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Review paper

Screening strategies for quorum sensing inhibitors in combating bacterial infections

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ABSTRACT

Interference with quorum sensing (QS) represents an antivirulence strategy with a significant promise for the treatment of bacterial infections and a new approach to restoring antibiotic tolerance. Over the past two decades, a novel series of studies have reported that quorum quenching approaches and the discovery of quorum sensing inhibitors (QSIs) have a strong impact on the discovery of anti-infective drugs against various types of bacteria. The discovery of QSI was demonstrated to be an appropriate strategy to expand the anti-infective therapeutic approaches to complement classical antibiotics and antimicrobial agents. For the discovery of QSIs, diverse approaches exist and develop in-step with the scale of screening as well as specific QS systems. This review highlights the latest findings in strategies and methodologies for QSI screening, involving activity-based screening with bioassays, chemical methods to seek bacterial QS pathways for QSI discovery, virtual screening for QSI screening, and other potential tools for interpreting QS signaling, which are innovative routes for future efforts to discover additional QSIs to combat bacterial infections.

1. Introduction

The emergence of antimicrobial resistance constitutes a major global health care concern and poses a significant challenge. Conventional antibiotic therapies targeting the synthesis of protein, DNA, RNA, and folic acid usually exert bacteriostatic or bactericidal effects on multiple targets, resulting in strong selective pressure on bacterial communities and subsequently giving rise to bacterial strains resistant to conventional antibiotics [1–5]. In response to this, alternative strategies to antibiotics for bacterial infections are generating significant interest. Among these, some innovative alternative therapies, such as quorum sensing (QS) disruption, have been intensively studied [6–10]. In terms of the extensive impacts on microbial physiology induced by QS, interference with QS has been demonstrated to be a promising approach to decreasing bacterial virulence and restoring antibiotic effectivity by controlling biofilm production, paving the way for future anti-infective treatment and dressing/coating agents in medical applications [11,12].

In the 1970s, Nealson found that the density of *Vibrio fischeri (V. fischeri)* and *Vibrio harveyi (V. harveyi)* was positively correlated with their bioluminescence, and confirmed that this phenomenon is controlled by the QS system in bacteria (first described as QS) [13]. QS is an intercellular chemical communication process in a cell-density-dependent manner in which bacteria coordinate the expression of QS-mediated genes based on the exchange of small signaling molecules defined as quorum sensors or autoinducers (AIs).

Chemically, QS is based on the synthesis, sensing, and uptake of AIs [14]. Once a particular threshold concentration of bacteria is reached, programmed changes that coordinate biological effects including biofilm formation, virulence secretion, swarming ability, sporulation, and protease production are motivated in a density-dependent manner. The signal molecules related to clinical pathogens have been reported and are listed in Table 1 and Fig. 1: 1) acyl homoserine lactones (AHLs), which exist in most Gram-negative bacteria except *Vibrio harringiensis* and *Mucoccus flavus*; 2) autoinducer peptides (AIPs), which are produced by most Gram-

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Table 1
Classification of autoinducer molecules.

Classification	Signal molecules	Regulatory factors	Microbial sources
AHLs	C4-HSL	RhlR/SwrR/AhyR/AsaR	*Pseudomonas aeruginosa/Serratia liquefaciens/Aeromonas hydrophila/Aeromonas salmonicida*
	C6-HSL	CviR/CepR/SwrR/–/YtbR/PhzR	*Chromobacterium violaceum/Burkholderia cenocepacia/Serratia liquefaciens/Pseudomonas aeruginosa/Yersinia pseudotuberculosis/Pseudomonas aureofaciens*
	C8-HSL	CepR/AinsR/YpsR/CepR	*Burkholderia cenocepacia/Vibrio fischeri/Yersinia Pseudotuberculosis/B. cepacia*
	3-OH-C4-HSL	LuxN	*Vibrio harveyi*
	3-OXO-C6-HSL	LuxR/Unkown/YpsR/YpeR/CarR/ExpR/EsaR	*Vibrio fischeri/Pseudomonas aeruginosa/Yersinia pseudotuberculosis/Yersinia pestis/Erwinia carotovora/Erwinia chrysanthemi/Erwinia stewartii*
	3-OXO-C8-HSL	TraR	*Agrobacterium tumefaciens*
	3-OXO-C10-HSL	PpuR/Unkown//YspR	*Pseudomonas putide/Pseudomonas aeruginosa/Yersinia pestis*
	3-OXO-C12-HSL	LasR	*Pseudomonas aeruginosa*
	7-cis-C14-HSL	CerR	*Rhodvacter sphaeroides*
AIPs	AIP-I, II, III, IV	AgrC	*Staphylococcus aureus/Bacilus subtilis*
	iAM373	–	*Enterococcus faecalis*
	CSP	ComD	*Streptococcus mitis/Streptococcus constellatus/Streptococcus anginosus/Streptococcus thermophilus/Streptococcus milleri/Streptococcus gordonii/Streptococcus pneumoniae*
	cAM373, cAD1, cCF10, cPD1, iPD1, iAD1, CF10	–	*Enterococcus faecalis*
	EDF	–	*Escherichia coli*
	CbnB2	CbnK	*Carnobacterium piscicola*
	$ComX_{RO-E-2}$	–	*Bacillus subtilis*
AI-2	AI-2	LsrR/LsrR/TlpB, CagA	*Vibrio harveyi/Escherichia coli/Helicobacter pylori*
	AI-2b	LsrR	*Vibrio harveyi*
Others	PQS	PqsR	*Pseudomonas aeruginosa*
	DKP	–	–
	IQS	IqsR	*Pseudomonas aeruginosa*

–: not available. AHLs: acyl homoserine lactones; HSL: homoserine lactone; AIP: autoinducer peptides; CSP: competence stimulating peptide; EDF: extracellular death factor; CbnB2: camobacteriocin B2; AI-2: autoinducer-2; PQS: quinolones; DKP: diketopiperazines; IQS: integrated quorum sensing signal.

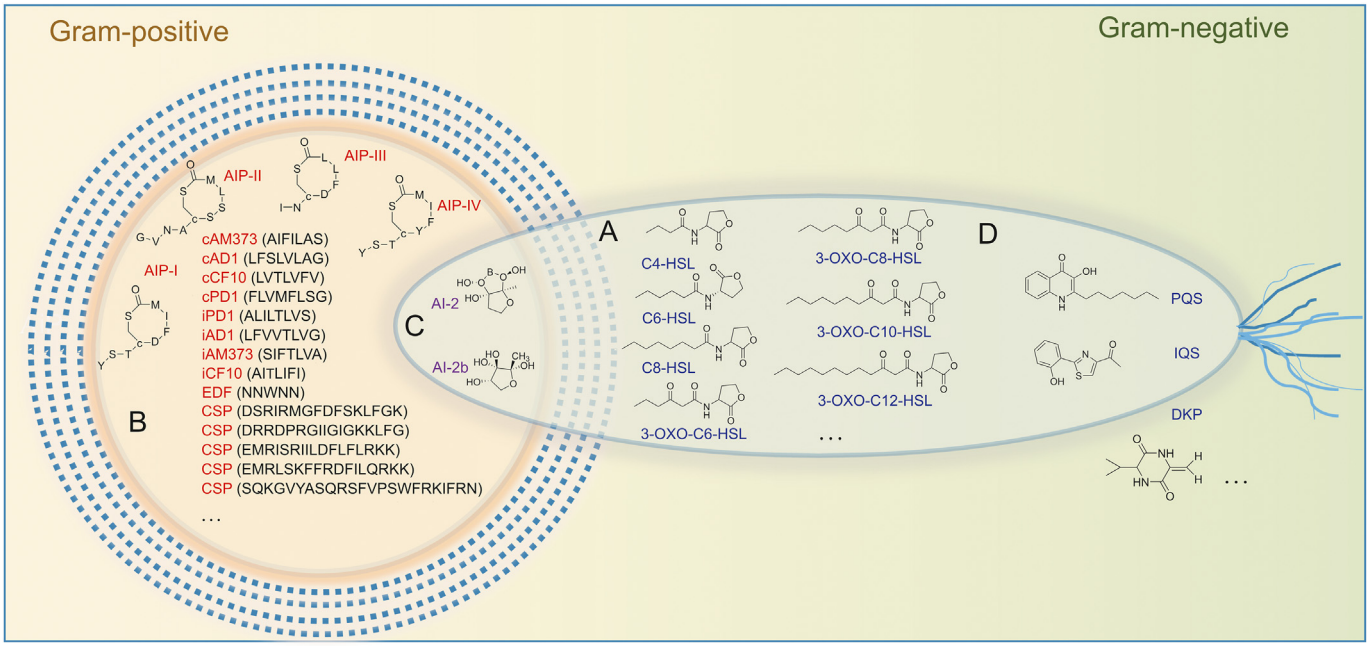

Fig. 1. Representation of AI molecules. (A) Acyl homoserine lactones; (B) autoinducer-2 furanones; (C) autoinducer peptides; (D) others. AIs: autoinducers; AIPs: autoinducer peptides; HSL: homoserine lactone; PQS: quinolones; DKP: diketopiperazines; IQS: integrated quorum sensing signal.

