Acyl-HSL signaling & Quorum Quenching: screening and design on a new frontier in antimicrobial action

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Contents

Abstract

Quorum sensing, the ability of many bacterial colonies and communities to modify gene transcription in a density-dependent manner, is the mechanism by which biofilm formation occurs. Biofilms, communities of bacteria surrounding themselves with a robust extracellular matrix, cause infections to be difficult to treat with antibiotics. The pressure is on to find alternative antimicrobial molecules for these pathogens. This work is a description of recent screening and design efforts to inhibit the most well characterized quorum sensing signaling system, which uses N-acylated homoserinelactones (acyl-HSLs) as signals. Acyl-HSL signal degradation enzymes such as Acyl-HSL acylases and lactonases are widespread in prokaryotes, and naturally occurring small molecule inhibitors are produced by many plants. As more acyl-HSL system homologues are elucidated, more inhibitory molecules are discovered, and more 3D structures are resolved, rational design and screening of quorum sensing inhibitors is rapidly being refined.

Introduction

In 1970, microbiologists were shocked to discover that bacteria could communicate with one another using signaling molecules[1]. This form of communication is based on the concentration of constitutively produced signals. At high enough cell densities, the signal molecules reach threshold concentrations that trigger auto-induction of production of these signals and the modulation of genes under the control of this communications system. This phenomenon is called quorum sensing (QS)[2], and is essentially a mechanism by which prokaryotes can modulate their gene transcription based on the size of their colony or community.

At first, only a small number of prokaryotic organisms capable of QS had been discovered, deemed curious outliers, however, over the span of three to four decades it has become clear that quorum sensing, if anything, is the norm rather than the exception in the microbial world[3]. Along with the plethora of QS organisms discovered, it was found that the functions that are under the control of QS vary greatly among species (See table 1), from bioluminescence to virulence. QS modulated virulence in human pathogens is often linked to biofilm formation.

Biofilms are comprised of aggregated communities of prokaryotes that are enveloped by a secreted hydrated matrix consisting of exopolysaccharides, DNA and proteins[4]. A biofilm is capable of adhering to diverse biotic and abiotic surfaces, and provides a protective, stable, sessile organization for its inhabitants. Biofilm formation triggered by quorum sensing is a common strategy employed in many environments, also by human pathogens. Some examples are cystic fibrosis dependent pneumonia, caused by *Pseudomonas aeruginosa*, where the organism colonizes the lungs of susceptible individuals (those suffering from cystic fibrosis) forming a biofilm[5], *Staphylococcus aureus* (wound infections) and *Escherichia coli* (urinary tract infections)[6].

The structural attributes of biofilms make biofilm mediated infections especially difficult to deal with.

One major factor is the fact that the biofilm matrix functions as a barrier for antibiotics[7]. The charged matrix traps and may even degrade antibiotic molecules. Although the extracellular matrix does not completely block antibiotic access, rate and degree penetration by antibiotics is reduced.[4]. This allows the bacteria extra time to respond by producing resistance factors upon recognition of the low dose of antibiotics that penetrates the biofilm. Additionally, the host's immune response is hindered by the formation of biofilms due to the obstruction of phagocytes[4]. In the face of these difficulties, the pursuit of QS inhibitors (QSI) has become not only fundamentally but also medically relevant.

An additional reason why the inhibition of QS is such an attractive line of enquiry is the idea that resistance as it pertains to antibiotics cannot occur[8]. Since antibiotics are aimed at killing cells, those cells that are resistant are provided with a larger environment to thrive in, leading quickly to the dominance of resistant strains. However, quorum sensing inhibition does not attempt to actually kill the target organism, but rather combat biofilm formation (or other QS functions). Therefore, the selective pressure on the target organisms will not be as potent as if it were a life-or-death pressure[7]. The organisms that are no longer capable of expressing their quorum induced virulence factors can still survive, but simply do not infect the host.

This text aims to outline the mounting knowledge on acyl-HSL dependent QS signaling, involved in pathogenic biofilm formation, and how it is being strategically applied to the discovery and design of quorum sensing inhibition mechanisms.

