Macromolecular Inhibition of Quorum Sensing: Enzymes, Antibodies, and Beyond

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1. Introduction

This review article focuses on quenching of bacterial quorum sensing (QS) signaling by macromolecules. Although various research efforts have led to the design and identification of a plethora of small molecules as QS inhibitors, the area of macromolecular QS quenching has been pioneered only recently. A variety of organisms have been identified in the past several years that are able to interfere with bacterial QS through enzymatic hydrolysis of quorum sensing molecules (QSMs). In fact, many organisms appear to have evolved these tools to actively interfere with the expression of bacterial genes involved in interspecies competition, e.g., virulence factors in bacterial pathogens. This phenomenon raises the following questions: does active interference with bacterial QS indeed protect eukaryotes from microbial invasion and colonization? What is the true biological value of such a strategy for an organism compared with conventional immunological strategies, such as antimicrobial peptides, and the complement system?

We will summarize research efforts that cover several aspects of QS attenuation by three types of proteins: (i) enzymes—both the discovery of naturally occurring enzymes that are able to hydrolyze QSMs and laboratory-evolved or engineered enzymes; (ii) antibodies that scavenge QSMs; and (iii) decoy receptors—both naturally occurring and engineered receptors. We will discuss the relevance of these attenuators in vivo and their potential use to prevent and combat bacterial disease.

Neri Amara was born in Israel in 1978, and a successful training as a professional "a la carte" chef inspired him to study Chemistry at Ben-Gurion University of the Negev in Be'er Sheva, Israel. After completion of his B.Sc. degree, he joined the Chemical Biology group of Michael Meijler for a combined M.Sc./Ph.D. track, and his research projects focus on inhibition of quorum sensing in bacteria and elucidation of signaling between bacteria and eukaryotes.

1.1. Coordination of Gene Expression

The term "quorum sensing" has been coined to describe the ability of a population of unicellular bacteria to act as a single multicellular organism in a cell-density-dependent manner.[1] Bacteria achieve this feat by using small diffusible molecules to exchange information among the members of the population. Examples of QS-controlled behaviors are bioluminescence, virulence factor expression, and biofilm formation. These processes are advantageous to a bacterial population only when they are carried out simultaneously by all its members; for example, bioluminescence produced by the marine bacterium *Vibrio fischeri* is beneficial to host organisms only if a sufficiently high number of bacteria synchronize their light production. An example of such a symbiotic relationship is that of *Euprymna scolopes* and *V. fischeri*. The squid provides nutrients and protection for the bacteria and, in turn, uses the light produced by *V. fischeri* for sexual mating rituals, hunting prey, and warding off predators. For a variety of marine inhabitants, normal development of the light organs requires colonization by *V. fischeri*.[2,3]

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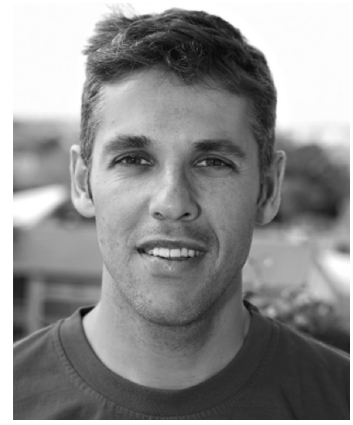
Bastiaan P. Krom was born in 1973 on the island of Curaçao (Netherlands Antilles). He studied molecular biology of fungi at the University of Groningen and the University of Texas at Austin. He completed his Ph.D. studies in Biochemistry at the University of Groningen with Dr. Lolkema and Professor Konings. After receiving a TALENT fellowship (NWO), he continued for postdoctoral studies at Georgetown University Medical Center, Washington, DC, in the group of Professor Cihlar. In 2004 he returned to The Netherlands to join the group of Professors Busscher and van der Mei at the University Medical Center Groningen, Netherlands, and in 2005 he was appointed as an Assistant Professor at the Department of Biomedical Engineering at the same institute. His research focuses on microbial biofilm formation, especially of *Candida albicans*, with emphasis on molecular unraveling of microbe−material and microbe−microbe interactions.

Gunnar F. Kaufmann was born in Germany and studied Human Biology in Marburg and Greifswald where he received his Diploma in 2001. He then joined Professor Kim D. Janda's group at The Scripps Research Institute and obtained his Ph.D in Macromolecular and Cellular Structure and Chemistry in 2006, working on generation and engineering of antihapten antibodies as well as bacterial quorum sensing as target of immunotherapy. During his postdoctoral studies, he investigated the effect of bacterial quorum sensing molecules on the mammalian host in a joint position between Professor Janda's group and the laboratory of Professor Richard J. Ulevitch at TSRI. Currently, he is an Assistant Professor in the Departments of Chemistry and Immunology & Microbial Science at TSRI working on the development of new active vaccines targeting bacterial quorum sensing systems.



Michael M. Meijler was born in 1970 in Amsterdam, The Netherlands. He combined studies in Chemistry and Organic Synthesis at the University of Amsterdam with competitive distance running (specializing in 3000 meters steeplechase). After completing his Ph.D. studies in Bioorganic Chemistry at The Weizmann Institute of Science in Israel with Professor Abraham Shanzer, he joined the group of Professor Kim Janda at The Scripps Research Institute in 2002 as a postdoctoral fellow and from 2005 to 2006 as Assistant Professor. By the end of 2006, he returned to Israel for a faculty position at the Department of Chemistry at Ben-Gurion University of the Negev in Be'er Sheva. His research interests focus on chemical communication within and between species and on developing chemical tools to elucidate the molecular mechanisms that guide these interactions.

During the past two decades, a large number of studies regarding intercellular communication among bacteria have been published. Different signaling systems have been discovered, and still more proteins and small molecules involved in QS are likely to be uncovered in the coming years. QS is mediated by autoinducers, which can be categorized into several major classes of compounds[4−6] (Figure 1): (i) oligopeptides, consisting of 5−10 amino acids, which are generally used by Gram-positive bacteria, e.g., autoinducing peptides (AIPs) in *Staphylococcus aureus*; (ii) derivatives of the sugar-like molecule dihydroxypentanedione (DPD), e.g., AI-2 in *V. harveyi*, which has been identified in both Gram-positive and Gram-negative bacteria and has been proposed to be a universal interspecies signaling molecule;[7] (iii) *N*-acyl homoserine lactones (AHLs), which are produced by over 70 species of Gram-negative bacteria (differences within this subgroup occur in the length and oxidation state of the acyl side chain (Figure 1)); and (iv) other small molecules that include the *Pseudomonas* quinolone signal (PQS),[8] 3-hydroxypalmitic acid methyl ester,[9] bradyoxetin,[10] the *V. cholerae* autoinducer (*S*)-3-hydroxytridecan-4-one,[11] AI-3 (a small molecule autoinducer with unknown structure, used by enterohemorrhagic *E. coli*),[12] and other molecules that do not fall within one of the major groups.