positive bacteria; 3) autoinducer-2 (AI-2) furanones, which are utilized by more than 40 species of Gram-positive and Gram-negative bacteria for communication and transmission [15,16]; 4) autoinducer-3 (AI-3), which is used by enterohemorrhagic *Escherichia coli*. Although the AI-3 structure is still not finely characterized, it has been found that epinephrine or norepinephrine could be considered as an alternative to AI-3; and 5) other signal molecules such as the *Pseudomonas* quinolone signal (Pqs) and ketopyridazines, which also play important roles in intraspecific QS. Some biosynthetic pathways regulating the generation of important QS signal molecules such as AHL and AI-2 involve numerous receptors and enzymes, which are summarized as: S-adenosyl methionine-dependent methyltransferase, LuxS, AHL synthase, LuxR receptor, histidine kinase response regulated LuxN protein, and AI-2 (LuxP and LsrB) receptor. For example, LuxI/LuxR are key regulatory factors in AHL-mediated QS signaling pathways. The AHL synthase (LuxI type) synthesizes AHL derivatives to generate QS signal molecules. The synthesized AHL entities subsequently diffuse in the

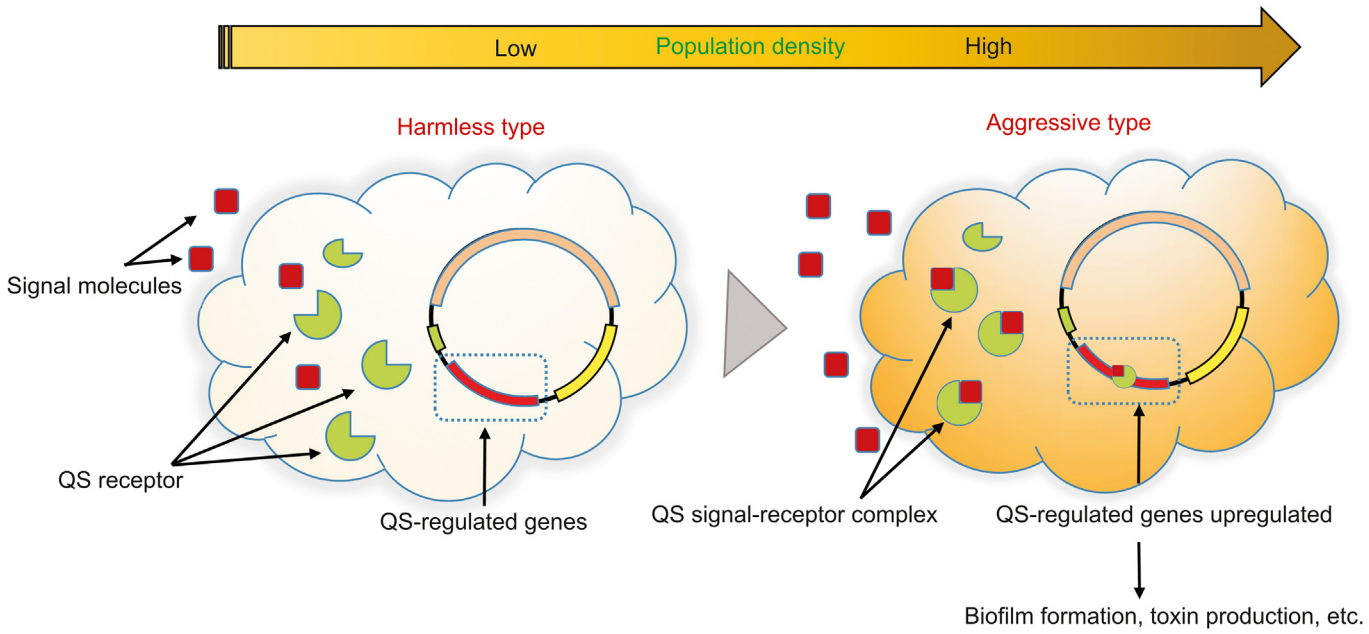

Fig. 2. Cyclic process of signal molecules. QS: quorum sensing.

adjacent environment and accumulate in a cell-density-dependent manner (Fig. 2) [17]. When the AHL level reaches the threshold, the AHL interacts with the LuxR-type receptor to activate target gene transcription in QS signaling pathways [18,19].

In Gram-negative bacteria, many QS circuits are based on the LuxR-LuxI homologs of *V. fischeri* that use AHL signal molecules. For example, *Pseudomonas aeruginosa* (*P. aeruginosa*) has two key QS systems, LasR-LasI and RhlR-RhlI, which make use of 3-oxo-C12-homoserine lactone and C4-homoserine lactone (HSL) as signal molecules, respectively. The QS is also mediated through the Pqs system, which senses Pqs (2-heptyl-3-hydroxy-4-quinolone). These three systems are hierarchically presented, because the Las system is at the top and LasR controls Rhl and Pqs QS circuits. The Pqs system, which intervenes between the Las and Rhl systems, can interfere with the levels of Las- and Rhl-controlled genes. The Las and Rhl systems coordinate various virulence factors, including exoproteases, siderophores, toxins, and biofilm formation, and apparently exert an influence on host immune systems.

Interference with QS, termed quorum quenching (QQ), was first discovered as an enzyme capable of degrading AHL signals to interrupt QS signaling in *Erwinia carotovora* [20]. Specifically, QQ can be achieved by preventing bacteria from producing or perceiving AIs using quorum sensing inhibitors (QSIs) screened by different means [21], eliminating AIs by QQ antibodies, or extracellular enzymatic hydrolysis of AIs by QQ enzymes [22]. To date, many QQ enzymes, QSIs, and QQ antibodies have been reported and extensively reviewed [18,23–25]. Because QQ enzymes or antibodies can act remotely and independently without entering the bacterial cells to degrade AHL signals, they are probably less resistance-prone than QSIs, which have to interact with an intracellular target or a receptor on the bacterial outer membrane. However, stability remains a major constraint that usually limits the application of QQ enzymes and antibodies. Moreover, most QQ enzymes discovered such as lactonase are limited in quantity and variety [26]. Compared with that of QQ enzymes, the biochemical nature of purified QSIs, originating from a host of organisms including microbes, plants, fungi, and animals in ecosystems, is highly diverse [27]. For the discovery of QSIs, diverse approaches exist in-step with the scale of screening as well as specific QS systems. Among them, basic activity-based screening strategies consist of screening organisms, cells, and chemical libraries by QS bacterial reporters as well as QS-based phenotypes, including biofilm formation and virulence secretion. However, these natural QSIs may present a weak efficiency or possible toxicity in the targeted environment. A promising solution to overcoming these limitations is most likely to chemically design and synthesize molecules according to natural QSIs. Additionally, alternative strategies involve structure-based screening for target-oriented discovery of QSIs as well as other novel biotechnical tools for interpreting QS signaling. Overall, different methods, relying on the scale of screening as well as specific QS systems, are necessary for conducting QSI screening, which benefits the discovery and utilization of bioactive QSIs and validation of the effects of some QSI candidates. This review highlights the recent findings in strategies and methodologies for QSI screening, which are promising routes for QSI discovery to control bacterial biofilm formation and virulence to combat bacterial infection.

2. Activity-based screening with bioassays

In activity-based screening with bioassays, the detection systems rely on the testing signal generated by certain reporter proteins involved in colorimetric, bioluminescence, chemilumin escence, and fluorescence effects. Regarding QS bacterial reporters, two approaches are currently being applied, including those relying on the natural QS molecule-triggered responses (Fig. 3A) and by constructing engineered strains based on genetic modification (Fig. 3B).