Table 1: Examples of the diversity of functions regulated by quorum sensing

Bacterial strain	Signal producer/ response regulator	Signal	Regulated function
Aeromonas hydrophilia	AhyI/AhyR	C4-HSL	Protease, biofilm development
Agrobacterium tumefaciens	TraI/TraR	3O,C8-HSL	Virulence plasmid (pTi) copy no. & conjugal transfer
Burkholderia cepacia	CepI/CepR	C8-HSL	Siderophore, protease
Pantoea stewartii	EsaI/EsaR	3O,C6-HSL	Exopolysaccharide
Pseudomonas aeruginosa	LasI/LasR, RhlI/RhlR	C4-& 3O,C12-HSL	Virulence & biofilm development, Virulence & rhamnolipids
Pectobacterium carotovorum	ExpI/ExpR, CarI/CarR	3O,C6-HSL	Exoenzymes, antibiotics
Pectobacterium chrysanthemi	ExpI/ExpR	3O,C6-HSL	Pectinases
Pseudomonas chlororaphis	PhzI/PhzR	C6-HSL	Phenazine
Rhodobacter sphaeroides	CerI/CerR	C14-HSL (C7=C8)	Aggregation
Vibrio anguillarum	VanI/VanR	3O,C10-HSL	NYI
Vibrio fischeri	LuxI/LuxR	3O,C6-HSL	Bioluminescence

Table 1: This table shows examples of quorum sensing dependent functions. All examples use acyl-HSL signaling. The signal producers (XxxI) are acyl-HSL-synthases, the response regulators (XxxR) are DNA binding proteins. Signal nomenclature: 3O indicates a carbonyl on the C3 of the acyl chain. C7=C8 indicates a double bond between C7 and C8 of the acyl chain. The 'X' in CX-HSL indicates the length of the acyl chain. Table adapted from Fuqua & Greenberg (2002)[3] and Uroz et al. (2009)[9]

Types of quorum sensing signals vary among organisms

In order to understand the mechanism behind the formation of biofilms and other QS effects with the aim of finding inhibition mechanisms, the signaling molecules responsible for bacterial cell-to-cell communication have been studied extensively. Contrary to initial expectations in the first works describing quorum sensing, a large amount of quorum sensing effects have been observed over a range of bacterial strains[3,10]. Lines of inquiry initiated by this realization include how different bacterial strains keep their 'conversations' private, or if there is cross-talk between strains[9]. To address this question, a large variety of signaling molecules responsible have been identified (Figure 1).

The two main types of signaling molecules responsible for quorum sensing are N-acyl homoserine lactones (acyl-HSL) among gram negative strains and oligopeptide signals among gram positive strains[11]. However, there are interesting outliers as well, such as PQS, a hydrophobic signal produced in *Pseudomonas*, and lipid based signals from organisms such as *Xanthomonas* and *Ralstonia*[9]. Acyl-HSLs, also being the first type of quorum signal discovered as mentioned previously, have been the most extensively characterized in structure and mechanism[3].

Figure 1. The diversity of QS signaling molecules

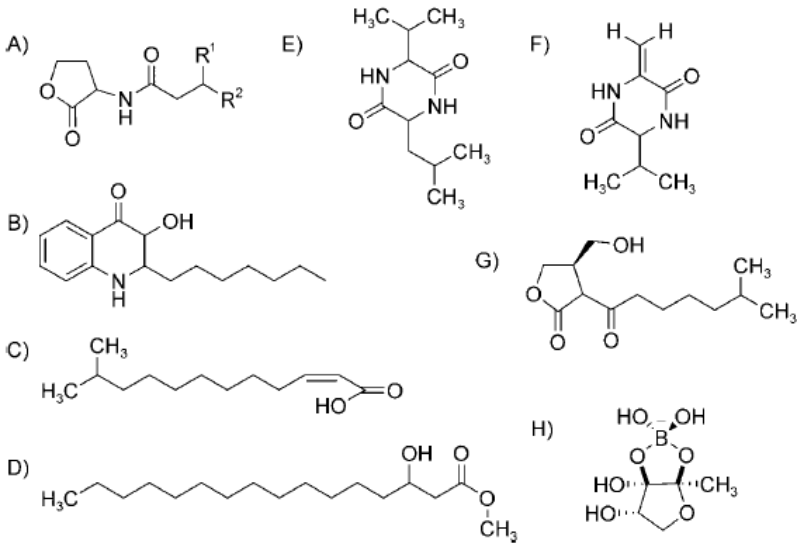

Figure 1. Structures of different signaling molecules involved in QS. A) General acyl-HSL structure, where R1 can be a hydrogen, carbonyl or hydroxyl group, R2 is the variable carbon chain; B) PQS from *Pseudomonas*; C) & D) Lipid based signals from *Xanthomonas* and *Ralstonia*respectively; E) & F) Peptide signals from *Pseudomonas*; G) γ-butyrolactone from Streptomyces; H) AI-2 from *Vibrio harveyi*. Figure adapted from Uroz et al. (2009)[9]

