MedChemComm

Accepted Manuscript

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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

Review

Attenuation of quorum sensing-mediated virulence in Gram-negative pathogenic bacteria: implications for the post-antibiotic era

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Received 19th August 2014
Accepted XXXX2014
DOI: 10.1039/b000000x[2]

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Quorum sensing is a cell-density dependent regulatory system which orchestrates Quorum-sensing (QS) systems use extracellular signals to modulate the expression of a particular gene(s) in a bacterial cell, which results in virulence gene expression or biofilm formation and occasionally causes deadly plant and animal diseases. The frequent use of antibiotics to treat deadly diseases has led to the development of multiple drug-resistant bacterial strains. The increasing presence of pathogenic bacteria has thus forced us to develop alternative methods of controlling pathogen virulence. One possible such method, quorum quenching (QQ), has emerged as an interesting approach. A variety of bioactive molecules or drugs from prokaryotic or eukaryotic sources have been identified as QQ molecules, some of which are chemically synthesised and determined the agonist or antagonist of their cognate receptor or metabolic intermediate. Current strategies to attenuate the virulence of gene expression can be grouped into the following categories: (a) block AHL– Lux-R-type binding sites, (b) inhibition of AHL–Lux-R- and Lux-I-type interactions, (c) inhibition of transporters, (d) degradation of existing AHLs by QQ enzymes and (e) inhibitor of enzyme involves in metabolic synthesis of QS molecules. This review summarises several potential QQ molecules that have been reported to attenuate QS-based virulence gene expression in serious Gram-negative pathogenic bacteria. These QQ molecules suggest possible ways of controlling the virulence effects of pathogenic bacteria in the post-antibiotic era.

1. Introduction

Quorum-sensing (QS) systems help bacteria to communicate and regulate gene expression in a population density-dependent manner through the accumulation of diffusible extracellular signals.[1,2] Gram-negative bacteria use different types of QS signal molecules, such as *N*-acyl homoserine lactone (AHL), indole, *Pseudomonas* quinolone signal (PQS; 2-heptyl-3-hydroxy-4-quinolone, HHQ), LuxR homolog SdiA, luxS/AI-2 and AI-3/epinephrine/norepinephrine, which are now known to modulate expression of the diverse range of genes involved in biofilm formation, motility, antibiotic production, virulence gene expression and genetic material exchange.[1-7] The QS mechanism is well characterised in some Gram-negative pathogenic bacteria as *Pseudomonas aeruginosa, Escherichia coli*, *Salmonella* and *Agrobacterium tumefaciens* (see Supplementary Figure 1), which suggests that the molecular mechanisms in these bacteria are highly conserved.[8,9]

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†Electronic Supplementary Information (ESI) available: **Image 1.**

Therefore, the review summarizing several potential QQ molecules that have been reported to attenuate QS-based virulence gene expression in *A. tumefaciens*, *Pectobacterium carotovora*, *P. aeruginosa*, *E. coli* and *Salmonella*.

There is an urgent need to identify new drugs or antimicrobial compounds that do not exert selective pressure on the pathogens that ultimately lead to drug-resistant strains such as vancomycin, linezolid and the latest beta-lactams.[10,11] In protecting against or curing bacterial infection, conventional antibiotics either stop bacterial growth or kill bacterial cells by interfering with the biosynthesis of essential molecules such as cell wall, DNA, RNA, proteins and vitamins.[12] The conventional approach, i.e. attempting to kill invading bacteria, thus induces strong selective pressure to develop resistance mechanisms. To minimise such selective pressure, new strategies are needed to avoid growth inhibitory effects of the novel compounds. One such strategy, the quorum-quenching (QQ) strategy, has been proposed as an alternative process that interferes with pathogen virulence without killing the invading bacteria. Nonbiocidal molecules inhibit QS signalling, thus providing another possible opportunity for developing new drugs capable of contesting virulence genes.

Several mechanisms for disrupting the QS systems in Gram-

negative bacteria through various QQ molecules have been reported (Table 1). Current strategies to attenuate the virulence of gene expression can be grouped into the following categories: (a) block AHL– Lux-R-type binding sites, (b) inhibition of AHL–Lux-R- and Lux-I-type interactions, (c) inhibition of transporters, (d) degradation of existing AHLs by QQ enzymes and (e) inhibitor of enzyme involves in metabolic synthesis of QS molecules. Till date available QQ molecules are belonged to naturally, synthetically and enzymatically mediated. Naturally occurring QQ molecules and biological enzymes are generally non-specific, whereas synthetic QQ molecules are more selective with their target sites (Table 1). QQ molecules should ideally be small molecules that are non-digestible by the host's metabolic system and exert no adverse effects on the bacteria or host but have the potential to block QS-dependent gene expression. In addition, these compounds should be more stable than natural QS signals and sufficiently selective to inhibit the virulence expression of pathogenic genes. QQ molecules can be divided into two categories based on their molecular action: competitive and non-competitive. Competitive inhibitors are plausibly structurally analogous to naive AHL signals and occupy or bind the same AHL-binding site, subsequently failing to activate the LuxR-type receptor, whereas non-competitive inhibitors often share no structural similarities with AHL signals and bind to different sites on the receptor protein.[13] The following sections summarise the QQ molecules reported to date and their mode of action against the aforementioned Gram-negative pathogenic bacteria.

2. QQ enzymes

The first AHL inactivation gene producing lactonase, *aiiA*, was identified from *Bacillus* isolate 240B1 and was found to attenuate virulence effect of the *P. carotovorum*.[14] Different types of enzymes from diverse sources were subsequently reported to quench the QS of bacteria, amongst them AHL-lactonase, AHL-acylase, aromatic carboxylic acid esters, paraoxonase, oxidoreductase and aminohydrolase (Fig. 1, Table 1).