2.1. Natural indicator strain for QSI screening

The easiest approaches to potential QSI screening involve colorimetric tests with natural indicator strains such as violacein formation in *Chromobacterium violaceum* (*C. violaceum*), bioluminescence in *Vibrio Ferdinans* and *Vibrio Harringtonii*, the prodigiosin

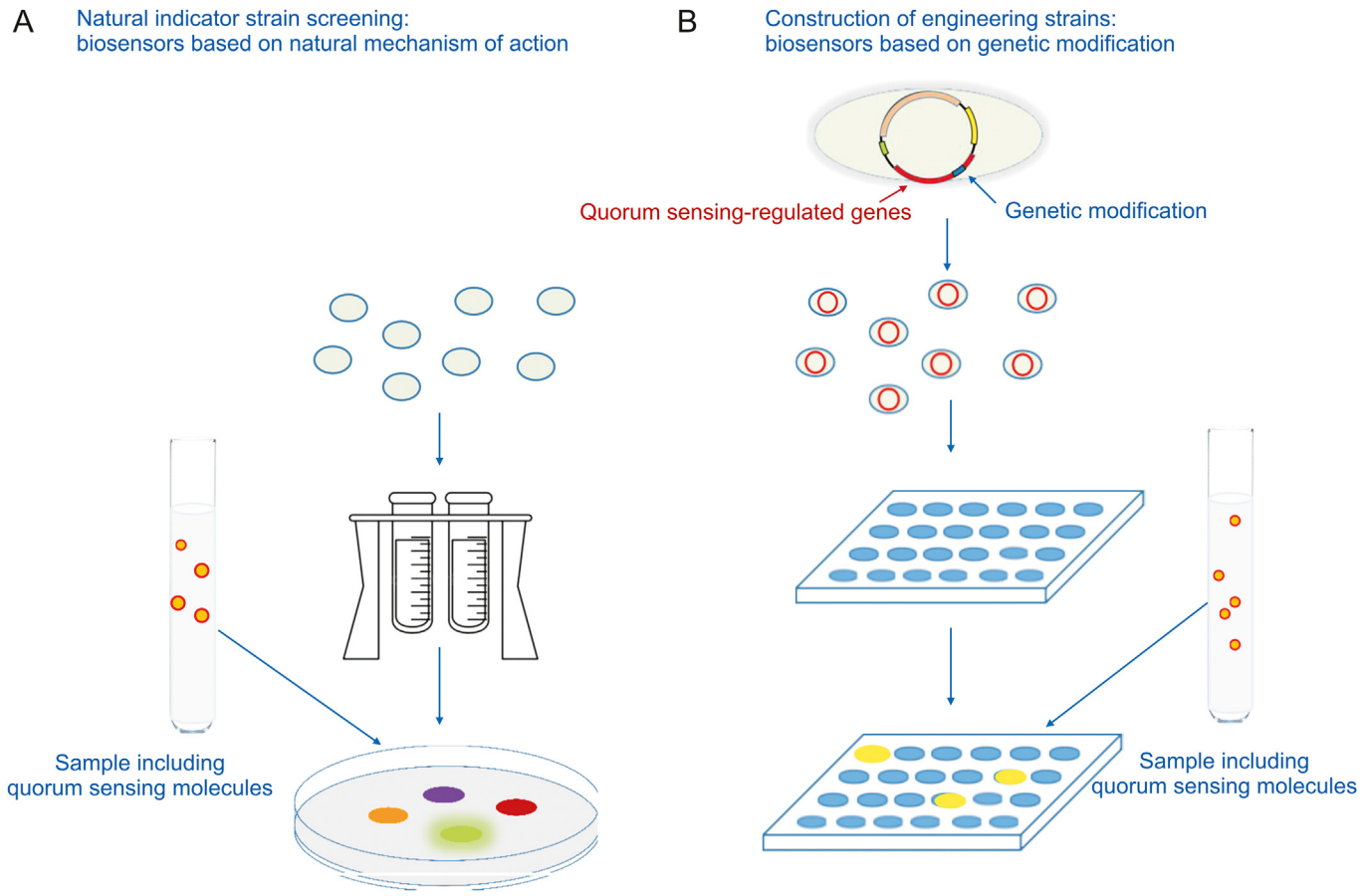

Fig. 3. Activity-based screening with bioassays. (A) The construction of engineering strains based on the naturally observed activity; (B) The construction of engineering strains based on genetic modification. The detection signal produced by QS is observed via colorimetric, fluorimetric, bioluminescent, and chemiluminescent analyses.

produced by *Serratia marcescens*, and the orange promethazine pigments produced by *Staphylococcus aureus* (*S. aureus*) (Fig. 3A) [28]. More specifically, *C. violaceum*, which produces the visible purple pigment violacein regulated by the CviI/CviR system, is a frequently used sensor strain for QSI screening. In addition, *V. harveyi* is a typical biosensor that regulates an easily observed QS-mediated phenotype termed bioluminescence and has well-characterized QS networks. Recently, it was reported that metabolite extracts of Gram-positive bacteria from diverse marine environments inhibit the bioluminescence produced by *V. harveyi*.

These studies usually started with an initial screen using an indicator strain or the combined tests against two QS reporter strains to estimate the anti-QS activity of candidates [29–33]. Analysis of QS-controlled phenotypes, such as the virulence factor assay and biofilm formation assay, has significantly decreased the potential candidates.

However, quantitative analyses of bioluminescence and violacein interference do not seem to adequately demonstrate that these inhibitors specifically disrupt QS signaling pathways. Molecular observations, including gene expression and in silico molecular docking, as well as biochemical tests of inhibitor agents, will be needed to explore the mechanisms and action modes of the inhibitory effects.

Notably, for the preparation of extracts for QSI screening, chromatography analysis by liquid chromatography-mass spectrometry or high-performance liquid chromatography (HPLC) fractionation is utilized for the extraction or purification of anti-QS compounds. It is also expected that the extraction approach used for QSI isolation may not be optimal for all types of inhibitory compounds. In some cases, metabolites with high hydrophilicity may not be fully soluble in ethyl acetate. In addition, producing fermentation extracts or isolating metabolites for further biological evaluation is often a laborious process.

It has been found that many microbes generate metabolites or fermentation extracts that regulate QS-dependent phenotypes. In this regard, co-cultivation assays offer a rapid screening approach to testing bacteria that are chemically innovative for QSI discovery. In one instance, an *Halobacillus salinus* isolate C42 isolated from a co-cultivation assay termed *Membrane Overlay* was discovered to generate *N*-(2-phenylethyl)-isobutyramide, which effectively inhibited the QS phenotypes in several Gram-negative biosensors [34]. This active compound represents a promising chemotype for inhibitors of QS phenotypes. These co-cultivation assays were applied to screen a host of organisms to inhibit QS phenotypes.

2.2. Construction of engineering strains for QSI screening

However, these colorimetric assays with natural strains cannot meet the requirement of large-scale QSI screening. To promote large-scale screenings, the QSI selector system, including a plasmid that involves a construct composed of a QS homolog gene such as LasI, LasB, and RhlA, as well as a phenotype or function regulated by a QS-mediated promoter, has been extensively developed [35–46].

2.2.1. QSIs-LasI biosensor

The QSIs-LasI biosensor emerged as an innovative strategy for QSI discovery, and diverse QSIs were identified by virtue of this biosensor. Using the *P. aeruginosa* QSIs-LasI biosensor combined with

Table 2
Activity-based screening with bioassays.