Oligopeptide mediated quorum signaling is common among gram positive strains such as *streptococcus, staphylococcus* and *bacillus*. The oligopeptides are generally recognized by membrane bound histidine kinases that modulate gene transcription through a two-component type signal transduction[11]. An example of such a signal is the 'auto-inducing peptide' AIP produced by the previously mentioned quorum modulated pathogenicity of *Staphylococcus aureus*[10], or the peptide based signals found in

Pseudomonas (Figure 1 E&F).

A unique signaling system among gram negatives is that of various *Streptomyces* strains (Figure 1 G). The filamentous soil dwelling *Streptomyces* synthesizes acylated lactones known as γ-butyrolactones. This signaling system has been show to modulate antibiotic production and morphological differentiation[1011]. Contrary to the signal molecules described above that have structural variation between species, there has been mounting evidence pertaining to an interspecies quorum signal. A furanosyl borate diester named autoinducer 2 (AI-2) produced by a variety of marine bacterial strains has been shown modulate bioluminescence in *Vibrio harveyi* [13], as well as be recognized by other organisms. It is possible that these bacteria use the signal to assess the competition in their environments [3].

Acylated homoserine lactones, the signal of the well defined LuxI-LuxR system

The predominant gram negative quorum signaling molecules are acyl-HSLs synthesized by a wide variety of proteobacteria. Acyl-HSLs are structurally (Figure 1A) comprised of a homoserine lactone connected by an amide group to an acyl chain, and can permeate the cell envelope to enter and exit the cell[11]. The acyl chain is subject to a large amount of variation between acyl-HSLs produced by different organisms, for instance, the presence of unsaturated carbon-carbon bonds in the chain and a carbonyl group or a hydroxyl group on the third carbon (Table 1)[14]. The first acyl-HSL signaling system found in the previously mentioned *Vibrio fischeri* was named the LuxI-LuxR system (Figure 2), the mechanism of which is an example of how acyl-HSL signals are transduced[1].