The probiotic use of the *Bacillus* species to control bacterial infection in aquaculture and animals have been proved much more effective than use of the antibiotics.[15,16] The AHL-lactonase production gene (*AiiA*) has also been reported in other *Bacillus* species, including *B. cereus*, *B. subtilis* and *B. thuringiensis*, which exhibit 90% identity in their amino acid sequencing.[17-21] The conserved motif His^{106}-X-Asp^{108}-His^{109}-59X-His^{169}-21X-Asp^{191} has been identified in the catalytic cavities of different AHL lactonases with little variance, and AHL-lactonase shown to be a metalloprotein containing Zn^{2+} in their active site.[22-25]AHL-lactonase inactivates AHLs by hydrolyzing the lactone bond to produce corresponding N-acyl homoserines (Fig. 1). The transformation of the *aiiA* gene into tobacco and potato genomes via *Agrobacterium*-mediated transformation has been reported to attenuate infection by *P. carotovorum*, and reduce pectinase and *polygalacturonase* activity.[17,26,27] Similarly, the heterologous expression of AHL-lactonase in *P. aeruginosa*, *E. coli* and *P. carotovora* has been shown to reduce disease symptoms in tobacco.[28] The transformation of *Bacillus* A24 *AiiA* lactonase into *P. aeruginosa* PAO1 reportedly blocks AHL accumulation and inhibits swarming motility and virulence factors.[28-29] Another QQ enzyme obtained from *Acinetobacter* strain C1010 was found to attenuate the virulence effects of plant pathogen *P. carotovora* during virulence testing.[30]

Notably, *A. tumefaciens* also has ability to encode AHL-lactonase BlaC (AttM) to degrade its own AHLs.[31-33]Although the AHL-lactonase of *A. tumefaciens* is produced during starvation conditions, it does not inhibit the QS-mediated transfer of conjugative plasmid-Ti.[34] During bacterial growth, *attM* is negatively repressed by transcriptional factor AttJ, and AHL accumulation occurs. During the stationary phase, in contrast, starvation signals suppress AttJ production, eventually activating AttM and reducing AHL availability. The plant product γ-aminobutyric acid has also been shown to induce lactonase activity during starvation.[35]

Another enzyme, AHL-acylase, has been identified in various bacterial sources and found to act on AHL amide bonds and release homoserine lactones and corresponding fatty acids (Fig. 1). It is a member of the *N*-terminal-hydrolase family and is present in both *Ralstonia* sp. XJ12B (AiiD) and *P. aeruginosa* PAO1 (PvdQ), despite the two sharing only 39% homology at the amino acid level.[36,37] The AHL-acylases produced from *Streptomyces* and *P. aeruginosa* have been shown to act specifically on AHLs with acyl chains featuring six or more carbons.[38-39] For example, the overexpression of *pvdQ* (an acylase) in *P. aeruginosa* PAO1 inhibits the accumulation of 3-oxo-C12-HSL via cleavage, whereas the concentration of C4-HSL decreases without degradation.[40] The heterologous expression of AHL-acylase (*aiiD*) in *P. aeruginosa* has been shown to significantly reduce virulence gene expression in a *Caenorhabditis elegans* model .[41]

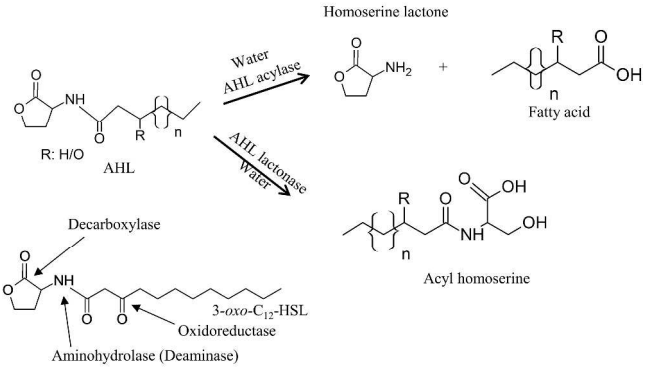

Fig. 1. The enzymatic degradation of acyl homoserine lactones signals produced from Gram-negative bacterial pathogens have a specific cleavage site of decarboxylase, oxidoreductase and aminohydrolase or deaminase. The figure shows the corresponding degradation mechanisms of AHL-lactonase and AHL-acylase. The dotted line indicates blockage sites.

Oxidoreductases (P-450/NADPH-P450 reductase and novel oxidoreductase BpiB09) have been reported to substitute the oxo-group at C3 of AHLs with the hydroxyl group, which may successively be degraded by amidohydrolase for conversion into homoserine lactone and hydroxydecanoic acid (Fig. 1).[42] Thus, oxidoreductases are not degraded AHLs, but rather modify the concentration of AHLs and inhibit virulence effects. Oxidation occurs primarily at the ω-1, ω-2 and ω-3 carbons of the acyl chains. A cytochrome P450 monooxygenase obtained from *Bacillus megaterium* has been shown to act as a CYP102A1-dependent mechanism capable of efficiently oxidising AHLs, and

in a later study metagenomic-derived oxidoreductase BpiB09 was transformed into *P. aeruginosa*, revealing a reduction in pyocyanin production and decreased motility while exhibiting poor biofilm formation.[43,44]

Mammalian paraoxonases (PONs) are known to perform hydrolytic activities in esters and lactones, which are related to drug metabolism and the detoxification of organophosphate, while hydrolysing the homoserine lactone ring of 3-oxo-C12-HSL signals in the A549 cells of human lungs and CaCo-2 cells of the human colon.[45-47] PONs have been reported in humans, rabbits, mice, horses, sheep and bovines.[48] The PON gene family is divided into three types, PON1, PON2 and PON3, with amino acid sequencing revealing that these enzymes share 79-95% sequence homology. Recombinant PON1-, PON2- and PON3-expressing mouse cells have been shown to display strong

AHL degradation activity, and the expression of human PON1 in fruit flies to result in the reduced lethality of *P. aeruginosa* with the degradation of 3-oxo-C12 HSL.[48,49] These catalysts thus appear to be the most active within long- rather than short-chain AHL signals. The results of a study in which a mouse model was infected with *P. aeruginosa* indicated that PON1 and PON3 are more potent than PON2 in attenuating virulence gene expression *in vivo*.[49] In contrast, PON2 demonstrates a preference for hydrolysing only the (*S*)-isomers of AHLs. With the exception of AHL lactonase, the role of these enzymes in the attenuation of QQ has yet to be thoroughly investigated.