Strain source	Compounds	Main routes	Target bacteria	Targets/pathways	Anti-QS effects	Refs.
Natural indicator strain	Isoprenyl caffeate	Violacein inhibition assay; Differential gene expression assay; In silico docking experiments; LC-MS; HPLC	*C. violaceum ATCC 12472*	vioABCDE operon, involved in violacein biosynthesis	Inhibition in the violacein biosynthesis as a competitor in VioA and VioD.	[29]
	Extracts of *Tremella fuciformis*	Quantifying violacein production	*C. violaceum CV026*	–	–	[30]
	SCS-KFD08	Quantitative analysis of violacein production	*C. violaceum CV026*	–	Inhibitory effects on QS and violacein production	[31]
	Culture supernatants of a *Pseudomonas* sp.	Virulence factor assay; Biofilm formation assay; HPLC	*C. violaceum CV026*	Rhl system	Inhibitory effects on Rhl-controlled pyocyanin, rhamnolipid, elastase, protease, and biofilm	[32]
	Phenethylamides and a cyclic dipeptide	Test colony on top assay; Membrane overlay assay; Bioassay-guided fractionation; Lactonase gene analysis	*V. harveyi* *C. violaceum*	–	Inhibitory effects on bioluminescence production against *V. harveyi* and violacein production by *C. violaceum*	[33]
	Phenethylamide metabolites	Cocultivation experiment; Bioassay-guided fractionation; Detection of lactonase genes; Mathematical modeling	*V. harveyi* *C. violaceum CV026* *E. coli JB525*	Acting as antagonists of QS	Interfering with the bioluminescence, biofilm formation, motility, antibiotic biosynthesis, and virulence production	[34]
Construction of engineering strains	Lyngbic acid	QS assays with *V. harveyi* JMH626 (ΔluxN luxQ::Tn5 cqsA::Cm) and *V. harveyi* KM413 (ΔluxS ΔluxM); 16S Illumina tag sequencing Luminescence	*V. harveyi*	Functioning as a natural antagonist of the CqsS/CAI-1 QS system	Inhibitory effect on luminescence and CAI-1-mediated QS	[35]
	Substitution of AHL molecules	Construction of QS luxCDABE reporters; Luminometry	*E. coli*	LuxR, LasR, and RhlR	These sensors can be used to characterize the AHL signal(s) involved in QS	[36]
	Equisetin	QSI screening; Motility assays; Transcriptional activity; Assays for virulence factor production; and biofilm formation; RT-PCR	*P. aeruginosa*	Las, Rhl, and Pqs systems	Suppression in the biofilm formation, swarming motility, and the production of virulence factors	[37]
	α-pyrones from metabolites of *Streptomyces* sp.	Diversity-oriented synthesis; Anti-QS activity assay	*P. aeruginosa*	LasI	Inhibitory effects on the QS-regulated gene expression in *P. aeruginosa* QSIs-LasI	[38]
	The crude extracts from 65 marine fungi	Agar well-diffusion assay Bioautographic TLC	*P. aeruginosa*	PlasI-sacB reporter	This fungus produced several QSI compounds other than penicillic acid or patulin	[39]
	Zeaxanthin	Monitor strains (LasB-gfp and RhlA-gfp); qRT-PCR; In silico molecular docking	*P. aeruginosa*	Las and Rhl systems	Downregulation in the QS-related genes and inhibitory effects on biofilm formation	[40]
	Evernic acid	QSI screenings by LasB-gfp or RhlA-gfp monitor strain; qRT-PCR	*P. aeruginosa*	LasB and RhlA	QS systems (LasIR and RhlIR) of *P. aeruginosa* were regulated	[41]
	Garlic extract and 4-nitro-pyridine-N-oxide	QSIs assays; TLC assay; GeneChip-based transcriptome analysis; Biofilm formation assay; *Caenorhabditis elegans* nematode model	*P. aeruginosa*	PhlA, LasR, and RhlR	These QSIs reduced biofilm tolerance to antibiotics and virulence	[42]
	Secondary metabolites of *Flustra foliacea*	Inhibition assay with AHL-sensor strains including *Pseudomonas putida* (pKR-C12), *P. putida* (pAS-C8) and *E.coli* (pSB403); Measurement of protease activity; GC-MS; HPLC	*P. aeruginosa PAO1* *P. fischeri*	LasB: elastase gene; LasR: encodes the cognate N-3-oxododecanoyl-L-homoserine lactone receptor; cepR: sensitive for N-octanoyl-L-homoserine lactone; luxR: highest sensitivity for the *P. fischeri* signal N-oxohexanoyl-L-homoserine lactone;	Specifically blocking AHL-regulated gene expression and reducing protease activity	[43]
	Secondary metabolites produced by marine *streptomyces*	QSI screening; Biofilm production assay; Scanning electron microscopy; Assessment of virulence factors motility, hemolysin, and urease activity	*Proteus mirabilis* (uropathogen)	Synthesis inhibition by blocking the AHL synthase LuxI-type proteins; Interference with the signal receptors or blocking the formation of AHL/LuxR complex	Inhibiting QS-regulated prodigiosin biosynthesis and the QS-dependent factors	[44]
	N,N'-alkylated imidazolium-derivatives	Pectobacterium AHL-biosensors; Virulence ssays	*P. atrosepticum*	ExpI: encodes the synthase for the biosynthesis of the AHL-signals; RsmA: downregulated by AHLs	Targeting virulence genes and decreasing the severity of the symptoms provoked by *P. atrosepticum*	[45]

(*continued on next page*)

Table 2 (*continued*)

Strain source	Compounds	Main routes	Target bacteria	Targets/pathways	Anti-QS effects	Refs.
	Coumarin	QQ biosensor assay; β-Galactosidase assay; Phenazine production; Assays for biofilm formation and motility; Bioluminescence of *A. fischeri;* Determination of protease activity	A broad spectrum of bacterial strains	RhlI promoter modulating RhlI/R-associated biosurfactant rhamnolipids; PqsA promoter in Pqs signaling system controlling the production of phenazine LuxI/R QS system controlling bioluminescence	Presenting abroad spectrum of activity against kinds of AHLs; Inhibiting biofilm, phenazine production and swarming motility	[46]

–: not available. TLC: thin-layer chromatography; QS: quorum sensing; QSI: quorum sensing inhibitor; RT-PCR: real time-polymerase chain reaction; GC-MS: gas chromatography-mass spectrometry; HPLC: high performance liquid chromatography; CAI: cholera autoinducer.

analysis of QS-controlled phenotypes and HPLC, equisetin in secondary metabolites from the marine fungus *Fusarium* sp. Z10 and four new alpha-pyrones from the secondary metabolites of *Streptomyces* sp. were discovered as the bioactive anti-QS components [37,38]. In one instance, Wang et al. designed an efficient screening system involving a QSIs-LasI selector based on the PlasI-sacB reporter. Plasmid pMHLASI harboring PlasI-sacB and Plac-LasR transcriptional fusion was constructed and transformed into a *P. aeruginosa* LasI-RhlI double mutant PAO-MW1 to generate the selector QSIs-LasI. They tested the crude extracts from 65 marine fungi, and then an isolate, *Penicillium Atramentosum* QJ012, was selected to exhibit an inhibitory effect on the QS system. The thin-layer chromatography assay of the fungal extracts for bioautographic identification by QSIs-LasI demonstrated that the fungus produced several valuable compounds with anti-QS effects [39].

2.2.2. LasB-gfp and RhlA-gfp biosensor

The LasB-gfp and RhlA-gfp monitor strains contain an LasB promoter regulated by LasR, an RhlA promoter regulated by RhlR, and genes controlling an unstable green fluorescent protein. Using this biosensor, active compounds such as zeaxanthin and evernic acid were discovered to downregulate QS-related genes and inhibit biofilm formation in *P. aeruginosa* [40,41]. Rasmussen et al. [42] constructed three novel QSI selectors for QSI screening, including QSIs1 phlA encoding the toxic gene product, the expression of which was controlled by LuxR; QSIs2, the LasR- and RhlR-regulating LasB promoter controlled expression of the sacB gene, which led to cell death in the presence of sucrose; and the QSIs3 system, which contained the npt and gfp genes, conferring kanamycin resistance and green fluorescence, respectively. By virtue of these QSIs systems, two potential QSIs, garlic extract and 4-nitro-pyridine-N-oxide, were identified. In addition to in vitro phenotypic assays, GeneChip-based transcriptome analysis and a *Caenorhabditis elegans* pathogenesis model were also used to evaluate the specificity of QS-regulated virulence genes and virulence in vivo.

2.2.3. AHL-biosensor and others

The application of biosensors for the detection of the inhibitory activity in AHL-mediated QS has been instrumental in the rapid and streamlined screening of the QSI in a broad spectrum of microbes, especially Gram-negative bacteria. The realization that structurally different AHLs exist, possessing diversity in the acyl side chain and having different activation profiles, has prompted the construction of the corresponding biosensors. These reporters could be utilized in further studies to characterize the AHL signals in QS in a broad spectrum of organisms.

Using AHL-biosensors to screen out from the QSI chemical library and AHL-analogs as well as secondary metabolites produced by microbes, marine *Streptomyces* and *Flustra foliacea*, a large number of novel QSIs including N,N′-alkylated imidazolium-derivatives, and some secondary metabolites were identified as potential QSIs [43–45].

In another study, coumarin was identified as a potential QSI using a novel screening system composed of three biosensors (*Serratia marcescens* SP15, *C. violaceum* DSM30191, and *Agrobacterium tumefaciens* NTL4 containing a plasmid pZLR4 carrying a traG:lacZ reporter fusion) to detect short, medium, and long AHLs, respectively. Subsequently, coumarin was demonstrated to significantly suppress biofilm formation and protease activity in other microbes and inhibit bioluminescence in *Aliivibrio fischeri* [46]. All cases mentioned above are shown in Table 2 [29–46].