Figure 2. The LuxI-LuxR system

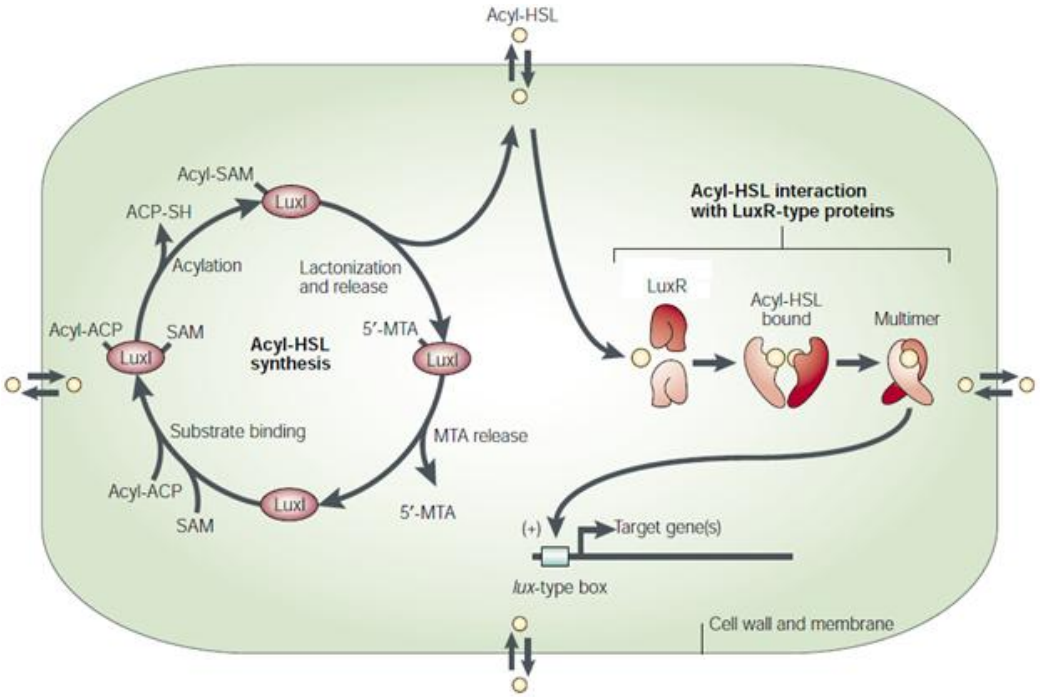

Figure 2. Signal transduction of acyl-HSLs as observed in *Vibrio fischeri*. Acyl-HSLs are shown passing through the membrane. Acyl-HSLs are shown interacting with response regulator LuxR, allowing LuxR to multimerize and bind upstream of the target genes of this system, which include *luxI*. The synthesis of acyl-HSLs by LuxI using SAM and acyl-ACP as substrates is shown in the left of the figure. Figure adapted from Fuqua & Greenberg (2002)[3].

In the LuxI-LuxR system, LuxI is the protein that synthesizes the acyl-HSL named auto-inducer 1 in this organism (AI-1). LuxR is the response regulator that, upon interaction with AI-1, activates expression of the Lux operon whose products are responsible for autoinduction of *luxI* and bioluminescence in *Vibrio fischeri*. There are many homologues of LuxI and LuxR in other organisms that have a similar function to activate (or in some cases, repress) those organisms' quorum effects[3].

The proteins responsible for synthesizing acyl-HSL mostly share high structural similarity, and are named LuxI-type proteins. There are also, however, AinS type proteins, which can use different acyl donors to LuxI-types and have no sequential similarity to LuxI-types[3]. The precursors to an acyl-HSL are an acyl group provided by carrier protein ACP and S-Adenosyl methionine (SAM). It is the recognition of different acyl-ACP precursors that defines the specificity of each LuxI/AinS type protein[3]. This specificity and the fact that these proteins utilize SAM in a unique manner compared to other SAM dependent proteins, provides opportunity for the screening for or design of specific anti quorum sensing molecules.

LuxR, the response regulator of the LuxI-LuxR system, and its homologues, have been shown to form oligomers of different orders (depending on the organism) in the presence of the appropriate acyl-HSL that associate with DNA. They have two distinct domains: conserved amino acid residues in the N-terminal domain are responsible for HSL binding, and the C-terminal domain has a helix-turn-helix motif responsible for binding DNA in its major groove[3]. The structural and mechanistic information gathered on these types of proteins are valuable targets for inhibition of quorum sensing.

Inhibition strategies for acyl-HSL based signaling

Antimicrobial action through the inhibition of quorum sensing is being sought after through exploitation of structural knowledge of LuxI-LuxR type systems described above. There are various lines of inquiry that can be pursued by screening techniques and rational drug design[8]. They can be broadly put into two categories. The first is degradation of acyl-HSLs, aimed at stopping them from reaching concentrations required to activate quorum sensing systems[9,15]. The second is inhibition of LuxR-type and LuxI-type proteins to block signal reception and production.

Acyl-HSL degrading enzymes

Proteins that can prevent the accumulation of acyl-HSLs are widespread among both prokaryotes and eukaryotes[8]. These come in the form of enzymes that degrade the lactone ring, called acyl-HSL lactonases, or hydrolyze the amide bond connecting the lactone ring to the acyl chain called acyl-HSL acylases[9,13] (Figure 3). Proteins that modify the acyl chain also exist, although so far their natural function has only been identified as part of the construction of specific acyl-HSLs[9].

