h of incubation [150]. The same coating when combined with enzyme α-amylase, to digest the exopolysaccharide (EPS) component of biofilms, in multiple layers, demonstrated a maximal reduction of 70% of biofilm formation in a catheterized rabbit, compared to the untreated catheter after 7 days of monitoring spontaneous bacterial infection in a dynamic environment [150]. New techniques for immobilization of QQ acylases on PVC catheters by using glycidyl methacrylate, without loss of enzyme activity, were recently demonstrated [151]. SsoPox was also successfully immobilized in polyurethane coatings using glutaraldehyde with no loss of activity in QQ applications with about a seven-fold reduction of biofilm formation by *P. aeruginosa* compared to uncoated control [123].

5. Conclusions

We have discussed the nature and key roles of main quorum sensing (QS) circuits and signals in regulating bacterial behaviors including pathogenicity and biofilm formation, in this review. Recent efforts have demonstrated the variety of QS interference strategies, and the numerous enzymatic ways to inactivate signaling molecules. Such enzymes, through the interference in QS, can effectively behave as biofilm and virulence inhibitors. This and their lack of cytotoxicity are making them appealing candidates to control bacteria. Several studies have highlighted their potential to prevent bacterial infections in a variety of *in vivo* systems. The identification of highly stable enzymes from extremophiles, and the improvement of their properties via molecular engineering may open the path for the use of the QS interference strategy in a variety of clinical settings.

6. Expert opinion

A significant part of the research work on QS is dedicated to the interference strategies that were discussed in this review. These strategies, using, for example, QQ enzymes, may allow bacterial control with little to no bactericidal activity, without the need to physically enter or contact the bacterial cells. The variety of the possible fields of applications for the anti-virulence and anti-biofilm activity of these enzymes is large, yet it will require significant improvements in the properties of these molecules to make them compatible with the constraints of industrial productions. For example, different enzymes demonstrate variable substrate specificities and activities, have stability issues under different application conditions such as extreme pH and temperature and dependence on the availability and nature of metal cofactors for optimal functionality. Recent efforts in these directions, such as the formulations of stable enzymatic materials [150,152,153] or preparation that can be used in the field [132,146,153], have led to promising observations. Indeed, recent work (discussed below) on microbial communities has highlighted the complex interconnection between bacteria producing and/or sensing different types of signaling molecules, and it may change traditional views about quorum sensing.

Interestingly, work using lactonases to interfere with complex biological processes revealed profound changes to the microbial population structure because of disruption of AHL signaling. This finding is surprising, since AHL interference was long studied mostly in the context of AHL producers and sensors, i.e. Gram-negative bacteria. These global changes likely pertain to the biological importance of these signaling molecules to complex microbial communities. Changes were observed in numerous environments: e.g. in the context of Membrane BioReactors (MBR) treated with beads embedded with lactonases. Analysis of the microbial population structure revealed global changes to surface communities [153,154]. In the waste sewage sludge system, the use of the lactonase AiiM altered the microbial community, favoring Gram-positive bacteria over Gram-negative species and subsequently decreasing methane production by over 400% while increasing fermentation leading to acetic acid production [155]. This insight may be consistent with most AHL-responders being Gram-negative bacteria.

Other studies, following the engineering of lactonases for higher activity and stability (e.g. Ssopox [152]), investigated the effects of signal disruption using a lactonase in the context of biocorrosion [156] and biofilm formation [157]. Both studies found that AHL signal disruption resulted in an inhibition of the tested biological processes (biofilm or biocorrosion formation) that was concomitant to significant changes in the microbial population structure (Figure 5(b)). Intriguingly, observed changes in microbiomes are not easily interpretable considering the ability of bacteria to produce/sense AHLs. Indeed, some AHLs producers/sensors as well as some bacteria known not to encode genes for AHL production and sensing were either positively or negatively affected by signal disruption.

This study suggests the intricacy and intimacy of microbial relationships. Altering the AHL-based quorum sensing systems has effects that goes beyond AHL-producer and sensor microbes, and numerous, possibly indirect effects are observed. This might include changes in Gram-positive bacterial composition and abundance, most of which are not using nor sensing AHLs, but also observation of changes related to larger organisms (in the context of biofouling, e.g. mussels, algae).

Interestingly, these global effects do not appear to be limited to autoinducer-1 (AHL) based systems, but were also

observed with autoinducer-2. The effects of the administration of bacteria producing AI-2 in a gut microbiome model. Because AI-2 is used for QS by numerous species of Firmicutes, administration of *E. coli* overproducing AI-2 was used to restore the population of Firmicutes and subsequent health of the microbiota [158,159]. Similarly, *Blautia obeum*, a gut commensal bacterium, produces the QS molecule DPO which can prevent colonization by *V. cholerae* [160]. Therefore, oral route of delivery of QQ enzymes to facilitate selective QQ and allow for healthy microbial composition in the human gut holds promise. However, there remain concerns that these enzymes could lose stability and activity once exposed to the extremes of pH and proteases in the gastrointestinal (GI) tract. These concerns could be alleviated by coating the enzymes inside specialized pH-responsive capsules [161] which limit their exposure to low pH and/or by bioengineering these enzymes for better protease and pH tolerance [90,91,146].

These discoveries may represent the tip of the iceberg in the understanding of the biological role(s) of signaling molecules in complex microbiomes, and the potential utilization of these molecules to alter them. Future discoveries in the field of QS and QQ will reveal the mechanisms for the cellular responses of bacteria to signaling molecules, including the context of stress (e.g. antibiotics [162] or phage infection [163]). New, efficient ways to interfere with other microbial languages will be identified (e.g. AI-2). More studies, focusing on complex biological processes and communities will likely provide unprecedented understanding of interactions, interdependences, cooperation, and competitions at the microbiome level. This will ultimately inform us about the biological importance of bacterial signaling in complex communities.

Funding

This work was supported by the National Institute of General Medical Sciences of the National Institutes of Health under award number R35GM133487. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Declaration of interest

M Elias deposited the patent WO2014167140 A1 that was licensed to Gene&GreenTK, a company that he co-founded in 2013. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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