3. Natural QQ molecules

Recent decades have seen a significant increasing in the application of natural products in therapeutic treatments designed to cure bacterial diseases by inhibiting QS-dependent virulence gene expression (**1**-**11**, Table 1). Halogenated furanones, a natural product derived from macroalgae (*Delisea pulchra*) might lead to discovery of the potentially new drugs which could treat numerous diseases.[50] *D. pulchra* is known to produce at least 30 different types of halogenated furanones that determine surface colonisation through pelagic bacteria and most common structure of halogenated furanone are shown as structure **1**-**4**.[51] Furanones are also reported to reduce AHL-mediated virulence gene expression in *V. fischeri* (bioluminescence) and *P. carotovora*, both of which are analogous to bacterial AHLs and compete with cognate AHL signals for their receptor binding site, LuxR.[50,52-54] They are also strong inhibitors of both AI-1- and AI-2-mediated QS systems at high AHL concentrations. In instance, it has been found that furanones are reduced the ability of *E. coli* cells to produce biofilms by inhibiting AI-2 activity and decreasing light emission in *Vibrio* species.[52-54] Compound **5** has been found to inhibits virulence gene expression in *A. tumefaciens* whereas compounds **6** and **7** attenuated QS dependent gene expression in *P. aeruginosa* at micro-molar concentration (Table 1). Another widely investigated natural compound is cinnamaldehyde **8**, which has been shown to affect many of the QS-regulated biofilm formation activities of *P. aeruginosa* in addition to reducing virulence in *Vibrio* species by interfering with the DNA-binding activity of QS response regulator LuxR.[55-56] Similarly, grapefruit products such as furocoumarins have been found to exert strong inhibiting affects on AI-1 and AI-2 of *V. harveyi* reporter strains BB886 and BB170, *E. coli* O157:H7, and *S. typhimurium* and *P. aeruginosa* biofilm formation.[57] Several flavonoids, including naringenin, quercetin and citrus limonoid, were also shown to strongly inhibit *V. harveyi* BB120 and *E. coli* O157:H7 biofilm formation in a later study.[58] Propolis extracts reportedly attenuate the production of LasA and LasB protease activities in *P. aeruginosa*.[59] Similarly, malabaricone C appears to successfully inhibit the lasR and rhlR systems in *P. aeruginosa*.[60] In another study using a mouse model, garlic extract (*Allium sativum*) demonstrated the ability to block QQ-dependent gene expression in *P. aeruginosa*, including virulence factors alginate, haemolysin and phospholipase C.[61] Interestingly, a lignin precursor *P*-coumaric acid (**9**) was shown broad spectrum activity against *A. tumefaciens* NTL4, *Chromobacterium violaceum* 5999 and *P. chlororaphis*, with no influence on cell viability.

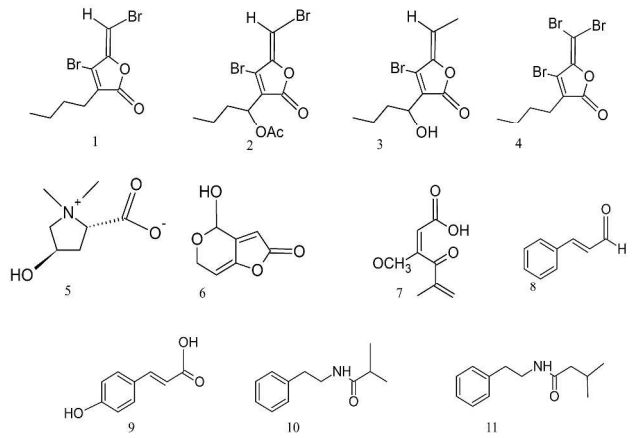

Fig. 2. Natural QQ compounds: (1-4) Halogenated furanone (IC_{50} 15 – 50 µM) extracted from the red alga *Delisea pulchra*, (5) betonicine obtained from the red alga *Ahnfeltiopsis flabelliformis*, (6) patulin and (7) penicillic acid extracted from *Penicillium*, (8) cinnamaldehyde extracted from *Cinnamomum zeylanicum,* (9) p-coumaric acid (a lignin precursor), (10) N-(2′-phenylethyl)-isobutyramide and, (11) 3-methyl-N-(2′-phenylethyl)-butyramide (bacterial secondary products).

Several bacterial secondary metabolites have also been evaluated to determine their QQ activity (compounds **10**, **11**, Table 1), with phenylethylamides found to structurally mimic AHL with antagonists for the receptor binding sites in *V. harveyi* BB120.[62] In addition, the potential QSI compounds reported from the cell-free supernatant of a *Hafnia alvei* culture were evaluated for *Salmonella enterica* serovar Enteritidis PT4, which is known to inhibit the early stages of biofilm development.[63] In another study, floral compound protoanemonin was observed to significantly inhibit QS-mediated gene expression, particularly that involved in the iron starvation response (i.e. pyoveidine and pyochelin) and such virulence factors as *lasA* (encoding LasA protease precursor) and *lasB* (encoding elastase LasB), in *P. aeruginosa*.[64] That study also posited that protoanemonin may be an example of a small-sized QSI that fits well into the LasR binding domain of LasR in a way similar to the lactone ring of AHLs. Therefore, halogenated furanones and protoanemonin have also been shown to prevent the correct formation of the hydrophobic core in the LasR system of *P. aeruginosa*.[65]

Table 1. Different quorum quenching molecules for inhibiting quorum sensing dependent virulence gene expression in Gram-negative pathogenic bacteria