2.3. Other activity-based bioassays

Other series of activity-based assays use known QS-mediated mechanisms for functional differentiation. The QS-regulated functions tested in these bioassays involve motility, pyocyanin or pyoverdin production, and biofilm formation in *P. aeruginosa*. In this regard, similar steps are applied in the preliminary stage, including diffusing potential QSIs into agar; measuring the area of pigment inhibition, which is visualized by the formation of a colorless but visible halo around the well; and the method of microbulion dilution, which quantitatively detects the level of pigment by measuring the optical density using a spectrophotometer [47,48]. Numerous studies have described the discovery of QSIs based on biofilm formation. Commonly used qualitative methods to evaluate the characteristics of the biofilm in these cases involve Congo red agar, the microtiter plate assay, or the crystal violet assay.

Another novel approach used for QSI discovery is proteomic analysis, which is a two-step procedure involving an initial quantitative 2D-difference gel electrophoresis and subsequent protein identification in each spot by mass spectrometry. This method has been proven to be beneficial for the examination of the impact of various factors on a range of proteomic and metabolomic changes mediated by QS in bacteria [49].

Transcriptome analysis provides deep insights into QS modulation in response to anti-QS compounds [50,51]. Comparing real-time polymerase chain reaction used in small-scale genes for QSI screening in early studies, this transcriptome analysis based on high-throughput sequencing methods achieved more quantitative analysis of comprehensive transcription profiles. The analysis included 1) total RNA extraction of bacterial samples, 2) RNA reverse transcription into a complementary DNA library for transcriptome sequencing, and 3) comparative analysis of differentially expressed genes by various genetic techniques. The subsequent differential expression analysis by bioinformatics approaches is beneficial for revealing and finally characterizing global gene expression patterns in response to anti-QS compounds.

In vivo models are widely used to evaluate the efficiency of potential QSIs for antivirulence activity. The *Caenorhabditis elegans*

model achieves in vivo high-throughput screening (HTS) and provides a better understanding of QS modulation and virulence regulation [52]. Additionally, mammalian models with general immune systems are also commonly employed to evaluate the anti-QS activities of potential QSIs on bacterial infections [53].

3. Chemical approaches to interrogating QS pathways for QSI discovery

3.1. Solid-phase synthesis for the identification of small molecule QS modulators by interaction with their target proteins/receptors

Each compound yielded by traditional synthesis strategies requires individual purification, identification, and bio-activity evaluation, rendering the acquisition process cumbersome and time-consuming. Unlike traditional synthesis methods, the update of combinatorial chemistry involving solid-phase synthesis significantly accelerates chemical synthesis and screening. Solid-phase synthesis, in which the reactant molecule is chemically bound to an insoluble material while reagents are added in the solution phase, is extensively applied in the high-throughput synthesis of biological molecules such as peptides, nucleic acids, oligosaccharides, and QSIs [54].

Geske et al. [55–57] constructed a library of AHL-analogs using a solid-phase synthesis technique. They adopted a novel synthetic strategy containing a series of microwave-assisted reactions followed by a CNBr cyclative-cleavage procedure on the polystyrene beads. Furthermore, through systematic screening of these analogs, numerous antagonists were identified to competitively bind to the LuxR-type protein. These compounds were found to attenuate QS-mediated phenotypes in bacterial culture as well as in eukaryotic host infections.

Novel solid-phase synthesis techniques such as macroarrays and microarrays were studied for the discovery of QSIs [58–64]. In small molecule macroarrays, libraries of varying sizes were formed by the spatial synthesis of small molecules on planar polymeric supports. This platform, with advantageous properties such as economics, stability, and compatibility, was proven to be a practical approach to the large-scale synthesis of small molecules including QSIs [65,66].

Not limited to macroarrays, microarray techniques were also investigated and utilized for the combinatorial synthesis of QSIs [67]. Sharing the analogous underlying principle with macroarray synthesis, the size and diversity of microarrays are apparently smaller and simpler [68,69]. Moreover, macroarrays and microarrays simultaneously provide platforms not limited to chemical synthesis, but many on-support biological tests are involved, further facilitating the discovery of bioactive anti-QS molecules.

3.2. Affinity chromatography approaches for identifying QS receptors based on ligand–receptor interactions

Not limited to synthetic AI analogs as QS modulators, ligand–receptor interactions in QS signaling have also been studied for target identification of QS receptors. Affinity chromatography based on ligand–receptor interactions, with its exquisite specificity, has been proven to be a valuable tool for the purification and identification of biomolecules. In affinity chromatography, one molecule is immobilized to a support, whereas its binding counterpart is soaked in the mobile phase. Under alternate conditions, molecular recognition between the two molecules allows the counterpart to be isolated from the eluent and separated from the support. This highly selective separation approach is of significant value in the identification, isolation, purification, pretreatment, or analysis of many biologically relevant molecules.

To identify AHL-binding receptors, Spandl et al. [70] synthesized biotin-labeled N-3-oxo-hexanoyl L-homoserine lactone (OHHL). The biotin tag, with powerful affinity and recognition to avidin, permits the ligand OHHL to be readily immobilized to an avidin-functionalized support. Biological tests and 2D-difference gel electrophoresis have also been pursued to investigate the target for OHHL. In a similar work, affinity resins were utilized to identify putative receptors for AHLs in eukaryotic cells. Two piperazine-modified N-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL) derivatives were synthesized and linked to agarose resin [71]. Subsequently, two proteins were successfully obtained by affinity assay by virtue of these OdDHL-modified resins from mammalian cell lysates. Such resins in affinity-based methods could be ultimately valuable for studying novel AHL-binding proteins.

3.3. Antibodies for quenching QS signaling

Another approach that has gained considerable attention for interrupting QS signaling is to apply antibodies to quench QS by competing with or interrupting QS signal molecules [72]. The AIs or derivatives thereof are delivered into an animal to activate monoclonal antibody formation. The yielded antibodies, which are substantially specific for the AI molecules, could catalytically inactivate or effectively sequester AIs by acting as the QS quenchers, resulting in the QS signaling interruption [73]. Usually, generating antibodies such as QQ needs a molecular template (or hapten) to immunize. Small QS molecules such as AHLs, with hydrolytic instability and nonproteinaceous nature, cannot complete antibody-based immune reactions by themselves because they are unlikely to be appropriately presented by the mammalian immune system. The antibody-based strategy has promising applications in future vaccine research and development.

In recent years, numerous studies have reported the design and evaluation of QQ antibodies to sequester AIs in vitro and in vivo [72,74]. Using an immunotherapeutic strategy for QQ, Janda and coworkers [75] pioneered eliciting immune responses against the synthetic 3-oxo-AHL analog RS2 in mice, and isolated an anti-AHL antibody (RS2-1G9). The RS2-1G9 efficiently inhibited QS phenotypes and QS pathways in *P. aeruginosa* by sequestering 3-oxo-C12-HSL. Furthermore, they utilized this approach to raise antibodies against a similar analog of AIP-IV. In this case, the native thioester in the AIP macrocycle was more hydrolytically stable using an ester bond. The antibody obtained, AP4-24H11, was shown to be an active QSI in *S. aureus* cultures [76].

Additionally, antibody-based QQ also involved other strategies, such as raising catalytic antibodies for degrading and thus inactivating AIs. Marin et al. [77] utilized this approach to screen and evaluate catalytic antibodies for lactonase activity. An antibody (XYD-11G2) capable of hydrolyzing OdDHL from an antibody library was identified and demonstrated to suppress QS in *P. aeruginosa* cultures.

3.4. Biotic polymers/polymeric materials

Materials-based approaches have also gained considerable attention for QS modulation. The first strategy is the sequestration of the AI signal by non-native polymeric materials. By computational modeling and prior affinity studies, Piletska et al. [78] selected a series of monomers with binding capacity with an AHL signal named OHHL produced in *V. fischeri*. Polymers based on one monomer, named itaconic acid (IA), showed higher affinity to OHHL and thus suppressed QS of *V. fischeri*. Subsequently, they generated a polymer production composed of a template molecule and polymers synthesized by utilizing molecularly imprinted polymers [79]. The formed IA-based polymer production served as high-

specificity binding pockets to sequester QS signaling and biofilm formation in *P. aeruginosa*. In another study, 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) was reported to downregulate the levels of QS-controlled genes in *Serratia marcescens* and *P. aeruginosa*. Then, cross-linked HP-β-CD was utilized to form sheets of 2-hydroxypropylcellulose to form an AHL-sequestering material. Subsequently, these HP-β-CD-linked sheets were reported to suppress the levels of QS-dependent genes when co-cultured with the two above-mentioned strains.

