Characterization of a novel quorum quenching enzyme and determination of autoinducer signal receptor specificity

A Thesis
SUBMITTED TO THE FACULTY OF THE
UNIVERSITY OF MINNESOTA
BY

Nathan D. Hoekstra

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE IN MICROBIAL ENGINEERING

Professor Mikael H. Elias, Advisor
Dr. Romas Kazlauskas and Dr. Michael Freeman, Committee Members
February 2021

Acknowledgements

I am grateful for the help of my advisor, Prof. Mikael Elias, who gave me an opportunity to work in his lab and learn from him. I've learned much regarding proteins from him, but additionally I've gained valuable skills under his guidance in areas such as scientific writing, literature review, and presentation of information in front of audiences. I know these skills will be useful tools no matter what field I pursue. I appreciate his patience and good-natured support.

I would also like to thank my colleagues in the Elias lab, namely Dr. Celine Bergonzi, Dr. Sudipta Shaw, Dr. Rakesh Sikdar, and Dr. Kathleen Mahan for their valuable input and their service as role models to an aspiring researcher. In addition, I collaborated with many people outside of the lab, including Lambros Tassoulas, Kathryn Crone, and Fredarla Miller and I benefited from their help and guidance.

I would like to dedicate this thesis to my parents, Nancy and Stephen Hoekstra, for telling me that graduate school was a good idea, and for their constant encouragement.

Nathan Hoekstra, Minneapolis, October 10, 2020

The work in this Thesis was supported by grants from Sea Grant Minnesota and BTI-MnDRIVE.

Abstract

Many bacterial species exhibit cell-density dependent traits that are most advantageous when expressed simultaneously throughout their population. Examples of these traits include the production of enzymes used to dismantle the defenses of a host organism, the formation of protective biofilm, and bioluminescence, among others. Detection of the required threshold cell density (a quorum) relies on chemical communication via the production of a variety of small molecule autoinducers such as *N*-acyl homoserine lactones (AHLs). This communication system is known as quorum sensing (QS), and its specificity depends on the ability of different bacteria to produce and sense distinct AHL molecules. In this work I focused on (i) the specificity of AHL receptors and (ii) the characterization of a novel enzyme to interfere with QS.

Specifically, I investigated 4 receptors from different bacteria, and report their preference for range of AHLs. In particular, combined with a literature survey, my data suggest that receptors have broader AHL preference than what is typically reported. Additionally, I show that one of these receptors can bind and respond to lactone signals other than AHLs.

Some bacterial enzymes can interfere with QS through the degradation of autoinducers, quorum quenching (QQ) the expression of the behaviors regulated by QS. The discovery of new enzymes capable of QQ is important to the development of fine-tuned control of bacterial communication and behaviors and can give hints to the biological importance of microbial signaling. Here we report the characterization of a novel quorum quenching enzyme, ZHD, from the fungi *Clonostachys rosea*, suggesting that interference in QS extends beyond bacteria. We found that ZHD is a broad spectrum AHL lactonase with k_{cat}/K_M values in the range of 10^4 to 10^5 $M^{-1} \cdot s^{-1}$. ZHD hydrolyzes AHLs of various acyl chain lengths as evidenced by pH indicator assay measurements, biosensor measurements and mass spectrometry data. Analysis of ZHD bound to C8-AHL allowed for the identification of the substrate binding mode and proposal of a catalytic mechanism. ZHD is likely a novel representative of the α/β-hydrolase family of lactonases.

Altogether, these results, i.e. the demonstration that AHL receptors that can respond to lactones other than AHLs and the identification of a quorum quenching enzyme from fungi, are evidence that the level of cross communication may not be limited to AHL-producing bacteria.

Contents

List of Figures

List of Tables

Chapter 1

1.1 Thesis Organization

Thesis Outline

Chapter 1 will introduce the topic of quorum sensing and will provide a summary of quorum sensing systems used by some bacteria, while focusing on the quorum sensing systems of the gram-negative bacteria *Pseudomonas aeruginosa*. There will also be a summary of strategies used to interfere with quorum sensing, with a focus on enzymes capable of degrading small communication molecules.

Chapter 2 will introduce different receptors used in quorum sensing systems and provide insight into their specificity towards their substrates. There will also be a discussion of my work examining the possible broad substrate specificity of some of these receptors towards small molecules not produced by species with those receptors, pointing towards a possible role in interspecies communication.

Chapter 3 will be presented mostly in a journal article format, as most of this chapter will form the basis for a paper to be published in the future regarding the characterization of a novel enzyme capable of degrading small molecules used in quorum sensing.

Notational Conventions

N-acylhomoserine lactones are here referred to as either AHLs or HSLs. The number of carbons in the acyl chain are designated using a number following “C”. For example, N-butyryl-homoserine lactone, with a 4 carbon acyl chain, is referred to as “C4-AHL” or “C4-HSL”. AHLs with an additional oxygen group at the third carbon of the acyl chain are referred to as 3-oxo-AHLs: for example, “3-oxo-C12-AHL” or “3OC12AHL”.

1.2 Introduction

Certain behaviors exhibited by microbial populations provide a greater benefit to the members of that population when expressed above a threshold population density[1]. Microbes thus have developed ways to communicate with one another, including the secretion of chemicals and electrical signaling through depolarization of their cell membranes[2]. Bacteria secrete a variety of small molecule signals, and as the density of the population increases, the concentration of these small molecules also increases. Some small molecules known as autoinducers upregulate their own production. After reaching a threshold density, these autoinducers are involved in regulating the behaviors of the population. Autoinducer detection, production, and modulation of behavior related to cell density are collectively known as quorum sensing (QS)[3].

Many QS regulated behaviors have negative impacts on a host organism. For example, the opportunistic pathogen *Pseudomonas aeruginosa* produces an extracellular biofilm partly due to the influence of two of its QS systems. This biofilm confers greater resistance to antibiotics and disinfectant chemicals, making biofilm disruption an important medical consideration[4]. Another pathogenic QS regulated behavior is adhesion to the surface of host cells via pili and flagella in *Salmonella, Vibrio,* and *E. coli,* and via production of rhamnolipids in *P. aeruginosa*[5]. In Gram-negative bacteria, increased resistance to antibiotics has been linked to QS regulated modifications of bacterial cell membranes[6]. *P. aeruginosa* also utilizes QS regulated production of a variety of phospholipases, elastases, and proteases to inactivate components of a host's immune response[7].

The first behavior studied in connection to quorum sensing was bioluminescence from the symbiotic gram-negative bacteria *Vibrio fischeri*, which colonizes the light organs of Hawaiian bobtail squid, *Euprymna scolopes*. Although individual *V. fischeri* luminesce to a lesser degree when present at low cell density, when present above a threshold density luminescence is greatly increased. Luminescence from the light organs obscures the shadow of *E. scolopes* in the water column providing it some camouflage from other organisms in the environment[8].

The QS systems in *V. fischeri* have several characteristics common to QS regulated gene expression in bacteria:

(1) an autoinducer molecule

(2) a transcription regulatory protein

(3) a synthase capable of producing more of the autoinducer

(4) interplay between different QS systems involving different synthases and autoinducers

A variety of autoinducers are used in quorum sensing systems and can be categorized based on their structure and whether they are produced by either gram-negative bacteria, gram-positive bacteria, or both. The first type of autoinducers (AI-1) are *N*-acyl homoserine lactones (AHLs, or HSLs) produced by gram-negative bacteria such as *P. aeruginosa* and vary in both the length of the acyl chain and in the oxidation state of the third carbon on that chain[9]. Autoinducing peptides (AIPS) consist of 5-10 amino acids and are produced by gram-positive bacteria. Dihydroxypentanedione (AI-2) is produced by both gram-negative and gram-positive bacteria. Autoinducers also include the *Pseudomonas* quinolone signal (PQS), and γ-lactones (**Figure 1**)[10].

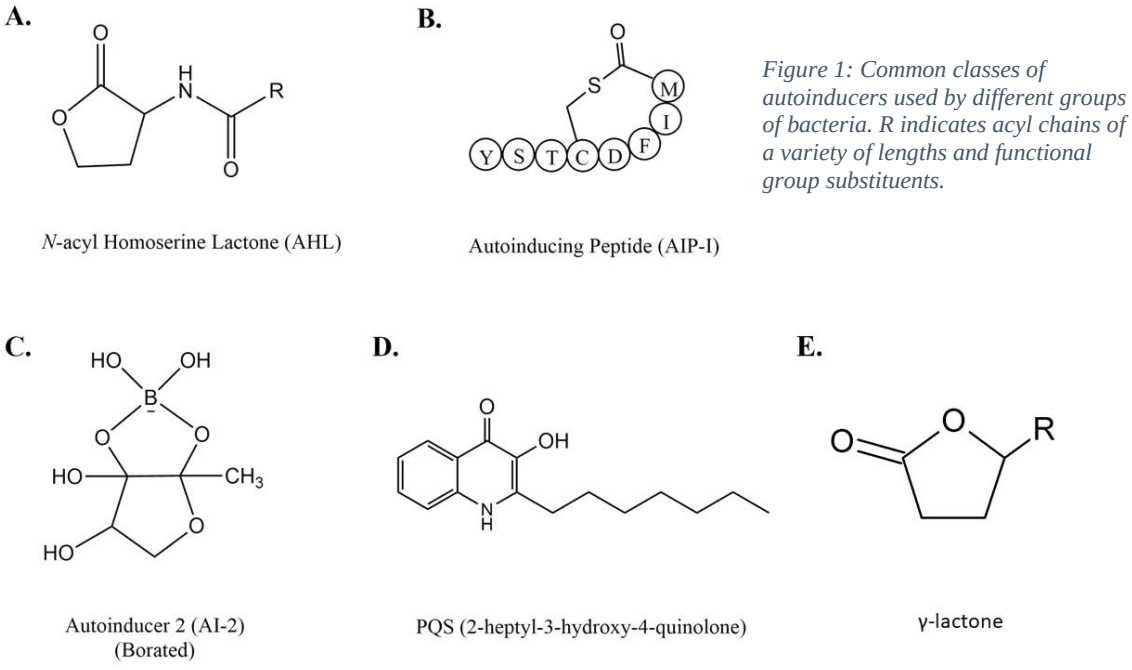


Figure 1: Common classes of autoinducers used by different groups of bacteria. R indicates acyl chains of a variety of lengths and functional group substituents.

1.3 Quorum sensing in gram-negative bacteria using homoserine lactones

Quorum Sensing in Vibrio fischeri *and* Agrobacterium tumefaciens

In *V. fischeri*, colonization and luminescence within the light organs are regulated by two QS systems involving two different autoinducers[11]. At low cell densities the repressor LuxO actively inhibits expression of LitR, a transcription factor that upregulates LuxR production, thus indirectly repressing luminescence (**See Figure 2**). At intermediate cell densities the concentration of N-octanoyl homoserine lactone (C8-HSL) increases due to production by the synthase AinS. A threshold concentration of C8-HSL has two effects. First, C8-HSL inactivates LuxO, allowing production of LitR and upregulation of LuxR. Secondly, C8-HSL can bind directly to LuxR, activating it and allowing it to bind to a promoter upregulating expression of the *lux* operon, including expression of LuxI. LuxI produces *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL), which binds to and more strongly activates LuxR. At high cell densities, a threshold concentration of LuxR bound 3-oxo-C6 HSL is reached, and the *lux* operon is strongly upregulated, leading to increased luminescence. 3-oxo-C6 HSL thus acts as an autoinducer by increasing its own production via binding to LuxR[12]. In this fashion, *V. fischeri* expresses colonization and luminescence behaviors in a stepwise fashion as it reaches specific cell densities.

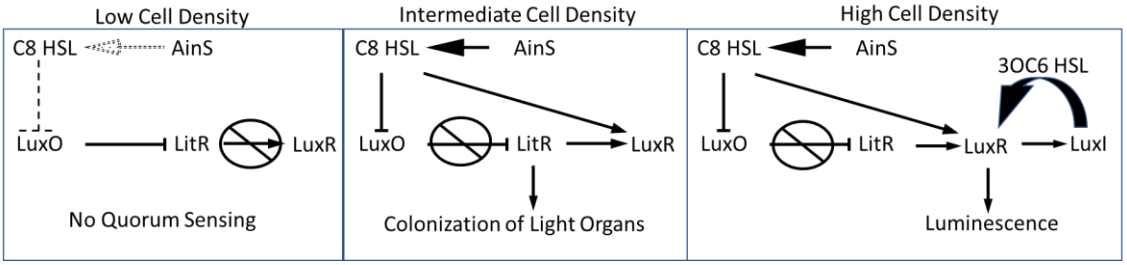


Figure 2: Stepwise activation of quorum sensing regulated behaviors by autoinducers produced by the AinS and LuxI synthases

AHLs are generally produced by synthases homologous to LuxI, and have been discovered controlling quorum behaviors in a variety of species.[13] In *Agrobacterium tumefaciens* the gene encoding a LuxI homologue, TraI, resides on the tumor inducing (Ti) plasmids. These plasmids contain genes required by *A. tumefaciens* for insertion of a segment of transfer DNA (T-DNA) directly into host plant cell nuclei. The expression of the inserted DNA causes the formation of

tumors and production of opine chemicals that the infecting bacteria can use as a nutrient source[14]. TraI synthesizes *N*-3-oxo-C8-octanoyl homoserine lactone (3-oxo-C8-HSL), which binds to and stabilizes a LuxR homologue, TraR, also encoded on the Ti plasmid. TraR upregulates genes related to replication of the Ti plasmid, transfer of that plasmid to other bacteria via conjugation, and transcription of TraI[15].

Quorum Sensing in Pseudomonas aeruginosa*: a model organism*

P. aeruginosa is a common cause of respiratory infections[4] and a highly studied model organism utilizing three characterized QS systems, each with their own autoinducer, autoinducer synthase, and transcriptional regulator. Two of these systems use a LuxI/LuxR synthase/regulator pair homologous to the TraI/TraR pair found in *A. tumefaciens.* These are the LasI/LasR and RhlI/RhlR

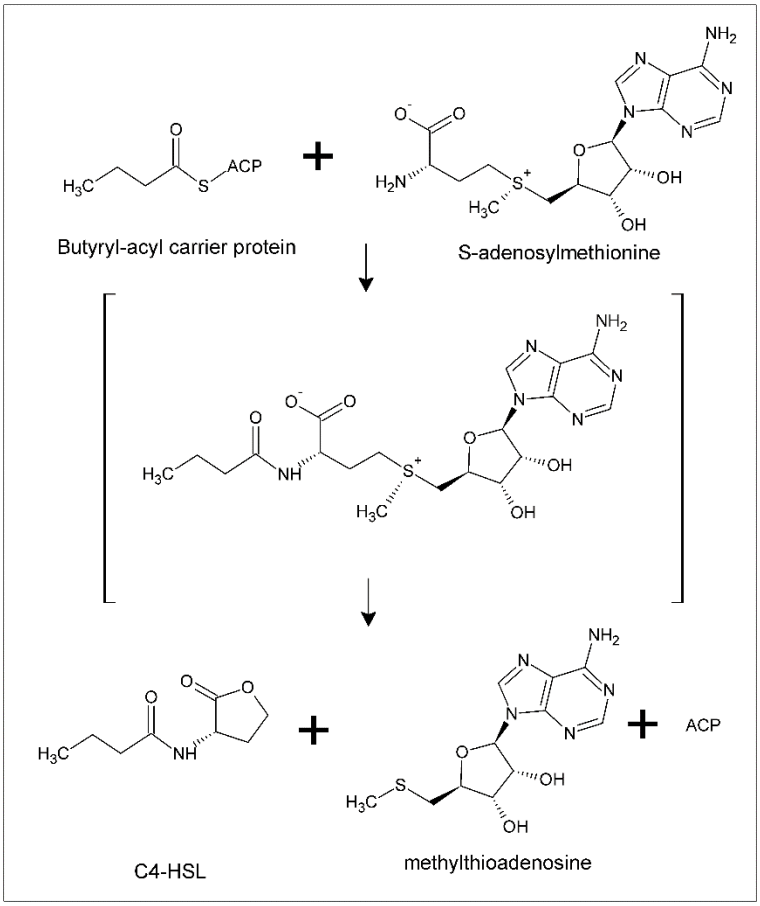


Figure 3: C4-HSL synthesis. C4-HSL is synthesized via the enzyme catalyzed transfer of an acyl group from a carrier protein to the amine group of S-adenosylmethionine. This transfer is then followed by the cyclization of the intermediate into the lactone product. The acyl carrier protein and methylthioadenosine are then released.

systems. LasI produces *N*-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL), while RhlI produces *N*-butyryl-L-homoserine lactone (C4-HSL), which bind to LasR and RhlR respectively. The larger binding tunnel of LasI permits binding to the long chain acyl-ACP construct, relative to the more restrictive structure of RhlI[16]. 3-oxo-C12-HSL-bound LasR is activated and will bind to *las* promoter boxes, while C4-HSL-bound RhlR will bind to *rhl* promoter boxes[17]. RhlI catalyzed production of C4-HSL from *S*-adenosylmethionine (SAM) has been characterized (**Figure 3**), and a similar reaction appears to be carried out by other LuxI homologues[18]. AHL-bound LasR and RhlR complexes regulate transcription of more than 300 genes in the *P. aeruginosa* genome[7].

An additional QS system of *P. aeruginosa* is the *Pseudomonas* quinolone signal (PQS) system[1]. PQS, or 2-heptyl-3-hydroxy-4-quinolone, is chemically distinct from AHLs used in systems with

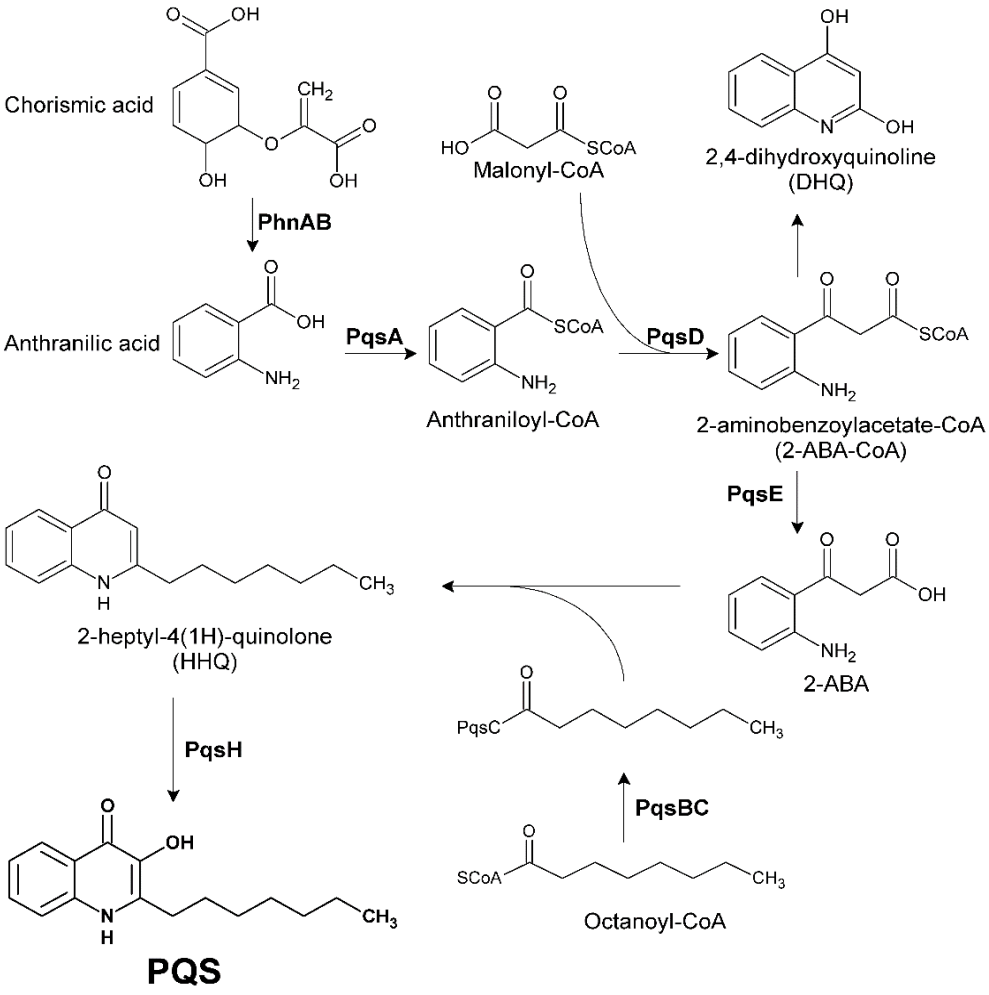


Figure 4: PQS biosynthesis pathway

a LuxR homologue, such as LasR or RhlR. PQS binds to and activates PqsR (previously known as MvfR), a transcriptional regulator that upregulates the *pqsABCDE* operon, *phnAN*, and *pqsH* leading to increased production of PQS[19]. Enzymes involved in PQS biosynthesis include PqsABCDE, PhnAB, and PqsH. Production of 2-*N*-alkyl-4(1H)-quinolones (AQs) such as PQS and its precursor 2-heptyl-4(1H)-quinolone (HHQ) begin with PhnAB-catalyzed synthesis of anthranilic acid, derived from the chorismic acid pathway (**Figure 4**)[20]. Biosynthesis from anthranilic acid involves the anthranilate-coenzyme A (CoA) ligase (PqsA), a heterodimer PqsBC that couples 2-aminobenzoylacetate (2-ABA) to an octanoyl moiety to produce HHQ, and an enzyme that has been proposed to form 2-aminobenzoylacetyl-CoA from anthraniloyl-CoA and malonyl-CoA (PqsD). PqsE is involved in limiting formation of side products of the AQs synthetic pathway, upregulating production of 2-ABA[21]. Addition of a hydroxyl group to HHQ, carried out by PqsH, gives the final PQS product[22].

Integrated Quorum Sensing System

A controversial proposed component of *P. aeruginosa* QS is the Integrated Quorum Sensing (IQS) system[1]. This system uses a unique autoinducer, 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde, or aeruginaldehyde[23], production of which is stimulated under low phosphate conditions. Upon discovery of this system, it was reported that a set of enzymes involved in the production of L-2-amino-4-methoxy-trans-3-butenoic acid (AMB), an antimetabolite that acts to repress plant seed germination[24] may be involved in the production of the IQS signal[25]. The AMB synthesis pathway involves the genes of the *ambABCDE* operon, and it was reported that Δ*ambBCDE* (but not Δ*ambA*) mutants expressed lower levels of PQS and C4-HSL used in the RhlI/R QS system but had no effect on the LasI/R system. PQS and C4-HSL production could be restored by adding the IQS signal molecule. Virulent behaviors such as pyocyanin production, generally controlled by QS systems, were also downregulated in Δ*ambBCDE* strains, and could be restored by addition of IQS. This was interpreted this as evidence that *ambBCDE* were involved in production of the IQS signal, not AMB as previously reported[25].