Figure 3. Two main types of enzymatic acyl-HSL degradation

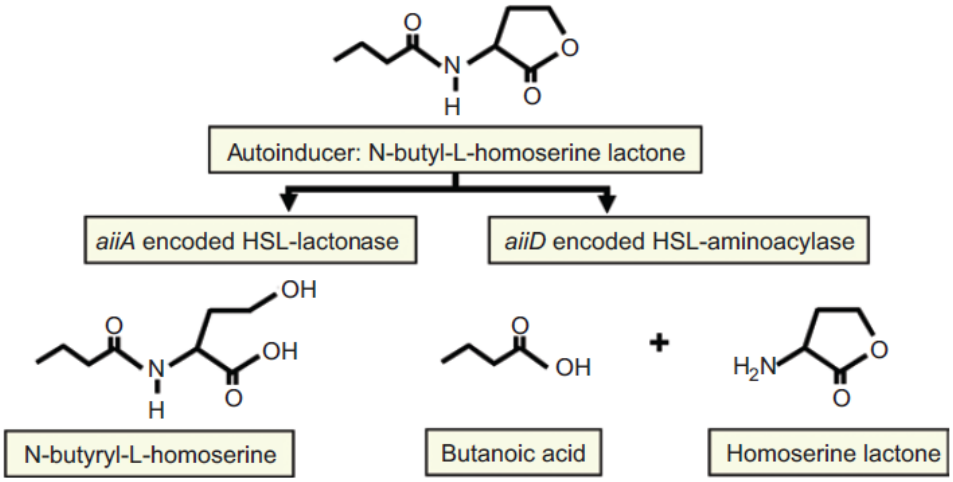

Figure 3. Illustrated are the two main types of enzymatic degradation reactions of acyl-HSLs . acyl-HSL acylases chydrolyze the amide bond connecting the acyl chain to the lactone ring, whereas acyl-HSL lactonases open the lactonase ring. Figure adapted from Kalia et al. 2011[13].

There are two major classes of lactonases, both of which are zinc dependent metalloenzymes. One Acyl-HSL lactonase, AiiA, is present across the *Bacillus* genus[9]. AiiA has a wide range of acyl-HSL targets, and many homologues of AiiA have been characterized from other prokaryotic sources, such as *Argobacterium* and other proteobacteria. The second acyl-HSL lactonase class, QsdA-types, is unrelated to AiiA-types and occurs only in the *Rhodoccocus* genus.

Acyl-HSL acylases, first discovered in *Variovorx paradoxus*, occur in many proteobacteria[13]. Overall these enzymes are more effective at degrading acyl-HSLs with long acyl chains. The variety of targets for acyl-HSL acylases is larger than that of lactonases, due to the fact that the acyl chain is being targeted, allowing for more substrate specificity in terms of the acyl chain length and substituents[13].

One of the possible evolutionary roles for these naturally occurring acyl-HSL degrading enzymes is that they perform a regulatory role within the organism's own signaling system, since some bacteria (such as *Argobacterium tumefaciens)* produce enzymes that degrade their own acyl-HSLs[9]. Another interesting possibility is that bacteria produce acyl-HSL degrading enzymes to disrupt QS signaling of competing bacteria[9]. Acyl-HSL lactonases with a distinct calcium dependent mechanism have also been found to be present in a range of eukaryotes, including in mammal serum, such as humans, mice and bovines, which may have evolved these enzymes for the purpose of combating QS bacteria[16].

Since acyl-HSL lactonases and acylases are found in many cell types that are routinely exposed to bacteria that use quorum sensing, there is a rich source to screen from[9] (Table 2). New acyl-HSL degradation enzymes are being discovered through environmental metagenomics analysis. For instance, metagenomics analysis of pasture soil samples in France[17] yielded a novel acyl-HSL degrading enzyme, QlcA. Although it is not known whether this is the main function of the enzyme, expression in a *Pectobacterium carotovorum* host showed inhibition of the host's QS mediated virulence towards potato tubers. Additionally, phylogenetic analysis suggests that this protein is related to already characterized acyl-HSL lactonases.