Name of QSI compounds / (Source)	Active against	Reference
Algae		
Halogenated furanone (*Delisea pulchra* a red algae) (IC_{50} 15 – 50 µM)	*Escherichia coli* *Pseudomonas aeruginosa* *Vibrio fischeri* *V. harveyi*	Inhibition of biofilm formation and swarming of *Escherichia coli* by (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone[54] New halogenated furanones from the marine alga *Delisea pulchra* (cf. fimbriata)[50], Chemical mediation of bacterial surface colonisation by secondary metabolites from the red alga *Delisea pulchra* [51], Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover[52] Eukaryotic interference with homoserine lactone-mediated prokaryotic signaling[96]
Floridoside, betonicine and isoethionic acid (*Ahnfeltiopsis flabelliformis* a red algae)	*Agrobacterium tumefaciens*	Quorum sensing inhibitors from the red alga, *Ahnfeltiopsis flabelliformis*[97]
Fungi		
Patulin (IC_{50} 0.56 µM) and penicillic acid (IC_{50} 29.85 µM) from *Penicillium*	*P. aeruginosa*	Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector[98]
Plants		
γ-hydroxybutyrate (*Arabidopsis* exudates)	*A. tumefaciens*	
Catechins (*Camellia sinensis*)	*E. coli*	Inhibition by epigallocatechin gallate (EGCg) of conjugative R plasmid transfer in *Escherichia coli*[99]
Cinnamaldehyde (*Cinnamomum zeylanicum*)	*P. aeruginosa, E. coli* and *V. harveyi*	Subinhibitory concentrations of cinnamaldehyde interfere with quorum sensing[55]
Flavonoid catechin (*Combretam albiflorum*)	*P. aeruginosa*	Quorum sensing in *Vibrio fischeri*: probing autoinducer-LuxR interactions with autoinducer analogs[100]
Furocoumarins (grapefruit juice)	*E. coli, P. aeruginosa* and *Salmonella typhimurium*	Grapefruit juice and its furocoumarins inhibits autoinducer signaling and biofilm formation in bacteria[57]
Extract of *Medicago truncatula*	*P. aeruginosa, Salmonella enterica*	Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals[101]
Rosamarinic acid (*Ocimum basilicum*)	*P. aeruginosa* PAO1	Evaluation of anti-quorum-sensing activity of edible plants and fruits through inhibition of the N-acyl-homoserine lactone system in *Chromobacterium violaceum* and *Pseudomonas aeruginosa*[102]
p-coumaric acid (a lignin precursor)	*P. chlororaphis*	Quorum sensing inhibition activity of garlic extract and p-coumaric acid[103]
Quercetin and naringenin	*V. harveyi* BB120 and *E. coli* O157:H7	Citrus limonoids interfere with *Vibrio harveyi* cell-cell signaling and biofilm formation by modulating the response regulator LuxO[58]
Malabaricone C (*Myristica cinnamomea*)	*P. aeruginosa* PAO1	Malabaricone C from *Myristica cinnamomea* exhibits anti-quorum sensing activity[104]
Extract of *Syzygium Aromaticum*	*P. aeruginosa* PAO1	Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* PAO1 by ayurveda spice clove (*Syzygium aromaticum*) bud extract[105]
Pyrogallol	*V. harveyi* (AI-2 analogue)	Pyrogallol and itsanalogs can antagonize bacterial quorum sensing in *Vibrio harveyi*[106]
Sulforaphane and erucin (Brocoli)	*P. aeruginosa* (antagonist LasR)	Sulforaphane and erucin, natural isothiocyanates from broccoli, inhibit bacterial quorum sensing[60]
Animals		
Manolide, manolide monoacetate and secomanoalide (*Luffariella variabilis*)	*P. aeruginosa*	Quorum sensing antagonism from marine organisms[107]
Bacteria		
N-(2′-phenylethyl)-isobutyramide, 3-methyl-N-(2′-phenylethyl)-butyramide and 2-N-pentyl-4-quinolinol	*Vibrio* species	Secondary metabolites produced by marine bacterium *Halobacillus salinus* that inhibit quorum sensing controlled phenotypes in gram-negative bacteria[62], 2-n-Pentyl-4-quinolinol produced by a marine *Alteromonas* sp. and its potential ecological and biogeochemical roles[108], Antagonistic interactions among marine bacteria impede the proliferation of *Vibrio cholerae*[109]
4-methylenebut-2-en-4-olide (protoanemonin)		Protoanemonin: a natural quorum sensing inhibitor that selectively activates iron starvation response[64]
Synthetic QSI or AHLs analogue		
Lactam analogue of 3OC12HSL	*P. aeruginosa* (markedly reduced acyl HSL activity)	Functional analysis of the *Pseudomonas aeruginosa* autoinducer PAI[75]
4-phenylbutanoyl HSL	*V. fischeri* (antagonizes LuxR) and *E. coli* (Binding of OHC6HSL harbouring LuxR)	Quorum sensing in *Vibrio fischeri*: probing autoinducer-LuxR interactions with autoinducer analogs[100] Comparative analyses of N-acylated homoserine lactones reveal unique structural features that dictate their ability to activate or inhibit quorum sensing[110]
Carbonyl substituent at 3 position with methylene of C8HSL	*A. tumefaciens* (AI of TraR activation)	Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*[74]
Meta-bromo-thiolactone	*P. aeruginosa* (Inhibits LasR and RhlR activity)	A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation[111]