Later work both revised the initial structure of the IQS signaling molecule and demonstrated that while aeruginaldehyde is involved in QS, it is most likely produced as a side product during the synthesis of a siderophore, pyochelin[23]. Other researchers however, have shown that knockouts of the *amb* and *pch* operons (for AMB and pyochelin synthesis, respectively) show no significant change in production of PQS, C4-HSL, or 3-oxo-C12-HSL, indicating that if these gene clusters

are important in IQS signal production, the IQS system has little to no interaction with other QS systems[26]. These mutants also showed no change in the regulation of *phz1* or *phz2* gene clusters involved in phenazine production (a precursor to pyocyanin)[26]. In short, while the signaling molecule of the IQS system has been identified as aeruginaldehyde, its synthesis[27], effects[26], and receptor[1] remain controversial and/or require further elucidation, as evidenced by conflicting literature reports on the subject[7].

1.4 Quorum sensing regulated behaviors of *Pseudomonas aeruginosa*

A wide variety of *P. aeruginosa* behaviors are regulated via QS systems (See **Table 1**), including motility, membrane permeability, antibiotic resistance, adhesion, biofilm production, enzyme and toxin production, and nutrient uptake. Motility and adhesion of *P. aeruginosa* are controlled through the production of rhamnolipids[28], which may have additional roles as surfactants and antimicrobial agents[29]. Production of rhamnolipids appears to under the direct control of the RhlI/RhlR QS system, but is also is indirectly controlled by LasR and PqsR[29].

Table 1: QS regulated behaviors of P. aeruginosa

Regulator Protein	Affected Regulatory Protein or Behavior	Regulated Genes	Response	Effect	Ref.
LasR, RhlR, PqsR	Biofilm Production		Formation of mature biofilms with exopolysaccharide matrix	+	[1]
LasR, RhlR	Elastase B Production	*lasB*	Proteolytic activity against host	+	[17]
LasR	Alkaline Protease Production	*aprA*	Proteolytic activity against host	+	[17]
LasR	Exotoxin A Production	*toxA*	Host cell death	+	[17,30]
RhlR	Rhamnolipid Production	*rhlABR*	Motility and adhesion	+	[17]
RhlR	Hemolysin Production		Lysis of cell membranes	+	[17]
LasR, RhlR, PqsR	Pyocyanin (phzABCDEFG, phzM)	*phzABCDEFG phzM*	Increase of oxidative stress on host cells	+	[17,31]
LasR, RhlR	Elastase A	*lasA*	Proteolytic activity against host	+	[31]
PqsR	Lectins LecA and LecB	*lecA, lecB*	Adhesion to host mucosa and cytotoxicity against host respiratory epithelia, biofilm formation	+	[31,32]
PqsR	Outer Membrane Vesicle (OMV) formation		Secretion of large molecules into extracellular environment	+	[7]
LasR, RhlR	Xcp-T2SS		Secretion of virulence factors elastase A/B and exotoxin A	+	[33]
RhlR	T3SS		Secretion of virulence factors,	-	[33,34]

			various exotoxins		
RhlR, LasR, PqsR	H2-T6SS		Secretion of virulence factors, iron transport	+	[33,35]
LasR	Phospholipase C-B (PLC-B)		Breakdown of lung surfactant, decreased lung function, hydrolyze lipids such as sphingomyelin and phosphatidylcholine in host cell membranes	+	[36]

Membrane permeability, resistance to antibiotics, and the capability to hide from a host's immune system are also mediated by QS, partially through modification of lipopolysaccharides (LPS) on the outer membrane of the bacteria. Increased membrane LPS acts as a barrier against antibiotics that would otherwise permeate and negatively affect *P. aeruginosa*. Some antibiotics target and bind to bacterial LPS, and QS systems are involved in modifying LPS structure to increase resistance. LPS and flagella of can also associate with host receptors, leading to an inflammatory response[7].

Protease and elastase production

LasR and RhlR also control production and secretion of proteins and toxins that increase virulence against the host. Genes controlling protease, elastase, and exotoxin A production are upregulated by 3-oxo-C12-HSL-bound LasR, while similar genes encoding proteases, elastases, and pyocyanin are upregulated by C4-HSL-bound RhlR[3]. PqsR mutants have also demonstrated lower production of elastase and exoprotein toxins[22]. Elastase B from *P. aeruginosa* is capable of degrading human and bovine elastin, a protein found in organ and tissues of vertebrates. Patients suffering from cystic fibrosis have been shown to have decreased elastin and collagen in their lung tissue due to elastase activity. Additionally, elastase B is capable of degrading several proteins of the human immune system, including antibodies IgA, IgG, and Surfactant Proteins A and D, which are involved in recognition of surface oligosaccharides of many bacteria[37]. Elastase B functions alongside several other *P. aeruginosa* proteases including Elastase A. In concert they have also been demonstrated to have activity in the degradation of the tight junctions[38], increasing the capability of the bacteria to infect epithelial cells. Elastase A has also demonstrated staphylolytic activity against *Staphylococci aureus*, possibly as a way of eliminating competition[39].

Exotoxin production

Pseudomonas Exotoxin A (PE) inhibits synthesis of proteins by the host organism. PE is translated as an unfolded precursor in the cytoplasm, cleaved to remove an *N*-terminal signal peptide, folds

in the periplasm, and then is secreted through the outer membrane. Two components (AB) make up PE: the cell binding component B and the enzymatic component A. Component B of binds to CD91, a receptor protein on the surface of host cells, and reaches the trans Golgi network (TGN) through either the KDEL-receptor mediated pathway or the lipid-dependent sorting pathway. The first pathway involves first internalization of PE, then cleavage into a 28 kDa fragment and a 37 kDa fragment (component A). The 37 kDa fragment contains the ADP-ribosylation domain that grants PE its toxicity. This fragment is then transported to the endoplasmic reticulum (ER) via binding to a KDEL receptor which moves between the TGN and the ER. The second pathway involves internalization via caveolar endocytosis, and then transport of PE by caveosomes into early endosomes. PE is cleaved inside the early endosomes into the same 28 kDa and 37 kDa fragments. In both pathways, the 37 kDa fragment is then secreted into the cytosol from the ER. Component A then ADP-ribosylates eukaryotic elongation factor-2 (eEF-2), inactivating it and preventing host protein synthesis[30].

Pyocyanin production

Pyocyanin (PYO) is a phenazine and a blue pigment that acts as an electron carrier for *P. aeruginosa*. Molecular oxygen is reduced by pyocyanin to form the superoxide anion O_2^- leading to the formation of reactive oxygen species (ROS). Because PYO can cross host cell membranes, it can oxidize host electron carriers such as NADH and NADPH, using the electrons to increase ROS concentrations. As well as decreasing the concentration of reduced NADH/NADPH available for host ATP production, the increased concentration of ROS places host cells in the respiratory epithelium under oxidative stress. As a result, both the innate immune system and epithelial cell gene expression are modified, leading to overproduction of mucin, inhibition of catalase activity, and overproduction of cytokines leading to recruitment and activation of neutrophils. Resulting damage to epithelial lung tissue can be observed in cystic fibrosis patients, in whose infections *P. aeruginosa* is dominant[40]. PYO production is regulated by both the LasI/LasR and RhlI/RhlR QS systems as well as by PQS. Phenazines in general are synthesized from chorismic acid as part of

the shikimic acid pathway **(**See **Figure 5,** modified from previous work[41,42]**)**. A variety of phenazine biosynthesis genes (*phzABCDEFGOMS*) are required[41].

Biofilm formation

After initially invading an area or infecting a host, bacteria can attach to both biological and non-biological surfaces. After attachment the bacterial will proliferate and may reach high cell densities, and will release a variety of extracellular polymeric substances products to form a protective matrix[7,43]. The bacteria living within this matrix are known as a biofilm[43]. Bacteria in biofilms have demonstrated resistance to host immune systems, antibiotics, and greater production of virulence factors that can negatively impact the host[44].

The process of biofilm formation involves adhesion to the surface, transition from a planktonic

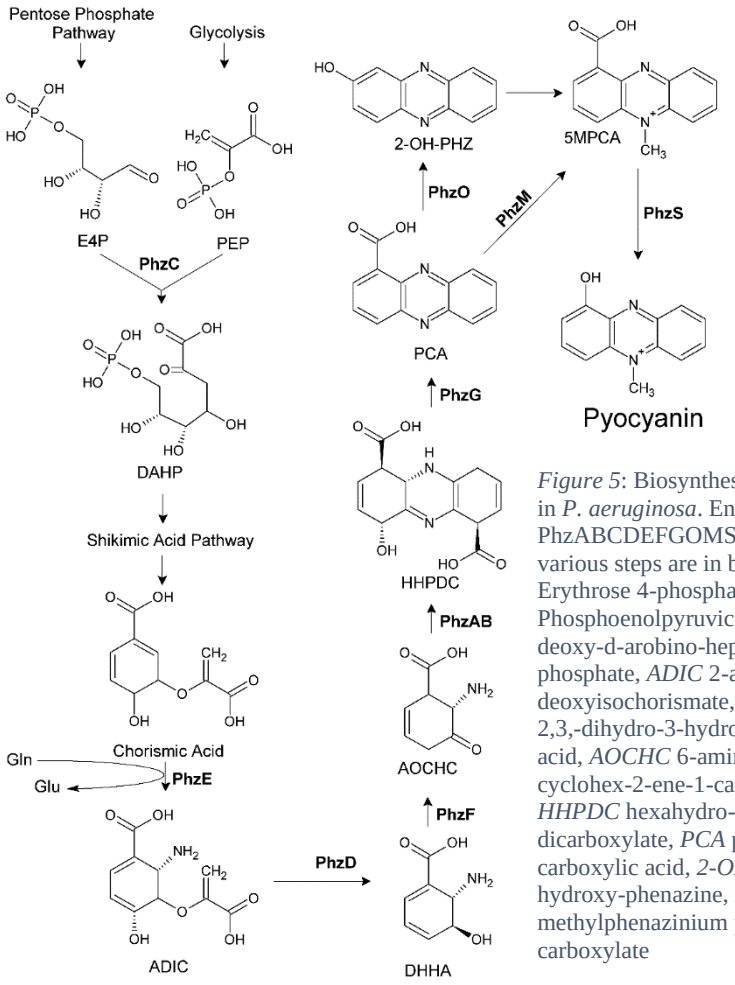


Figure 5: Biosynthesis of Pyocyanin in *P. aeruginosa*. Enzymes PhzABCDEFGOMS catalyzing various steps are in bold. *E4P* Erythrose 4-phosphate, *PEP* Phosphoenolpyruvic acid *DAHP* 3-deoxy-d-arobino-heptulosonate 7-phosphate, *ADIC* 2-amino-2-deoxyisochorismate, *DHHA* trans-2,3,-dihydro-3-hydroxyanthranilic acid, *AOCHC* 6-amino-5-oxo-cyclohex-2-ene-1-carboxylic acid, *HHPDC* hexahydro-phenazine-1,6-dicarboxylate, *PCA* phenazine-1-carboxylic acid, *2-OH-PHZ* 2-hydroxy-phenazine, *5MPCA* 5-methylphenazinium phenazine 1-carboxylate

(motile) to sessile lifestyle, proliferation, differentiation of different cells, and then finally release of some of the cells in order to infect new surfaces. Initial attachment to surfaces can involve specific receptors on a host's cell, the action of the invading bacteria's pili and flagella, and the hydrophobicity of the surface[43]. Bacteria can also produce adhesin proteins that can modify and attach to specific host membrane proteins[44]. In *Salmonella, Vibrio,* and *E.* coli, the production and function of pili and flagella are controlled by QS systems, but in *P. aeruginosa* adhesin is instead controlled by QS through its regulation of rhamnolipid production. Rhamnolipid is a glycolipid that is key for *P. aeruginosa* biofilm formation, maintenance, and virulence against both its competitors and its host[7].

After adhesion, cells will proliferate, mature, and differentiate into metabolically distinct micro-communities connected by pores and channels within the biofilm. They will also produce polysaccharides, lipids, proteins, and DNA that combine with water to form a protective matrix[7]. Upon reaching sufficient cell density within the biofilm, the concentration of autoinducers will reach threshold levels and upregulate QS controlled behaviors. Finally, mechanical forces or production of enzymes capable of degrading the extracellular matrix will lead to the release of microbes from the community in order to colonize new areas[43]. Biofilm formation in *P. aeruginosa* is controlled by the LasR, RhlR, and PQS QS systems[1,17].

1.5 Interdependence among the Las, Rhl, PQS, and IQS quorum sensing systems of *Pseudomonas aeruginosa*

Autoinducer-bound LuxR homologues in *P. aeruginosa* upregulate not only the production of their matching LuxI homologe but also regulate expression levels of genes involved in the other QS systems. 3-oxo-C12 AHL-bound LasR upregulates RhlI expression. Production of the PQS signal is positively regulated by the LasR system through upregulation of the *pqsH* and *pqsR* genes involved in the PQS synthesis pathway (see **Figure 4**). The PQS system is negatively regulated by RhlR system through repression of *pqsABCD* and *pqsR* in the same pathway[3]. **Table 2** and **Figure 6** summarize the interactions and behaviors controlled by each system.

Table 2: Effects of the Las, Rhl, PQS, and IQS QS systems on one another

Regulator Protein	Affected System or Behavior	*Regulated Genes*	Effect, Role	Affect	Notes	Ref.
LasR	LasR/I QS	*lasI*	3-oxo-C12-HSL production	+	Results in autoinduction	[1]
RhlR	RhlR/I QS	*rhlI*	C4-HSL production	+	Results in autoinduction	[1]
PqsR	PQS QS	*pqsABCDE, phnAB*	PQS production	+	Results in autoinduction	[22]
IqsR (putative)	IQS system	*Controversial (see section on IQS)*	Aeruginaldehyde production	+	Results in autoinduction	[23,31]
LasR	RhlR QS	*rhlR, rhlI*	C4-HSL production	+		[31]
LasR	PQS QS	*pqsR, pqsABCDH*	PQS production	+		[1,31]
PqsR	RhlR QS	*rhlI*	C4-HSL production	+		[17,19]
RhlR	PQS QS	*pqsR, pqsABCD*	PQS production	-		[17,19]
IQS	PQS, RhlR/I QS	*Unknown*	PQS production C4-HSL production	?	Unknown mechanism	[26,27,31]

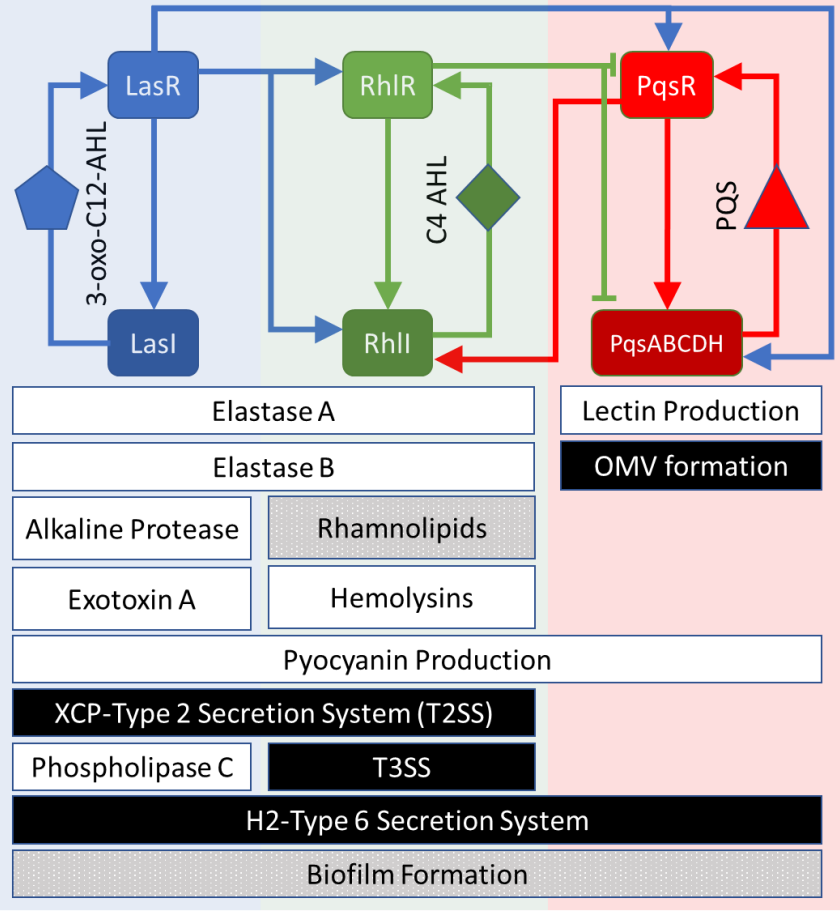


Figure 6: Hierarchal control of the quorum sensing systems of P. aeruginosa. *Arrows symbolize upregulation, while inhibition is symbolized with a line. Autoinducers are indicated with different shapes: pentagon: 3-oxo-C12-AHL, diamond: C4-AHL, triangle: PQS. Products whose expression has found to be inhibited in strains with mutations in each QS system are listed underneath the appropriate system (see table 1 and 2 for references and further details). Products in white boxes are virulence factors that attack the host in a variety of ways, black boxes indicate a role in secretion, and grey boxes a role in biofilm formation and adhesion. The putative IQS system has not been included.*

1.6 Quorum sensing using autoinducer peptides and γ-butyrolactone derivatives

While quorum sensing utilizing *N*-acyl homoserine lactones in gram-negative bacteria is the main focus of my research, other small molecules such as autoinducer peptides (AIPs) and γ-butyrolactone derivatives are also used in quorum sensing.

Autoinducer peptides

AIPs include simple linear peptides, cyclic lactones and thiolactones, and peptides with complex posttranslational modifications (See **Figure 1**). Unlike the AHLs, AIPs cannot freely diffuse through the cell membrane in order to act as an extracellular signal, and thus AIP production and export in gram-positive bacteria is generally more complex than AHL synthesis in gram-negative bacteria. AIP biosynthesis, modification, and export systems fall into three groups, exemplified by systems in *Bacillus*, *Enterococcus*, and other gram-positives. These systems generally involve cytoplasmic production of a precursor peptide followed by processing by either integral or extracellular peptidases[3].

Production of autoinducer peptides

In *Bacillus*, a precursor peptide is produced that contains an *N*-terminus secretion sequence. Following secretion, extracellular proteases remove the signal sequence. *Enterococcus* AIP production involves a precursor with a lipoprotein C-terminal tail and an *N*-terminus secretion signal, both of which are removed by integral proteases. Cyclic peptide signal precursors contain a leader sequence and a charged C-terminal tail[45]. The peptidase responsible for removing the C-terminal also catalyzes the cyclization of the peptide[46].

Response to autoinducer peptides

Response to produced AIPs occurs through two general mechanisms: either the AIP is transported directly into the cell directly binding to and activating a transcriptional regulator, or it indirectly activates a regulator by binding to a membrane-bound kinase that then activates a transcription regulating protein via phosphorylation. Either method causes an activated regulatory protein to bind to a promoter, thus upregulating expression of genes associated with QS behaviors and production of the precursor AIP[3].

Use of γ-butyrolactone derivatives as signaling molecules

Other gram-positive bacteria of the *Rhodococcus* and *Streptomyces* genera can utilize γ-butyrolactone derivatives (**Figure 1**) as quorum sensing molecules that regulate gene expression[47]. In various *Streptomyces* species, γ-butyrolactones structural groups known as A-factor, IM-2, and VB bind to repressor proteins, changing their conformation and allowing expression of regulated

genes for virginiamycin, pigment, actinorhodin, and undecylprodigiosin production[48]. Further work has shown that *Rhodococcus jostii* produces an isomer of A-factor γ-butyrolactone that can bind to receptor proteins in *Strepomyces griseus,* indicating a possible role in communication between *Streptomyces* and *Rhodoccous* species[47].

1.7 The diversity of signaling molecules and their receptors demonstrates the complexity of microbial communication systems

As previously mentioned, LuxR and its homologues such as RhlR, LasR, and TraR respond to subsets of the different *N*-acyl homoserine lactones. Complexes of these receptors with their cognate ligands are responsible for DNA binding as dimers (See **Figure 7)**, regulating complex microbial behaviors including increased production of their autoinducer[49,50]. However, research has shown that each receptor is also capable of responding to signaling molecules other than those produced by their matching LuxI synthase. Some receptors, such as QscR in *P. aeruginosa* are receptors capable of regulating gene expression after binding to AHLs, but do not have a matching synthase[51]. Receptors reported as being AHL specific may also be capable of responding to non-AHL lactones such as γ- and δ-lactones, a possibility that is investigated as part of my research.

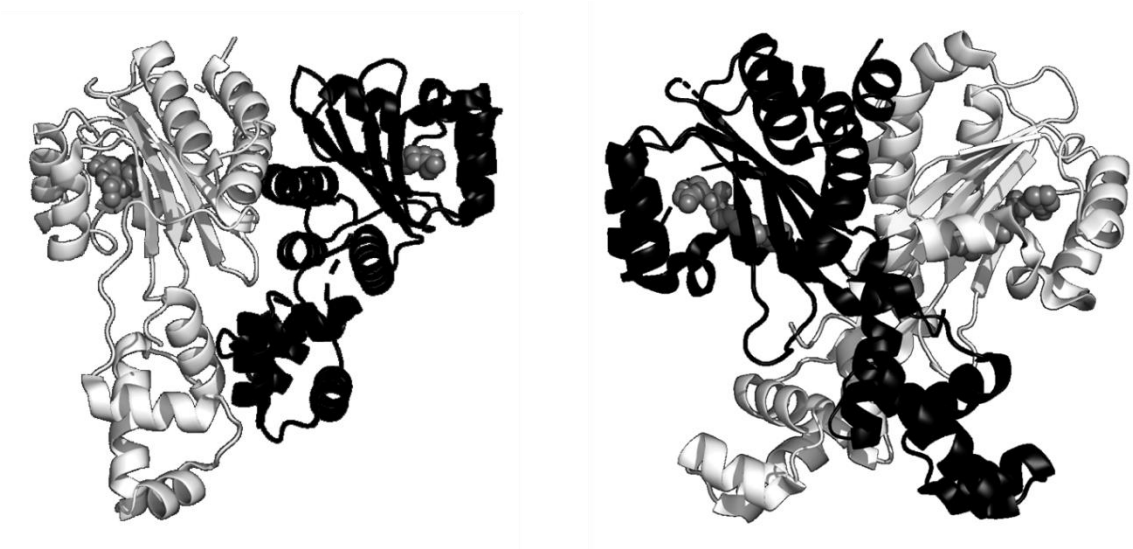


Figure 7: Structures of TraR (left) in complex with N-(3-oxo-octanoyl)-L-homoserine lactone, and QscR (right) in complex with N-(3-oxo-dodecanoyl)-L-homoserine lactone. Each receptor forms a dimer (monomers white and black), with the DNA binding site oriented towards the bottom in this figure. Each lactone is show with carbons as grey spheres. TraR structure reported in Vannini et al, 2002 and QscR structure in Lintz et al, 2011.