Another strategy is to utilize polymer materials for the spatial accumulation of QS modulators and thus to prevent the release of QS modulators into the surroundings. Poly lactide-co-glycolide is employed as a material to regulate the release and delivery of AHLs. Both the AHL concentration and the time of AHL release could be readily controlled without affecting its biological activity [80]. These studies indicated a novel material-based strategy for modulating QS signaling that can be applied in a series of contexts (e.g., biofilm prevention and coatings on the surface of clinical implants). All those mentioned above are shown in Table 3 [56,57,64,66,69,76–78,80].

4. Virtual screening for QSI screening

As described above, limited QSIs have been identified using traditional methods from natural resources. Computer-assisted screenings, especially structure-based virtual screening (SB-VS), have been developed as an efficient paradigm for lead discovery that fits in well alongside HTS to promote large-scale screenings, which can complement traditional methods.

The SB-VS approach, which is used extensively in the pharmaceutical industry, has also been applied for novel QSI discovery that antagonized AHL sensing in recent years: 1) against Las, Rhl, and Pqs systems: the screening of natural compounds and recognized drugs/compounds by in-silico docking analysis and SB-VS in *P. aeruginosa* [81,82]; 2) against the LasR and RhlR receptor proteins: the identification of five active compounds using an SB-VS of 1920 natural compounds [83]; 3) against LasR receptors: two compounds discovered by virtual screening and pharmacophore- and structure-based approaches and six novel potential QSIs screened with parameters including Lipinski's rule, and toxicity as well as absorption, distribution, metabolism, excretion (ADME) from the ZINC database [84,85]; 4) with the crystal structure of LuxP: the screening of cinnamaldehyde derivatives [86], boronic acid derivatives [87], and traditional Chinese medicine (TCM) databases [88] by SB-VS; 5) other targets/systems: the identification of baicalein in 51 bioactive components from TCMs against TraR protein [89], the discovery of eight lead-like molecules from the ZINC database by combined analysis including virtual screening, molecular docking as well as ADME and toxicity studies against FabI which plays the important role of enoyl-acyl carrier protein reductase related to the synthesis of 3-oxo-C12 HSL [90], the screening of most plant compounds and all NSAIDs to find the Z-phytol and lonazolac molecules which can suppress the SdiA as a homolog of LuxR in the QS pathway [91].

To sum up, SB-VS approaches represent an economical and rapid alternative to the traditional methods and HTS approaches. Screening strategies for SB-VS can be limited to commercially available compound libraries to avoid time-consuming and expensive chemical syntheses and utilized to dock compounds from known drugs/natural product libraries to circumvent problems in complicated testing stages, owing to toxicity or poor ADME properties, which would substantially reduce the costs, compared to conventional resource-consuming HTS processes. However, SB-

Table 3
QSI discovery by chemical techniques.

Compounds	Routes	Target bacteria	Receptors	Anti-QS effects	Refs.
90 non-native AHLs	A solid-phase synthetic route	*A. tumefaciens* *P. Aeruginosa* *V. fischeri*	TraR LasR LuxR	Showing agonistic or antagonistic activity in all species	[56]
Majority of the natural AHLs and a small test library of non-natural AHLs	A solid-phase synthetic route to both natural and non-natural AHLs	*P. aeruginosa,* *R. leguminosarum* *Y. pseudotuberculosis* *B. pseudomallei,* *S. meliloti,* *R. capsulatus,* *V. fischeri* *A. tumefaciens,* *V. anguillarum*	TraR LasR LuxR	Antagonizing LuxR-type protein and TraR	[57]
A 39-member library E	A microwave-assisted synthetic route to AHLs	*A. tumefaciens,* *P. aeruginosa,* *V. fischeri*	TraR LasR LuxR	Antagonists of TraR and LuxR	[64]
Macrocyclic peptide-peptoid hybrids (peptomers) as analogs of AIP-I	A solid-phase synthetic route includes microwave-assisted reactions followed by a tandem macrocyclization-cleavage step	*S. aureus*	AgrC receptor	Stimulating biofilm formation	[66]
A number of QS analogs	3D microarray platform: 3D microarray slides were probed with fluorescently labeled ligand-binding domains of the LuxR homolog CarR	*Serratia* *P. aeruginosa.*	LuxR homolog CarR	As potent inhibitors of AHL mediated QS phenotypes in *Serratia* and *P.aeruginosa.*	[69]
3-oxo-AHL analogue RS2 and AP4-24H11	An immunotherapeutic strategy for QQ to elicit immune responses against the synthetic 3-oxo-AHL analogue RS2 and against a close analogue of AIP-IV in mice	*P. aeruginosa* *S. aureus*	–	Sequestration of 3-oxo-C12-HSL, efficiently inhibition in QS phenotypes and QS signaling in vitro.	[76]
XYD-11G2	Screening catalytic antibodies in an existing library of antibodies known to catalyze the hydrolysis of the insecticide paraoxon	*P. aeruginosa*	–	Capable of hydrolyzing OdDHL and suppression of QS in *P. aeruginosa* cultures	[77]
A set of rationally designed polymers with affinity toward a signal molecule of *V. fischeri*	Functional monomers were selected based on the computational modeling. All polymers were prepared by thermal polymerization	*V. fischeri*	–	Computationally designed polymers could sequester a signal molecule of *V. fischeri* and prevent QS-controlled phenotypes	[78]
A synthetic AHL modulator of bacterial QS	A polymer-based approach to the release of a synthetic AHL	*V. fischeri*	–	Modulating QS in the marine symbiont *V. fischeri*	[80]

–: not available. AIP-I: autoinducing peptide I; QQ: quorum quenching; OdDHL: N-(3-oxo-dodecanoyl)-L-homoserine lactone.

Table 4
Virtual screening for QSI screening.

System	Compounds	Routes	Targets/pathways	Bioactive components	Target bacteria	Anti-QS effects	Refs.
Las, Rhl and Pqs	3040 natural compounds and their derivatives	SB-VS; Live reporter assay for QS; (iTRAQ)-based proteomic analysis; Molecular docking; Elastase assay; AHL competition assay; Glass-slide biofilm assay	LasR and LasB: controlled and encoded elastase; Pqs system: regulates release of extracellular DNA	5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d] pyrimidin-7-one	*P. aeruginosa* *E. coli*	Inhibition of Rhl system and Pqs system; Efficient in inhibiting elastase production and eDNA release in *P. aeruginosa* biofilms	[81]
	147 recognized drugs/ compounds	SB-VS; Molecular docking; Measurement of virulence factors and biofilm development	LasR, RhlA, PqsA	Salicylic acid, nifuroxazide, and chlorzoxazone	*P. aeruginosa.*	Significant, dose-dependent inhibition of QS-controlled gene expression and phenotypes	[82]
Las and Rhl	1,920 natural compounds/ drugs	SB-VS; Molecular docking studies; Measurement of virulence factors production; Biofilm inhibition assay; Swarming assay; Protein degradation assay	LasR and RhlR receptors in Las and Rhl dependent virulence factors production	Rosmarinic acid, naringin, chlorogenic acid, morin, and mangiferin	*P. aeruginosa.*	Inhibit biofilm related behaviors and virulence factors production	[83]
Las	122 compounds identified via in silico screening	Virtual screening; Pharmacophore- and structure-based approaches; QS inhibition and activation via cell-based assays	LasR, also bind to RhlR and TraR	Two compounds, named ZINC 2060666 and ZINC 2989037	*P. aeruginosa.*	Showing in vitro QS inhibition	[84]
	About 2,603 compounds from ZINC database	Virtual screening; Lipinski's rule, ADME and toxicity studies; Molecular docking	LasR	Six novel potential QS inhibiting compounds	*P. aeruginosa.*	–	[85]
Lux	Cinnamaldehyde derivatives	Molecular docking; e-Pharmacophore based virtual screening; Bioluminescence and biofilm inhibition assay; Swimming and swarming assays	LuxR: cognate receptor of AI LuxI/LuxR QS system controlling a wide range of cellular processes	3-(2,4-dichlorophenyl)-1-(1H-pyrrol-2-yl)-2-propen-1-one	*V. harveyi*	Inhibitory effects on the bioluminescence production in a dose dependent manner; Inhibition in biofilm formation and motility in *V. harveyi*	[86]
	Five boronic acid derivatives	Three docking protocols: RRD, IFD, and QPLD; Virtual Screening; Bioluminescence Inhibition Assay; Biofilm Inhibition Assay; Swimming and Swarming	LuxP: periplasmic binding protein (LuxP) binds to AI-2 to activates the biosynthetic pathway that is responsible for the production of AIs	Bicyclo[2,2,1]hept-5-ene-2,3-dicarboxylic acid-2,6-dimethylpyridine 1-oxide	*V. harveyi*	Dose-dependent inhibition in bioluminescence and biofilm formation	[87]
	A TCM database	Virtual screening and molecular docking; QS inhibitory assay; Production of extracellular enzymes and siderophores; Swimming and swarming motility; Impact on AHL and EPS content	LuxI-and LuxR-type proteins LuxI/LuxR QS system trolling a wide range of cellular processes	Benzyl alcohol, rhodinyl formate and houttuynine	*P. fluorescens P07*	Inhibitory effects on swimming and swarming motility, production of extracellular enzymes and siderophores, AHL content and biofilm formation	[88]
Others	51 bioactive components from TCMs	Degradation of TraR protein; Biofilm formation	The signal receptor TraR	Baicalein	*P. aeruginosa* *E.coli*	Inhibiting biofilm formation of *P. aeruginosa*; Promotion in proteolysis of the signal receptor TraR protein in *E.coli*	[89]
	75 natural compounds from the ZINC database	Virtual screening; ADME and toxicity studies; Molecular docking	FabI: the significant role of enoyl-acyl carrier protein reductase in the synthesis of 3-oxo-C12 HSL	Eight lead-like molecules	*P. aeruginosa.*	–	[90]
	Most plant compounds and all NASIDs	Molecular docking; Biofilm formation; Prediction of absorption, solubility and permeability of evaluated compounds	SdiA (a homolog of LuxR)	The Z-phytol and lonazolac molecules	*Salmonella*	Inhibition of QS mediated by AI-1 and biofilm formation in *Salmonella*	[91]