In general, bacteria secrete autoinducers at a constant rate, resulting in an increase in local concentration of the autoinducer that corresponds to the increase in population size. Upon reaching a certain threshold concentration, or population size, the corresponding QS receptor is bound effectively and undergoes activation, which results in the expression of QS regulated genes. This process is described in detail for AHLs and AI-2-like autoinducers in accompanying review articles by Stevens et al. and Spring et al., respectively.

2. Enzymatic Quenching of Quorum Sensing

Interactions between plants and microorganisms are ubiquitous. The ascent of plants on land and its subsequent first interactions with soil microbes has been estimated to have occurred around 500 million years ago,[13] and the ability of plants to defend themselves against continuous attacks of pathogens has been paramount to their evolutionary success. Given the importance of QS for bacteria as a mechanism to regulate colonization of plants (and other eukaryotes),[14−16] it should be no surprise that, in the arms race between different bacterial strains and between bacteria and plants,

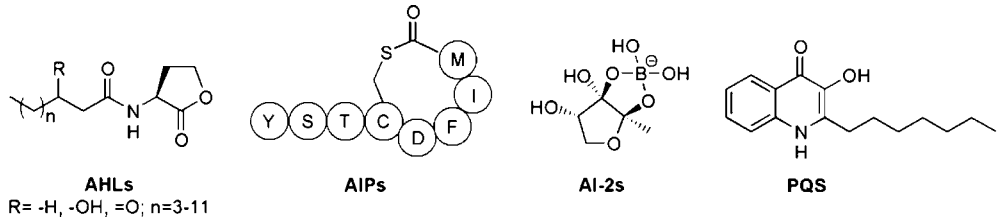


Figure 1. Examples of well-described quorum sensing molecules.

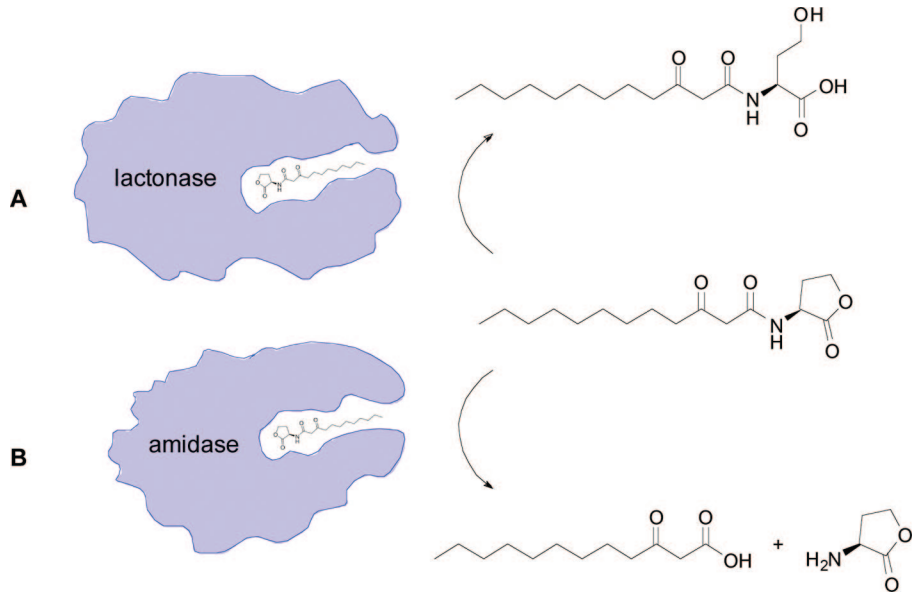


Figure 2. Two main modes of enzyme-catalyzed hydrolysis of *N*-acylhomoserine lactones: (a) lactone ring hydrolysis and (b) amide bond hydrolysis.

proteins evolved that aid one organism in disrupting the coordination of attacks by a competing organism. Indeed, enzymes that quench QS through degradation of the autoinducers (quorum quenching (QQ) enzymes) have been identified in bacteria as well as in a variety of different eukaryotes.[17]

2.1. AHL Degrading Enzymes

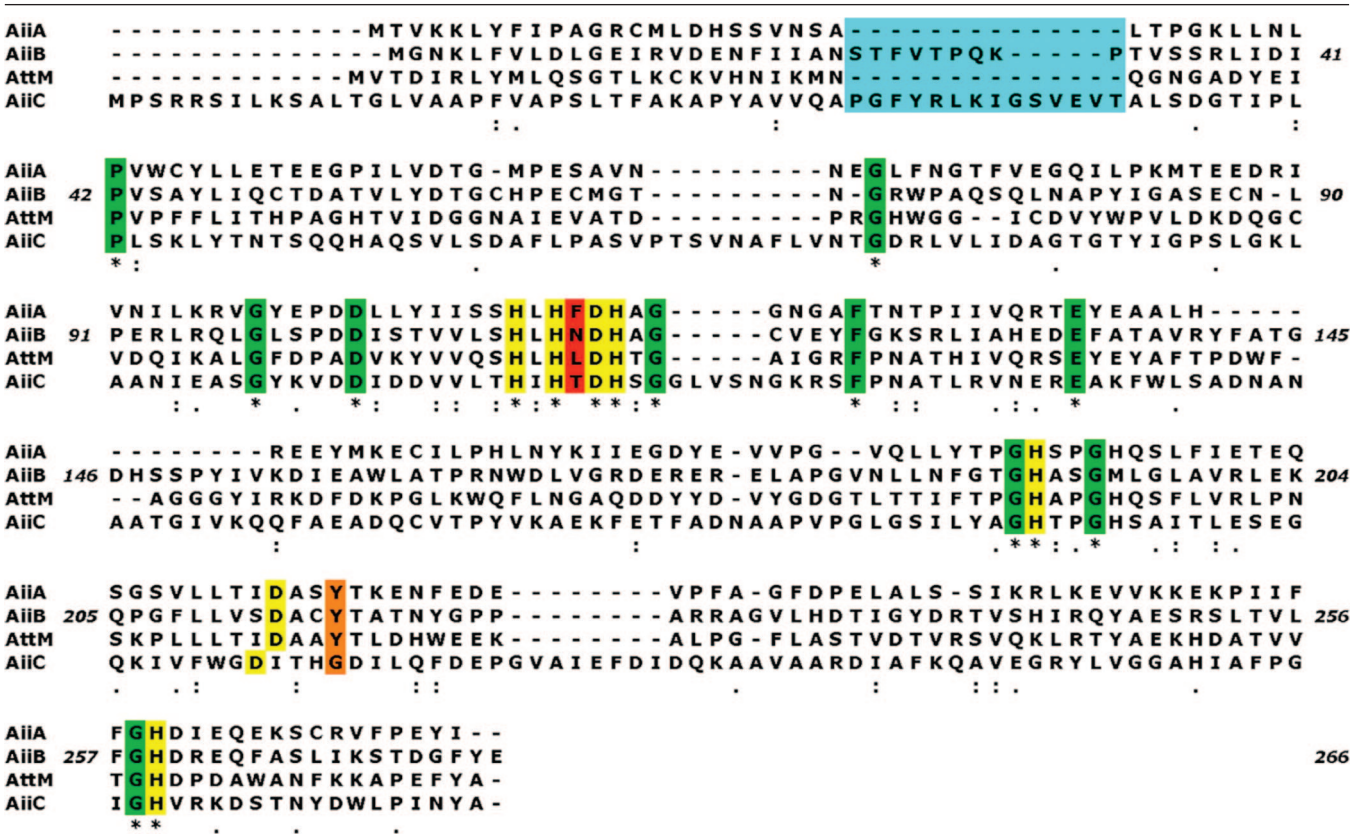
Of the different types of QS molecules, AHLs are the largest and most extensively studied family.[18−22] A large number of organisms have been identified that utilize this chemical class of molecules as the primary or dominant QS autoinducer. AHLs typically have an aliphatic acyl chain fused to a homoserine lactone ring via an amide bond. AHLs vary between different species in the acyl chain length and in the oxidation state at the beta position to the amide carbonyl, and in most cases the (*S*)-enantiomer is the active species. AHLs were found to be relatively unstable in aqueous media, being labile to hydrolysis of the lactone ring, as well as to elevated temperatures.[23] Kaufmann et al. showed that 3-oxo-AHLs can also undergo a Claisen-like intramolecular cyclization that yields an acylated tetramic acid, a compound that belongs to a class of known antibacterial compounds.[24] The rates of lactone hydrolysis and tetramic acid formation proved these reactions to be competing with reaction rates of the same order (4.77×10^{-6} S^{-1} and 1.49×10^{-6} S^{-1}, respectively, for 3-oxo-C_{12}-HSL). Both of these reactions were shown to have faster rates as the length of acyl chain decreases and as the temperature increases.[24,25] Lipid-substituted antibiotics have been shown to accumulate in the cell membrane surrounding of Gram-positive bacteria, thus inflicting significant bactericidal activity as opposed to their action on Gram-negative bacteria.[24,26,27] It was proposed that an additional role of Gram-positive QQ enzymes as QS interfering agents may be their ability to decrease the concentration of tetramic acids as products of AHL rearrangement.[24,28]