1.8 Implications of quorum sensing dependent bacterial behaviors on human activities

Bacterial virulence is regulated by quorum sensing in numerous pathogens infecting humans.

Medical complications due to bacterial infections are a major concern. *P. aeruginosa* is a common bacteria found in infections of soft tissue burns, the urinary tract, the respiratory system, and corneas[4]. Strains of *P. aeruginosa* resistant to a wide variety of antibiotics have been characterized in patients suffering from diabetic foot ulcers[52] as well as infections of the ear in patients with malignant external otitis (swimmer's ear)[53]. Gram positive bacteria such as *S. aureus* use autoinducer peptides to regulate expression of virulence factors such as biofilm formation, adhesion, and hemolysins. Strains of *S. aureus* with knock out mutations of genes important in its QS systems have demonstrated reduced virulence. The majority of strains involved in human infections have active QS systems[54].

Bacteria will form biofilms on both implanted devices and other medical equipment such as

catheters and contact lenses[43]. The formation of an extracellular matrix protects a biofilm from both a host's immune system and antibiotics, and sessile biofilm can be 10-1000 times as resistant to antimicrobial agents as planktonic bacteria[55]. Antimicrobial resistance can be explained via several mechanisms. The first involves the limited diffusion of some antibiotics through the exopolysaccharide matrix of some microbes and absorption of antimicrobial agents to the matrix itself. Mature biofilms also tend to exhibit slower bacterial growth, a characteristic that is usually accompanied by higher antibiotic resistance[56].

Ventilator associated pneumonia (VAP) is caused by formation of biofilm on the inner surface of endotracheal tubes used to intubate hospital patients[55]. As the ventilator cycles, parts of the biofilm itself, and secretions from that biofilm, are moved deeper into the respiratory system until the infection can colonize the lower airways. 9-27% of intubated patients suffer from VAP, which is associated with increased mortality and longer hospital stay[57]. Biofilm infections occurring in VAP have some similarity to those occurring in cystic fibrosis (CF) patients[55]. CF occurs due to mutations in the cystic fibrosis conductance regulator gene, and corresponding malfunction of the chloride channel of the lung mucosa. As a result, non-inflammatory removal of pathogens via ciliary clearance of mucous is impaired, and inflammatory responses occur. Chronic *P. aeruginosa* infection occurs in 80% of CF patients[58]. Before early eradication strategies involving inhalation of colistin and oral ciproflaxin, chronic *P. aeruginosa* infection would cause mortality in 50% of patients within 5 years[59]. Quorum sensing mediated biofilm formation has been linked to increased tolerance to tobramycin, kanamycin, and hydrogen peroxide[58].

Bacterial virulence is regulated by quorum sensing in numerous pathogens infecting plants

Plant pathogens such as *Agrobacterium tumefaciens* (discussed earlier), *Pectobacterium carotovorum*, *Pectobacterium atrosepticum*, and *Pantoea stewartii* have a variety of negative effects on human crops[14,60]. QS regulated behaviors in *Pectobacteria* include the production of cellulases, pectate lyases, and pectin methyl esterase collectively known as plant cell wall degrading enzymes (PCWDE). These PCWDE cause soft rot disease in plants, a disease responsible for yearly losses of 5-7% of potato crops in eastern Europe and some Asian countries, with a yearly cost in Europe estimated at 200 million euros[61]. Different strains of *P. carotovorum* and *P. atrosepticum* produce different ratios of autoinducers – strains in Class I produce mostly *N*-3-

oxooctanoyl-L-homoserine lactone (3-oxo-C8-AHL), and lesser amounts of *N*-3-oxohexanoyl-L-homoserine lactone (3-oxo-C6-AHL), while Class II strains produce mostly 3-oxo-C6-AHL and negligible amounts of 3-oxo-C8-AHL[62]. QS regulation of PCWDE allows *Pectobacteria* species to colonize a plant at low cell densities without activating host defenses[61]. Upon reaching a threshold density, AHL autoinducers bind to ExpR or other LuxR homologues found in *Pectobacteria*[62]. The AHL-ExpR complex inhibits the production of an mRNA binding protein, RsmA, which is responsible for blocking the translation of mRNA transcribed from the genes encoding PCWDE. In the absence of RsmA activity, PCWDE and other virulent behaviors are expressed and begin to macerate plant tissue[63].

Other QS regulated systems are found in bacteria in symbiosis with plants. For example, the LuxR/I homologues PhzR/I found in *Pseudomonas chlororaphis* regulate production of phenazines capable of broad-spectrum antifungal activity[64]. Species of *Bradyrhizobium* form symbiotic relationships with many legume crops, and behaviors such as nodulation and biofilm formation are partially controlled by QS systems. In *Bradyrhizobium japonicum* the expression of the *nod* gene, a component of a signal involved in nodulation, is controlled via a QS signal known as a cell density factor (CDF), or bradyoxetin[65].

Quorum sensing is involved in biofilm formation and biofouling

Marine bacteria colonize and form biofilms on the surfaces of ships hulls, desalination plants, and industrial equipment exposed to seawater. Biofilms generally form in several stages involving first microcolonization with marine bacteria and diatoms, and then macrofouling as larger organisms such as algae, mussels, and barnacles attach (see **Figure 8**)[66]. These biofilms increase operating

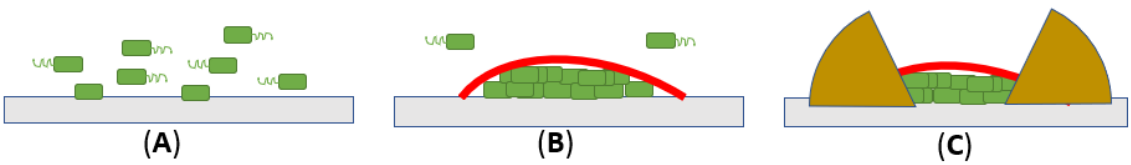


*Figure 8: Biofilm formation occurs in stages. First, bacteria adhere to surfaces using a variety of membrane proteins and lipids (**A**). Second, as population density increases, bacteria secrete a protective coating of polysaccharides and other components (**B**). Finally, larger organisms attach to the new biofilm (**C**).*

costs due to higher fuel costs related to greater drag on ship hulls and increased maintenance costs related to cleaning and removal of biofilms from surfaces[67].

Biofouling is also a major concern for the use of membrane bioreactors (MBRs)[68]. MBRs include an activated sludge reactor in which degradation of nitrogenous and phosphate waste is carried out by microorganisms and a membrane for filtration of solids and microorganisms from the effluent. The membrane can be submerged in the bioreactor or can be placed externally. External membranes require pumping of treated wastewater out of the reactor and through the membrane, while submerged membranes operate by applying a vacuum to the a submerged membrane chamber[69]. While MBRs have some advantages over conventional systems such as smaller footprints and higher quality water treatment, biofouling due to the growth of biofilms reduces membrane performance and requires costly maintenance or replacement of membranes[70]. A mixture of chemical and physical methods have had limited success in controlling biofouling, but disruption of quorum sensing mediated biofilm formation may be a more cost-efficient strategy[68]. Encapsulation of autoinducer-degrading bacteria into beads or sheets incorporated into MBRs has been shown to reduce biofilm formation and decrease the frequency of required membrane cleaning[68,71].

1.9 Interfering with microbial quorum sensing and communication

QS systems evidently play an important role in a variety of microbial behaviors, many of which are detrimental to human activities. The ability to selectively interfere with microbial communication has the potential to inhibit all behaviors regulated by QS, and thereby benefit human industry, medicine, and agriculture[72]. Interference with QS utilizing AHLs has been the focus of much of this work and will be discussed further here. In pursuit of this goal, three basic strategies have been proposed: (1) inhibition of the autoinducer synthase, (2) inhibition of the autoinducer receptor, and (3) degradation of the autoinducer to prevent its accumulation[60,61] (See **Figure 9**). A brief discussion of strategies (1) and (2) follows, but the focus of this work is development of enzymatic methods to degrade autoinducers, as in strategy (3).

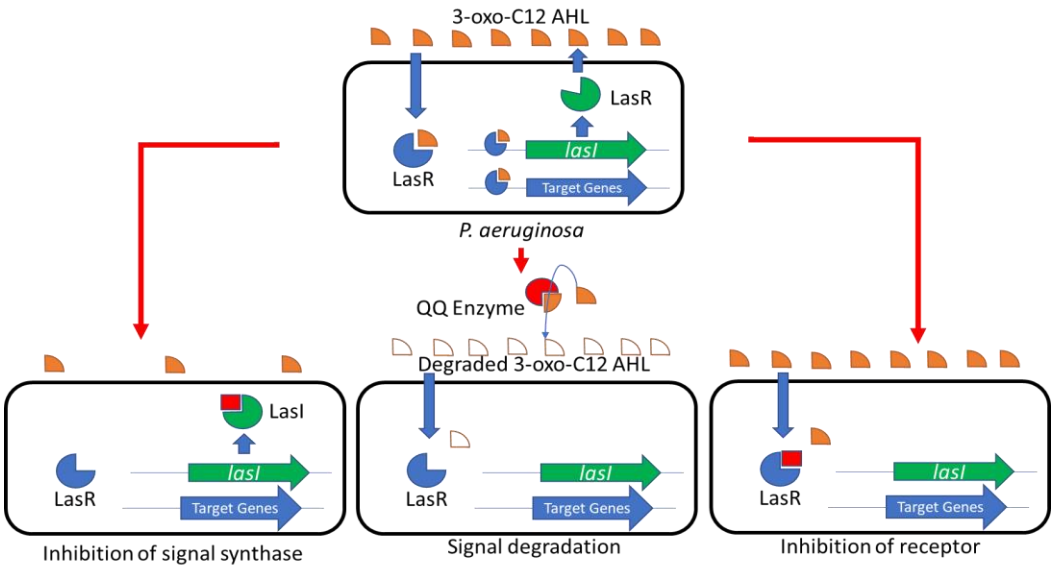


Figure 9: Proposed strategies for interfering with bacterial communication and quorum sensing. The LasR/I system of P. aeruginosa *is used as an example.*

Small Molecule Quorum Sensing Inhibitors (QSIs)

The first two strategies involve the use of small molecules capable of inhibiting QS, with the advantage of being able to infiltrate established biofilms, aiding in their dispersal and downregulation of virulent behaviors[73]. In order to develop the first strategy of inhibiting autoinducer synthesis, quorum sensing inhibitors (QSI) antagonistic towards these synthases have been characterized. One study tested 114 compounds from plant extracts and 10 synthetic compounds, discovering that salicylic acid, tannic acid, and trans-cinnamaldehyde inhibited production of AHLs as detected by a *Chromobacterium violaceum*. Salicylic acid was shown to decrease, but not eliminate AHL production, and the effects of tannic acid were attributed to unknown mechanisms instead of direct inhibition of the LasI or RhlI, but trans-cinnamaldehyde decreased production of both 3-oxo-C12-AHL and pyocyanin in *P. aeruginosa*[74]. Other plant extract small molecule QSIs include pyrogallol, L-canavanine, curcumin, furocoumarins, limonoids, and flavanoids[75].

In pursuit of the second strategy, that of interfering with autoinducer reception, many compounds with antagonistic effects towards LuxR homologues have been discovered and synthesized. Some quorum sensing inhibitors (QSI) with structures based on AHLs have been synthesized and their

capability to modulate QS related to the activity of LuxR homologues has been measured[75]. Reviews of the literature regarding these AHL analogues have shown that modifying certain moieties present in AHLs affects the antagonistic or agonistic effects of these molecules, including the length of the acyl chain, modification of the group attached to the 3-carbon of the acyl chain, lactone ring modifications, and addition of aromatic groups[76]. PQS inhibitors have also been synthesized and have demonstrated antagonistic capability against PqsR[1].

Naturally occurring small molecule QSI are produced by a wide variety of marine organisms such as algae, sponge, coral, fungi, and bacteria[60]. One class of small molecule QSI are the halogenated furanones, originally isolated from marine algae extracts. Originally isolated with bromine, additional halogenated furanones have also been synthesized and have demonstrated QS inhibition[76]. Brominated furanones have been shown to inhibit biofilm formation in *Streptococcus mutans*, which colonizes tooth surfaces[77]. These furanones may bind to the LuxS synthase responsible for production of 4,5-dihydroxy-2,3-pentanedione (DPD), the precursor to AI-2[1]. Brominated furanones with various chemical modifications inhibit biofilm formation in *P. aeruginosa* and *Escherichia coli*[78], despite the lack of a LuxS synthase gene in *P. aeruginosa*[1].

Quorum Quenching Through Degradation of Autoinducer Signals

A third strategy for control of QS involves the use of enzymes capable of degrading autoinducers. The majority of these quorum quenching enzymes (QQE) have been isolated from bacteria capable of degrading AHLs (and in some cases, γ-lactones), and include lactonases capable of opening the lactone ring, acylases capable of removing the acyl chain, and oxidoreductases catalyzing the hydroxylation of the ω-1, 2, or 3 carbon of the acyl chain[75]. (**Figure 10**).

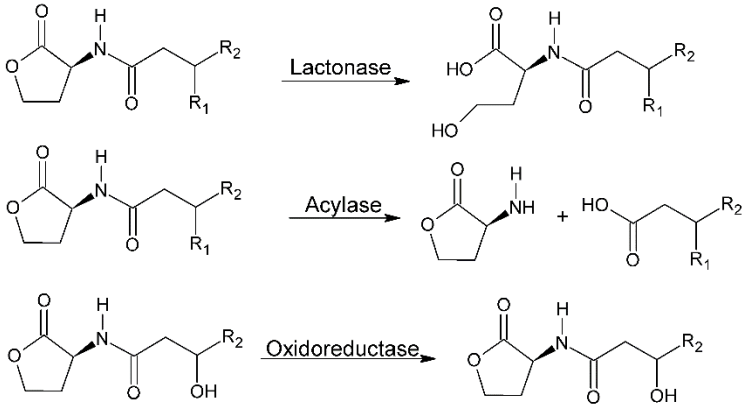


Figure 10: Enzymatic strategies for degradation of AHLs. R_1 represents -H, -OH, or =O groups, while R_2 represents a saturated hydrocarbon chain between 1 to 9 carbons in length.

AHL lactonases include several families of enzymes: the paraoxonases (PONs), the phosphotriesterase-like lactonases (PLLs), the metallo-β-lactamase like lactonases (MLLs)[79], and the α/β-hydrolase family lactonases. Each of these families has a unique protein fold, and some incorporate metal cations[80]. See **Figure 11** for specific examples and folds of lactone degrading enzymes. PON1 was isolated from mammalian liver[81], SsoPox[82] and GcL[79] from thermophilic bacteria, and ZHD from *Clonostachys rosea*, a fungus (which will later be covered in more detail)[74].

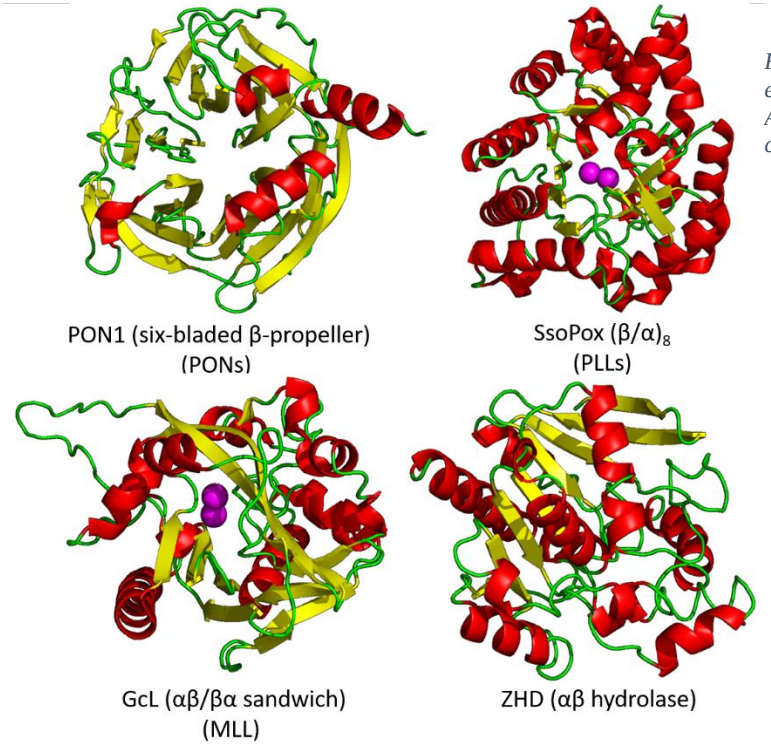


Figure 11: Families of enzymes capable of degrading AHL autoinducers. Metal cations shown as spheres.

Discovery and characterization of novel naturally occurring quorum quenching enzymes is an ongoing area of research, as these enzymes have demonstrated the promising capability to degrade a variety of small molecules used in quorum sensing, interfering with microbial behaviors that can be detrimental to human activities. Protein engineering approaches are also being pursued to improve the stability, specificity, and catalytic ability of these enzymes[83].

Chapter 2

2.1 Introduction

LuxR type receptors have diverse mechanisms and substrate specificities

A wide variety of receptors responding to the autoinducer signals used in quorum sensing have been characterized. Here I will discuss some of the LuxR type receptors that have been characterized along with their reported cognate ligand, and then discuss my characterization of some of these receptors and their response to previously unstudied potential ligands. Systems utilizing autoinducers other than homoserine lactones (such as autoinducer peptides and PQS) will not be discussed here.

Each QS system utilizing LuxR homologues consists of a synthase producing an endogenous ligand that binds to a receptor protein capable of modulating gene expression (See **Table 3**). The receptors have both a *N*-terminus autoinducer binding domain and an C-terminus DNA binding domain[84]. Receptors are diverse. As already discussed, the two AHL based QS systems of *P. aeruginosa* are the LasI/R and the RhlI/R systems. Both LasR[54] and RhlR[85] form homodimers after binding with their cognate ligand and then modulate gene expression by binding to a regulatory sequence (*las* and *rhl)* respectively[1]. Others, such as EsaR can dimerize and bind to their regulatory sequences in the absence of AHLs, and are actually inactivated and monomerized in the presence of AHLs[62], while SdiA from *E. coli* lacks a synthase partner and can bind to AHLs as a monomer[86].

*Table 3: LuxR homologous receptors paired with their reported ligands and the synthase producing that ligand. HSL: homoserine lactone, 3O: 3-oxo, C4/6/8/10/12: number of carbons in the acyl chain (**refer back to figure 1** for the structure of these compounds).*

Organism	Synthase	Receptor(s)	Proposed Endogenous Ligands	Reference(s)
P. aeruginosa	RhlI	RhlR	C4-HSL	[17,87]
	LasI	LasR	3OC12HSL	[16,17]
P. syringae	AhlI	AhlR	3OC6HSL	[88]
P. syringae pv. *tabaci*	PsyI	PsyR	C6-HSL, 3OC6HSL	[89]

E. carotovora	CarI	CarR, ExpR, VirR	3OC6HSL	[65,90]
P. stewartia	EsaI	EsaR	3OC6HSL	[91]
B. glumae	TofI	TofR	C8HSL	[92]
A. tumefaciens	TraI	TraR	3OC8HSL	[14]
C. violaceum	CviI	CviR	C10HSL	[93]
S. liquifaciens	SwrI	SwrR	C4-HSL, C6-HSL	[94]
V. fischeri	LuxI	LuxR	3OC6-HSL	[11,12]
	AinS	AinR	C8-HSL	[11,12]
E. coli	NA	SdiA	C6-HSL, 3OC6-HSL	[86]
A. hydrophilia	AhyI	AhyR	C4-HSL	[95]
A. salmonicida	AsaI	AsaR	C4-HSL	[95]
M. tianshanense	MrtI	MrtR	3OC12-HSL, 3OC14-HSL	[96]

Some researchers have proposed that LuxR homologues can be sorted into classes based on the effect of AHLs on protein folding, dimerization, and the regulatory state of the bound receptor (See **Table 4**) [97]. For example, Class I receptors such as TraR require bound 3-oxo-C8-HSL during translation in order to fold and then regulate gene expression as a dimer. 3-oxo-C8-HSL binds to each monomer of a TraR dimer, stabilizing the protein and protecting it from proteases[49].

Table 4: Functional classes of LuxR homologues[97]

Class	Example	Characteristics and mechanism of action
I	TraR	Irreversibly binds AHL during translation, binds DNA as dimer
II	LuxR	Reversibly binds AHL during translation, binds DNA as dimer
III	MrtR	Does not require AHL during translation, ligand-receptor complex binds DNA as dimer
IV	EsaR	Does not require AHL during translation, dimerized in absence of AHL but is nonfunctional when AHL-bound
V	SdiA	Does not require AHL during translation, binds DNA as a monomer in complex with AHL

LuxR type receptors can exhibit different ligand specificity

Receptors are also diverse regarding their specificity. Some receptors, such as SdiA, have an unrestrictive binding pocket that permits a wide range of ligand binding[97]. Behaviors beneficial at threshold population cell densities have been assumed to require high fidelity receptors capable responding to endogenous autoinducers for accurate quorum sensing[1]. The endogenous ligands reported in **Table 3** were determined using similar methods. C6-HSL was identified as the ligand of CviR after HPLC and mass spectrometry analysis of the culture supernatant from a quorum sensing deficient strain of *Chromobacterium violaceum* was compared to the quorum sensing strain[98]. The endogenous ligands C4-HSL of AhyR and AsaR[95] and 3-oxo-C6-HSL of LuxR in *V. fischeri*[13] were determined via a similar method.

Research has shown that some LuxR-type receptors are promiscuous and capable of binding to several AHLs[99]. CviR from *C. violaceum* regulates production of the purple pigment violacein in response to AHLs with acyl chains ranging from four to eight carbons in length, which allows the use of this bacteria in the detection of these autoinducers[100]. Evidently LuxR-type receptor responses are not restricted to endogenously produced autoinducers[101]. In fact, a census of LuxR homologous genes[102] found that 2698 out of 3550 *luxR* genes did not have an adjacent *luxI* homologues. The receptors encoded by these "solo" *luxR*-type genes may respond to exogenous signals from the wider microbial community, or possibly to autoinducers produced by non-adjacent *luxI*-type genes[102]. While exogenous signal molecules are sometimes referred to as "non-cognate AHLs"[85], the capability of LuxR-type receptors to respond to exogenous signals may actually have an important role in interspecies communication in natural microbial communities[103].