–: not available. EPS: extracellular polymeric substances; NSAIDs: nonsteroidal anti-inflammatory drugs; TCM: traditional Chinese medicine; ADME: absorption, distribution, metabolism, excretion; SB-VS: structure-based virtual screening; RRD: rigid receptor docking; IFD: induced fit docking; QPLD: quantum polarized ligand docking.

VS approaches, relying on the prediction by the docking software, have one major problem of false positives and false negatives. As more novel and advanced algorithms are developed, false hits may be minimized. Moreover, experimental validation is required to combine SB-VS for in vitro inhibition efficacy.

The SB-VS approaches promote the identification of targeted QSIs, and future directions include extending SB-VS to more novel targets and performing combined tests with quantitative structure-activity relationship studies to promote rational drug design. All cases mentioned above are shown in Table 4 [81–91].

5. Other potential tools for interpreting QS signaling

5.1. Three-dimensional (3D) printing

A microscopic 3D printing strategy was established to organize multiple populations of bacteria within essentially any 3D geometry. Using this approach, a distinct core–shell arrangement, which was composed of a single species at vastly differing densities as well as polymicrobial communities including motile and non-motile bacteria, was found in microbial populations. For example, Connell et al. [92] also demonstrated that a picoliter-volume aggregate of *S. aureus* had the ability to produce substantial resistance to β-lactam antibiotics by nesting a unique core–shell arrangement enclosed with a shell composed of *P. aeruginosa*.

5.2. Immobilized hybrids

Using an operationally simple dip-and-rinse procedure, Gomes et al. [93] synthesized natural product hybrids featuring an AHL inducer as well as a nitrodopamine and subsequently immobilized the hybrids onto biocompatible TiO_2 surfaces. The formed immobilized hybrids were demonstrated as effective QS activators by

Table 5
Recently authorized patents on the discovery of new bioactive agents, analogs and approaches interfering with QS signaling.

Year	Invention	Description/Methodology	Application	Refs.
2020	Compounds that affect QS in *S. aureus* and related *Staphylococcus* species	Modulation includes inhibition or activation of one or more of four AgrC receptors	In combination with one or more antibiotics to treat bacterial infections	[95]
2019	A novel brominated furanone derivative	An effective inhibitory activity of the biofilm formation and QS	Useful effects on oral diseases or inflammatory diseases, e.g., periodontal diseases	[96]
2019	Methods and compositions for the inhibition of biofilm formation	A method for the inhibition of biofilm formation with a bifunctional ligand comprising a QS-peptide-binding region and a protease-binding region	Inhibition of biofilm formation on the surface	[97]
2019	Applications of 3,4,5-methyl trihydroxybenzoate in inhibition of the activity of a bacterial QS system	A compound capable of significantly reducing the expression of related pathogenic factors in *P. aeruginosa* and *C. violaceum*	Controlling drug resistance	[98]
2019	D-galactose in QS inhibition	A composition for inhibiting QS	Inhibiting QS and treating oral bacterial diseases	[99]
2018	A preparation method and application of a camphor essential oil-based bacterial QSI	The preparation method comprises preparing essential oil and preparing the bacterial QSI which is a novel antibacterial substance based on bacterial QS inhibition in *E.coli*, purple *bacillus*, *S. aureus* and *P. aeruginosa*	The raw material is easy to obtain and the preparation method of the novel bacterial QSI is simple and reliable; the bacteriostatic agent does not generate drug resistance and has no toxic effects	[100]
2018	A QSI comprising at least one of wood particles	Wood particles from trees and grass	As feed additive for antibiotic-free prophylaxis of infectious diseases and modulating body temperature under heat stress conditions in farm animals	[101]
2018	A trackable moiety can be attached to a QS molecule to form a QS modulating conjugate	QS modulating conjugates retain their activity for QS manipulation and are able to be detected by imaging techniques	Diagnostic applications by enabling pinpointing of specific bacteria at infection sites	[102]
2018	Banana pseudostem-based liquid extracts	Results in disc diffusion method showed that antibacterial and QS inhibition activity in the extracts, especially in the autoclaved aqueous forms	As an antimicrobial against *E. coli, Salmonella typhimurium* and *S. aureus*, and as a QS inhibition agent against *P. aeruginosa.*	[103]
2018	A synergist for food biological preservatives and a method of the synergist	The combination of biofilm degrading enzyme and a QSI to interfere with the biofilm formation of food putrefying bacteria and inhibit the QS information exchange between putrefying bacteria	The target spot of the synergist is the biofilm and QS system of the putrefying bacteria, and the action site is accurate, not a lethal effect	[104]
2017	Application of pyrimidine derivative in preparing medicine for inhibiting a bacterial QS system	The pyrimidine derivative can inhibit QS signal molecules to finally reduce bacterial biofilm formation and effectively inhibit pathogenicity of virulence factors	Computer virtual screening is a compound screening method with high efficiency and low cost in studying QS medicine	[105]
2017	Novel apicidin methods and compositions for the QS inhibition	–	Treating a *staphylococcal* infection	[106]
2017	The modulation of the flora of bacteria in an environment by inhibiting the QS of a specific bacteria by administering a QS control composition	QS control agents include a sorbent material, sorbent mineral or non-porous mineral such as phyllosilicate clays, silica, calcite, zeolites, diatomaceous earth, smectite, activated carbon, a nanoparticle or a combination of any of the foregoing	Inhibiting the spoilage of food stuff and preventing vibriosis in fish or shell fish	[107]
2017	Methods for modulating QS in certain Gram-negative bacteria having multiple QS systems including Las, Rhl, and Pqs with associated receptors (LasR, RhlR, and PqsR)	Certain combinations of Las, Rhl, and Pqs exhibit improved inhibitory effects on virulence	Modulating QS in *Pseudomonas* and *Buckholderia*	[108]
2017	A novel application of lotus plumule extracts in the preparation of QS inhibitory drugs	The lotus plumule extracts provide good inhibition for both *P. aeruginosa* and drug-resistant *P. aeruginosa.*	Decreasing bacterial virulence and pathogenicity and controlling drug resistance	[109]
2017	Synthetic cyclic peptide modulators of the AgrC QS system of *S. epidermidis*	Compounds capable of either pan-group or group-selective AgrC receptor inhibition in *S. epidermidis* were identified	Treating infections of *S. epidermidis* and related *Staphylococcus* by administering a therapeutically effective amount of one or more compounds herein to an individual in need thereof	[110]
2017	Certain compounds of general formula A-W-HG having various carbocyclic and heterocyclic head groups and various tail groups	The compounds are useful in methods of modulating QS in Gram-negative bacteria, particularly in *Pseudomonas*. Compositions including certain RhlR modulators are useful for decreasing the virulence of Gram-negative bacteria	Pharmaceutical compositions comprising certain RhlR modulators are useful for treatment of infections of Gram-negative bacteria	[111]

–: not available.