Truncated AIPII (trAIP-II) B14, Cmp5 and Cmp9	*P. aeruginosa* (LasR-reporter) *A. tumefaciens* (TraR-reporter)	Comparative analyses of N-acylated homoserine lactones reveal unique structural features that dictate their ability to activate or inhibit quorum sensing[110], Theoretical and structural analysis of the active site of the transcriptional regulators LasR and TraR, using molecular docking methodology for identifying potential analogues of acyl homoserine lactones (AHLs) with anti-quorum sensing activity[112]
Azithromycin (MIC- 2 mg/L)	*P. aeruginosa*	Azithromycin inhibits quorum sensing in *Pseudomonas aeruginosa*[113], Regulatory effects of macrolides on bacterial virulence: potential role as quorum sensing inhibitors[114],
Aryl substituted AHLs and C8HSL	*V. fischeri* *P. aeruginosa*	New synthetic analogues of N-acyl homoserine lactones as agonists or antagonists of transcriptional regulators involved in bacterial quorum sensing[115], Library screening for synthetic agonists and antagonists of a *Pseudomonas aeruginosa* autoinducer[116], Induction and inhibition of *Pseudomonas aeruginosa* quorum sensing by synthetic autoinducer analogs[117]
4-hydroxy cis or trans analogues of HSL ring of 3OC8HSL	*V. fischeri* (potent activators of LuxR-based QS reporter system)	Synthesis of new 3- and 4-substituted analogues of acyl homoserine lactone quorum sensing autoinducers[118]
Furanone C-30 (IC_{50} - 3 to 9μM)	*P. aeruginosa* (virulence factors- elastase, protease, rhamnolipid production, phenazine biosynthesis, hydrogen cyanide and chitinase.) *V. anguillarum* (virulence factors)	Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors [67] An inhibitor of bacterial quorum sensing reduces mortyalities caused by Vibriosis in Rainbow trout (*Oncorhynchus mykiss*, Walbaum)[119]
3-oxo-C12-(2-aminophenol)	*P. aeruginosa* (GFP production in QS circuit)	Library screening for synthetic agonists and antagonists of a *Pseudomonas aeruginosa* autoinducer[116]
3-oxo-C12-(2-aminocyclohexanol) (IC_{50}- 29.4 μM)	*P. aeruginosa* (inactive in LasR activation but active against RhlR)	Induction and inhibition of *Pseudomonas aeruginosa* quorum sensing by synthetic autoinducer analogs[117]
N-sulfonyl-HSL (with a pentyl chain)	*V. fischeri* (antagonises LuxR)	N-Sulfonyl homoserine lactones as antagonists of bacterial quorum sensing[72]
pClPhT–DADMe–ImmA DADMe-Immucillins	*E. coli* and *Streptococcus pneumoniae* (5′-methylthioadenosine/S-adenosylhomocysteine hydrolase)	Transition state structure of 5-methylthioadenosine/S-adenosylhomocysteine nucleosidase from *Escherichia coli* and its similarity to transition state analogues[77] Structure and inhibition of a quorum sensing target from *Streptococcus pneumoniae* [78]
Fimbrolide (F1)	*A. tumefaciens* (TraR mediated *β*-galactosidase)	The molecular structure and catalytic mechanism of a quorum-quenching *N*-acyl-L-homoserine lactone hydrolase[23]
4-Nitro-pyridine-N-oxide (IC_{50} – 2 μM)	*P. aeruginosa* (virulence)	Inhibition by epigallocatechin gallate (EGCg) of conjugative R plasmid transfer in *Escherichia coli* [98]
N-(indole-3-butanoyl)-L-HSL	*P. aeruginosa* PAO1	
Novel quinazolinone (IC50 1.260.4 mM	*P. aeruginosa* (antagonist PqsR)	Structural basis for native agonist and synthetic inhibitor recognition by the *Pseudomonas aeruginosa* quorum sensing regulator PqsR (MvfR) [120]
Furanyl hydrazide	*P. aeruginosa* (QS transcriptional regulator)	Quorum sensing inhibition: targeting chemical communication in gram-negative bacteria[121]
N-(heptyl-sulfanyl acetyl)-L-HSL (IC_{50} - in nM)	*P. aeruginosa* and *V. fischeri* (inhibits transcriptional regulator — LasR)	Rational design and synthesis of new quorum-sensing inhibitors derived from acylated homoserine lactones and natural products from garlic[122]
Tetrazole (IC_{50}-30 nM) and V-06-018 (IC_{50}-10 μM)	*P. aeruginosa* (affects LasR-dependent gene expression)	Novel *Pseudomonas aeruginosa* quorum-sensing inhibitors identified in an ultra-high-throughput screen[123]
Brominated furanone (IC_{50}-2.4 – 3.5 μM)	*Salmonella enterica* serovar *Typhimurium* (Biofilm formation) and *V. harveyi* BB152	Brominated furanones inhibit biofilm formation by *Salmonella enterica* serovar *typhimurium*[124]
Analogues of *S*-ribosyl-L-homocysteine (modified at the ribose C3 position)	*Vibrio harveyi* (LuxS)	S-Ribosylhomocysteine analogues with the carbon-5 and sulfur atoms replaced by a vinyl or (fluoro)vinyl unit[125], Inhibition of S-ribosylhomocysteinase (LuxS) by substrate analogues modified at the ribosyl C-3 position[126]
3,5,6-trideoxy 6-fluorohex-5-enofuranose (luck C-3 hydroxyl group)	*V. harveyi* (LuxS)	2-methoxycyclopentyl analogues of a *Pseudomonas aeruginosa* quorum sensing modulator[127]
3-nitro phenylacetanoyl HSL (IC_{50}-0.3 μM)	*P. aeruginosa* (inhibitory to LasR-reporter) *A. tumefaciens* (TraR-reporter)	Comparative analyses of N-acylated homoserine lactones reveal unique structural features that dictate their ability to activate or inhibit quorum sensing[110]
Diastereomeric 2-methoxycyclopentyl	*P. aeruginosa* (agonist with significantly less activity pigmentation)	2-methoxycyclopentyl analogues of a *Pseudomonas aeruginosa* quorum sensing modulator[127]
4,5-dihydroxy-2,3- pentanedione (DPD) (IC_{50}-1.7 μM), Hexyl-DPD (IC_{50}-10 μM), (4S,5R)-DHD (IC_{50}-0.65 μM) and C4-PrO-HPD (IC_{50}-0.15 μM)	*V. harveyi* (bioluminescence)	Revisiting AI-2 quorum sensing inhibitors: direct comparison of alkyl-DPD analogues and a natural product fimbrolide[128], Defining the mode of action of tetramic acid antibacterials derived from *Pseudomonas aeruginosa* quorum sensing signals[129], Uncharacterized 4,5-dihydroxy-2,3-pentanedione (dpd) molecules revealed through nmr spectroscopy: implications for a greater signaling diversity in bacterial species[135]

Furanone F2 (IC_{50} -15μM), F3 (IC_{50} -10μM) and F4 (F3 and F4 structurally similar to F2 but with a nitro group instead of amine moiety)	*P. aeruginosa* (3OC12HSL dependent QscR)	Inhibitors of the *Pseudomonas aeruginosa* quorum sensing regulator, QscR[130]
Analogous of anthranilate (MA, 6FABA, 6CABA, and 4CABA)	*P. aeruginosa* (antagonist PqsA)	Interference with Pseudomonas quinolone signal synthesis inhibits virulence factor expression by *Pseudomonas aeruginosa*[89], Inhibitors of pathogen intercellular signals as selective anti-infective compounds[90]
Benzamidobenzoate	*P. aeruginosa* (PqsD)	Benzamidobenzoic acids as potent PqsD inhibitors for the treatment of *Pseudomonas aeruginosa* infections[136]
Hydroxamic derivative (compound 11)	*P. aeruginosa* (antagonist PqsR)	Identification of small-molecule antagonists of the Pseudomonas aeruginosa transcriptional regulator PqsR: biophysically guided hit discovery and optimization[137]
Compounds KM-03009 (IC_{50} -35μM) and SPB-02229 (IC_{50} -55μM)	*V. harveyi* (antagonist AI-2)	Structure-based discovery and experimental verification of novel AI-2 quorum sensing inhibitors against *Vibrio harveyi*[138]
Piperidine-C12 diastereoisomers of 3-oxo-C12	*P. aeruginosa* (antagonist LasR)	Tailor-made LasR agonists modulate quorum sensing in Pseudomonas aeruginosa[137]
Thiophenone and furanone	*E. coli* O103:H2	Thiophenone and furanone in control of *Escherichia coli* O103:H2 virulence[131]
Non-specific inhibitors (source)	Enzymes	
Bacillus sp. strain 240B1	Lactonase	AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*[17]
Bacillus thuringiensis	Lactonase	The molecular structure and catalytic mechanism of a quorum-quenching *N*-acyl-L-homoserine lactone hydrolase[23]
Ralstonia sp. XJ12B	AHL-acylase	Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes[37]
Pseudomonas aeruginosa PAO1	Acylase	Utilization of acyl-homoserine lactone quorum signals for growth by a soil pseudomonad and *Pseudomonas aeruginosa* PAO1[40]
Agrobacterium tumefaciens	Lactonase	The quormone degradation system of *Agrobacterium tumefaciens* is regulated by starvation signal and stress alarmone (p)ppGpp31, The Ti plasmid of *Agrobacterium tumefaciens* harbors an attM-paralogous gene, *aiiB*, also encoding N-acyl homoserine lactonase activity[33]
Arthrobacter sp. IBN110	Lactonase	Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates[134]
Rhodococcus erythropolis strain W2	Amidohydrolase, Acylase and Lactonase	N-acyl homoserine lactone producing *Rhodococcus* with different AHL-degrading activities[38], *N*-acylhomoserine lactone quorum-sensing molecules are modified and degraded by *Rhodococcus erythropolis* W2 by both amidolytic and novel oxidoreductase activities[42]
Halomonas sp. strain 33 *Oceanobacillus* strains 30, 97-2 and 172	Lactonase	Uncharacterized 4,5-dihydroxy-2,3-pentanedione (DPD) molecules revealed through nmr spectroscopy: implications for a greater signaling diversity in bacterial species[135]
Hyphamonas sp. DG895	Acylase/Lactonase	
Alteromonas sp. strain 168	Acylase	
Burkholderia strain GG4	Oxidoreductase	Characterization of *N*-acylhomoserine lactone-degrading bacteria associated with Zingiber *officinale* (ginger) rhizosphere: co-existence of quorum quenching and quorum sensing in *Acinetobacter* and *Burkholderia*[20]
Mammalian	Paraoxonases	Dominant role of paraoxonases in inactivation of the *Pseudomonas aeruginosa* quorum-sensing signal N-(3-oxododecanoyl)-L-homoserine lactone [47]