Biosensors incorporating LuxR-type receptors can be used to measure gene expression in response to lactones

Plasmid biosensors constructed by other researchers are capable of quantifying lactone concentration in solution, and generally contain a gene for the production of the LuxR homologue, a promoter region to which the AHL-bound LuxR homologue can bind, a reporter gene regulated

by that promoter, and a resistance marker (see **Fig. 12**). Incorporation of different receptor genes including AhyR, RhlR, LasR, and LuxR (See **Table 6**) allow detection and quantification of AHLs[99] due to their capability to easily diffuse through the cell membrane of reporter strains with these reporter plasmids[104]. The orphan LuxR homologue SdiA found in *E. coli* (**Table 3**) can interfere with detection of AHLs by these reporter plasmids. *E. coli* sdiA(-) strains have also been transformed with biosensor plasmids. Other researchers have constructed plasmids containing a *lux*-type promoter but lacking *luxR*-type gene in order to measure background expression of the regulated reporter gene even in the absence of the receptor (**Table 5**, pAL102, 104, 106)[105]. Cultures of bacteria transformed with reporter plasmids are incubated with small volumes of the solution to be quantified, and the expression of the reporter gene (fluorescence or luminescence) is compared to standard curves relating known concentrations of the AHL and reporter response[99]. Degradation of AHLs can also be measured relative to an inert control such as BSA by first incubating the AHL in question with a putative lactonase or acylase and then measuring the remaining AHL concentration using the same method[106].

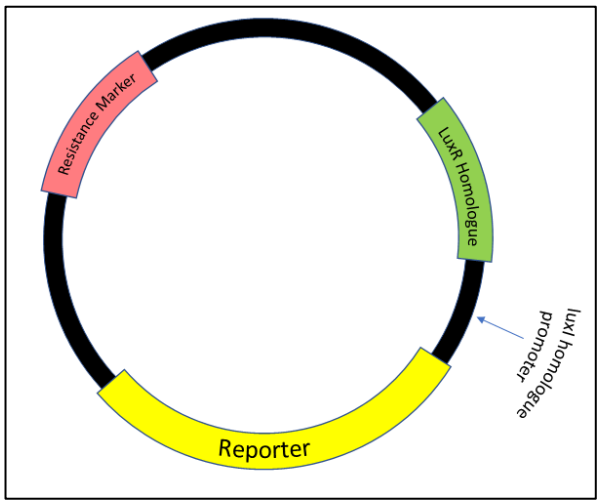


Figure 12: Components of reporter plasmids.

Table 5: Characteristics of biosensor plasmids used to reporter lactone concentration. Strain JLD271 is a SdiA(-) strain of E. coli.

Plasmid	Receptor /Promoter Pair	Promoter regulates expression of:	Notes	Bacterial strain	Ref
pSB403	LuxR/*lux*	*lux* operon		***E.coli MT102***	[99]
pSB401	LuxR/*lux*	*lux* operon		***E.coli JM109***	[99]
pSB536	AhyR/*ahy*	*gfp*		***E.coli JM109***	[99]
pSB406	RhlR/*rhlI*	*lux* operon		***E.coli JM109***	[99]
pSB1075	LasR/*las*	*lux* operon		***E.coli JM109***	[99]
pAL101	RhlR/*rhl*	*lux* operon		***E. coli JLD271***	[105]
pAL102	*rhl*	*lux* operon	Negative control for pAL101	***E. coli JLD271***	[105]
pAL103	LuxR/*lux*	*lux* operon		***E. coli JLD271***	[105]
pAL104	*lux*	*lux* operon	Negative control for pAL103	***E. coli JLD271***	[105]
pAL105	LasR/*las*	*lux* operon		***E. coli JLD271***	[105]
pAL106	*las*	*lux* operon	Negative control for pAL105	***E. coli JLD271***	[105]
pJNL *pPROBE$_{rsaL}$	LasR/*rsaL*	*gfp*	pJNL contains *lasR,* for constitutive expression	***E. coli DH5α***	[103]
pJNR *pPROBE$_{rhlA}$	RhlR/*rhlA*	*gfp*	pJNR contains *rhlR* for constitutive expression	***E. coli DH5α***	[103]
pJNQ *pPROBE$_{PA1897}$	QscR/*PA1897*	*gfp*	pJNL contains *qscR* for constitutive expression	***E. coli DH5α***	[103]
*The plasmid pPROBE is a template plasmid containing *gfp*. Promoters can be added to regulate GFP expression, shown as subscripts.					

2.2 LuxR homologues in reporter plasmids have nonspecific broad AHL specificity

The biosensors listed in **Table 5** often express measurable responses to a variety of AHLs[99,105], with the caveat that this response sometimes occurs at relatively high concentrations of some AHLs. This is interesting in that these receptors all have a matching synthase that primarily produces one

endogenous ligand, as previously described, and is another indicator that cross-talk between species using different AHLs may be an important factor in microbial communities.

In order to confirm these findings, and to generate standard curves measuring reporter culture response to AHLs, overnight cultures of *E. coli* with reporter plasmids were grown in LB media with the appropriate antibiotic, the diluted 1:50 (v:v) into fresh culture. After 1 hour, 2 µL of a stock 100x AHL solution was diluted with 198 µL of biosensor culture, and then further incubated for 2 hours. After 2 hours, either the fluorescence or luminescence was measured and then

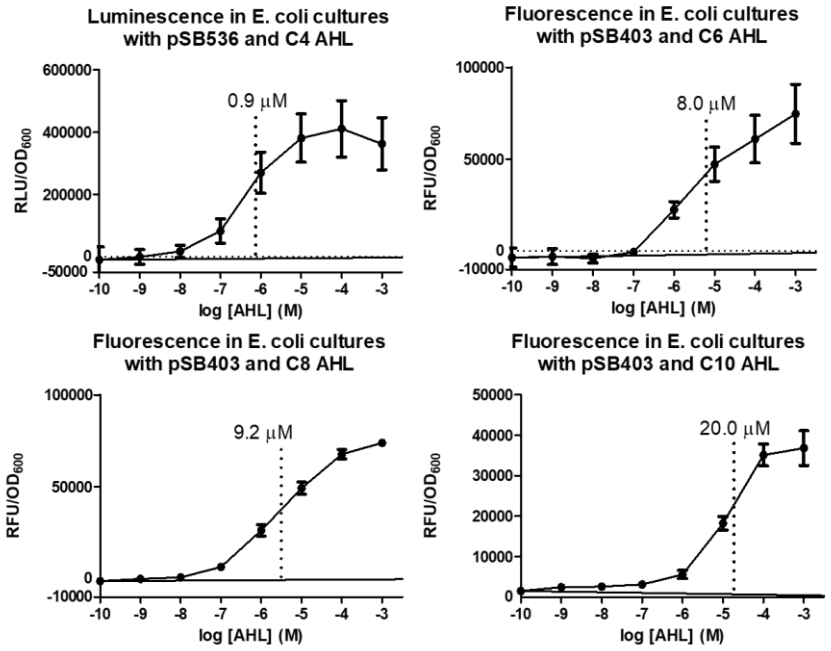


Figure 13: Dose response of E. coli cultures transformed with AhyR (pSB536) and LuxR (pSB403) reporter plasmids to AHLs with acyl chains of different lengths. Expression was measured in Relative Luminescence (RLU) or Relative Fluorescence (RFU) and normalized by OD_{600} of the cultures. SEM of biological quadruplicates shown as error bars. Background signal from samples not treated with ligands was subtracted. EC_{50} values are shown.

normalized by the OD_{600} of the cultures, and then the background luminescence from controls lacking AHLs was subtracted from those values. As can be seen in **Figure 13**, signal becomes significantly greater than background at AHL concentrations between 100 nM to 1 µM. Biosensors utilizing *rhl*, *las*, or *lux* promoters have shown to allow some expression of the regulated genes even in the absence of ligand-bound receptor proteins[107].

It is interesting to note that while the canonical cognate ligand for LuxR is 3-oxo-C6 AHL, **Figure 13** demonstrates that the biosensor can respond to C6, C8, and C10-HSL at similar concentrations. The measured maximum fluorescence with C10-AHL was roughly ~70% that of C6 and C8-AHL, and the threshold concentration for a response was an about an order of magnitude higher, but the receptor is responding to C10-AHL. These biosensors are thus useful tools for investigating the

response of LuxR-type receptors to a variety of small molecules with the caveat that the response is occurring via heterologous expression in *E. coli*. While most receptors have a ligand to which they will respond at relatively low concentrations (See **Table 6**), our survey of the literature shows that most receptors show wide substrate selectivity in their capacity to respond to a variety of AHLs of different acyl chain length[103].

Table 6: Approximate concentrations of different AHLs eliciting ~50% maximum response using various constructed biosensor plasmids. See Table 5 for more details regarding each plasmid. pJNL, pJNR, and pJNQ allowing constitutive expression of each receptor were co-transformed with pPROBE plasmids containing GFP and the promoter to which each receptor binds. Some sensors did not reach maximum signal, so their EC_{50} was not resolved "UNR". Ligands were not tested marked "-", and some gave no detectable response marked "ND". The reported endogenous ligand for each receptor is highlighted. QscR is an orphan receptor with no endogenous ligand, but appears to respond most strongly to C10-AHL[103]. indicates values that were determined by examining a graph or chart, as raw data was not available.*

	Biosensor plasmid(s) with incorporated LuxR-type receptor								
	pSB1075 LasR	pAL105 LasR	pJNL LasR	pSB406 RhlR	pAL101 RhlR	pJNR RhlR	pSB401 LuxR	pAL103 LuxR	pJNQ QscR
Lactone	EC_{50} Approximate concentration of ligand yielding 50% of maximum response (log[M])								
C4 AHL	-5	UNR	ND	UNR	UNR	-4	-4	-	ND
3OC4 AHL	-7.5	-	-	UNR	-	-	-4	-	-
C6 AHL	-5	-	ND	-8*	-	-4	-7	-	UNR
3OC6 AHL	-6	-	ND	UNR	UNR	UNR	-8.5	-8	UNR
C8 AHL	-5	-	UNR	-8*	-	UNR	-6	-	-6
3OC8 AHL	-6.5	-	-5	UNR	-	UNR	-8	-	-7
C10 AHL	-7	-	-5	-7*	-	ND	-6	-	-9
3OC10 AHL	-8.5	-	-7	-8*	-	ND	-7	-	-7
C12 AHL	-8.5	-	-6	-7*	-	ND	-6	-	-8
3OC12 AHL	-9.5	-8	-8	-7*	-	ND	-6	-	-7
C14 AHL	-	-	-7	-	-	ND	-	-	-7
3OC14 AHL	-9	-	-8	-7*	-	ND	-6	-	-8
Reference(s)	99	105	103	99	105	103	99	105	103

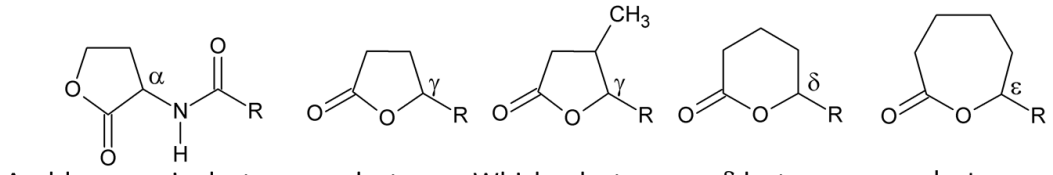
Table 6 reveals some interesting patterns. While the reported endogenous "cognate" of each ligand appears to have an EC_{50} lower than other ligands, each receptor seems to have a wide substrate selectivity. In addition, RhlR in pSB406 appears to require a relatively high concentration (~100 μM) of its reported C4-AHL cognate in order to reach half of maximum signal but is capable of responding to much lower concentrations of other AHLs. While LasR, LuxR, and QscR biosensors reach signal saturation, RhlR biosensor signal continues to increase with some ligands even at high concentrations, making their EC_{50} impossible to resolve with this receptor[103].

LuxR homologues may respond to γ, δ, and ε-lactones

Much work has been done to identify synthetic antagonists capable of inhibiting AHL binding to

LuxR-type receptors (see earlier discussion), **but little work has been done to identify natural small molecules other than AHLs capable of inducing a response from these receptors.** As mentioned previously, gram-positive bacteria such as *Streptomyces* and *Rhodococcus* can use γ-

Figure 14: AHL and γ, δ, ε -lactone structure



butyrolactone derivatives for quorum sensing and microbial communication[48]. Thus, it may be possible that LuxR and its homologues such as LasR and AhyR may engage in cross-talk with these species, and the receptors incorporating these receptors may be capable of responding γ, δ, and ε-lactones despite their different chemical structures. The γ, δ, and ε-lactones have an acyl chain from the γ, δ, and ε carbons of the lactone ring, respectively, while the acyl chain of AHLs is attached to the α carbon (**Figure 14**). In order to examine this possibility, we performed biosensor assays measuring the expression of reporter genes regulated by LuxR homologues in response to γ, δ, and ε-lactones[99].

To test whether these biosensors responded to non-AHL lactones, γ, *δ*, and ε-lactones at 1 mM concentration in solutions of dimethyl sulfoxide (DMSO) were incubated with 198µL of reporter culture with six replicates in the same method as the AHLs. Interestingly, the LuxR biosensor showed a statistically significant response to some non-standard lactones, while the LasR and AhyR biosensors showed a similar pattern of inhibition relative to the background signal with DMSO (See **Figure 15**). This may indicate that some non-standard lactones, such as γ-nonalactone, are capable of binding to LuxR productively, while possibly inhibiting expression of the reporter gene in LasR and AhyR regulated biosensors. Low levels of active LasR dimers have been detected in cultures in the absence of 3-oxo-C12-HSL, suggesting that these regulator proteins are capable of folding into an active conformation in the absence of signal[101]**.** The γ-lactones with longer acyl chains appear to have a greater effect on inhibiting background signal. The LasR and AhyR biosensors also appear to show a similar pattern in their responses to γ-, *δ*-, and ε-lactones and there is only ~28% similarity between the ligand binding domains of these proteins as seen in protein-protein BLAST[108]. This may indicate that these lactones are affecting reporter gene expression through a mechanism unrelated to the receptor. Controlling for these effects will require using a reporter plasmid lacking the gene encoding a LuxR homologue.

Figure 15: Response of LuxR type receptors to 1mM non-AHL lactones. (A) pSB1075 with LasR receptor, (B) pSB536 with AhyR receptor, (C) pSB403 with LuxR receptor. Lactones present at 1mM. The horizontal line and second column indicate background signal in the absence of lactone addition. The first column indicates signal from the same concentration of AHL as a positive control. Y-axis ranges are unique to each biosensor.

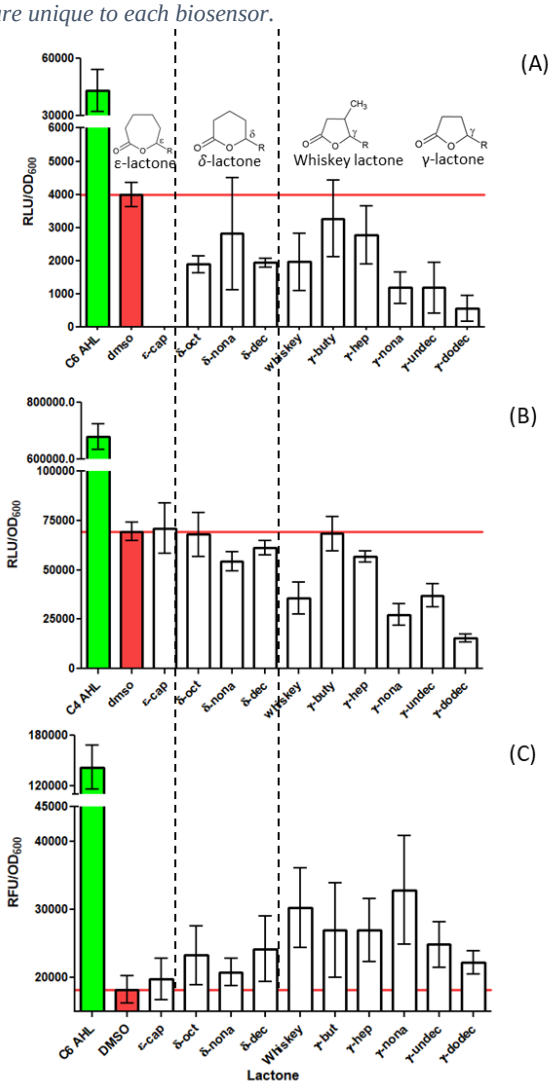
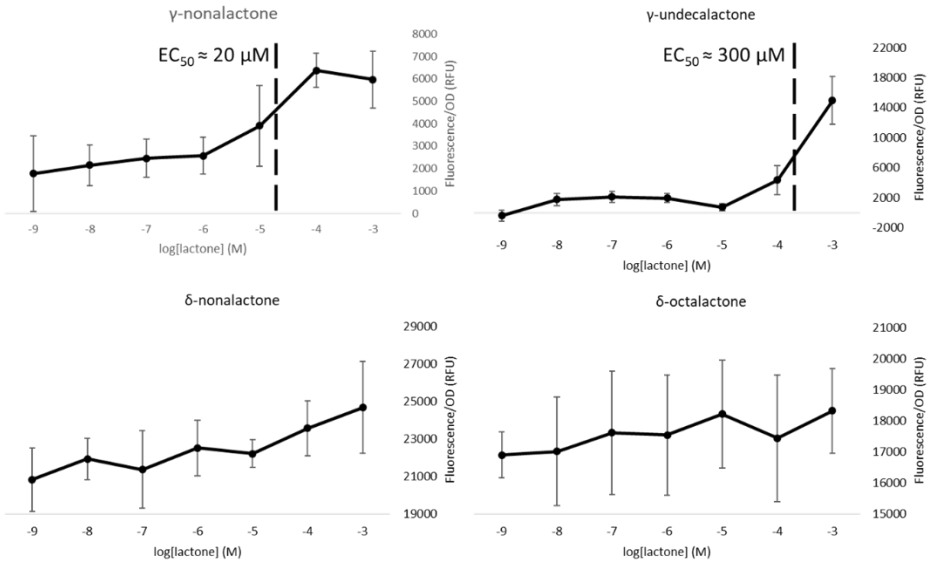


Figure 15C indicates that 1mM of some γ and δ lactones may upregulate production of the GFP reporter in pSB403. In order to examine whether this response was correlated to lactone concentration, we carried out a similar dose response assay using the LuxR containing pSB403 with different concentrations of several representatives of γ and δ lactones (**Figure 16**). Only γ-lactones appeared to cause a response, and only at relatively high concentrations (EC_{50} was approximately 20 μM and 300 μM for γ-nonalactone and γ-undecalactone, respectively) compared to the response to ~10nM of 3-oxo-C6 AHL. The addition of γ-undecalactone or γ-nonalactone at increasing concentrations was correlated with a clear increase in fluorescence.

Figure 16: Dose response of LuxR biosensor to γ and δ lactones. Fluorescence signal from reporter cultures was normalized by culture OD and background signal from cultures without addition of lactones was subtracted. Error bars display SEM for biological replicates in quadruplicate. Y-axis scales are different for each lactone.



Because the production of the signal reports on the productive binding (i.e. binding and activation of the receptor, it does not inform on non-productive binding events. Therefore, we also examined the effects of competition between γ lactones and an AHL (see **Figure 17**). For these experiments increasing concentrations of γ lactones were added alongside a constant concentration of C10-AHL.

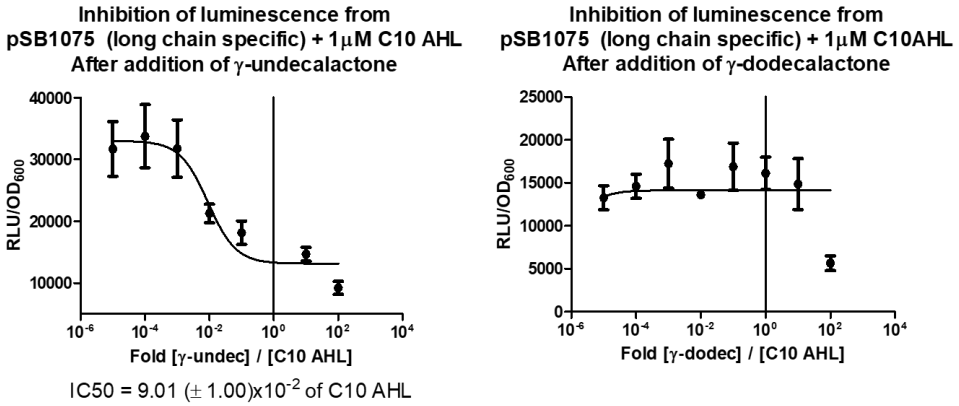
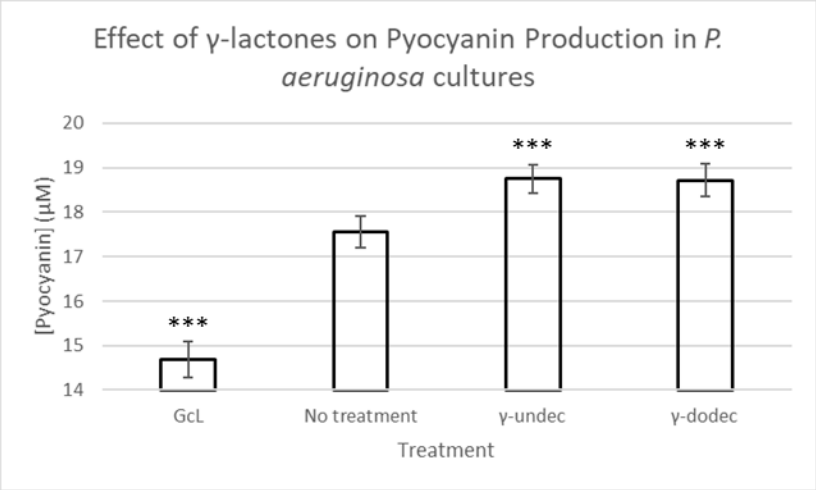


Figure 17: Effects on relative luminescent signal from pSB1075 reporter with LasR with addition of 1μM C10-AHL and increasing concentrations of γ-undecalactone and γ-dodecalactone. The x-axis is the [γ-lactone]/[C10-AHL], with a vertical line to indicate a 1:1 ratio of both lactones. Error bars display the SEM for biological replicates performed in quadruplicate. Y-axis scales are different between the two experiments.