Most AHL-degrading enzymes that have been identified thus far are either lactonases that hydrolyze the ester bond in the lactone ring or acylases that hydrolyze the amide bond, fusing the acyl chain and the homoserine lactone (Figure 2), although a few enzymes were shown to inactivate AHLs by oxidizing the ω-end of the acyl chain or by reducing the beta-keto carbonyl of 3-oxo-acyl-homoserine lactones.[29,30] AHL lactonases yield acyl-homoserines, the product of aqueous hydrolysis. The reaction is reversible, and at acidic pH (pH < 2) the active AHLs are regenerated. Any change in the structure of AHLs will significantly lower their affinity for their response regulators, diminishing their ability to affect gene regulation.

2.1.1. AHL Lactonases

The first QSM-inactivating enzyme was identified by Dong et al. by screening treated soil samples and laboratory bacterial collections.[31] The gene (*aiiA*) encoding the enzyme was cloned from a Gram-positive *Bacillus* sp. 240B1. The gene product, a 250 amino acid protein, was later characterized as a lactonase, catalyzing the hydrolysis of the ester bond in the lactone ring of a wide variety of AHLs.[32] Although no homology could be found to any of the known enzyme families, the enzyme was characterized as a zinc metalloprotease based on a conserved short sequence of HXHXDH∼60aa∼H, which is known as a zinc-binding motif shared by several zinc−metalloproteases such as

Table 1. Amino Acid Sequence Alignment of Lactonases[a]



[a] Residues that coordinate to the zinc ions are highlighted in yellow. Other residues that are completely conserved are labeled in green. A sequence insert found in AiiB but not AiiA is highlighted in blue. The positions of two residues in the AiiB active site, Y216 and N114, are highlighted in orange and red, respectively. Beneath the alignments, "*" indicates identical residues, ":" indicates conservative substitutions, and "." indicates semiconservative substitutions.[42]

glyoxalase II, arylsulfatase, and β-lactamase families. Overexpression of *aiiA* in *Erwinia carotovora* resulted in reduced AHL accumulation in the medium culture to undetectable levels, as well as significantly decreased the secretion of extracellular pectolytic enzymes. In a plant infection model, the *aiiA* overexpression strain was inoculated into intact Chinese cabbage, cauliflower, and tobacco plants. Maceration of plant tissue was prevented in all plant subjects even up to a week following inoculation, whereas wild-type infected plants showed severe maceration after one day.[26,31]

Shortly after this initial discovery, many similar AiiA-like enzymes were found and isolated from different *Bacillus* species, sharing more that 90% sequence homology in most cases.[26,33,34] AHL lactonases are present in *B. thuringiensis*, *B. cereus*, *B. anthracis*, and *B. mycoides*, suggesting that all *aiiA* enzymes have evolved from the same ancestral gene within the *Bacillus* genus.

The use of AHL-degrading enzymes has been proposed as a strategy to attenuate bacterial virulence. Reimmann and co-workers reported that overexpression of *aiiA* homologues of two *Bacillus* spp in *P. aeruginosa* PAO1 resulted in decreased production of elastase (LasB), a known QS-regulated phenotype controlled mainly by the 3-oxo-C_{12}-HSL-based LasR/LasI system.[34] Reduced production of rhamnolipids (LasA), another QS phenotype controlled mainly by the C_4-HSL-based RhlR/RhlI QS circuit, was observed. AiiA expression also affected production of HCN, a metabolite that is considered a virulence factor for its role in paralysis and killing of *Caenorhabditis elegans*. These results are similar to those obtained by Dong and co-workers for the expression of AiiA in *E. carotovora*.[31,32]

The AHL lactonase enzyme family possesses hydrolytic activity toward a broad spectrum of AHLs, regardless of the acyl chain length and the oxidation state at the C_3 position of the acyl chain. These enzymes are highly specific toward AHLs with little to no activity toward nonacylated homoserine lactones and noncyclic esters.[33,35] A catalytic mechanism was proposed for this family of enzymes based on the site-directed mutagenesis and two independent crystal structures of an AHL lactonase of the *B. thuringiensis* subsp. *Kurstaki*,[36] suggesting that two zinc ions are present in the binding site, coordinated by five histidines and two aspartates, which belong to the conserved HXHXDH∼60aa∼H motif (Table 1). An activated water molecule, bridged by the two zinc ions, attacks the substrate's carbonyl carbon, which is also polarized by this interaction with the zinc ions. This nucleophilic attack results in the formation of a negatively charged intermediate, resulting in cleavage of the carbon−oxygen bond of the lactone ester to yield the carboxylic acid product.