4. Synthetic analogues

QS signals, natural QQ molecules, AHL precursors and modified native AHL structures, all hold promise for the synthesis of novel QSI molecules (Compounds **12-30**, Table 1). For example, synthesized brominated furanone (substituted in the acyl chain at the C-3 position and a bromine substitution at the C-4 position figure 4a and b) are known to attenuate QS-mediated gene expression in the biofilms of several pathogens.[66,67] Further, synthesized furanone compound C-30 (**12**) repressed QS gene expression in *P. aeruginosa*, virulence factor genes *lasB* (encoding for elastase), *lasA* (encoding for protease) and *chiC* (chitinase production) in particular. This compound has also been found to block the activities of *rhl* AB operon (rhamnolipid production), *ph3-A-G* operon (phenazine biosynthesis) and *hcnABC* operon (HCN production) (Table 1). Moreover, C-30 is known to be a repressed transcription of the *phnAB* operon-encoding anthranilate synthase that is related to the *Pseudomonas* quinolone signalling system.[67] Wu et al. used a pulmonary mouse infection model with *P. aeruginosa* PAO1 to investigate the effects of furanone compounds C-30 and C56 (**12, 13**) on persistent *P. aeruginosa* biofilm formation in chronic infections.[68] They found that infected mice injected with C-30 and C56 to experience a significantly reduced bacterial load compared with the control group, in addition to a significantly prolonged survival time.

Synthetic QQ molecules do not have to be based on natural compounds, but can also be synthesised independently. One of the most commonly used antibiotics is azithromycin, a subclass of macrolides derived from erythromycin with a methyl-substituted nitrogen atom incorporated into the lactone ring. Azithromycin is used to prevent the bacterial infections that cause strep throat, pneumonia, typhoid, bronchitis and sinusitis, and has recently been observed to exhibit QSI activity.[69] Kohler et al. monitored QS system-dependent gene expression in the *lasR* mutant and wild-type *P. aeruginosa* in the presence and absence of azithromycin in tracheal aspirates.[70] Their results suggest that azithromycin reduces the growth rate of wild-type *P. aeruginosa*,

but not that of the *lasR* mutant. They thus concluded that this antibiotic significantly reduces the expression of both QS circuit (*lasI* and *rhlA*) genes, but exerts no influence on the expression of QS-independent gene *trpD in vitro* or *in vivo*.

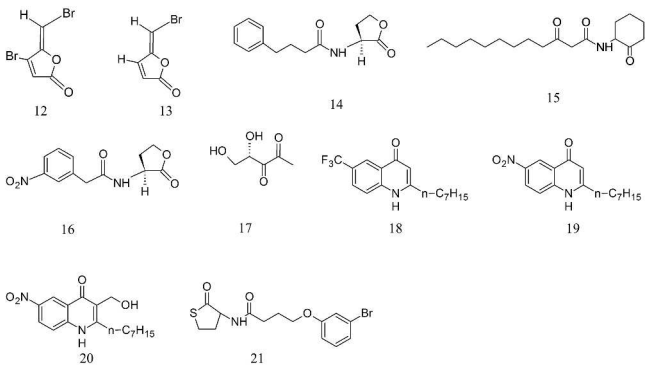

Fig. 3. Synthesized QQ compounds: (12) Furanone C30, (13) furanone C56, (14) 4-phenylbutanoyl HSL, (15) 3-oxo-C_{12}-acHone, (16) 3-nitro phenylacetanoyl HL, (17) 4,5-dihydroxy-2,3-pentanedione (DPD) (18) compound 1, (19) compound 2, (20) compound 16 and (21) meta-bromo-thiolactone analogue of LasR and RhlR.

Several, AHLs agonists and antagonists have been synthesized which are inhibited either virulence transcription of genes or biofilms formation. These agonists or antagonists are summarised in Table 1. AHL analogues are generally synthesised by replacing a carboxamide bond with a sulfonamide and substituting aryl for AHL, which results in the inhibition of bioluminescence in *V. fischeri*.[71,72] Palmer et al. synthesised AHLs in a manner analogous to modification of the acyl side chain of AHLs such as *P*-bromo-phenylacetanoyl L-homoserine lactones (HLs), *p*-bromo phenylpropanoyl HLs and *N*-(3-oxo)-dodecanoyl L-HLs.[27] The study found these synthesised compounds to be the most potent antagonists in attenuating the QS signalling systems of the mutant strains (AC5094, which lacks a functional ExpI gene) and wild type of *P. carotovora* when analysed in a potato host (*Solanum tuberosum*). Although their findings failed to reveal the mechanism of this attenuation phenomenon, Palmer et al. did successfully determine the times at which the agonist and antagonist should be administered to the host potato plant to interfere with attenuation of the virulence effect.[27] Other researchers discovered that *N*-acyl cyclopentyl amines, an AHL analogue, are the most effective QSI against Las and Rhl QS systems in *P. aeruginosa*.[73] Different types of AHL analogues have also been developed by modifying the naturally occurring or chemically synthesised AHLs (compounds **14-21**) that attenuate QS-dependent gene expression in various infectious Gram-negative pathogenic bacteria as mentioned in Table 1. For example, Zhu et al. found that substitution in the acyl chain of 3OC8-HSL (TraR) at the 3-position with a methylene of *A. tumefaciens* transforms the acyl chain into a QQ agent.[74] Modification in the lactam ring of AHLs has also been found to markedly reduce activity of the 3OC12-HSL in the *P. aeruginosa*.[75]