Table 2: Examples of acyl-HSL degredation enzymes

Organism	Enzyme, Gene	Targets
Acidobacteria	acyl-HSL-lactonase, *qlcA*	C6HSL,C7HSL,C8HSL
Anabaena (Nostoc) sp.PCC7120	acyl-HSL-acylase, *aiiC*	C10HSL, OC10HSL, OHC10HSL, OC12HSL, OHC12HSL, C14HSL, OC14HSL, OHC14HSL,
Bacillus sp.	acyl-HSL-lactonase, *aiiA*	C(4/6/8)HSL, 3OC(4/6/8/10/12)HSL, 3OHC4HSL
Klebiella pneumoniae	acyl-HSL-lactonase, *ahlK*	C6HSL, 30C6HSL, C7HSL, C8HSL
Rhodococcus erythropolis	acyl-HSL-lactonase, *qsdA*	C(6-14)HSL, with or without 3O/3OH substitution, acyl-HSL analogues (aromatized acyl chain, lack of lactone ring)
Ralstona solanacearum	acyl-HSL-acylase, *aac*	C(7/8/10)HSL, 3OC8HSL
Streptomyces sp. strain M664	acyl-HSL-acylase, *ahlM*	C(8/10/12)HSL, low activity on shorter acyl chains

Table 2: A selection of bacteria that produce acyl-HSL lactonases/acylases, their genes and target specificities. Table adapted from Kalia et al. (2011)[13] and Uroz et al. (2009)[9]

Inhibition of LuxI -type proteins

Inhibition of LuxI-type proteins can be approached by designing small molecules that compete with the precursors of acyl-HSLs and either inactivate the protein or lead to the synthesis of acyl-HSL-like products that are not biologically active[18]. The main issue with this approach is designing small molecules that specifically inhibit LuxI-type proteins, since the precursors SAM and acyl-ACP participate in a wide range of biological functions, which could very well be present in either beneficial bacteria or eukaryotic cells. However, the mechanism of SAM utilization by LuxI-types, as mentioned before, is unique among SAM dependent proteins[3].

Since the binding of LuxI-type proteins to their substrates has not been elucidated to great detail, rational design for inhibitors is hindered. However, screening can yield such an inhibitor. Two Acyl-HSL analogues produced from a library by Chung et al. (2011) were found to function as quorum sensing inhibitors in *B. glumae*, which has its main virulence factor involved in rice grain rot under the control of quorum sensing [19]. The library consisted of 55 derivatives of previously characterized quorum sensing

inhibitors. After its identification, one of acyl-HSL analogue hits was shown to interact with LuxI-type protein from *B. glumae*, TofI, through x-ray crystallographic analysis. The molecule occupies the space where the acyl donor is recognized by TofI.

Inhibition of LuxR type proteins

Inhibition of LuxR-type proteins can be approached by designing small molecules that mimic specific acyl-HSLs yet in some manner do not induce the correct conformational changes to allow LuxR-types to modulate transcription[9]. Some acyl-HSLs have been shown to inhibit quorum sensing functions of certain organisms (*C. violaceum, V. fischeri, A. tumefaciens*), possibly through competitive inhibition of LuxR-type proteins. A range of small molecules have been found to function in a similar way from various bacterial and plant sources[9].

The variety of LuxR-type proteins and the lack of characterization of structure-binding relationships between them and their ligands make it difficult to rationally design inhibitors based on that interaction[18]. Focus has been on approaching the problem by making analogues of known acyl-HSLs or any naturally occurring quorum sensing, supplemented with structural information for the specific LuxR-type protein in question if available (in recent years more and more X-ray crystal structures have been elucidated for LuxR-type proteins[20]), to construct large or more focused libraries of candidates to screen[18]. The screening process involves either reporter gene assays[21] or observation of phenotypic quorum effects[20]. Examples of reporter gene assays include plasmid constructs in which a lethal protein is under the control of a QS promoter, or alternatively, QS mediated repression of an antibiotic resistance gene. Cells transformed with these constructs will only be able to grow if an effective QSI is present at appropriate concentrations[21]. An example of a phenotypic assay is the measurement of colored or chemically active virulence factor production in *Pseudomonas aeruginosa* when exposed to QSIs[20].

An example of rational design of small quorum sensing inhibitor molecules is the synthesis of furanone derivatives [22]. These furanone derivatives were designed to have structural similarities to Patulin. Patulin is a LuxR-type protein inhibitor produced by *Penecillium* strains, which have been shown to inhibit quorum sensing in *P. aeruginosa*[22]. Six of these small molecules were found to inhibit quorum effects in *P. aeruginosa*. The mechanism of inhibiton was explored using molecular modeling techniques.