A different family of AHL-lactonases was identified in several species including *Agrobacterium tumefaciens (attM), Klebsiella pneumoniae (ahlK)*, and *Arthrobacter sp. (ahlD)*, which shares about 30−58% homology in amino acid sequence, within the family.[37,38] While the two families share only up to 25% homology (for AiiA and AttM for example), both have the same conserved zinc-binding motif;[39] therefore, it is likely that the catalytic mechanism is similar for both enzyme families.

In *A. tumefaciens, attM* plays a role in autodegradation of AHLs. Conjugated transfer of the Ti-plasmid is QS-regulated and is initiated at midlog phase, maximizes at midexponential phase, but then rapidly declines at late-exponential to early

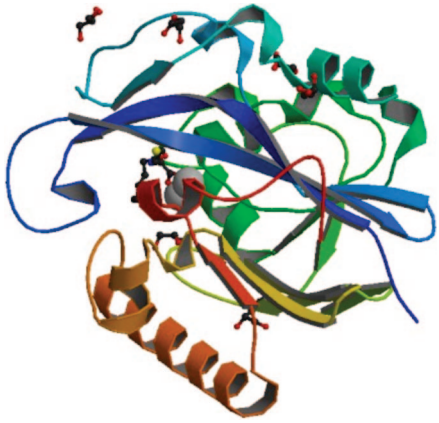


Figure 3. Crystal structure of AiiB.

stationary phase levels. Zhang et al. attributed the dramatic decrease in AIs in the culture media to the AHL lactonase encoded by *attM*. The authors proposed that this may be a mechanism by which the bacteria exits the QS mode; *attM* is negatively controlled by the transcription factor *attJ*, until the start of the stationary phase.[38] Similar behavior was observed in *E. carotovora*, but it was shown that the decline of AHLs at the beginning of the stationary phase in that instance was a result of subtle elevation in the pH of the culture medium rather than specific hydrolysis by degrading enzymes.[23,40]

A different AHL lactonase, encoded by *aiiB*, was identified in *A. tumefaciens* by Carlier et al. This enzyme shares a 28% amino acid sequence identity with $AiiA_{240B1}$ from *Bacillus sp.*, which excludes it from the AiiA family (genes within this cluster share 90−95% homology).[41] AiiB prefers substrates with longer acyl chain compared to AiiA; the overall activity is also lower toward 3-oxo-substituents. Crystallization of AiiB yielded a dimer,[42] which shows a $\alpha\beta/\beta\alpha$ fold for each monomer, similar to that of AiiA and the metallo-β-lactamase superfamily (see Figure 3). The overall structure and amino acid sequence alignment of AiiB resembles that of AiiA with two zinc ions forming a binuclear metal center, which is located on top of two β-sheets that form the binding site; however, AiiB contains a sequence insertion of nine amino acid residues, forming an unstructured loop.

This additional loop forms stabilizing interactions between the two monomers, which distinguishes between the monomeric AiiA and the dimeric AiiB. Dimers that formed in the crystalline sample were shown not to block the binding site of each monomer and are believed to exist in solution as well. Furthermore, the crystallographic structure suggests that contributions from each monomer could form an extended binding cavity. Although no AHL lactonases were found to form dimers, there are examples of dimeric members of the metallo-β-lactamase superfamily.[43] The fact that both genes, *aiiB* and *attM*, are located on plasmids may explain the phylogenic diversity observed for this class of enzymes.[41]

A fourth distinct class of AHL lactonases was identified from *Rhodococcus erythropolis*, which is unique to the *Rhodococcus* genus. The 323 aa enzyme encoded by *qsdA* (for quorum sensing signal degradation) is a member of the phosphotriesterase (PTE) superfamily. PTEs are zinc−metalloproteases that were originally identified for their ability to hydrolyze phosphotriester-containing organophosphorus compounds, but recently more members of this family were found to possess lactonase and amidohydrolase activity as well.[44,45] QsdA is related to the phosphotriesterase-like lactonase (PLL) subfamily of PTEs, out of which SsoPox, isolated from the archaebacterium *Sulfolobus solfataricus*, is the representative member. A mechanism for the lactonase activity of this enzyme was proposed from its crystal structure bound to a lactone mimic.[46] SsoPox is a globular α/β barrel, with a binding site that has the shape of an elongated cavity composed of a hydrophobic channel leading to a binuclear-iron and cobalt−metal center. The substrate's aliphatic chain accommodates the hydrophobic channel while the lactone ring is positioned closely to the metal center. The carbonyl oxygen of the ring interacts with the cobalt ion while the thioester sulfur of the analogue interacts with the iron ion. QsdA differs from SsoPox by having two zinc ions and a slightly altered metal-binding motif. An activated water molecule bridging the two ions participates in the nucleophilic attack. Although similar in function and in several key elements (such as two zinc ions in the catalytic site and their ability to degrade AHLs with acyl chains ranging from C_6 to C_{12}) in the mechanism of catalysis, SsoPox and PLLs do not share any sequence similarity with the AiiA or the AttM clusters. A phylogenetic study[44] revealed that QsdA homologues are abundant among the *Rhodococcus* genus with members in the *erythropolis*, *corynebacteriodes*, and *rhodochrous* subsp., which readily hydrolyze AHLs ranging in acyl chain length from 6 to 14 carbons, regardless of the oxidation state in the C3 carbon position.

Recently a novel group of genes, named *bpiB* (for biofilm phenotype inhibiting genes), were discovered through metagenomic analysis of soil samples.[47] Three *bpiB* genes, originating from *Nitrobacter* sp. strain Nb-311A, *P. fluorescence*, and *Xanthomonas campestris*, encode BpiB01, BpiB04, and BpiB07, respectively, which were found to play a role in degradation of 3-oxo-C_8-HSL, although sequence analysis revealed no resemblance to any known AHL-degrading gene cluster. Their activity was characterized as lactonase by the identification of 3-oxo-C_8-HSL as the primary hydrolysis product. Low concentrations of zinc or calcium were necessary for the hydrolytic activity, suggesting that all three enzymes are metallohydrolases. Virulence attenuation was observed, as reduction in biofilm formation and motility, in *A. tumefaciens* and in *P. aeruginosa*—as other lactonases have shown a certain degree of promiscuity in recognizing HSLs with varying alkyl chain lengths, BpiBs can be expected to recognize 3-oxo-C_{12}-HSL—however, a mechanism for the degradation has not yet been deduced.