Based on the reduction of background luminescence in cultures containing 1mM γ-undecalactone and γ-dodecalactone seen in **Figure 15**, these two lactones were examined first. Interestingly, γ-undecalactone appears to inhibit ~50% of luminescence when present at roughly 1/100-fold of the concentration of C10-AHL, whereas the structurally close γ-dodecalactone only inhibited luminescence when present at 100-fold the concentration of C10-AHL. We hypothesize that γ-undecalactone may be capable of productive binding with LuxR but may bind to LasR antagonistically.

If γ- and δ-lactones are capable of binding to LuxR homologues, they may also be capable of inducing or inhibiting behaviors regulated by those homologues. In order to examine this possibility, we measured pyocyanin production from cultures of *P. aeruginosa* PA14 with addition

Figure 18: Concentration of pyocyanin present in cultures of P. aeruginosa PA14 *3 hours after addition of GcL (a lactonase), 100 µM γ-dodecalactone or 100 µM γ-undecalactone. Error bars display the SEM for biological replicates in quadruplicate.*** *indicates p<.001 relative to "no treatment".*



of GcL, a lactonase that should degrade autoinducers and downregulate pyocyanin production, as well as 100 µM γ-dodecalactone or γ-undecalactone. Cultures of PA14 with the added γ-lactones demonstrated a significant increase in pyocyanin production (See **Figure 18**). We hypothesize that high concentrations of γ- and δ- lactones may bind productively to some LuxR-type receptors but not as productively as their endogenous ligands. The effects of adding non-AHLs need to be confirmed through *in vivo* measurement of other virulence factors such as biofilm formation, elastase/protease production, and others. Additional confirmation using qPCR to measure expression of genes regulated by receptors involved in quorum sensing will also be carried out.

2.3 Conclusion

It seems clear that most LuxR-type receptors, despite their widespread association with a specific cognate ligand, can in fact respond to a range of different acyl homoserine lactones and may also be capable of responding to some γ- and δ-lactones. This response occurs both in assays using reporter cultures and *in vivo* when measuring pyocyanin production in *P. aeruginosa*, a behavior regulated by quorum sensing systems. The capability of these receptors to respond to exogenous

signals may indicate a role in interspecies communication in microbial communities. Understanding the complex nature of these communication networks is essential to their manipulation and the modification of quorum sensing regulated bacterial behaviors. In future work we will continue to characterize key LuxR-type receptors and perform structural studies to discover determinants of ligand specificity. We expect these findings to help us better understand which types of signaling molecules bacterial species can sense and respond.

2.4 Future work

While the changes in the biosensor signals, apparent competition, and increased pyocyanin production may indicate binding of γ-, *δ*-, and ε-lactones to LuxR homologues, this needs to be further confirmed with proper controls. If confirmed, the relationship between the structure of the lactone (including the length of the acyl chain and the size of the lactone ring) and its capability to bind to LuxR homologues will be examined. Crystallization of the receptor protein complexed with non-standard lactones would provide insight into the mechanisms that allow classes of molecules with somewhat different properties to bind to a single active site. Additionally, the effect of non-standard lactones on expression of other behaviors (biofilm formation, protease production, etc.) in *P. aeruginosa* would also be measured.

Chapter 3

3.1 Introduction

As mentioned previously, a variety of enzymes are capable of degrading AHLs and preventing the detection of a quorum by a bacterial population. These enzymes are referred to as quorum quenching enzymes (QQE) and use a variety of mechanisms (see **Figure 10**). Here we will focus on the discussion of the enzymes that act as a lactonase, opening the lactone ring of their target molecule. These lactonases are generally split into different families based on their protein folds, and they originate from a variety of different bacteria, archaea, and mammals[109]. Some lactonases have broad substrate selectivity against lactones with a variety of attached moieties, while others are relatively specific. Some are highly thermostable while others are sensitive to solvents and temperature.[81]

Quorum quenching lactonases are divided into four major families, the paraoxonases (PONs), the metallo-β-lactamase-like lactonases (MLLs), the phosphotriesterase-like lactonases (PLLs), and the αβ hydrolase fold lactonases. An overview of these families is given below, followed by a description and characterization of zearalenone hydrolase, a member of a new potential lactonase family capable of quorum quenching.

Members of the PONs family with highly conserved sequences have been found among a variety of different mammals, as well as some invertebrates[10] and bacteria[109]. This family is characterized by a six-bladed β-propeller fold and a central tunnel. A catalytic calcium cation is involved in aligning and stabilizing the bound ligand and its intermediate[110]. PONs were studied due to their capability to degrade paraoxon, a metabolite of parathion, an insecticide. They can also degrade other organophosphates and neurotoxins such as sarin and soman[111]. However, PON1 paraoxonase activity is relatively low, while other closely related (~60% homologous) variants in this family, such as PON2 and PON3, have almost no paraoxonase activity[110]. Thus, it is likely that this activity is not the main function of these enzymes. PON1 and PON2 exhibit similar activity profiles against AHLs with acyl chain lengths ranging from 7 to 14 carbons for the homoserine lactones, and from 6 to 12 carbons for the 3-oxo-homoserine lactones. PON3 has a tighter activity profile and is more active against the longer chain 3-oxo-homoserine lactones such as 3-oxo-C12 AHL[81].

MLLs exhibit a αβ/βα fold and a conserved HXHXDH motif that permits a bi-metallic active site, and are found in bacteria, archaea, and eukaryotes[109]. Examples of this class of lactonases include AaL[109], GcL[112], MomL[113], AidC, AiiB, and AiiA[112]. These lactonases are proficient enzymes with a broad substrate selectivity[109].

PLLs belong to the amidohydrolase superfamily, and exhibit a $(\alpha/\beta)_8$ TIM barrel fold with a bimetallic active site located at the C-terminus of the barrel. Four histidine residues coordinate two metal cations which activate a bridging water molecule which opens the lactone ring of the substrate through nucleophilic attack[112]. PLLs have been isolated from bacteria and archaea and are often highly thermostable. They tend to show a preference for AHLs with longer acyl chains[109].

The α/β-hydrolase fold lactonases were isolated from bacteria, and have a wide substrate specificity[109]. The enzymes of this class do not contain a bimetallic active site. Instead they rely on a serine residue to attack the carbonyl carbon of the substrate's lactone ring. The serine is part of a catalytic triad with histidine and glutamate. Histidine acts as a base, deprotonating serine and

allowing it to form an enzyme/substrate bond through nucleophilic attack. The resulting tetrahedral intermediate is stabilized by an oxyanion hole, followed by the breaking of the weakened carbon-oxygen bond of the ring. An example of this class is AidH[114].

3.2 Characterization of a novel quorum quenching lactonase from fungi

As noted, most lactonases have a broad substrate specificity, and are equally capable of degrading AHLs with short and long acyl chains. An example of this is GcL from *Parageobacillus caldoxylosilyticus*, which catalyzes with similar efficiency the degradation of 3OC12 AHL and C4-AHL. However, some enzymes demonstrate a narrower substrate specificity. A member of the PLL-family, variant SsoPox W263I from *Saccharolobus solfataricus*, has a 930-fold greater catalytic efficiency towards 3OC12 AHL than with C4-AHL[115]. In *P. aeruginosa*, C4-AHL is the autoinducer produced by RhlI and activating RhlR, while 3OC12 is produced by LasI and activates LasR[1]. LasR regulates virulence factors such as the production of elastase, protease, and exotoxins[3], while RhlR regulates rhamnolipid production and swarming motility[28], as well as pyocyanin production[87] (See **Figure 6** for other regulated behaviors).

Lactonases with broad or narrow substrate selectivity have different effects on quorum sensing systems. The addition of SsoPox W263I and GcL to cultures of *P. aeruginosa*, both together and separately, has been shown to quench the expression of various virulence factors. Each of these enzymes reduced the production of pyocyanin, protease, and elastase, and when added in combination produced a synergistic effect, quenching these behaviors to a greater degree than either enzyme alone. SsoPox W263I showed a greater capability to disrupt biofilm formation and increase the antibiotic susceptibility of cultures of *P. aeruginosa* towards antibiotics. The addition of this enzyme also strongly reduced the expression of *pqsA*[115], a gene responsible for the production of the PQS autoinducer[19]. SsoPox W263I also decreased virulence of *P. aeruginosa* in an amoeba infection model[115]. When clinical isolates of *P. aeruginosa* from patients with cystic fibrosis were treated with either enzyme, the expression of virulence factors in some isolates was inhibited by one enzyme and not the other[116].

Engineering Quorum Quenching Enzymes

While a variety of QQE have been characterized, their use as biocontrol agents may have

unintended consequences as QS molecules are used to regulate beneficial and detrimental behaviors[81]. Expression of the lactonase *aiiA* has been shown to reduce nodule formation in *Sinorhizobium meliloti* (a N_2 fixing bacterium) due to degradation of long-chain AHLs produced by that species' LuxI homologue, SinI[10]. The same lactonase expressed in *Pseudomonas chlororaphis* has been shown to reduce the anti-fungal capabilities of this strain and decrease its ability to protect plants[81]. Apart from specificity, affinity, and activity, other considerations for engineering QQE include practical considerations such as thermostability, high yield, solubility, and immunogenecity[83].

To avoid interfering with potentially beneficial bacterial interactions, we propose that systems that are less wide-spread among bacteria could be targeted, or enzymes could be engineered for greater specificity towards certain small molecule autoinducers using both rational design and directed evolution[81]. The WT acylase PvdQ poorly degrades AHLs with side chains of less than 10 carbons, but researchers were able switch its specificity through the introduction of a L146W and a F24Y mutation. The resulting mutant degraded C8-AHL but not 3-oxo-C12 AHL, and as C8-AHL is used to modulate virulent behaviors in the pathogen *Burkholderia*, this mutant can be used to quench those behaviors[106]. The PLL orthologue enzyme MCP from *Mycobacterium avium* subspecies *paratuberculosis* shows little activity against C6 AHL, and no detectable activity against C4 and 3-oxo-C6 AHL, but a single mutant N266Y showed novel activity against C4 and 3-oxo-C6 AHL, as well as increased activity against C6-AHL and 3-oxo-C8-AHL[117].

The mechanisms underlying the specificity of enzymes and their mutants toward different substrates have been examined for several enzymes. In the case of the PvdQ work mentioned previously, crystallographic analysis of both the wild type PvdQ and PvdQ L146W F24Y showed that the introduction of a bulky tryptophan side chain in place of leucine decreased the size of the hydrophobic binding pocket, excluding the binding of longer chain AHLs such as 3-oxo-C12 and increasing affinity for C8-AHL. The replacement of phenylalanine with tyrosine introduced a new hydroxyl group that formed a hydrogen bond with the amine of Trp146, stabilizing its conformation[106]. The importance of the binding cleft size in conferring specificity has been demonstrated with other QQE such as the promiscuous lactonases AaL and GcL, in which the acyl chain of longer AHLs is exposed to the solvent, and the active site is relatively accessible[112,118]. In addition, AaL has a unique hydrophobic patch that may contribute to lower K_M values[118]. QQE engineering has demonstrated success in producing mutants with modified specificity and greater

ability to impact targeted QS regulated bacterial behaviors. However, concerns over unintended effects on beneficial host microbes and the development of resistance to QQE or small molecule inhibitors indicate that more research is needed in this field[83].

Thus, controlling quorum sensing regulated behaviors may require the use of multiple enzymes of different specificities. Engineering and discovery of quorum quenching enzymes may allow us to better understand the structural determinants of substrate specificity and eventually fine-tune our control of quorum sensing in microbial communities. To that end, we characterize in this work the substrate specificity and binding mode of zearalenone hydrolase, a lactonase capable of degrading AHL autoinducers.

Structural and Kinetic Characterization of a Quorum Quenching Lactonase from Fungi

Zearalenone (ZEN) is a mycotoxin produced by *Fusarium* fungal species, specifically *F. graminearum* and *F. culmorum*[119]. These species infect wheat and corn crops meant for human consumption in a disease known as *Fusarium* head blight[120]. Contamination of these crops is problematic, as ZEN is a mimic that competes with estrogen, binding to its receptors and causing a variety of reproductive disorders[121]. *Fusarium* species may produce ZEN to inhibit the growth of competing fungi[122].

Clonostachys rosea is a soil fungus that has shown potential as a biocontrol agent of *Fusarium*[123], but the mechanism of biocontrol was unknown until 2002, when a hydrolase capable of degrading ZEN was discovered[124]. Zearalenone hydrolase (ZHD) degrades the large lactone ring of ZEN to produce 1-(3,5- dihydroxy-phenyl)-10'-hydroxy-1'-undecen-6'-one, a much less potent toxin[125] (See **Figure 19**).

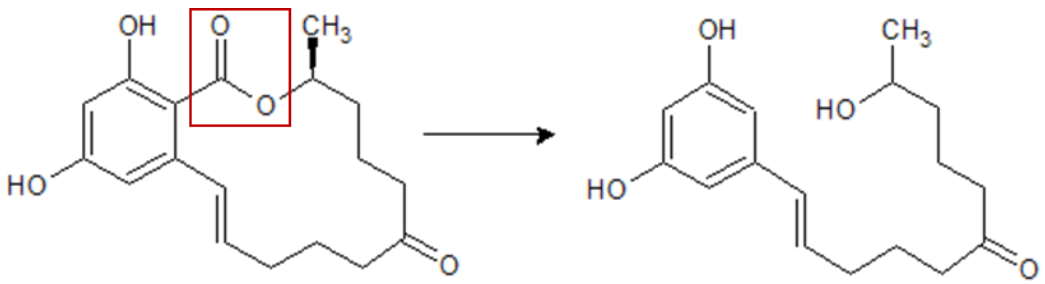


Figure 19: Cleavage of zearalenone by ZHD. Lactone group is highlighted.

Although the chemical structures of ZEN and AHLs are different (see **Figure 23**), both contain a lactone ring. It had been proposed that ZHD may have a role in quorum quenching and may be an example of an α/β-hydrolase fold lactonase that does not require a metal cation for catalytic activity. The crystal structure of ZHD in complex with ZEN[74] and the hydrolysis product 1-(3,5- dihydroxy-phenyl)-10'-hydroxy-1'-undecen-6'-one[126] has been solved, and a catalytic mechanism has been proposed involving nucleophilic attack by a serine residue as part of a catalytic triad with histidine and glutamic acid[74], similar to another α/β-hydrolase, AidH[114].

Here we provide data showing that ZHD is a broad-spectrum lactonase. We verified its ability to degrade AHLs using pH colorimetric assays, biosensor assays, and mass spectrometry. Contrary to some other characterized lactonases, no promiscuous activity was observed for paraoxon. The resolution of ZHD structure bound with an AHL molecule allowed us to establish the substrate binding mode of the substrate in this enzyme. The following work is presented in the format of a journal article as it is being prepared for future publication.

Materials and Methods

Cloning, expression, and purification of the protein ZHD.

The protein was produced in *Escherichia coli* strain BL21(DE3)-pGro7/GroEL (TaKaRa). A His tag (LEHHHHHH) was added to the C-terminus of the amino acid sequence which was then inserted into the expression plasmid pET22b(+) using the XhoI/NdeI restriction sites, and ordered from Genscript (New Jersey, USA). The protein was produced at 37º C in 6 L of autoinducer media ZYP along with 100 $\mu g \cdot ml^{-1}$ ampicillin, 34 $\mu g \cdot ml^{-1}$ chloramphenicol, and 1 mM $MgSO_4$. When the OD_{600} of the culture reached the exponential growth phase, it was induced with 0.2% L-arabinose. After induction the cultures were grown at 18º C overnight, and then were centrifuged at 4400 x g for 20 minutes at 4º C to pellet the cells, which were then frozen at -20º C. Frozen pellets were then resuspended in lysis buffer (10 mM Tris, 100 mM NaCl, pH 7.5, .1mM PMSF, 25 mg/mL

lysozyme, and 10 µg/mL DNAse I) and left on ice for 45 minutes. Then, cells were sonicated for 35 seconds (1 second pulse-on, 1 second pulse-off) at amplitude 35 (Q700 Sonicator, Qsonica, USA) until the lysate viscosity was low and no visible cell pellet remained. Sonication was followed by centrifugation of the lysate (27000 x g, 45 minutes, 4º C). The lysate supernatant was then filtered first through a .8 µm and then a .45 µm syringe filter. The sample was then loaded onto a 5 ml HisTrap HP chromatography column (GE Healthcare) in ZHD binding buffer (10 mM Tris, 100 mM NaCl, pH 7.5) at room temperature. The column was then washed with 5 column volumes of binding buffer, and then with 2 column volumes of 90% binding buffer and 10% elution buffer (10 mM Tris, 100 mM NaCl, pH 7.5, 300 mM imidazole). The % elution buffer was then increased linearly from 10% to 60% over 5 column volumes. Upon observation of a UV peak corresponding to protein release from the column, the percent elution buffer was held constant over a period sufficient to completely elute the protein. After confirmation of the protein's monomeric size and purity using Coomassie-stained SDS-PAGE, the protein sample was filtered through a 0.2 µm syringe filter and loaded onto a size exclusion column (Superdex 75 16/60, GE Healthcare) to further purify the protein. Final purity and size of the protein was again confirmed via Coomassie-stained SDS-PAGE.

Kinetic Measurements

ZHD catalytic activity was measured using a microplate reader (Synergy HTX, BioTek, USA), using Gen5.1 software, in a 96 well plate with a path length of 0.58 cm, each well containing 200 uL reaction volume at room temperature. Kinetic parameters were calculated by fitting the data to the Michaelis-Menten equation using Graph-Pad Prism 5.0 software. Measurements were performed in quadruplicate at minimum.

Biosensor assay

Biosensor plasmids containing reporter genes regulated by LuxR/I or AhyR/I were used to detect the concentration of AHLs present in solution. The two reporter plasmids used were pSB403 and pSB536[99]. Each plasmid contains one regulatory gene (R) without a functional AHL synthase gene (I). pSB403 encodes LuxR, regulating expression of green fluorescent protein, while pSB536 encodes AhyR regulating a *lux* operon. pSB403 was transformed into *Escherichia coli* strain MT102, while pSB536 was transformed into *E. coli* strain JM109. Cultures of each transformed strain were grown overnight for 18 hours at 30 ºC with appropriate antibiotics (10 µg/ml

tetracycline for pSB403, and 100 μg/mL ampicillin for pSB536) in LB, and then diluted 1:50 in fresh LB and grown for 1 hour before addition of lactones. 180 μL of culture was then combined with 20 μL of lactone solution and incubated for 3 hours on a rocking platform at 37 ºC. Luminescence was measured with a microplate reader (Synergy HTX, BioTek, USA), while fluorescence was measured at 509 nm after excitation at 488 nm. Standard curves of luminescent or fluorescent response were generated using GraphPad Prism 5.0 software. The LuxR/I pair responds to a wider variety of AHLs with various chain lengths, and so pSB403 was used with C6, C8, and C10-AHL. pSB536 with the AhyR/I pair was used with C4-AHL due to its stronger signal with that lactone.

Quorum Quenching Assay

Degradation of lactones by Bovine Serum Albumin (BSA) ZHD, and GcL, was quantified by incubating 495 μL of 100 μM of each lactone with 5 μL of .1mg/ml enzyme solution in LB media for 2 hours at 30 ºC. GcL and SsoPox W263I were used as positive controls due to their known activity against AHLs[79,127]. A blank without lactone was used to subtract background signal. After incubation, 20 μL of the media was added to 180 μL of the appropriate biosensor culture as detailed in the previous section, after which the luminescent or fluorescent signal was measured.

Lactonase assay

Hydrolysis of the lactone ring of AHLs generates a carboxylic acid, which lowers the pH of the solution. The rate of hydrolysis can thus be indirectly measured by reading a colorimetric absorbance change at 577 nm of a pH indicator, *m*-cresol purple as previously described[82,112]. The rate factor of this indicator was determined by titration with acetic acid at a range of concentrations from .35 mM to .05 mM. Reaction volumes contained 2.5 mM Bicine, 150 mM NaCl, .25 mM *m*-cresol purple, and 5% dimethyl sulfoxide at pH 8.3. Lactone substrates were added at 8 concentrations in the range of 10 μM to 1000 μM. Substrates tested included *N*-acyl homoserine lactones of different acyl chain lengths of 4, 6, 8, and 10 carbons (C4,6,8, or 10-AHL), δ-lactones, and γ-lactones. AHL substrates were purchased from Cayman Scientific, while γ- and δ-lactones were obtained from Sigma.

Thiolactonase assay

Hydrolysis of the lactone ring of homocysteine lactones was measured using the Ellman assay (1961) following the reaction of 5,5'dithio (bis-2 nitro benzoic acid, DTNB) with sulfhydryl

groups[128,129]. The resulting production of 5-thio-2-nitrobenzoic acid (TNB) is followed by measuring the A_{412nm} due to increasing yellow coloration. Ellman assays were carried out in phosphotriesterase (PTE) buffer (50 mM HEPES, 150 mM NaCl, pH 8.0). The extinction coefficient of TNB at pH 8.0 is $1.36x10^4\ M^{-1}\cdot s^{-1}$. In short, 145 µL of PTE buffer was combined with 40 µL of Ellman solution (4mg/mL DTNB in PTE buffer), with 5 µL of the enzyme solution, and 10 µL of the thiolactone solution (in DMSO) for a 200µL reaction volume. A large volume mixing was performed before beginning the time course measurement of a A_{412nm}.

Paraoxonase assay

Ethyl-paraoxon hydrolysis by ZHD at 25 ºC was measured by following the production of *p*-nitrophenolate at 405 nm (ε_{405nm} = 17000 $M^{-1}\cdot cm^{-1}$) in *PTE buffer* (50 mM HEPES, 150 mM NaCl, pH 8) as previously described[82,112].

Dihydrocoumarin Hydrolase Assay

Dihydrocoumarin (DHC) hydrolase activity was measured by following the production of 3-(2-hydroxyphenyl)propionate through measurement at A_{270nm} at pH 7, 25 ºC, in 50 mM potassium phosphate buffer as described previously[130]. Assays were performed in quadruplicate, and were compared to activity in an enzyme with measured DHC hydrolytic activity (SsoPox W263I)[127].