The alteration of the acyl chain moiety in acyl-HSLs has produced many insights[20] into binding selectivity with LuxR-type proteins. Mattmann et al. (2010) found that the acyl chain length and its flexibility are both highly important in the binding between an acyl-HSL and LasR. They formed a library of possible QSIs by constructing analogues of known acyl-HSLs with modified acyl chains (Figure 4). Screening efforts have yielded analogues that inhibit quorum sensing in one system (QscR in *Pseudomonas aeruginosa*) yet leave others unmodulated (LuxR). In a later study the hits of this library were used to determine possible structure-activity relationships to aid the construction of a second generation library to screen[23]. For instance, the bulkiness of acyl chains and the presence of aromatic groups contributed to antagonistic effects on QscR.

Figure 4. Screening of acyl chain modifications to produce inhibitory acyl-HSL analogues

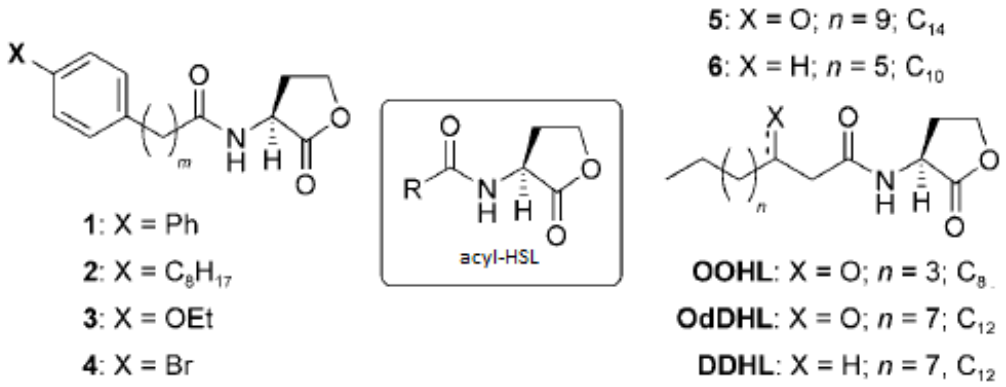

Figure 4. The permustations of the acyl chain of acyl-HSL studied by Mattmann et al. (2011)[23]

Persson et al. (2005) reported designing new quorum sensing inhibitors based on LuxR-acyl-HSL binding mechanisms and structural insights provided by natural quorum sensing inhibitors from garlic[18]. In pursuit of an inhibitor for the LasR protein, involved in controlling the virulence of *P. aeruginos,* the homologue TraR from *A. tumefaciens* provided the structural information on which the design was based, since although LasR and TraR do not share much sequence similarity, the amino acid residues responsible for ligand binding are conserved[18]. A noteworthy approach to designing inhibitory acyl-HSL analogues is the replacement lactone group of acyl-HSLs with a thiolactone[24]. This modification has been shown to swap agonistic effects to antagonistic effects on *P. aeruginos* quorum sensing and vice-versa for both natural and synthetic acyl-HSLs.

Discussion & perspectives

When one considers the advantage of quorum inhibition over antibiotics in terms of evolutionary pressures to develop resistance, one may wonder whether bacteria can not just evolve resistance to quorum sensing inhibitors. Although this cannot be dismissed, it must be noted that since quorum sensing is a 'group effort', a mutant with for instance different acyl-HSL specificity/synthesis would have to grow to high densities in order to express quorum dependent genes. If we are considering pathogenic bacteria, this means that all the quorum inhibition sensitive organisms are still alive competing with the resistant mutants for resources in the host for example[8].

One thing to note, before eventual implementation of QSIs (enzymes or small molecules) to combat biofilm based infections, is the problem of beneficial bacteria and their possible sensitivity to quorum sensing inhibition[25]. This could prove to be a major drawback of using QSIs clinically, and is one of the lines of inquiry that must be pursued should QSIs become the new antimicrobial standard.

The progress in the field of QSI design is bottlenecked by 3D structure acquisition of both the signal synthesis proteins and response regulator proteins. As more of these types of proteins are characterized through X-ray crystallography, the ability to rationally examine active sites and binding pockets will

increase. This will allow the scientific community to design more numerous and more specific inhibitory molecules that are tailored for inhibition of specific acyl-SHL dependent QS functions. In terms of enzymatic degradation, the 3D structure elucidation of acyl-AHL acylases and lactonases opens up the possibility of protein engineering/improvement.

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