2.1.2. AHL Acylases

A second major group of AHL-degrading enzymes, acylases, target the amide bond connecting the fatty acyl chain to the homoserine lactone (Figure 2B). Acylases were first identified by Leadbetter and Greenberg, who discovered that a strain of *Variovorax paradoxus* is able to utilize AHLs as a sole source of energy and nitrogen.[48] This strain does not produce AHL molecules; however, it evolved to grow on a variety of saturated AHLs varying in acyl chain length from 4 to 12 carbons long as well as 3-oxo-C_8-HSL and homoserine. The ability to grow on media containing different AHLs correlated with the length of the acyl chain, suggesting that the acyl group and not the lactone is used as an energy source. Furthermore, disappearance of HSL from the culture medium suggests that other enzymes may be involved in degrading HSL to benefit from the nitrogen. Similar behavior was observed also for *R. erythropolis*, which

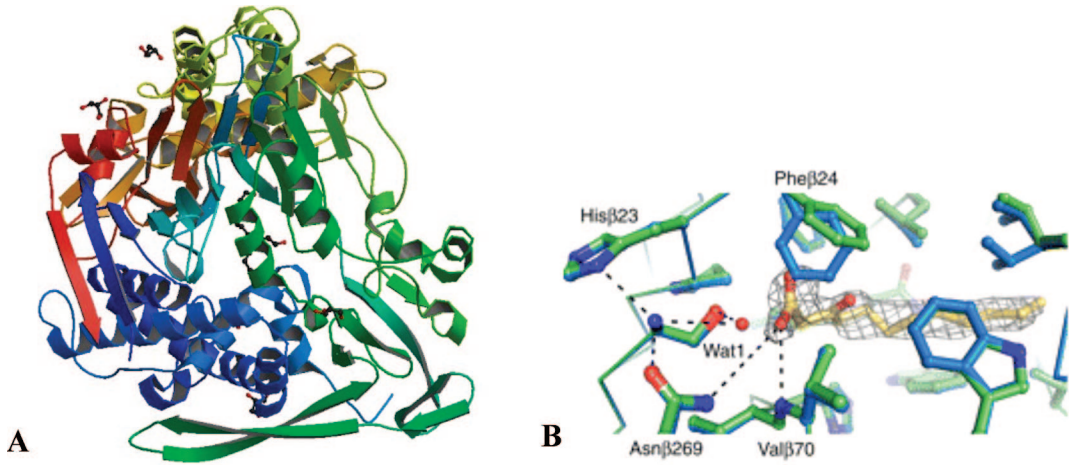


Figure 4. Crystal structure of PvdQ (A) and its binding site with bound 3-oxo-dodecanoic acid (B). Reproduced with permission from ref 61.

possesses at least three types of AHL inactivating and degrading enzymes;[29] for *Arthrobacter* spp., that was shown to grow in cocultures with *Variovorax* spp. to benefit from the degradation products fatty acids and HSL[49] and for *Ralstonia*.[50]

The AHLacylase isolated from *Ralstonia* strain XJ12B was the first to be characterized and represents a family of HSLacylases named AiiD. The AiiD enzyme from *Ralstonia* shares a.a. sequence homology with members of the N-terminal nucleophile hydrolase (Ntn-hydrolase) superfamily, including cephalosporin and penicillin acylases. Ntn-hydrolase is a group known for undergoing post-translational cleavage, yielding two subunits that form the active enzyme. In silico analysis predicted that AiiD has features similar to other Ntn-hydrolases such as a hydrophobic signal peptide followed by an α-subunit, a spacer, and a *β*-subunit recognized as necessary for the autoproteolytic event, as well as several conserved residues such as a glycine and serine pair that were proven to be essential for the acylase activity.[50] It has been postulated that AiiD is a membrane-anchored or membrane-associated protein located in the periplasm, as is the case for its family counterparts penicillin acylase and cephalosporin acylase.[51,52] This is consistent with both having a hydrophobic signal protein used for transport and the observation that activity of AiiD appears to cofractionate with lysed cell debris. In several instances, the hydrolysis product HS was detected in the culture medium, but the possibility that it may have been transported or diffused out of the cell cannot be ruled out.[48,50] Recombinant expression of AiiD in *P. aeruginosa* proved to prevent AHL accumulation in the culture medium, reduce virulence, and impair swarming motility as well as attenuate paralysis and killing of *C. elegans*. Sequence alignments against genome databases revealed several homologues that may encode for similar HSL acylases with a.a. sequence overlaps that range between 38 and 83%. Such homologues were found in the genome of *R. solanacearum, R. metallidurans, Deinococcus radiodurans, P. aeruginosa, P. putida, P. syringae*, and *P. fluorescence*.[50]

The *P. aeruginosa* homologue, encoded by PA2385, was previously designated *pvdQ* for its role in the biosynthesis of the siderophore pyoverdin.[53] Huang et al. reported that two strains of *P. aeruginosa* can grow on HSLs as a sole source of energy, but in contrast to the broad substrate specificity exhibited by *Variovorax* and *Ralstonia* spp, these isolates utilized only HSLs with acyl chain longer than 8 carbons.[54] PvdQ was responsible for degradation of AHLs displaying acylase activity as HSL accumulated as the degradation product, while no acylated homoserine could be detected (see Figure 4). Overexpression of *pvdQ* in *P. aeruginosa* PAO1 inhibited accumulation of 3-oxo-C_{12}-HSL. However, a *pvdQ* knockout strain was still able to utilize HSLs as a sole source of carbon, implying that another enzyme is involved in the degradation process.[54] Indeed, a second AHL acylase was found in *P. aeruginosa* and was named *quiP* for quorum signal utilization and inactivation. QuiP shares 21% a.a. sequence homology with PvdQ and 23% homology with AiiD from *Ralstonia* spp., and it is another member of the Ntn-hydrolase family, with substrate preference for long-chain AHLs.[55] In a related experiment, expression of *pvdQ* in *P. aeruginosa* was shown to abolish not only accumulation of 3-oxo-C_{12}-HSL but also accumulation of PQS, a quinolone that serves as the third QS signal. In contrast, accumulation of C_4-HSL was not affected.[56] Exogenous addition of the enzyme has the same effect, namely, preventing signal accumulation as well as elastase and pyocyanin production. Reduction of virulence was also tested in vivo using a *C. elegans* infection model.[57]

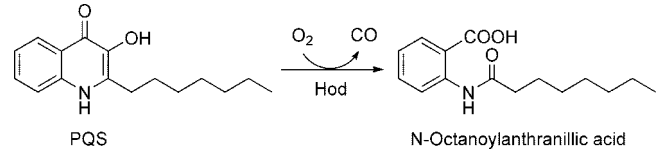
The multitude of QS regulation mechanisms that have been uncovered in *P. aeruginosa* may be explained by a need for bacteria to carefully, flexibly, and efficiently regulate the flow of information needed for efficient groupwise decision making under varying environmental circumstances. Autodegradation of QSMs was previously observed for *A. tumefaciens* and proposed as a timing mechanism to terminate the energy-consuming process of conjugational transfer of Ti-plasmid.[58] The role of autodegradation of 3-oxo-C_{12}-HSL in *P. aeruginosa* has not yet been determined, although some clues may arise from the dual function of PvdQ. This enzyme is involved in the regulation of synthesis of pyoverdin, the main iron chelator used by *P. aeruginosa*. *PvdQ* is located in the *pvd* locus, and as such it is expressed under iron-limiting conditions.[59] When a deletion mutant (Δ*pvdQ*) of *P. aeruginosa* strain PA14 was tested for its effect on virulence in an iron-deprived environment, virulence was dramatically reduced, both in a plant infection model and in a *C. elegans* fast-killing assay. The effect of the absence of pyoverdin, a known virulence factor for itself, was more pronounced than the effect of having higher levels of 3-oxo-C_{12}-HSL. An expected increase in virulence phenotypes due to higher levels of QS signals was not observed, however, which may be explained by overall decrease in virulence due to a lower ability to transport iron.[60]