Liquid Chromatography – Mass Spectroscopy of lactone degradation products

200 uL solutions of C4--AHL, C8-AHL, γ-butyrolactone, γ-decalactone, δ-octanolactone, δ-undecanolactone were prepared at concentrations of 1 mg/mL. The AHL solutions contained 5% DMSO to improve solubility of the lactone, while the γ- and δ-lactones contained 1% DMSO. These samples were incubated with 5 µL of ZHD enzyme solution at 10 mg/mL. A second set of identical lactone solutions were incubated with 5 µL of .5 mg/mL GcL, a lactonase with broad spectrum activity, as a positive control[112], while a third set without enzyme treatment was also tested. Each enzyme/substrate solution was incubated for 4 hours, then frozen overnight before being sent for LC/MS. The solutions were then diluted 1/100 in water with 5% acetonitrile (ACN), and .1% formic acid. 10µL of each sample was then injected onto a reverse-phase, positive mode column (Water, Acquity BEH C-18 column, 2.1 x 100 mM, 1.7 µm particle size), at 40 °C, with a flow rate of .4ml/minute, with the effluent flowing directly into the mass spectrometer. Buffer A consisted of water with 0.1% formic acid, while buffer B was ACN with 0.1% formic acid. A gradient elution method was used, in which % buffer B was increased linearly from 1 to 40% over

4 minutes, followed by a rapid linear increase from 40 to 95% buffer B, after which buffer B was held at 95% for one minute, then decreased to 2% over the next 30 seconds. Finally, buffer B was held at a 2% for the last minute and a half.

Mass spectrometric analysis was performed on a ThermoScientific, Q Exactive, Quadrupole, Orbitrap with Heated-Electrospray Ionization probe source (HESI-II). Ion spray voltage was 3400 V positive, sheath gas flow rate was 50 mL/min, capillary temperature was 320 ºC, with an auxiliary gas heater at 400 ºC. MS method was full MS-SIM, with data collection duration of 8 minutes. Chromatographic peak width was 4 seconds, with positive polarity, 70,000 resolution, automatic gain control (AGC) target was 106. Maximum injection time was 200 ms. Scan range was 70 to 1050 m/z.

Quantitative analysis of lactones from LC/MS

Examination of mass spec data was performed using Thermo Xcalibur software. Background signal from samples lacking lactones were subtracted before comparison of curves from lactone solutions. Peaks for both the intact homoserine lactone and the lactonase degradation product were found using their accurate isotope mass, and the peak intensities for each species in the non-enzyme treated, ZHD treated, and GcL treated samples were compared. Accurate isotopic masses used were: C4-AHL: 172.09737 m/z, C4 homoserine: 190.10793 m/z, C8-AHL: 228.15997 m/z, C8 homoserine: 246.17053 m/z.

Crystallization

Conditions for crystallization were hanging-drop vapor diffusion at 293 K with a protein concentration 30 mg/ml. Protein solution consisted of *ZHD buffer* (10 mM Tris, 100 mM NaCl, pH 7.5). Reservoir solution contained 1.4 M ammonium dibasic phosphate, 200 mM KCl, 100 mM imidazole pH 7.5. Crystals in the shape of long rectangular prisms appeared after 2 or 3 days, with sizes ranging from .3 x .05 x .05 mm to 2 x .25 x .25 mm. Cryoprotectant solution (.1 M imidazole, .2M KCl, .6 M ammonium dibasic phosphate, and 30% glycerol) including substrate appropriate to the complex crystallized was added at a ratio of 1:1 to the crystal drops prior to flash freezing in liquid nitrogen. Concentration of substrate used in both co-crystallization and cryoprotectant solutions were near their respective maximum solubilities in aqueous solution to increase occupancy of the active site. C4-AHL was present at 35 mM, C8-AHL at 8 mM, and γ-nonalactone at 20 mM.

X-ray diffraction data collection and protein-ligand complex modelling

X-ray diffraction data for each complex were collected at 100 K using synchrotron radiation at the 23IDB beam line (Advanced Photon Source, Argonne National Laboratory, USA). X-ray diffraction data were integrated and scaled with the XDS package[131]. The phases were obtained using the structure of ZHD (PDB code 5c7y) as a starting model for molecular replacement with MOLREP. WinCoot[132] was used for building models which were then refined with REFMAC5[132]. PyMOL v2.3.0 was used to generate structure diagrams[133].

Melting point determination for ZHD

Melting point determination was carried out using SYPRO Orange dye as detailed by Huynh and Partch[134] with some modifications. Briefly, this assay involves the use of a dye that fluoresces after binding to the exposed hydrophobic regions of denatured proteins. A RT-PCR machine is used to change the temperature of the solution, denaturing the protein and increasing the fluorescence of the dye. 5 µM ZHD was combined with 3X SYPRO Orange dye in triplicate, while a blank triplicate without the protein was used to subtract background fluorescence. The reaction mixture was incubated for 3.5 minutes at 25° C, and then increased from 25° C to 95° C at the rate of 0.5° C every 30 seconds while measuring fluorescence at 570 nm.

Results and Discussion

ZHD is a quorum quenching enzyme.

Biosensor responses to samples treated with ZHD were less than those from samples treated with BSA (**Figure 20**).

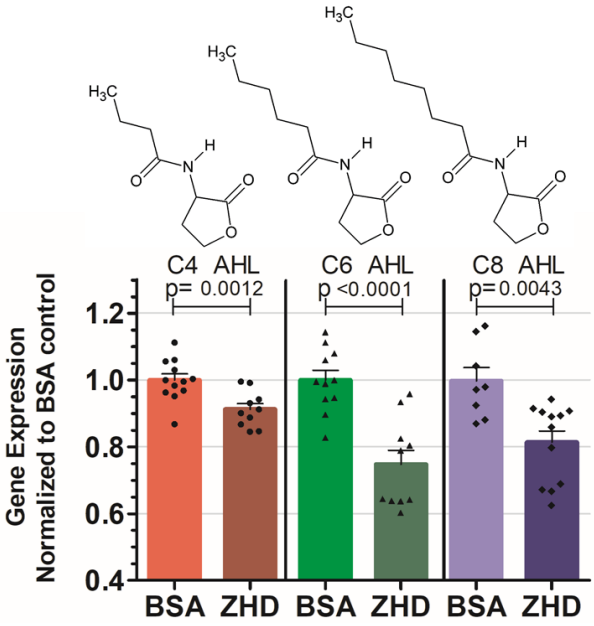


Figure 20: Quenching of luminescent (C4-AHL) or fluorescent (C6, C8) signal from reporter plasmid pSB536 or pSB403 respectively after incubation of 100µM lactone solution with BSA control or ZHD for 3 hours. BSA was used as a negative control, One-tailed P values comparing BSA and ZHD treated samples are shown.

Degradation of C4 and C8-AHL by ZHD was further confirmed via LC/MS of lactone solutions incubated with the enzyme (**Figures 21 and 22**). Samples were treated with ZHD or GcL, a highly active AHL lactonase used as a positive control. GcL treated samples showed large peaks corresponding to the degraded homoserine products for both C4 and C8-AHLs, with a corresponding decrease in the peak associated with the intact AHL. ZHD treated samples showed a similar pattern confirming the lactonase activity yet suggesting that it is slower than GcL.

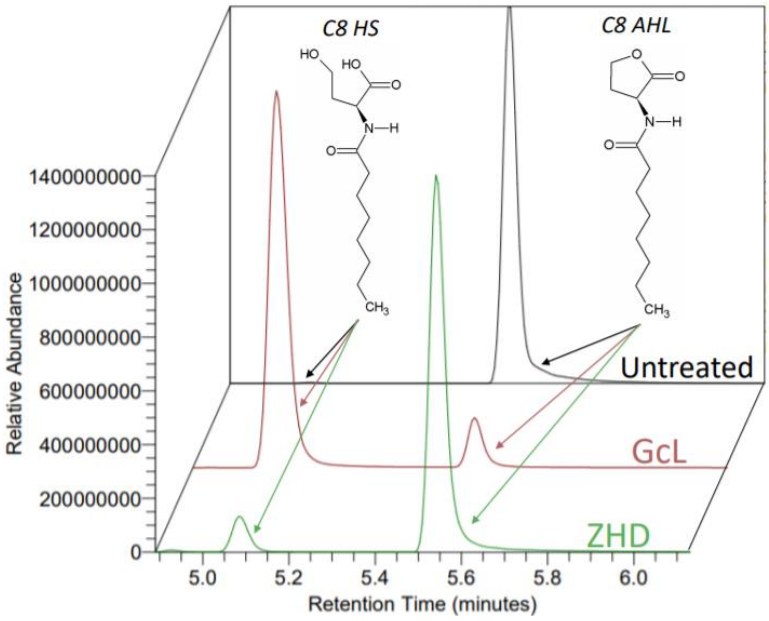


Figure 21: Mass spectroscopic analysis of solutions of C8-AHL after incubation without enzyme, with GcL, or with ZHD. The peak with a retention time of 5.08 minutes corresponds to the hydrolyzed C8-AHL, while the peak at 5.54 minutes corresponds to the intact C8- AHL.

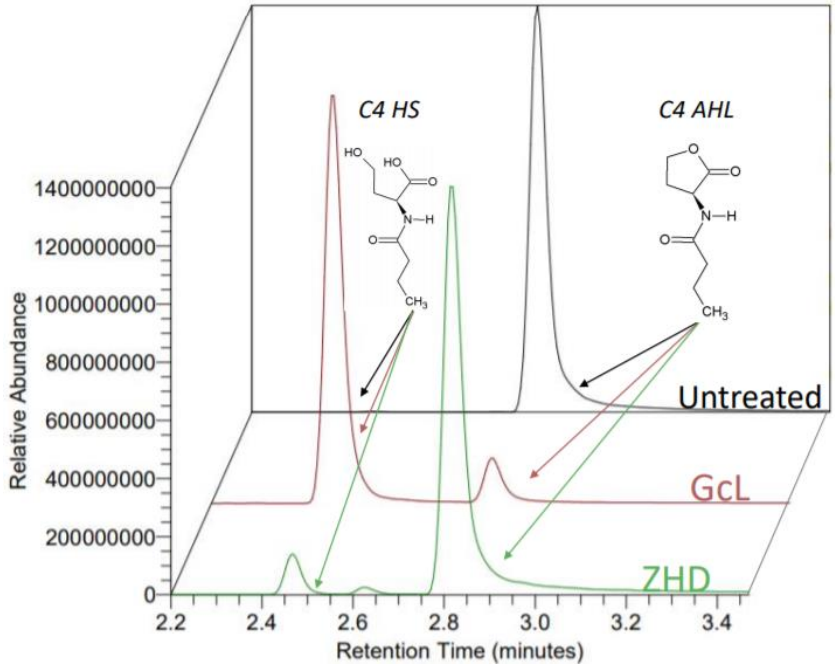


Figure 22: Mass spectroscopic analysis of solutions of C4-AHL after incubation without enzyme with GcL, or with ZHD. The peak with a retention time of 2.47 minutes corresponds to the C4 homoserine, while the peak at 2.81 minutes corresponds to the intact C4 homoserine lactone. The small peak at 2.62 minutes is an unknown contaminant with the formula $C_{10}H_{22}O_9$

ZHD is a broad-spectrum lactonase.

The activity of ZHD against a variety of substrates was measured (**Table 3**). Along with zearalenone (**A**), ZHD degrades a broad spectrum of AHLs (**B**), γ-lactones (**D**), and δ-lactones (**E**). No activity was found for ZHD against homocysteine lactones, also known as homothiolactones (HTLs, **C**), dihydrocoumarin (**F**), or paraoxon-ethyl (**G**). The kinetic parameters were determined and are reported below in **Table 7**. ZHD demonstrated broad spectrum activity against a variety of AHLs, γ-lactones, and δ-lactones, with a k_{cat}/K_M in the range of 10^4 to 10^5 $M^{-1} \cdot s^{-1}$. These kinetic parameters are consistent with previous observations showing that

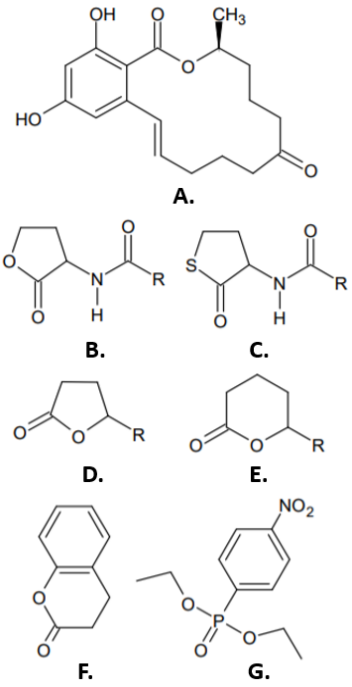


Figure 23: Potential substrates of ZHD. R-groups indicate carbon chains of different lengths.

A. Zearalenone (ZEN)
B. Acyhomoserine Lactone (AHL)
C. Acylhomocysteine Lactone (HTL)
D. γ-lactone
E. δ-lactone
F. Dihydrocoumarin
G. Paraoxon-ethyl

Substrate		ZHD		
	Name	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} M^{-1})
AHLs	C4-AHL (L)	8.9 ± 0.4	50.3 ± 12.9	1.8 (± 0.5)x10^5
	C6-AHL (L)	4.9 ± 0.4	8.2 ± 5.1	5.9 (± 3.3)x10^5
	C8-AHL (L)	8.9 ± 0.5	9.5 ± 3.5	9.3 (± 3.5)x10^5
	C10-AHL (L)	6.9 ± 1.0	150.1 ± 50.6	4.6 (± 2.6)x10^4
γ-lactones	γ-butyrolactone	10.7 ± 1.0	49.4 ± 20.8	2.2 (± 0.9)x10^5
	γ-nonalactone	19.0 ± 1.1	95.6 ± 22.0	2.0 (± 0.5)x10^5
	γ-undecanolactone	9.3 ± 0.4	16.8 ± 4.1	5.5 (± 1.4)x10^5
δ-lactones	δ-nonalactone	19.2 ± 1.7	85.2 ± 24.0	2.3 (± 0.7)x10^5
	δ-decalactone	21.6 ± 2.1	72.3 ± 32.8	2.9 (± 1.9)x10^5
HTLs	C1-HTL (DL)	ND	ND	ND
	C4-HTL (DL)	ND	ND	ND
	C8-HTL (DL)	ND	ND	ND
Others	Dihydrocoumarin	ND	ND	ND
	Paraoxon	ND	ND	ND
	Zearalenone[α]	0.23 ± 0.01	8.5 ± 2.1	2.71(± .7)x10^4

Table 7: Kinetic constants for ZHD. [α]Kinetics with zearalenone at pH 8.5 were determined in 2004 by Takahashi-ando, et al[123].

ZHD is less active than GcL, which exhibits rates up to 10^6 $M^{-1} \cdot s^{-1}$. It had no detectable activity against some substrates that are commonly degraded by other classes of known lactonases (PONS, PLLs, MLLs) such as homocysteine lactones, dihydrocoumarin, or phosphotriesters such as paraoxon ethyl.

ZHD has moderate thermal stability, but appears to lose activity rapidly in solution

We measured the melting temperature of ZHD (**Figure 24**) using a SYPRO Orange dye assay[134] and determined that the melting temperature of ZHD was 50.80 ± .38 ºC. Despite this relatively high melting temperature, ZHD appears to lose activity after less than 20 minutes in solution with C4 and C8 AHL, for unknown reasons that need to be examined further (mass spec data not shown). This loss of activity could explain the large difference in peak area from LCMS observed between ZHD and our positive control, the highly active GcL enzyme (**Figures 21** and **22**).

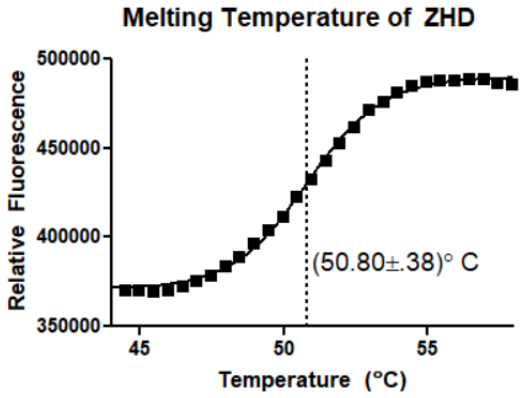


Figure 24: Fluorescence of SYPRO Orange in solution with ZHD with increasing temperature.

Crystallization and modelling of ZHD in complex with an AHL and a γ-lactone

In order to examine whether ZHD is capable of hydrolyzing lactones commonly used in quorum sensing (the AHLs as well as some γ lactones), we crystallized ZHD in complex with both C8 AHL and γ-nonalactone and modeled these structures using x-ray diffraction. The crystal structures of ZHD in complex with C8 AHL and γ-nonalactone were determined at resolutions of 1.90 Å and 1.80 Å respectively using molecular replacement with a previously determined model of ZHD[74]. Size exclusion chromatography of ZHD demonstrates that it is monomeric in solution. It is very similar in structure to AidH, a member of the α/β-hydrolase family (RMSD = 2.06). Like AidH, ZHD has a core α/β-hydrolase fold core and a cap domain. Data collection and refinement statistics

for these modeled complexes can be found in **Table 8**, while models of the active site of ZHD bound to each ligand are shown in **Figure 25**.

Table 8: Data collection and refinement statistics for ZHD complexes

Structure	**ZHD bound to C8 AHL**	**ZHD bound to γ-nonalactone**
DATA COLLECTION STATISTICS		
PDB ID		
Diffraction source	APS Argonne 23ID-B	APS Argonne 23ID-B
Wavelength (Å)	.991840	.991840
Detector	DECTRIS EIGER 16M	DECTRIS EIGER 16M
Rotation range per image (°)	0.5	0.5
Total rotation range (°)	250	250
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell parameters (Å)	a = 74.80	a = 74.19
	b = 91.30	b = 91.84
	c = 113.50	c = 113.45
	α = 90.000	α = 90.000
	β = 90.000	β = 90.000
	γ = 90.000	γ = 90.000
Resolution range (Å) (last bin)	1.90 (1.90-2.00)	1.80 (1.80-1.90)
Total N° of reflections (last bin)	530767 (76749)	389702 (57815)
N° of unique reflections (last bin)	60380 (8474)	71500 (10568)
Completeness (%) (last bin)	99.9 (99.9)	99.8 (99.7)
Redundancy (last bin)	8.79 (9.06)	5.45 (5.47)
⟨I/σ(I)⟩ (last bin)	25.61 (4.29)	22.84 (3.75)
Rsym (%) (last bin)	5.4 (53.3)	7.9 (57.1)
CC(1/2) (last bin)	99.9 (92.0)	99.9 (93.0)
REFINEMENT STATISTICS		
R_{free}/R_{work} (%)	19.88/16.51	18.31/15.91
N° of total model atoms	4880	5032
Ramachandran favored (%)	95.44	94.95
Generously allowed rotamers (%)	2.73	3.21
Ramachandran outliers (%)	1.82	1.83
Rmsd from ideal		
Bond lengths (Å)	.013	.013
Bond angles (°)	1.727	1.827

Figure 25: Active site residues of ZHD in complex with C8 AHL (a) and the product of γ-nonalactone hydrolysis (b). The perspective has been rotated 180° horizontally between (a) and (b). The substrates are shown as sticks with a radius thicker than the side chains of the residues. The main chain portion of S102, S103, and G32 are also shown.

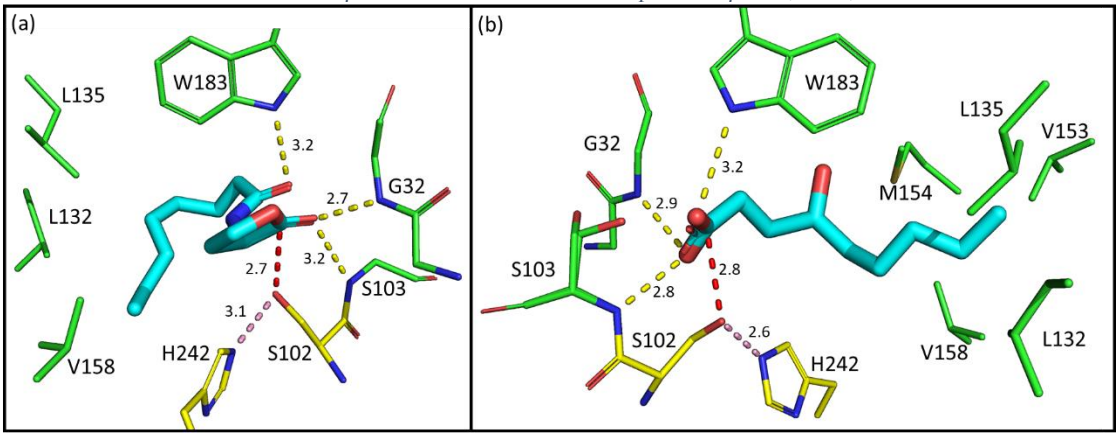


Figure 25(a) shows the interactions present between the intact C8 AHL in the active site of ZHD and nearby residues. Ser102 is located at a distance of 2.7 Å from the carbonyl carbon of the lactone ring, well situated to perform a nucleophilic attack on this carbon in the formation of a tetrahedral transition state. Two members of the catalytic triad are shown (Ser102 and His242). The carbonyl oxygen of the lactone ring is 2.7 Å and 3.2 Å from the main chain amide groups of Gly32 and Ser103 in a position that allows these residues to stabilize the oxyanion of the transition state after nucleophilic attack. Trp183 forms a 3.2 Å hydrogen bond with the carbonyl oxygen of the acyl chain amide group of the substrate. On the left side of **Figure 25(a)** are several hydrophobic residues (Leu135, Leu132, and Val158) that accommodate the side chain.

ZHD was also crystallized with γ-nonalactone (**Figure 25(b)**), and the hydrolyzed product could be modelled. A similar pattern of interactions occurs with this substrate, with Ser102 situated for attack on the carbonyl carbon of the lactone ring, and an oxyanion hole formed by the main chain amid groups of Gly32 and Ser103. Trp183 forms a hydrogen bond with one of the oxygens of the carboxylic acid group formed as a result of hydrolysis. A variety of residues accommodate the hydrocarbon tail of γ-nonalactone, including Leu135, Leu132, Val158, Val153, and Met154.