Alfaro et al. designed substrate analogues of LuxS inhibitors that block the β-elimination steps catalysed by LuxS (Al-2 signalling).[76] They synthesised two substrate analogues that are competitive inhibitors, *S*-anhydroribosyl-L-homocysteine and *S*-homoribosyl-L-cysteine, and prevent the first and last steps of the catalytic mechanism, respectively. Several LuxS inhibitors have subsequently been synthesised from compounds 10 and 11.[77-79] These two compounds are synthesised during replacement of the unstable enediolate moiety of a predicted enediolate intermediate with a stable hydroxamate group. They are stereoisomeric inhibitors (S_N1 transition), with *Ki* values in the submicromolar range targeting *E. coli* and *S. pneumonia*, and are bound to a homocysteine-binding pocket of LuxS to interact with the active-site metal ion, which suggests that this interaction is crucial to high-affinity binding and LuxS inhibition.[79] The most potent inhibitor of enzyme 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN) found to date is *p*-chlorophenylthio-DADMe-ImmA (*p*ClPhT-DADMe-ImmA), which binds more tightly to the MTAN of *E. coli* than that of *S. pneumoniae*.[78] Researchers have also developed rational peptide-designed methods that make use of synthesis of inhibitors targeting the LuxS of the *Streptococcus suis* serotype 2 (SS2).[79] Phage-encoded peptides generally interact with the LuxS enzyme, making it a good target for the creation of QQ molecules. Han et al. generated one such peptide inhibitor (TNRHNPHHLHHV) of LuxS after three rounds of phage display that partially (25%) inhibited the enzyme's activity.[80] Very few studies have examined QQ in *E. coli* and *S. pneumonia*, and most have adopted *in vitro* screening methods using purified LuxS. There is thus an urgent need to identify the strong QQ molecules of LuxS and establish their efficacy *in vivo*.

Recently, some advancement has been made for development of QQ against PQS signaling in *P. aeruginosa*.[81-83] This signal is actively involved in the bacterial virulence and biofilm formation.[3] Notably, PQS and HHQ activate PqsR, subsequently enhancing the expression of their biosynthetic operon *pqsABCDE* (Supplementary Fig. 1).[3] Thus, inhibiting HHQ production via blockage of biosynthesis pathway or receptor reduces virulence and biofilm of *P. aeruginosa*.[82,83] Storz et al. followed ligand based approach and designed an inhibitor 2-nitrophenyl(phenyl)methanol (compound 22) which blocks activity of a key enzyme of PQS biosynthesis, called PqsD and significantly inhibited biofilm formation of *P. aeruginosa* PA14 at IC_{50} 3.2 μM.[84] Additionally, two other compound 1 and 2 (**23-24**) was also designed but compound 1 did not show significant effect on biofilm reduction of *P. aeruginosa* PA14.[85] This enzyme is catalyzed formation of the 2-aminobenzoylacetate from condensation of anthraniloyl-CoA and malonyl-CoA.[85,86] Subsequently, formation of the HHQ/PQS is carried out by another enzyme PqsC through unknown mechanism.[87,88] It has also suggested that 2NPPM irreversibly binds to near active site of PqsD.[87] Some other inhibitors such as methylanthranilate (Compound **27**) and halogenated analogs of anthranilate (compound **28-30**) are significantly inhibited production of HHQ, PQS and HAQs via blocking the activity of PqsA.[89,90] Interestingly, FabH inhibitors (compounds **25, 26**) were reported for attenuation inhibiting activity of PqsD.[82] Thus, inhibition of QS molecules production is also considered as a novel approach for the development of anti-virulence and anti-biofilm compounds.85

Structure-activity relationship (SAR) between ligand and

receptor in the gram-negative bacteria has been also led to discovery of several antagonists of cognate receptor.[91,92] In instance, this approach has been recently proved using antagonist of PqsR in *P. aeruginosa*. Lu et at. discovered two antagonists, compound 1 and 2 (**18,19**) of PqsR with nanomolar range which are reduced amount of the pyocyanin production in the *P. aeruginosa*.[91] Further optimization with their solubility and antagonist activity, a novel compound 16 (**20**) has been found which is more potent than previously reported.[92] Particular finding is also suggested that the carbonyl group at 4-position is determined the interactions between ligand and receptor and the substituents at 3-position is critical role for the ligand activation.[92] Therefore, SAR study can be led to better understanding of receptor - ligand interaction and could lead to development of high potential receptor analogous compounds for drug design.

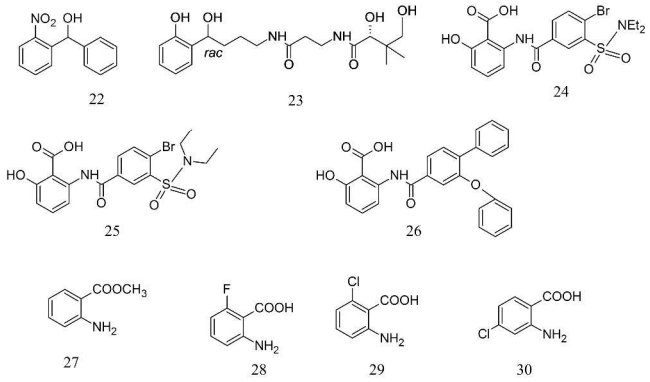

Fig. 4. Synthesized enzymes inhibitors: Inhibitors of PqsD, (22) compound 1, (23) compound 2 (24) compound 3, (25) compound 4 and (26) compound 5. Inhibitors of PqsA, (27) methylanthranilate, (28) 2-amino-6-fluorobenzoic acid (6FABA), (29) 2- amino-6-chlorobenzoic acid (6CABA), and (30) 2-amino-4-chlorobenzoic acid (4CABA).