Table 6
Quorum-sensing inhibitors in clinical evaluation [112].

NCT No.	Title	Condition or disease	Interventions	Phase	Year	Status
NCT00610623	Azithromycin as a QSI for the prevention of *P. aeruginosa* ventilator-associated pneumonia	Pneumonia, ventilator-associated *Pseudomonas* infections	Drug: azithromycin Drug: placebo	II	2008	Terminated
NCT01201577	Biological modulation of bacterial QSSMs, innate and adaptive immunity by antibiotics, probiotics and prebiotics in healthy individuals	QS Prebiotics Probiotics Sepsis	Dietary supplement: *Bifidobacterium longum* BB536 Dietary supplement: active hexose correlated compound Dietary supplement: *Bifidobacterium longum* BB536 and active hexose correlated compound Dietary supplement: corn starch placebo capsule Drug: azithromycin	I	2011	Completed

QSSM: quorum sensing signaling molecules.

Table 7
Recent anti-biofilm agents under clinical evaluation [112].

NCT No.	Condition or disease	Status	Intervention	Phase	Year
NCT04254835	High caries risk patients	Recruiting	Drug: cervitec F, ivoclar vivadent - Schaan Liechtenstein Drug: fluor protector, ivoclar vivadent - Schaan Liechtenstein	IV	2020
NCT03683563	End-stage kidney disease; Renal dialysis; Central venous catheter; Biofilms	Unknown	Other: 4% sodium citrate Other: 30% sodium citrate	–	2018
NCT03686904	Wound infection	Active, not recruiting	Drug: benzalkonium gel Other: standard of care topical gel Procedure: debridement Drug: benzalkonium irrigation Other: saline irrigation (SOC irrigation)	IV	2018
NCT03678012	Dental caries	Completed	Drug: ferumoxytol/hydrogen peroxide Drug: hydrogen peroxide; Drug: water	Early I	2018
NCT03213249	Breast implant infection; Mammoplasty	Completed	Other: normal saline; Drug: cefazolin Procedure: skin biopsy Procedure: bilateral skin- or nipple-sparing mastectomies Device: tissue expander; Device: breast implant Procedure: autologous flap Other: acellular dermal matrix sling Drug: gentamicin Drug: bacitracin	I	2017
NCT02946801	Biofilms; Essential oils; Periodontitis	Not yet recruiting	Drug: essential oils Drug: essential oils without alcohol Drug: sterile water	IV	2016
NCT02946814	Biofilms; Substantivity; Essential oils,	Unknown	Drug: essential oils Drug: Eessential oils without mouthwash Drug: sterile water	IV	2016
NCT03146390	Oral biofilm; Dental plaque; Periodontitis	Recruiting	Drug: essential oils Drug: alcohol free essential oils Other: water	IV	2017
NCT02052973	Streptococcal infections; Saliva altered	Completed	Other: propolis varnish	I and II	2015
NCT02545244	Gingivitis	Completed	Dietary supplement: black tea Dietary supplement: green tea Drug: 0.12% chlorhexidine mouthwash	III	2015
NCT02656251	Dental plaque	Completed	Drug: 0.12% clorhexidine with alcohol Drug: placebo Drug: 0.12% clorhexidine without alcohol	III	2015
NCT02486458	Dental caries	Completed	Drug: 5% sodium fluoride varnish Drug: 1.23% sodium fluoride acidic gel	Early I	2015

–: not available. SOC: soil organic carbon.

gradually releasing them from the TiO2 surface. This approach is a straightforward and useful strategy for interrupting the QS signaling pathway by the preparation of coated surfaces in medical devices in a wide variety of bacterial species.

5.3. Biological nanofactories

Hebert et al. [94] constructed a biological nanofactory to regulate the behaviors of bacteria on the surface of human epithelial

cells. The biological nanofactory construct activated the surface to produce the bacterial QS signaling molecule, AI-2. Biological nanofactories include several functional subassemblies: the cell targeting module with the targeting element CD26 antibody, the fabrication, and the synthesis modules. The biosynthesis component implemented is the fusion protein His_6-protein G-LuxS-Pfs-Tyr_5 (HGLPT). The HGLPT consisted of both synthesis and fabrication domains, which facilitated flexible self-assembly of the functional units. The HGLPT utilized the substrate S-adenosylhomocysteine to synthesize AI-2 in a two-step process using enzymes such as Pfs and LuxS. This tool would be beneficial for modulating signaling activities associated with the microbiome present in the human gastrointestinal tract and other environments.

6. Challenges in QSI discovery and some criteria for QSIs

To date, many recently authorized patents focused on the discovery of new bioactive agents, analogs, and approaches interfering with QS signaling highlight the application of QS inhibition in the fields of plant pathogens, aquaculture, antifouling, and medical devices (Table 5) [95–111]. However, very few QSI compounds have reached human clinical trials. Until now, QSI therapeutic studies for clinical QS-induced bacterial infections are still undergoing preclinical phases and have not been extensively developed. In total, there are only a few clinical trials on azithromycin (a QSI for preventing *P. aeruginosa* ventilator-associated pneumonia and treatment of cystic fibrosis) (Table 6) [112]. Some beneficial QSI effects, such as the ability to prevent biofilm formation, bacterial virulence, and innovative medical devices equipped with QSI molecules have attracted the most attention. Various inhibitors/agents capable of QS-controlled biofilm formation are ongoing clinical evaluations (Table 7) [112] and have been published with outcomes [113–118]. Moreover, QSIs were primarily designed for functionalizing catheters. The increasing range of QSIs fuel efforts to develop innovative medical devices and diversify their applications.

Unlike antibiotics, QSIs act by interfering with QS-mediated gene expression controlling virulence traits that are not necessary for bacterial growth, which probably produces a limited selective pressure to develop bacterial resistance. However, some studies have pointed out the risk of inducing selective pressure and developing resistance in QS disruption conditions by anti-QS agents [119,120]. However, the literature manifestly indicates that resistance to QS inhibition may emerge, but likely at a much lower level than that which conventional antibiotics could induce with their massive use. To date, there have been few reports regarding the potential QQ-related resistance mechanisms that various bacteria could induce to support this possibility, but it cannot be completely ignored.

At present, only a few QSIs were finely clarified for the anti-QS mechanisms supporting the related anti-QS activity at a subcellular level and functionally evaluated regarding their biological role in vivo. In this regard, QSIs may not be as active as antibiotics, and are in realistic conditions, which becomes a major bottleneck for their potential for antibacterial treatments to reach clinical trials. In some cases, QSIs have been described as having potential medicinal effects on bacterial communities in the human body [121]. The impacts on the hose microbiome were quite likely to influence human health and induce metabolic disturbance [122].

Overall, to prevent the negative influence on hosts and minimize the development of QSI resistance, some criteria for QSIs should be considered: 1) low molecular weight; 2) high stability and specificity; 3) no side effects on hosts and no interference with the host microbiome; and 4) unlikely to develop drug resistance.

7. Conclusions

Over the past two decades, numerous studies have reported that QS circuitry and potential QSIs have significant impact as anti-infective agents against a host of bacteria. The discovery of QSIs and their initial success represent a promising target for the design of novel anti-infective drugs. The discovery of QSIs is appealing in large part to expand the anti-infective therapeutic arsenal to complement classical antibiotics and antimicrobial agents. With regard to QSI discovery, some problems that need to be solved, including the QSI molecular target identification, the molecular targeting delivery, the QSI cytotoxicity evaluation at the organism, cellular, and molecular levels. These queries on the above points are certainly promising trails for further efforts to discover QSIs and diversify their applications.

CRediT author statement

Lan Lu: Project administration, Funding acquisition, Writing - Original draft preparation, Supervision; **Mingxing Li:** Investigation, Writing - Reviewing and Editing; **Guojuan Yi and Li Liao:** Conceptualization, Methodology, Software; **Qiang Cheng, Jie Zhu and Bin Zhang:** Conceptualization, Investigation; **Yingying Wang, Yong Chen and Ming Zeng:** Visualization.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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