As briefly discussed earlier, ZHD has been crystallized in complex with zearalenone (ZEN) substrate as well as its hydrolysis product. A variety of residues in the active site hydrogen bond with ZEN, while others (Ser102, His242, and Glu126) have been proposed as members of a catalytic triad, with Ser102 acting as a nucleophile and attacking the ester carbon of the lactone

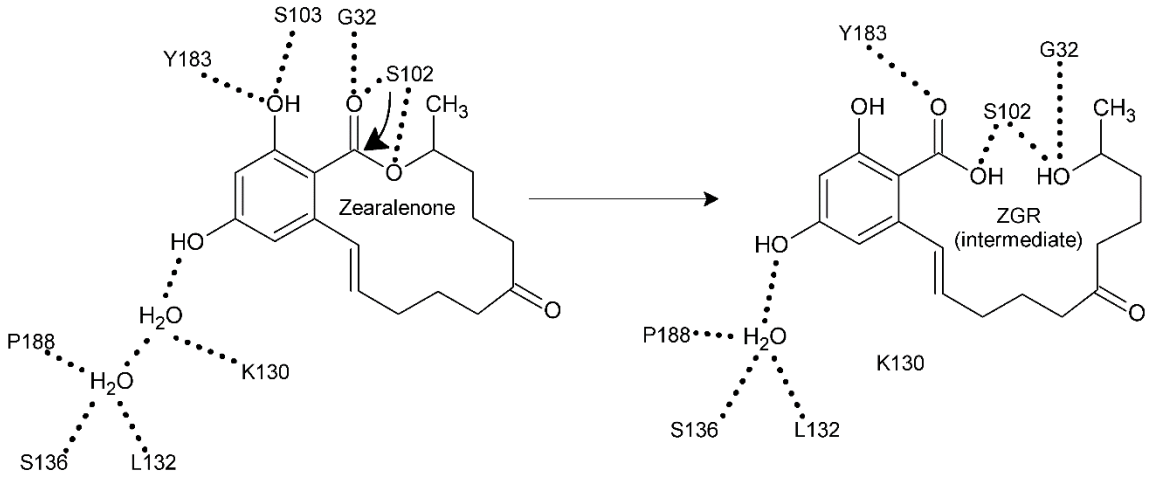


Figure 26: ZHD binding mode to ZEN. Dotted lines represent hydrogen bonding coordination in the active site of ZHD, while the curved arrow represents nucleophilic attach of serine on the ester carbon of the lactone ring.

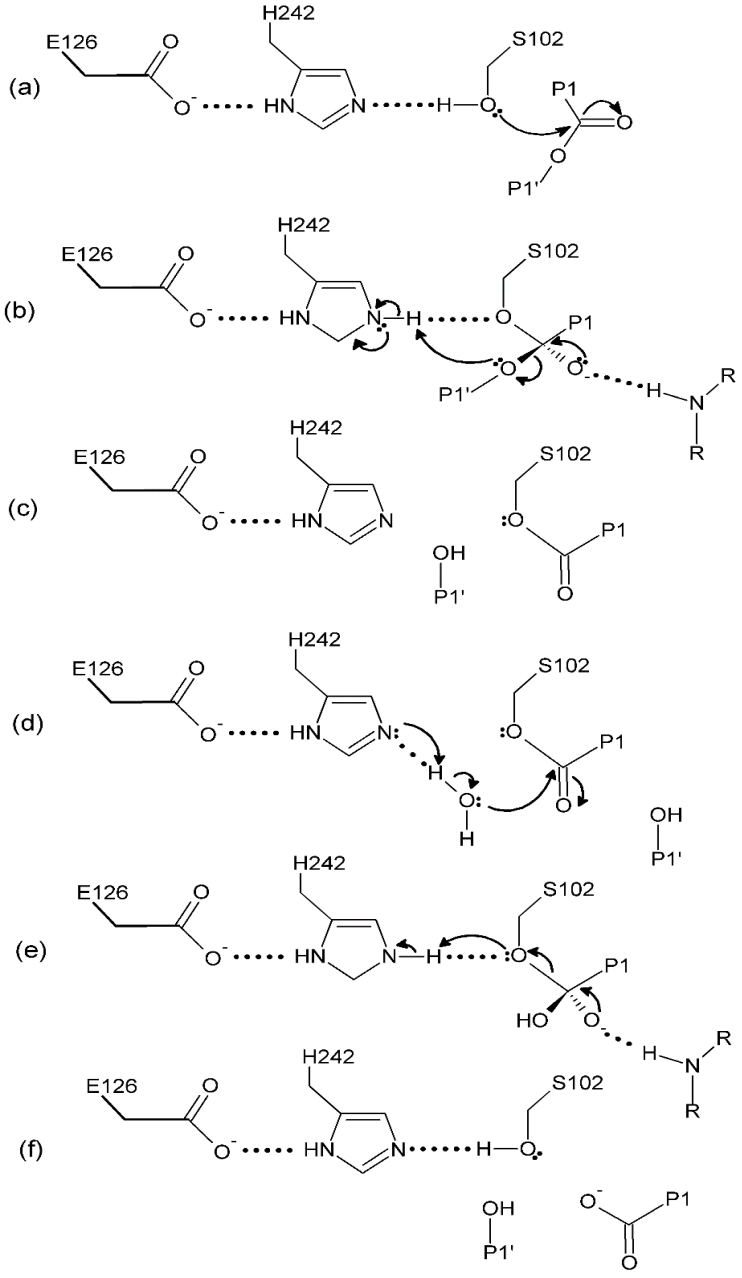
ring[74] (see **Figures 26** and **27**). AidH, another α/β-hydrolase, has a similar catalytic triad (Ser102, His248 and Glu219) and has demonstrated activity against a variety of homoserine lactones[114].

The catalytic triad shown in **Figure 26** is well known, but a brief description of its mechanism follows. An aspartate or glutamate forms a hydrogen bond with a histidine, stabilizing the positive charge on the histidine, allowing the histidine to deprotonate the nucleophile (a). This nucleophilic serine can then attack the ester carbon of a lactone ring. A tetrahedral transition state is formed (b) followed by donation of a proton by histidine to the leaving group P1' (c). Water then attacks the carbon of the ester group (d), and then the catalytic serine is regenerated (e). The final products are an alcohol-bound moiety (P1') and a carboxylic acid bound moiety (P1) (f). The members of the catalytic triad in **Figure 26** are labeled as those from ZHD. The tetrahedral transition states (b and e) are stabilized by an oxyanion hole consisting of backbone amide groups[135].

The relatively wide binding channel for ZHD, with few large residues that could block the active site helps explain the wide substrate specificity of ZHD toward a variety of AHLs as well as γ- and *δ*-lactones. The acyl chains of both molecules are bound differently in the active site (see **Figure 25**), as the AHL and γ-lactone acyl chains are attached at the α and γ positions of the lactone ring.

The catalytic Ser102 seems well situated for nucleophilic attack on the lactone ring of these substrates, and the main chain amide groups of Gly32 and Ser103 provide stabilization for the resulting oxyanion transition state. This binding mode is consistent with the proposed mechanism in **Figure 27**, and consistent with the proposed binding mode of Zearalenone to ZHD[74].

Figure 27: Catalytic triad of ZHD



3.3 Zearalenone Hydrolase - Conclusion

ZHD degrades a variety of small molecules that are used by both gram-negative and gram-positive bacteria as part their quorum-sensing systems, demonstrating a broad substrate specificity with a catalytic efficiency in the same range as other lactonases belonging to distinct families.

Quorum quenching activity has arisen from a variety of protein folds and families. ZHD is not a member of the three main lactonase families, the phosphotriesterase-like lactonases, the paraoxonases, and the metallo-β-lactamase-like lactonases. Respectively, the proteins of these families utilize six-bladed β-propeller, αβ/βα, and a$(\alpha/\beta)_8$ TIM barrel fold, while ZHD exhibits a α/β-hydrolase fold. AidH[114], a different member of the α/β-hydrolase family, also demonstrates lactonase activity with mechanism similar to ZHD but little sequence similarity exists between ZHD and AidH. Convergent evolution of various families has thus produced enzymes capable of degrading similar substrates, while parallel evolution of ZHD and AidH has produced two enzymes with similar functional and structural characteristics but no sequence identity (See **Figure 28**)[136]. ZHD is thus a novel lactonase with a fold unique from the major lactonase families, and a sequence with no similarity to the other characterized α/β-hydrolase lactonase.

Bacteria compete for nutrients with fungal hyphae in the soil, and the formation of biofilm on the hyphae can prevent nutrient uptake by those hyphae. Fungi, such as *Clonostachys rosea* from which

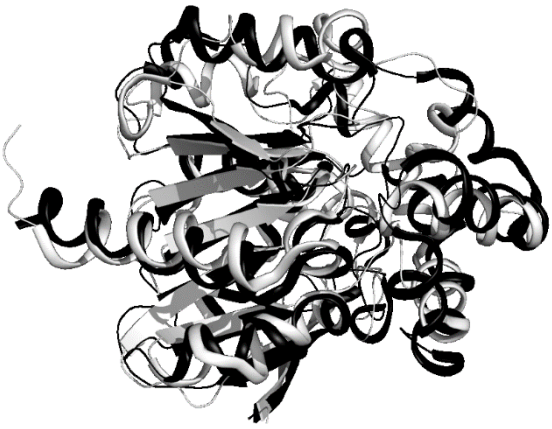


Figure 28: Superimposition of AidH (white) and ZHD (black). These proteins share no detectable sequence similarity but have a similar structure. Both hydrolyze AHLs.

ZHD was isolated, use a variety of strategies to prevent bacterial growth and biofilm formation. These strategies include the secretion of antimicrobial molecules and interference with microbial

communication[137]. As ZHD has a demonstrated capability to degrade AHLs used in bacterial communication, it may have a role in fungal interference in quorum sensing among competing gram-negative bacteria.

4. Conclusion

Quorum sensing is a complex phenomenon, and receptors used by bacteria to regulate their behaviors can respond to a specific or wide range of molecules. Here we have shown that LuxR homologue regulated biosensors capable of responding to AHLs display some wider substrate selectivity toward some γ-lactones. This response was further verified *in vivo* through the measurement of pyocyanin production in *P. aeruginosa*. We have also demonstrated that LuxR homologous receptors vary in their specificity to substrates with the *N*-acyl homoserine lactones. The wider substrate selectivity of some receptors may indicate that they have a role in interspecies communication in addition to their capability to detect and respond to autoinducers produced by their host species.

Bacteria and fungi have evolved a range of enzymes capable of interfering with quorum sensing through the degradation of these molecules. Control of the behaviors regulated by quorum sensing through the use of quorum quenching enzymes can be a useful tool, and the discovery of novel proteins with different sequences, structures, and mechanisms for the degradation of autoinducers provides a variety of starting points for engineering enzymes that may be useful in industry, agriculture, and medicine. Here we have shown that ZHD, isolated from a fungus, is capable of degrading AHLs used for quorum sensing in bacterial populations, crystallized it in complex with a representative AHL, and proposed a mechanism by which it catalyzes AHL degradation. Zearalenone hydrolase is a representative of the α/β-hydrolase family of quorum quenching enzymes that offers a novel sequence with the potential for further engineering and application.

Continuing research in quorum sensing has demonstrated that bacterial communication involves autoinduction among individuals of the same species, but also that the receptors used by these species can respond to signaling molecules produced by the wider microbial community. Behaviors regulated by these different small molecules are diverse and targeting specific behaviors will be greatly facilitated through both a better understanding of these communication networks and the discovery and improvement of enzymes capable of interfering with quorum sensing.

Bibliography

1. Papenfort K, Bassler BL. Quorum sensing signal–response systems in gram-negative bacteria. *Nat Publ Gr*. 2016;14:576-588. doi:10.1038/nrmicro.2016.89

2. Prindle A, Liu J, Asally M, Ly S, Garcia-Ojalvo J, Suel GM. Ion channels enable electrical communication in bacterial communities. *Nature*. 2015;527:69-63. doi:10.1038/nature15709

3. Rutherford ST, Bassler BL. Bacterial quorum sensing: Its role in virulence and possibilities for its control. *Cold Spring Harb Perspect Med*. 2012;10(5):1-25. doi:10.1101/cshperspect.a012427

4. Gellatly SL, Hancock REW. Pseudomonas aeruginosa: New insights into pathogenesis and host defenses. *Pathog Dis*. 2013;67:159-173. doi:10.1111/2049-632X.12033

5. Verbeke F, Craemer S De, Debunne N, Janssens Y. Peptides as quorum sensing molecules: Measurement techniques and obtained levels in vitro and in vivo. *Front Neurosci*. 2017;11(April):1-18. doi:10.3389/fnins.2017.00183

6. Maldonado RF, Isabel S, Valvano MA. Lipopolysaccharide modification in gram-negative bacteria during chronic infection. *FEMS Microbiol Rev*. 2016;40(March):480-493. doi:10.1093/femsre/fuw007

7. Turkina M V, Vikström E. Bacteria-host crosstalk: Sensing of the quorum in the context of Pseudomonas aeruginosa infections. *J Innate Immun*. 2018;11:263-279. doi:10.1159/000494069

8. Verma SC, Miyashiro T. Quorum sensing in the squid-vibrio symbiosis. *Int J Mol Sci*. 2013;14:16386-16401. doi:10.3390/ijms140816386

9. Parsek MR, Val DL, Hanzelka BL, Cronan Jr. JE, Greenberg EP. Acyl homoserine-lactone quorum-sensing signal generation. *Proc Natl Acad Sci North Am*. 1999;96(April):4360-4365. doi:10.1073/pnas.96.8.4360

10. Amara N, Krom BP, Kaufmann GF, Meijler MM. Macromolecular inhibition of quorum sensing: Enzymes, antibodies, and beyond. *Chem Rev*. 2011;111:195-208. doi:10.1021/cr100101c

11. Lupp C, Ruby EG. Vibrio fischeri LuxS and AinS: Comparative study of two signal synthases. *J Bacteriol*. 2004;186(12):3873-3881. doi:10.1128/JB.186.12.3873

12. Lupp C, Ruby EG. Vibrio fischeri uses two quorum-sensing systems for the regulation of early and late colonization factors. *J Bacteriol*. 2005;187(11):3620-3629. doi:10.1128/JB.187.11.3620

13. Liu J, Fu K, Wu C, Qin K, Li F, Zhou L. “In-group” communication in marine Vibrio: A review of n-acyl homoserine lactones-driven quorum sensing. *Front Cell Infect Microbiol*. 2018;8(May):1-17. doi:10.3389/fcimb.2018.00139

14. White CE, Winans SC. Cell–cell communication in the plant pathogen Agrobacterium tumefaciens. *Philos Trans R Soc*. 2007;(362):1135-1148. doi:10.1098/rstb.2007.2040

15. Costa ED, Chai Y, Winans SC. The quorum-sensing protein TraR of Agrobacterium tumefaciens is susceptible to intrinsic and TraM-mediated protelytic instability. *Mol Microbiol*. 2012;84(5):807-815. doi:10.1111/j.1365-2958.2012.08037.x

16. Gould TA, Schweizer HP, Churchill MEA. Structure of the Pseudomonas aeruginosa acyl-homoserinelactone synthase LasI. *Mol Microbiol*. 2004;53(4):1135-1146. doi:10.1111/j.1365-2958.2004.04211.x

17. Lee J, Zhang L. The hierarchy quorum sensing network in Pseudomonas aeruginosa. *Protein Cell*. 2014;6(1):26-41. doi:10.1007/s13238-014-0100-x

18. Rhli P, Raychaudhuri A, Tullock A, Tipton PA. Reactivity and reaction order in acylhomoserine lactone formation by Pseudomonas aeruginosa RhlI. *Biochemistry*. 2008;47:2893-2898. doi:10.1021/bi702009n

19. Wade DS, Calfee MW, Rocha ER, et al. Regulation of Pseudomonas quinolone signal synthesis in Pseudomonas aeruginosa. *J Bacteriol*. 2005;187(13):4372-4380. doi:10.1128/JB.187.13.4372

20. Milot S, He J, Mindrinos MN, Tompkins RG, Rahme LG. Analysis of Pseudomonas aeruginosa 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proc Natl Acad Sci North Am*. 2004;101(5):2-7. doi:10.1073/pnas.0307694100

21. Drees SL, Fetzner S. PqsE of Pseudomonas aeruginosa acts as pathway-specific thioesterase in the biosynthesis of alkylquinolone signaling molecules. *Chem Biol*. 2015;22(5):611-618. doi:10.1016/j.chembiol.2015.04.012

22. Lin J, Cheng J, Wang Y, Shen X. The Pseudomonas quinolone signal (PQS): Not just for quorum sensing anymore. *Front Cell Infect Microbiol*. 2018;8(July):1-9. doi:10.3389/fcimb.2018.00230

23. Ye L, Cornelis P, Guillemyn K, Ballet S, Christopherson C, Hammerich O. Structure revision of N-Mercapto-4-formylcarbostyril produced by

Pseudomonas fluorescens G308 to 2-(2-Hydroxyphenyl)thiazole-4- carbaldehyde [aeruginaldehyde]. *Nat Prod Commun*. 2014;9(6):789-794. doi:10.1177/1934578X1400900615

24. Allard P, Chahtane H, Fu TN, et al. The plant pathogen Pseudomonas aeruginosa triggers a DELLA-dependent seed germination arrest in Arabidopsis. *Elife*. 2018;7(August):1-34. doi:10.7554/eLife.37082

25. Lee J, Wu J, Deng Y, et al. A cell-cell communication signal integrates quorum sensing and stress response. *Nat Chem Biol*. 2013;9(March):339-343. doi:10.1038/nchembio.1225

26. Sun S, Zhou L, Jin K, Haixia J, He Y. Quorum sensing systems differentially regulate the production of phenazine-1-carboxylic acid in the rhizobacterium Pseudomonas aeruginosa PA1201. *Sci Rep*. 2016;(February):16-18. doi:10.1038/srep30352

27. Cornelis P. Putting an end to the Pseudomonas aeruginosa IQS controversy. *Microbiologyopen*. 2020:1-2. doi:10.1002/mbo3.962

28. Caiazza NC, Shanks RMQ, O'Toole GA. Rhamnolipids modulate swarming motility patterns of Pseudomonas aeruginosa. *J Bacteriol*. 2005;187(21):7351-7361. doi:10.1128/JB.187.21.7351

29. Reis RS, Pereira AG, Neves BC, Freire DMG. Gene regulation of rhamnolipid production in Pseudomonas aeruginosa – A review. *Bioresour Technol*. 2011;102(11):6377-6384. doi:10.1016/j.biortech.2011.03.074

30. Michalska M, Wolf P. Pseudomonas Exotoxin A: Optimized by evolution for effective killing. *Front Microbiol*. 2015;6(September):1-7. doi:10.3389/fmicb.2015.00963

31. Defoirdt T. Quorum-sensing systems as targets for antivirulence therapy. *Trends Microbiol*. 2018;26(4):313-328. doi:10.1016/j.tim.2017.10.005

32. Tielker D, Hacker S, Loris R, et al. Pseudomonas aeruginosa lectin LecB is located in the outer membrane and is involved in biofilm formation. *Microbiology*. 2005;151(5):1313-1323. doi:10.1099/mic.0.27701-0

33. Pena RT, Blasco L, Ambroa A, et al. Relationship between quorum sensing and secretion systems. *Front Microbiol*. 2019;10(June):1-14. doi:10.3389/fmicb.2019.01100

34. Sun Y, Karmakar M, Taylor PR, Pearlman E. ExoS and ExoT ADP Ribosyltransferase activities mediate Pseudomonas aeruginosa keratitis by promoting neutrophil apoptosis and bacterial survival. *J Immunol*. 2012;188:1884-1895. doi:10.4049/jimmunol.1102148

35. Sana TG, Hachani A, Bucior I, et al. The second type VI secretion system of Pseudomonas aeruginosa strain PAO1 is regulated by quorum sensing and Fur and modulates internalization in epithelial cells. *J Biol Chem*. 2012;287(32):27095-27105. doi:10.1074/jbc.M112.376368

36. Wargo MJ, Gross MJ, Rajamani S, et al. Hemolytic phospholipase C inhibition protects lung function during Pseudomonas aeruginosa infection. *Am J Respir Crit Care Med*. 2011;184(14):345-354. doi:10.1164/rccm.201103-0374OC

37. Hoge R, Pelzer A, Rosenau F, Wilhelm S. Weapons of a pathogen: Proteases and their role in virulence of Pseudomonas aeruginosa. *Curr Res Technol Educ Top Appl Microbiol Microb Biotechnol*. 2010;45:383-395. https://www.semanticscholar.org/paper/%22Weapons-of-a-pathogen%3A-Proteases-and-their-role-in-Hoge-Pelzer/25158e1219b7bc5f1fbaee01b4ede37b1ff8fdb5.

38. Nomura K, Obata K, Keira T, et al. Pseudomonas aeruginosa elastase causes transient disruption of tight junctions and downregulation of PAR-2 in human nasal epithelial cells. *Respir Res*. 2014;15(21):1-13. doi:https://doi.org/10.1186/1465-9921-15-21

39. Kessler E, Safrins M, Olson JC, Ohman DE. Secreted LasA of Pseudomonas aeruginosa ss a staphylolytic protease. *J Biol Chem*. 1993;268(10):7503-7508. https://www.jbc.org/content/268/10/7503.short.