The recently solved crystal structure of PvdQ led to its characterization as a member of the Ntn-hydrolase family, with a slightly elongated hydrophobic pocket resembling the QsdA lactonase. PvdQ is first expressed as a proenzyme with a $\alpha\beta\beta\alpha$-fold structure common to all Ntn-hydrolases. Autoproteolysis yields the formation of two chains that appear as a bowl-shaped structure with a deep, solvent-accessible crevice in the middle. The proteolytic cleavage unveils an N-terminal serine residue that is located in the bottom of the crevice and serves as a nucleophile for the catalytic event. A hydrophobic pocket is constructed on top of the N-terminal serine, involving bulky hydrophobic residues from two α-helices and two β-sheets and several unstructured loops. The loops fold away from a central β-sheet and a phenylalanine residue serves as a gate, closing off the hydrophobic pocket from the solvent. Following binding the substrate 3-oxo-C_{12}-HSL, the phenylalanine shifts position in alignment with several other residues to enable the accommodation of the long molecule. Upon elucidation of the crystal structure, a mechanism for the acylation reaction was proposed by Bokhove et al.[61] The N-terminal serine, located in the bottom of the hydrophobic binding pocket, acts as a nucleophile activated by a water molecule. The terminal α-amine serves as a base that through hydrogen bonding with the water molecule deprotonates the serine hydroxide. The activated hydroxylate is positioned in close proximity to the carbonyl carbon of the amide bond fusing the acyl chain and the homoserine lactone ring, which upon the nucleophilic attack forms a reactive oxyanion supported by a tetrahedral transition state that can be stabilized by a backbone amide and an asparagine residue. The water molecule then protonates the amide nitrogen and promotes the elimination of the HSL from the binding cavity. This results in the collapse of the transition state into an ester intermediate, which is hydrolyzed by the water hydroxylate, relieving a new hydroxyl, which is protonated by the terminal α-amine to regenerate a water molecule. This proposed mechanism is similar to that proposed for penicillin G acylase.[61]

AHLacylases were also found in the Gram-negative *Streptomyces sp.* strain M664, suggesting that AHLacylases are as ubiquitous among diversified bacteria genera as AHL lactonases are. *AlhM* encoding an acylase shares 35% and 32% a.a. sequence homology with AiiD from *Ralsonia* strain XJ12B and PvdQ from *P. aeruginosa* PAO1, respectively. AlhM was characterized as an additional member of the Ntn-hydrolase superfamily as it shares many of the characteristic features, such as a signal peptide, an α-subunit followed by a spacer, and a β-subunit. The catalytic preferences of AlhM are quite similar to those of PvdQ, with decreasing activity toward AHLs that are shorter than 8 carbons in the acyl chain. Accumulation of 3-oxo-C_{12}-HSL in the culture medium was strongly decreased when purified enzyme was exogenously added to the *P. aeruginosa* culture. Accumulation of butyryl homoserine lactone (C_4-HSL) was also decreased, although the enzyme does not degrade the molecule. This effect, however, is likely due to the correlation between C_4-HSL production and LasR activation. A concomitant decrease in virulence was also observed at early stages of the stationary phase, with a lower degree of reduction at the late stationary phase.[62]

A different acylase, AiiC, was isolated from *Anabaena* sp. strain PCC7120.[63] This enzyme shares 29% a.a. sequence homology with QuiP of *P. aeruginosa* and is considered another member of the Ntn-hydrolase family, although it differs from the other members by its low specificity and its ability to hydrolyze AHLs with 4−16 carbons in the acyl chain, with or without the 3-oxo moiety. The sequence of AiiC includes a signal peptide and a transmembrane domain located in the N-terminal region. Its periplasmic location correlates with the absence of acylase activity in the cell free culture supernatant. Another similar AHL acylase was found in *Comamonas* strain D1, which shows a very wide specificity for different AHLs, although it was reported that *Comamonas* could not grow on AHLs as a sole source of carbon or nitrogen.[64] The similarity to other known acylases could not be determined, as the coding gene remains unknown.

Scheme 1. Cleavage of PQS to CO and *N*-Octanoylanthranillic Acid, Catalyzed by Hod, a Dioxygenase from Arthrobacter Nitroguajacolicus

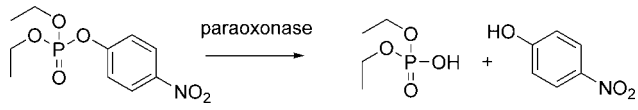


Five distinct prokaryotic classes of AHL lactonases have been discovered and characterized so far, in which the representative members are AiiA, AttM, AiiB, QsdA, and BpiB. All of these lactonases share a binuclear zinc metal center character, with one of the zinc ions contributing to the stability of the protein structure while the second ion participates in the catalytic event. AHL acylases, on the other hand, belong to the same Ntn-hydrolase superfamily and may have evolved from the same ancestral enzyme. The occurrence of AHL-degrading enzymes in prokaryotes is prevalent throughout most bacterial genera as well as some archaeal species. Strikingly, evidence of similar inactivating enzymes with hydrolase activity toward other QS signal molecules such as AIPs and AI-2 has not been reported so far. However, a few examples of enzymatic oxidation of QS molecules can be found for AIPs (section 4) and the *P. aeruginosa* QSM 2-heptyl-3-hydroxy-4(1H)-quinolone (*Pseudomonas* quinolone signal, PQS). In an elegant study by the groups of Williams and Fetzner,[65] this molecule was found to be oxidized to *N*-octanoylanthranillic acid in the presence of Hod, a dioxygenase from the soil bacterium *Arthrobacter nitroguajacolicus* (Scheme 1).