5. Antibodies as QS molecules

The antibody-based QQ approach has also been used to attenuate virulence gene expression in Gram-negative bacteria. For example, Kaufmann et al. reported a hapten design strategy for obtaining specific monoclonal antibodies in the AHLs of *P. aeruginosa*, with RS2-1G9 demonstrated to suppress virulence gene expression (pyocyanin production) through 3-oxo-C12-HSL sequestration *in vitro*.[93] Miyairi et al. evaluated the effect of a 3-oxo-C12-HSL-BSA conjugate on acute *P. aeruginosa* lung infections in mice, and found very similar survival rate increases in immunised and non-immunised mice, regardless of the number of bacteria in the lungs, although they observed significantly lower levels of cytokine tumour necrosis factor R in the immunised mice.[94] Miyairi et al. thus hypothesised that immunisation protected the mouse macrophages from the cytotoxic effects of 3-oxo-C12-HSL, which suggests that the antibodies targeting AHL are responsible for protection during acute *P. aeruginosa* infection.[94] Marin et al. concurrently discovered a catalytic antibody, XYD-11G2, that hydrolyses 3-oxo-C12-HSL and inhibits *P. aeruginosa* pyocyanin production *in vitro*.[95] Together, these results suggest that immunotherapy is a viable approach to attenuating QS-mediated virulence gene expression in Gram-negative bacterial pathogens *in vivo*.

6. Conclusions and future prospects

Gram-negative bacteria use QS systems to induce virulence gene expression and opportunistically cause serious diseases. Some bacterial species are much more resistant to traditional antibiotics and native immune systems than others, and are thus forced to adopt QQ strategies to control the infectious virulence gene expression of bacteria. The QS system mechanisms available in pathogenic bacteria help to develop QQ molecules, including QQ enzymes and other analogous small molecule inhibitors that target LuxI-type synthases and LuxR-type receptors. Although the QQ approach remains in the developmental stage, the literature review presented herein demonstrates that it is a potentially fascinating way of controlling bacterial infection in the post-antibiotic era. Although there is sufficient evidence to show that the expression of genes encoding AHL-degrading enzymes directly in hosts controls AHL-dependent virulence expression, genetic modifications in plants or animals may impose unforeseen difficulties. The intriguing findings on existing enzymes discussed herein thus constitute only the beginnings of our *in vivo* understanding. Considerable work remains to be done to develop pharmaceutical applications and to determine the stability, efficacy, toxicity and side effects of enzyme delivery systems. Synthesised small QQ molecules are more potent and selective than natural compounds in attenuating QS systems. Further *in vivo* investigation of the natural and synthesised QQ compounds reported to date is needed to determine their effect on bacterial gene expression and on the host lifecycle with a view towards eventual pharmaceutical applications. A few studies have developed antibody-mediated QQ molecules and successfully established their effects *in vitro*, thus laying the foundation for the development of more potent antibody-based QQ molecules for AI-1- and AI-2-mediated virulence gene expression. Further research is needed to establish their specificity *in vivo*, an essential requirement in medical microbiology.

Acknowledgment

First author would like to thanks Professor Micha Ilan, Tel Aviv University for his valuable suggestion and discussion.

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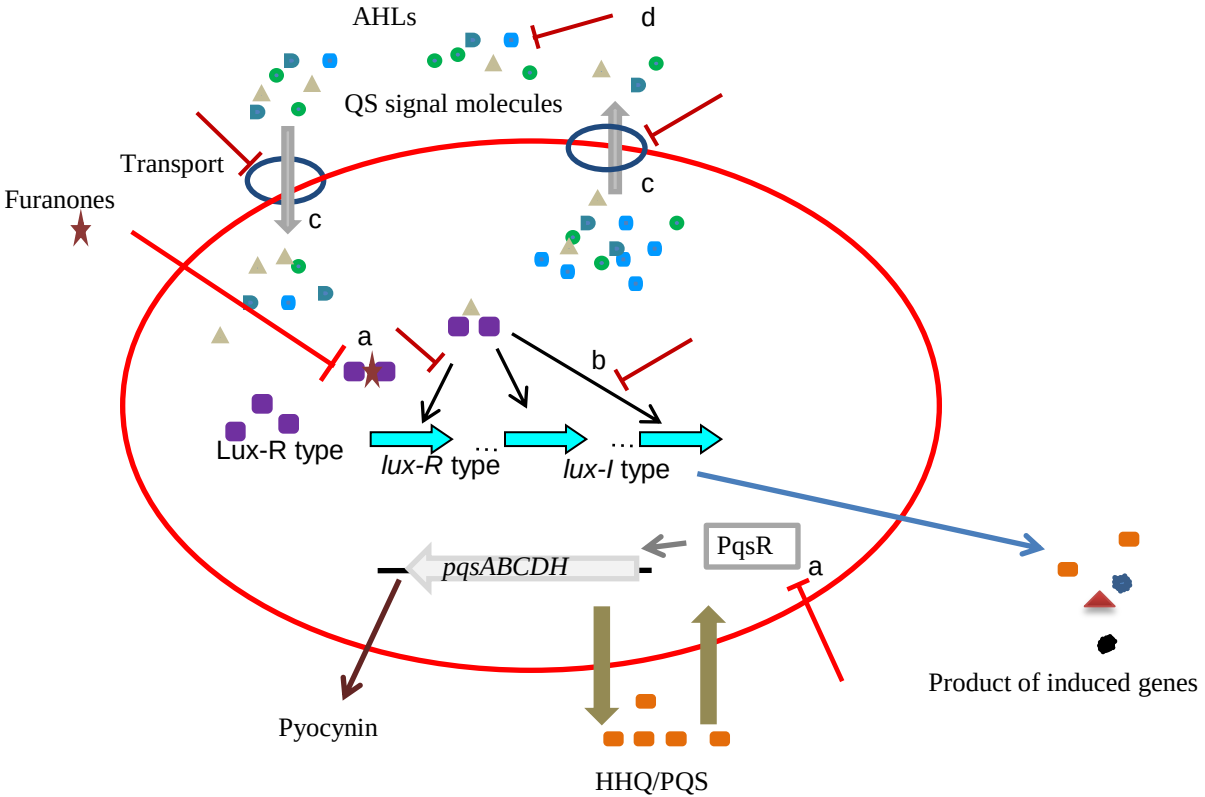

Quorum quenching molecules are attenuated quorum sensing by several sites (a,b, c and d)