40. Leto TL, Rada B. Pyocyanin effects on respiratory epithelium: relevance in Pseudomonas aeruginosa airway infections. *Trends Microbiol*. 2013;21(2):73-81. doi:10.1016/j.tim.2012.10.004

41. Pierson LSI, Pierson EA. Metabolism and function of phenazines in bacteria: Impacts on the behavior of bacteria in the environment and biotechnological processes. *Appl Microbiol Biotechnol*. 2010;86:1659-1670. doi:10.1007/s00253-010-2509-3

42. Bilal M, Guo S, Iqbal HMN, Hu H, Wang W. Engineering Pseudomonas for phenazine biosynthesis, regulation, and biotechnological applications: A review. *World J Microbiol Biotechnol*. 2017;33(10):1-11. doi:10.1007/s11274-017-2356-9

43. Jamal M, Ahmad W, Andleeb S, et al. Bacterial biofilm and associated infections. *J Chinese Med Assoc*. 2018;81(1):7-11. doi:10.1016/j.jcma.2017.07.012

44. Chen L, Wen Y. The role of bacterial biofilm in persistent infections and control strategies. *Int J Oral Sci*. 2011;3(February):66-73. doi:10.4248/IJOS11022

45. Thoendel M, Horswill AR. Biosynthesis of peptide Signals in gram-positive bacteria. *Adv Appl Microbiol*. 2010;71(10):91-112. doi:10.1016/S0065-2164(10)71004-2

46. Wang B, Zhao A, Novick RP, Muir TW. Key driving forces in the biosynthesis of autoinducing peptides required for staphylococcal virulence. *Proc Natl Acad Sci North Am*. 2015;112(34):10679-10684. doi:10.1073/pnas.1506030112

47. Ceniceros A, Dijkhuizen L, Petrusma M. Molecular characterization of a Rhodococcus jostii RHA1 molecule and its main biosynthesis gene gblA. *Nat Sci Reports*. 2017;7(December):1-13. doi:10.1038/s41598-017-17853-6

48. Kato J, Funa N, Watanabe H, Ohnishi Y, Horinouchi S. Biosynthesis of γ-butyrolactone autoregulators that switch on secondary metabolism and morphological development in Streptomyces. *Proc Natl Acad Sci*. 2007;104(7):2378-2383. doi:10.1073/pnas.0607472104

49. Vannini A, Volpari C, Gargioli C, et al. The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA. *EMBO J*. 2002;21(17):4393-4401. doi:10.1093/emboj/cdf459

50. Lintz MJ, Oinuma K, Wysoczynski CL, Peter E, Churchill MEA. Crystal structure of QscR, a Pseudomonas aeruginosa quorum sensing signal receptor. *Proc Natl Acad Sci*. 2011;108(38):15763-15768. doi:10.1073/pnas.1112398108

51. Oinuma K, Greenberg EP. Acyl-homoserine lactone binding to and stability of the orphan Pseudomonas aeruginosa quorum-sensing signal receptor QscR. *J Bacteriol*. 2011;193(2):421-428. doi:10.1128/JB.01041-10

52. Sivanmaliappan TS, Sevanan M. Antimicrobial susceptibility patterns of Pseudomonas aeruginosa from diabetes patients with foot ulcers. *Int J Microbiol*. 2011;2011:1-4. doi:10.1155/2011/605195

53. Giamarellou H. Therapeutic guidelines for Pseudomonas aeruginosa infections. *Int J Antimicrob Agents*. 2000;16(2):103-106. doi:10.1016/s0924-8579(00)00212-0

54. Castillo-juárez I, Maeda T, Mandujano-tinoco EA, et al. Role of quorum sensing in bacterial infections. *World J Clin Cases*. 2015;3(7):575-599. doi:10.12998/wjcc.v3.i7.575

55. Fricks-lima J, Hendrickson CM, Allgaier M, et al. Differences in biofilm formation and antimicrobial resistance of Pseudomonas aeruginosa isolated from airways of mechanically ventilated patients and cystic fibrosis patients. *Int J Antimicrob Agents*. 2011;37(4):309-315. doi:10.1016/j.ijantimicag.2010.12.017

56. Mah TC, Toole GAO. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol*. 2001;9(1):34-39. doi:https://doi.org/10.1016/S0966-842X(00)01913-2

57. Hunter JD. Ventilator associated pneuomonia. *BMJ*. 2012;3325(May):1-7. doi:10.1136/bmj.e3325

58. Ciofu O, Tolker-nielsen T, Østrup P, Wang H, Høiby N. Antimicrobial resistance, respiratory tract infections and role of biofilms in lung infections in cystic fibrosis patients. *Adv Drug Deliv Rev*. 2015;85:7-23. doi:10.1016/j.addr.2014.11.017

59. Hansen CR, Pressler T, Høiby N. Early aggressive eradication therapy for intermittent Pseudomonas aeruginosa airway colonization in cystic fibrosis patients: 15 years experience. *J Cyst Fibros*. 2008;7(6):523-530. doi:10.1016/j.jcf.2008.06.009

60. Galloway WRJD, Hodgkinson JT, Bowden S, Welch M, Spring DR. Applications of small molecule activators and inhibitors of quorum sensing in gram-negative bacteria. *Trends Microbiol*. 2012;20(9):449-458. doi:10.1016/j.tim.2012.06.003

61. Cirou A, Uroz S, Chapelle E, et al. Quorum sensing as a target for novel biocontrol strategies directed at Pectobacterium. In: Gisi U, Chet I, Gullino ML, eds. *Recent Developments in Management of Plant Diseases*. Dordrecht: Springer Netherlands; 2009:121-131. doi:10.1007/978-1-4020-8804-9_10

62. Põllumaa L, Alamäe T, Mäe A. Quorum sensing and expression of virulence in pectobacteria. *Sensors*. 2012;12:3327-3349. doi:10.3390/s120303327

63. Monson RE, Apagyi K, Bowden SD, et al. The rsmS (ybaM) mutation causes bypass suppression of the RsmAB post-transcriptional virulence regulation system in enterobacterial phytopathogens. *Sci Rep*. 2019;9:1-14. doi:10.1038/s41598-019-40970-3

64. Morohoshi T, Wang W, Suto T, et al. Phenazine antibiotic production and antifungal activity are regulated by multiple quorum-sensing systems in Pseudomonas chlororaphis subsp. aurantiaca StFRB508. *J Biosci Bioeng*. 2013;116(5):580-584. doi:10.1016/j.jbiosc.2013.04.022

65. Lasarre B, Federle MJ. Exploiting quorum sensing to confuse bacterial pathogens. *Microbiol Mol Biol Rev*. 2013;77(1):73-111. doi:10.1128/MMBR.00046-12

66. Carvalho CCCR De. Marine biofilms: A successful microbial strategy with economic implications. *Front Mar Sci*. 2018;5(April):1-11. doi:10.3389/fmars.2018.00126

67. Neoh KG, Kang ET. Combating bacterial colonization on metals via polymer coatings: Relevance to marine and medical applications. *ACS Appl Mater Interfaces*. 2011;3(8):2808-2819. doi:10.1021/am200646t

68. Maqbool T, Jamal S, Waheed H, Lee C, Hashmi I, Iqbal H. Membrane biofouling retardation and improved sludge characteristics using quorum quenching bacteria in submerged membrane bioreactor. *J Memb Sci*. 2015;483:75-83. doi:10.1016/j.memsci.2015.02.011

69. Marrot B, Moulin P, Roche N. Industrial wastewater treatment in a membrane bioreactor: A review. *Environ Prog Sustain Energy*. 2004;23(1):59-68. doi:10.1002/ep.10001

70. Miura Y, Watanabe Y, Okabe S. Membrane biofouling in pilot-scale membrane bioreactors (MBRs) treating municipal wastewater: Impact of biofilm formation. *Environ Sci Technol*. 2007;41(2):632-638. doi:10.1021/es0615371

71. Hyun C, Choi D, Kwon H, et al. Application of quorum quenching bacteria entrapping sheets to enhance biofouling control in a membrane bioreactor with a hollow fiber module. *J Memb Sci*. 2017;526(December 2016):264-271. doi:10.1016/j.memsci.2016.12.046

72. Defoirdt T, Brackman G, Coenye T. Quorum sensing inhibitors: How strong is the evidence? *Trends Microbiol*. 2013;21(12):619-624. doi:10.1016/j.tim.2013.09.006

73. Oppenheimer-shaanan Y, Steinberg N, Kolodkin-gal I. Small molecules are natural triggers for the disassembly of biofilms. *Trends Microbiol*. 2013;21(11):594-601. doi:10.1016/j.tim.2013.08.005

74. Peng W, Ko T, Yang Y, et al. Crystal structure and substrate-binding mode of the mycoestrogen-detoxifying lactonase ZHD from Clonostachys rosea. *RSC Adv*. 2014;4(107):62321-62325. doi:10.1039/c4ra12111b

75. Kalia VC. Quorum sensing inhibitors: An overview. *Biotechnol Adv*. 2013;31(2):224-245. doi:10.1016/j.biotechadv.2012.10.004

76. Galloway WRJD, Hodgkinson JT, Bowden SD, Welch M, Spring DR. Quorum sensing in gram-negative bacteria: Small-molecule modulation of AHL and AI-2 quorum sensing pathways. *Chem Rev*. 2011;111(1):28-67. doi:10.1021/cr100109t

77. He Z, Wang Q, Hu Y, et al. Use of the quorum sensing inhibitor furanone C-30 to interfere with biofilm formation by Streptococcus mutans and its luxS mutant strain. *Int J Antimicrob Agents*. 2012;40:30-35. doi:10.1016/j.ijantimicag.2012.03.016

78. Shetye GS, Singh N, Gao X, Banyopadhyay D, Yan A, Luk Y-Y. Structures and biofilm inhibition activities of brominated furanones for Escherichia coli and Pseudomonas aeruginosa. *Med Chem Commun*. 2013;4(7):1079-1084. doi:10.1039/c3md00059a

79. Bergonzi C, Schwab M, Elias M. The quorum-quenching lactonase From Geobacillus caldoxylosilyticus: Purification, characterization, crystallization and crystallographic cnalysis. *Acta Crystallogr*. 2016;F(72):681-686. doi:10.1107/S2053230X16011821

80. Wang W, Morohoshi T, Ikenoya M, Someya N, Ikeda T. AiiM, a novel class of n-acylhomoserine lactonase from the leaf-associated bacterium Microbacterium testaceum. *Appl Environ Microbiol*. 2010;76(8):2524-2530. doi:10.1128/AEM.02738-09

81. Fetzner S. Quorum quenching enzymes. *J Biotechnol*. 2015;201:2-14. doi:10.1016/j.jbiotec.2014.09.001

82. Ng FSW, Wright DM, Seah SYK, Icrobiol APPLENM. Characterization of a phosphotriesterase-like lactonase from Sulfolobus solfataricus and its immobilization for disruption of quorum sensing. *Appl Environ Microbiol*. 2011;77(4):1181-1186. doi:10.1128/AEM.01642-10

83. Murugayah SA, Gerth ML. Engineering quorum quenching enzymes: Progress and perspectives. *Biochem Soc Trans*. 2019;47(3):793-800. doi:10.1042/BST20180165

84. Pappas KM, Weingart CL, Winans SC. Chemical Communication in Proteobacteria: Biochemical and structural studies of signal synthases and receptors required for intercellular signalling. *Mol Microbiol*. 2004;53(3):755-769. doi:10.1111/j.1365-2958.2004.04212.x

85. Ventre I, Ledgham F, Prima V, Lazdunski A, Foglino M, Sturgis JN. Dimerization of the quorum sensing regulator RhlR: Development of a method using EGFP fluorescence anisotropy. *Mol Microbiol*. 2003;48(1):187-198. doi:10.1046/j.1365-2958.2003.03422.x

86. Houdt R Van, Aertsen A, Moons P, Vanoirbeek K, Michiels CW. N-acyl-L-homoserine lactone signal interception by Escherichia coli. *FEMS Microbiol Lett*. 2006;256(1):83-89. doi:10.1111/j.1574-6968.2006.00103.x

87. Chang C, Krishnan T, Wang H, Chen Y, Yin W, Chong Y. Non-antibiotic quorum sensing inhibitors acting against n-acyl homoserine lactone synthase as druggable target. *Sci Rep*. 2014;4:1-8. doi:10.1038/srep07245

88. Scott RA, Lindow SE. Transcriptional control of quorum sensing and associated metabolic interactions in Pseudomonas syringae strain B728a. *Mol Microbiol*. 2016;99(6):1080-1098. doi:10.1111/mmi.13289

89. Ichinose Y, Tasaka Y, Yamamoto S, Inoue Y, Takata M, Nakatsu Y. PsyR, a transcriptional regulator in quorum sensing system, binds lux box-like sequence in psyI promoter without AHL quorum sensing molecule and activates psyI transcription with AHL in Pseudomonas syringae pv. tabaci 6605. *J Gen Plant Pathol*. 2020;86:124-133. doi:10.1007/s10327-019-00893-3

90. Azimi S, Klementiev AD, Whiteley M, Diggle SP. Bacterial quorum sensing during infection. *Annu Rev Microbiol*. 2020;74(September):201-219. doi:10.1146/annurev-micro-032020-093845

91. Beck von Bodman S, Majerczak DR, Coplin DL. A negative regulator mediates quorum-sensing control of exopolysaccharide production in Pantoea stewartii subsp. stewartii. *Proc Natl Acad Sci USA*. 1998;95(June):7687-7692. doi:10.1073/pnas.95.13.7687

92. Chen R, Barphagha IK, Karki HS, Ham JH. Dissection of quorum-sensing genes in Burkholderia glumae reveals non-canonical regulation and the new regulatory gene tofM for toxoflavin production. *PLoS One*. 2012;7(12):1-15. doi:10.1371/journal.pone.0052150

93. Stauff DL, Bassler BL. Quorum sensing in Chromobacterium violaceum: DNA recognition and gene regulation by the CviR receptor. *J Bacteriol*. 2011;193(15):3871-3878. doi:10.1128/JB.05125-11

94. Labbate M, Queck SY, Koh KS, Rice SA, Givskov M, Kjelleberg S. Quorum sensing-controlled biofilm development in Serratia liquefaciens MG1. *J Bacteriol*. 2004;186(3):692-698. doi:10.1128/JB.186.3.692

95. Swift S, Karlyshev A V, Fish L, et al. Quorum sensing in Aeromonas hydrophila and Aeromonas salmonicida: Identification of the LuxRI

homologs AhyRI and AsaRI and their cognate n-acylhomoserine lactone signal molecules. *J Bacteriol*. 1997;179(17):5271-5281. doi:https://pubmed.ncbi.nlm.nih.gov/9286976/

96. Yang M, Giel JL, Cai T, Zhong Z, Zhu J. The LuxR family quorum-sensing activator MrtR requires its cognate autoinducer for dimerization and activation but not for protein folding. *J Bacteriol*. 2009;191(1):434-438. doi:10.1128/JB.01247-08

97. Stevens AM, Queneau Y, Soule L, Bodman S Von, Doutheau A, Soule L. Mechanisms and synthetic modulators of AHL-dependent gene regulation. *Chem Rev*. 2011;111(1):4-27. doi:10.1021/cr100064s

98. Mcclean KH, Winson MK, Fish L, et al. Quorum sensing and Chromobacterium violaceum: Exploitation of violacein production and inhibition for the detection of N-acyl homoserine lactones. *Microbiology*. 1997;December(143):3703-3711. doi:10.1099/00221287-143-12-3703

99. Winson M, Swift S, Fish L, et al. Construction and analysis of luxCDABE-based plasmid sensors for investigating N-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol Lett*. 1998;163:185-192. doi:10.1111/j.1574-6968.1998.tb13044.x

100. Swem LR, Swem DL, Loughlin CTO, et al. A quorum-sensing antagonist targets both membrane-bound and cytoplasmic receptors and controls bacterial pathogenicity. *Mol Cell*. 2009;35(2):143-153. doi:10.1016/j.molcel.2009.05.029

101. Hawver LA, Jung SA, Ng W. Specificity and complexity in bacterial quorum-sensing systems. *FEMS Microbiol Rev*. 2016;40(5):738-752. doi:10.1093/femsre/fuw014

102. Hudaiberdiev S, Choudhary KS, Alvarez RV, Ligeti B, Lamba D, Pongor S. Census of solo LuxR genes in prokaryotic genomes. *Front Cell Infect Microbiol*. 2015;5(March):1-6. doi:10.3389/fcimb.2015.00020

103. Wellington S, Greenberg P. Quorum sensing signal selectivity and potential for interspecies cross talk. *Am Soc Microbiol*. 2019;10(2):1-14. doi:10.1128/mBio.00146-19

104. Yung-Hua L, Xiaolin T. Quorum sensing and bacterial social interactions in biofilms. *Sensors*. 2012;12:2519-2538. doi:10.3390/s120302519

105. Lindsay A, Ahmer BMM. Effect of sdiA on biosensors of n-acylhomoserine lactones. *J Bacteriol*. 2005;187(14):5054-5058. doi:10.1128/JB.187.14.5054

106. Koch G, Nadal-jimenez P, Reis CR, Muntendam R, Bokhove M, Melillo E. Reducing virulence of the human pathogen Burkholderia by altering the substrate specificity of the quorum-quenching acylase PvdQ. *Proc Natl Acad Sci*. 2014;111(4):1568-1573. doi:10.1073/pnas.1311263111

107. Chen Y, Ho JML, Shis DL, et al. Tuning the dynamic range of bacterial promoters regulated by ligand-inducible transcription factors. *Nat Commun*. 2018;8(64):1-8. doi:10.1038/s41467-017-02473-5

108. Altschul SF, Madden TL, Schäffer AA et al. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res*. 1997;25(17):3389-3402. doi:10.1093/nar/25.17.3389

109. Sikdar R, Elias M, Sikdar R, Elias M. Expert review of anti-infective therapy quorum quenching enzymes and their effects on virulence, biofilm, and microbiomes: A review of recent advances. *Expert Rev Anti Infect Ther*. 2020;18(12):1-13. doi:10.1080/14787210.2020.1794815

110. Harel M, Aharoni A, Gaidukov L, et al. Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. *Nat Struct Mol Biol*. 2004;11(5):412-419. doi:10.1038/nsmb767

111. Anh Q, Le T, Kim S, Chang R, Kim YH. Insights into the lactonase mechanism of Serum Paraoxonase 1 (PON1): Experimental and quantum mechanics/molecular mechanics (QM/MM) studies. *J Phys Chem*. 2015;119(30):9571-9585. doi:10.1021/acs.jpcb.5b03184

112. Bergonzi C, Schwab M, Naik T, Elias M. The structural determinants accounting for the broad substrate specificity of the quorum quenching lactonase GcL. *ChemBioChem*. 2019;20:1848-1855. doi:10.1002/cbic.201900024

113. Bokhove M, Jimenez PN, Quax WJ, et al. Penicillin V acylases from gram-negative bacteria degrade N-acylhomoserine lactones and attenuate virulence in Pseudomonas aeruginosa. Cornelis P, ed. *PLoS One*. 2015;7(6):4342-4353. doi:10.1371/journal.pone.0038492

114. Gao A, Mei G, Liu S, et al. High-resolution structures of AidH complexes provide insights into a novel catalytic mechanism for N-acyl homoserine lactonase. *Acta Crystallogr*. 2013;69:82-91. doi:10.1107/S0907444912042369

115. Rémy B, Plener L, Decloquement P, et al. Lactonase specificity is key to quorum quenching in Pseudomonas aeruginosa. *Front Microbiol*. 2020;11(April):1-17. doi:10.3389/fmicb.2020.00762

116. Mahan K, Martinmaki R, Larus I, Sikdar R, Dunitz J, Elias M. Effects of signal disruption depends on the substrate preference of the lactonase. 2020;10(January):1-11. doi:10.3389/fmicb.2019.03003

117. Chow JY, Wu L, Yew WS. Directed evolution of a quorum-quenching lactonase from Mycobacterium avium subsp. paratuberculosis K-10 in the amidohydrolase superfamily. *Biochemistry*. 2009;48(20):4344-4353. doi:10.1021/bi9004045

118. Bergonzi C, Schwab M, Naik T, Daudé D, Chabrière E, Elias M. Structural and biochemical characterization of AaL, a quorum quenching lactonase with unusual kinetic properties. *Nat Sci Reports*. 2018;(June):19-21. doi:10.1038/s41598-018-28988-5

119. Caldwell RW, Tuite J, Stob M, Baldwin R, Al CET. Zearalenone production by Fusarium species. *Appl Microbiol*. 1970;20(1):31-34.

https://aem.asm.org/content/20/1/31.

120. Popiel D, Koczyk G, Dawidziuk A, Gromadzka K, Blaszczyk L, Chelkowski J. Zearalenone lactonohydrolase activity in Hypocreales and its evolutionary relationships within the epoxide hydrolase subset of a/b-hydrolases. *BMC Microbiol*. 2014;14(82):1-12. doi:10.1186/1471-2180-14-82

121. Brook S. Interaction of naturally occurring nonsteroidal estrogens with expressed recombinant human estrogen receptor. *J Steroid Biochem Mol Biol*. 1994;49(2):153-160. https://www.sciencedirect.com/science/article/pii/0960076094900051.

122. Kosawang C, Karlsson M, El H V, et al. Zearalenone detoxification by zearalenone hydrolase is important for the antagonistic ability of Clonostachys rosea against mycotoxigenic Fusarium graminearum. *Fungal Biol*. 2014;8. doi:10.1016/j.funbio.2014.01.005

123. Takahashi-ando N, Ohsato S, Shibata T, Hamamoto H, Yamaguchi I, Kimura M. Metabolism of zearalenone by genetically modified organisms expressing the detoxification gene from Clonostachys rosea. *Appl Environ Microbiol*. 2004;70(6):3239-3245. doi:10.1128/AEM.70.6.3239

124. Takhashi-Ando N, Kimura M, Kakeya H, Osada H, Yamaguchi I. A novel lactonohydrolase responsible for the detoxification of zearalenone: Enzyme purification and gene cloning. *Biochem J*. 2002;6(365):1-6. doi:10.1042/BJ20020450

125. Kakeya H, Takahashi-ando N, Kimura M, Onose R, Yamaguchi I, Osada H. Biotransformation of the mycotoxin, Zearalenone, to a non-estrogenic compound by a fungal strain of Clonostachys sp. *Biosci Biotechnol Biochem*. 2014;8451(May):2723-2726. doi:10.1271/bbb.66.2723

126. Qi Q, Yang W, Zhou H, Ming D, Sun K. The structure of a complex of the lactonohydrolase zearalenone hydrolase with the hydrolysis product resolution of zearalenone at 1.60 Å research communications. *Acta Cryst F*. 2017:376-381. doi:10.1107/S2053230X17007713

127. Hiblot J, Gotthard G, Elias M, Chabriere E. Differential active site loop conformations mediate promiscuous activities in the lactonase SsoPox. *PLoS One*. 2013;8(9):1-14. doi:10.1371/journal.pone.0075272

128. Ellman GL, Courtney DK, Andres VJ, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol*. 1961;7(2):88-95. doi:10.1016/0006-2952(61)90145-9

129. Owusu-Apenten R. Colorimetric analysis of protein sulfhydyl groups in milk: Applications and processing effects. *Crit Rev Food Sci Nutr*. 2007;45(20):1-23. doi:10.1080/10408690590900126

130. Kataoka M, Honda K, Shimizu S. 3,4-Dihydrocoumarin hydrolase with haloperoxidase activity from Acinetobacter calcoaceticus F46. *Eur J Biochem*. 2000;267:3-10. doi:10.1046/j.1432-1327.2000.00889.x

131. Kabsch W. Integration, scaling, space-group assignment and post-refinement. *Acta Crystallogr*. 2010;D66:133-144. doi:10.1107/S0907444909047374

132. Emsley P, Lohkamp B. Features and development of Coot. *Acta Crystallogr*. 2010;D66:486-501. doi:10.1107/S0907444910007493

133. The PyMOL Molecular Graphics System, Version 2.3.0 Schrödinger, LLC.

134. Huynh K, Partch CL. Analysis of protein stability and ligand interactions by thermal shift assay. *Curr Protoc Protein Sci*. 2016;79(28):1-19. doi:10.1002/0471140864.ps2809s79.Current

135. Voet D, Voet JG, Pratt CW. *Fundamentals of Biochemistry*. 4th ed.; 2013.

136. Elias M, Tawfik DS. Divergence and convergence in enzyme evolution: Parallel evolution of paraoxonases from quorum-quenching lactonases. *J Biol Chem*. 2012;287(1):11-20. doi:10.1074/jbc.R111.257329

137. Stöckli M, Lin C, Sieber R, Plaza DF, Ohm RA, Künzler M. Coprinopsis cinerea intracellular lactonases hydrolyze quorum sensing molecules of gram-negative bacteria. *Fungal Genet Biol*. 2017;2017(102):49-62. doi:10.1016/j.fgb.2016.07.009