2.2. Interkingdom Quorum Quenching

The wide diversity of bacterial phyla that have evolved AHL-degrading enzymes, including proteobacteria (e.g., *Agrobacterium sp., Comamonas, Ralstonia, Variovorax, Pseudomonas sp.* and *Klebsiella*), firmicutes (e.g., *Arthrobacter* and *Bacillus*), cyanobacteria (e.g., *Anabaena sp.*), and actinobacteria (e.g., *Rhodococcus* and *Streptomyces sp.*),[66] testifies to the potential importance of the enzymatic degradation of AHLs as a mechanism to interfere with QS. Although the evolutionary purpose of AHL-degrading enzymes is yet to be fully understood, it is clear that bacteria benefit from interspecies as well as intraspecies manipulation of QS signal levels. It is only natural that such mechanisms would be adopted for interkingdom manipulation of QS signaling. Eukaryotes are continuously exposed to QS molecules, secreted by diverse microbial flora present in varying eukaryotic habitats, such as in mammals, in the rhizosphere of plants, or in the vicinity of fungi. Different types of reaction to the presence of QS molecules were observed in different eukaryote hosts.[14,17,67]

Scheme 2. Paraoxon Hydrolysis Catalyzed by Paraoxonase



Evidence for a mammalian response to AHLs includes apoptosis, inhibition of smooth muscle contraction, and immunomodulatory effects, whereas in plants the production of AHL decoy molecules, uptake of AHLs into the shoot of the plant, increase of stomatal conductance, and stimulated root growth have been observed.[68−71]

2.2.1. AHL-Degrading Enzymes in Eukaryotes

Recently, several plants, mostly legumes including alfalfa, clover, lotus, and yam beans, have been reported to possess AHL-degrading capabilities.[72,73] The observed depletion of C_6-HSL from the growth media was independent of the pH, and QS-inhibiting compounds were not detected in seed-germination assays. However, temperature-dependency suggests that degradation is enzymatic. Specificity appears to be tolerant for longer acyl chains including 3-oxo-acyl-HSLs, as was tested for the *Lotus corniculatos* plantlets. The biochemical nature of the enzymes was not verified, and the a.a. sequence remains unknown, but the comparison of the AHL-to-HS ratio in yam beans suggests that in this case the degrading activity can be related to lactonases. Hydrolytic activity was also found in root-associated fungi collected from forest environments. Three subspecies of the *Ascomycota* and *Basidomycota* lineages were shown to degrade C_6-HSL and 3-oxo-C_6-HSL with lactonase-degradation patterns. Phylogenetic analysis revealed strong homology (96−100%) to other fungi from 9 different genera suggesting that lactonases may be abundant throughout the phyla.[74]

In mammals, it was found that human airway epithelial cells can hydrolyze AHLs. Greenberg and co-workers have shown that hydrolysis is cell membrane associated and of enzymatic lactonase activity.[75] Similar inactivation of AHLs was observed when 3-oxo-C_{12}-HSL was exposed to mammalian serum obtained from rabbit, mouse, horse, goat, bovine, and human, although interestingly not in samples from fish and chicken.[76] The serum-degrading activity was also of enzymatic lactonase nature, including Ca^{2+} dependence suggesting metalloprotease activity. The enzyme characteristics of both serum and cell-associated lactonases are highly reminiscent to those of paraoxonases (PONs). These PONs are a family of mammalian hydrolyzes that exhibit diverse activities, including hydrolysis of various organophosphates such as nerve gases and pesticides; hydrolysis of aromatic esters, preferably those of acetic acid; and hydrolysis of aliphatic lactones as well as some lactonizing ability.[77−79] The name paraoxonases was chosen for their ability to hydrolyze paraoxon (Scheme 2), a potent insecticide that is also highly toxic to humans.

PONs are also known for their role in the inactivation of drug molecules and in the prevention of atherosclerosis.[80] Recently evidence has accrued suggesting the native activity for PONs to be that of lactonases, which evolved to decrease bacterial virulence through hydrolysis of homoserine lactones.[78,79,81] The PON gene family has three members, *PON1*, *PON2*, and *PON3*, which are highly conserved in mammals sharing 79−95% a.a. sequence homology between species. Human PON1 is produced in the liver and excreted into the blood, where it associates with high-density lipoprotein (HDL). PON2 is a cell membrane-associated protein expressed in different tissues including brain, liver, kidney, and testis. PON3 is also produced in the liver, with small amounts found in the kidney, and excreted to the blood, although in lower levels than PON1.[77,82] PON-like enzymes are widely spread in mammals, such as rat, rabbit, mouse, and humans, but also some invertebrates, such as *C. elegans* and the fruit fly *Drosophila melanogaster*;[77] however, paraoxonase activity is not seen or only weakly seen in fish and avian species.[83]

A detailed study of the PON-like enzyme sequence in *C. elegans* shows that the conserved residues required for catalytic activity are missing, suggesting that this variant lacks enzymatic activity.[84] A similar lack of enzymatic activity was also shown for *Drosophila melanogaster*.[81] The first crystal structure of PON1 was solved by Tawfik and co-workers, showing that PON1 is a six-bladed β-propeller with 4 strands composing each of the propellers.[80] The central cavity is a tunnel-shaped binding pocket with two calcium ions (Ca1 and Ca2) at the bottom and two α-helices that form a canopy-like pocket with connecting loops that result in a closed binding cavity. Ca1 seems to participate in the catalytic mechanism while Ca2 serves a role in the overall stability of the enzyme. Ca1 is bound by side chain oxygen of five a.a. residues: three aspargines, aspartate, and glutamic acid. Two remaining sites of the calcium ion coordinate a water molecule and a phosphate ion that appear to mimic the oxyanion tetrahedral intermediate formed in the hydrolysis of the lactone ring. His115 is proposed to act as a base deprotonating the water molecule to obtain a reactive hydroxide, with His134 assisting with proton transfer to increase the basicity of His115. This His−His dyad is a newly described mechanism, although there is a certain resemblance to the His−Asp dyad reported for phospholipase A2 (PL2) and the His−Glu dyad present in diisopropylfluorophosphatase (DFPase) from *Loligo vulgaris*.[85,86]

Specificity for AHLs was tested in all three PONs, showing that PON2 preferably hydrolyses AHLs, but only the (*S*)-isomers. Aliphatic lactones were hydrolyzed better than aliphatic esters, with faster rates for six-membered ring lactones than their five-membered ring counterparts.[78,79] In vivo tests showed that mouse blood serum containing PON1 and PON3 degraded 3-oxo-C_{12}-HSL, while serum from mice with targeted disruption of *PON1* exhibited impaired degradation. Furthermore, by adding purified recombinant PON1 to ΔPON1 mouse serum, proper activity was restored. In a *P. aeruginosa* infection model in mice, wild-type PAO1 infected mice exhibited 55% mortality 100 h after infection, while 10% mortality was observed after infection with a Δ*lasI P. aeruginosa* strain (LasI is its 3-oxo-C_{12}-HSL synthetase). Surprisingly, PON1-deficient mice were protected from the infection, with 100% of the mice surviving after 50 h as opposed to 50% of the wild-type mice. A possible explanation is that increased expression levels of PON2 and PON3 compensated for the deficiency in PON1.[87] Stoltz et al., on the other hand, chose *Drosophila melanogaster* as a model to assess the ability of PON1 to protect animals from infection with *P. aeruginosa*. Drosophila flies do not express an enzymatically active PON-like enzyme, and when infected with *P. aeruginosa*, >80% mortality was observed 24 h after infection. When PON1 transgenic flies were infected with *P. aeruginosa*, 18 h postinfection 87% of the flies were still alive, confirming that PON1 provides protection from *P. aeruginosa* killing. This lack of virulence

was comparable to that of a Δ*lasI*/*rhlI* knockout strain used to infect wild-type flies. The same protective effect was observed when infected transgenic flies were tested for expression levels of four known QS-dependent genes, including *lasA*, *lasB*, *aprA*, and *toxA*, showing markedly reduced levels of expression.[81]

2.3. Quorum Quenching As an Antivirulence Strategy

Even though quorum quenching appears to be widely spread among prokaryotes and eukaryotes, the biological relevance of this phenomenon has not been fully understood in most cases, as was carefully reviewed by Roche et al.[28] Further research is needed to elucidate the physiological role of QQ enzymes, but already a vast amount of evidence has been collected indicating that these enzymes can effectively reduce bacterial QS-regulated infection phenotypes, thus priming enzymatic QQ as a relevant therapeutic strategy. Indeed, QQ strategies have been adapted by researchers to reduce and possibly eradicate QS-related bacterial infection phenotypes. Several approaches have been developed to utilize QS signal-degrading enzymes for the purpose of attenuating bacterial virulence. None of these novel strategies however has yet ripened to a practical approach that is ready to be employed in agricultural or clinical settings.

2.3.1. Genetic Engineering

Heterologous expression of QQ enzymes has been shown to be effective both in bacterial and in plant models. Dong et al. inserted *aiiA* into the genome of potato and tobacco plants, resulting in enzymatic degradation of 3-oxo-C_6-HSL in the environment of the plants; this proved effective in reducing plant maceration by *E. carotovara*.[32] Enhanced expression of AiiA in the mutated plant correlated with enhanced resistance to infection of *E. carotovora*, as maceration areas appeared dramatically smaller, with up to 95% decrease 20 h after inoculation. However, protection was not complete and depended on inoculation density. High inoculation density correlated strongly with plant maceration, although affected areas were still smaller that those observed for nontransgenic control plants. Similar results were obtained by other plant models infected by *E. carotovora*.[88,89]

Sinorhizobium meliloti is a nitrogen-fixing bacterium that forms symbiotic relationships with plants. Leguminous plants are known to form symbiotic nodules with rhizobia in nitrogen-deficient environments. These root nodules are responsible for conversion of nitrogen from the atmosphere to ammonia, which can be taken up by the plant; the formation of the symbiotic nodules is QS regulated,[90] and QS-deficient strains—lacking either AHL synthase or AHL receptors—are still able to produce nodules, although with a reduced rate and efficiency.[91] Insertion of *aiiA* in a wild-type strain of *S. meliloti* resulted in deactivation of long-chained AHLs produced by SinI, its main AHL synthase.[92] Symbiotic cultures of the *aiiA*-overexpressing strain of *S. meliloti* and a germinating seedling of the legume *Medicago truncatula* (Barrel Clover) were producing ∼50% less nodules compared to wild-type symbionts, due to substantially lower rate or efficiency of nodule initiation during the first 12 h. However, it was also found that the presence of AiiA in the symbiotic culture resulted in degradation of AHL-mimic molecules that are naturally produced by the *M. truncatula*, and the effect of this interference with the symbiosis dynamics is not clear.

Gene insertion of QQ genes in host organisms was shown to be efficient in quenching QS in an animal model as well, when *P. aeruginosa* infection symptoms where dramatically reduced in transgenic fruit flies,[81] or in *C. elegans*[50,57,60] as discussed previously. Notably, disruption of AHL communication does not affect bacterial growth.[89] The advantage of expressing AHL-degrading enzymes directly in hosts that are exposed to the effects of virulent pathogens that employ AHLs as QS molecules is clear; however, genetic modifications of plants or animals may pose yet unforeseen difficulties. While reducing or blocking virulence used by pathogens, the unintentional attenuation of unknown positive or even essential effects for the host mediated by symbiotic bacteria is inevitable. Although this concern could in theory be addressed by engineering the specificity of the QQ enzyme toward one specific AHL, in practice this may prove more difficult. In a directed-evolution approach, Chow et al. recently generated lactonases to obtain several enzymes with various specificities for AHLs, but the differences were rather small.[93]

2.3.2. Biocompetition

In a biocompetion strategy, bacteria that display native QQ ability are incorporated or cocultivated in virulence-sensitive environments. This more subtle approach has proven to be successful in several instances and can be useful for manipulating biological activities that are regulated by AHL-dependent QS.[94,95] Cocultures of *Acinetobacter* strain C1010, which expresses an AHL-degrading enzyme, with plant pathogens *Burkholderia glumae* and *E. carotovora*, the causative agent of rot disease, have been successful in reducing virulence and disease symptoms. Growth of the bacteria was not altered by *Acinetobacter*, nor did any symptoms appear on the plant model.[96] *P. chlororaphis* O6 is used as a "biological control" bacterium, producing phenazine in a QS-regulated mechanism, which inhibits the growth of phytopathogenic fungi. Cocultures of *P. chlororaphis* O6 with *Acinetobacter* strain C1010 reduced phenazine production. Co-inoculation of the plant pathogen *Pectobacterium carotovorum*, another soft-rot disease causative, and *R. erythropolis* strain W2—which is able to quench QS through expression of lactonase, acylase, and oxidoreductase—into potato tubers totally prevented maceration of the tissues.[97] Pathogenesis was prevented even when high inoculation densities were used, probably owing to the combination of the three degrading and processing enzymes.[29,98] Although these examples convincingly demonstrate that biocompetition (biocontrol) can be a useful strategy in manipulating the behavior of certain plant pathogens, the disruption of AHL-communication networks may have profound effects on the plant rizhosphere, and beneficial traits such as phenazine production of *P. chlororaphis*—as well as other yet unknown symbiotic relationships—may be lost.

3. Antibody-Mediated Quenching of Quorum Sensing

As highlighted in this review, there have been numerous reports of prokaryotic and eukaryotic enzymes that quench bacterial QS signaling. Additionally, a few species have been shown to possess orphan receptors for QS molecules that might be used to sequester QSMs released by other microbial

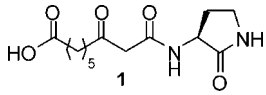


Figure 5. Chemical structure of the RS2 hapten.

organisms, thus potentially quenching QS signaling as well by scavenging the autoinducers. One might expect that the mammalian adaptive immune system would also respond to the presence of QS molecules by generating antibodies (immunoglobulins) against the various chemical entities. However, the nonproteinaceous nature (AHLs and DPD) as well as the hydrolytic instability and low molecular weight of the QS molecules effectively prevent an antibody-based immune response as these molecules cannot be properly processed and presented by the mammalian immune system. This phenomenon exhibited by small molecules was initially discovered by Karl Landsteiner, and he coined the term "hapten" for molecules that are too minute to elicit an immune response by themselves.[99]

Notably, recent research also revealed that bacterial QS molecules of the AHL family exert potent biochemical effects on mammalian cells, including induction of apoptosis and modulation of NF-κB activity, a key regulator of innate immunity.[100−102] These adverse effects of bacterial autoinducers on mammalian cells provide a reasonable foundation to regard them as simple bacterial small-molecule toxins. In light of these findings, the neutralization of the QS signaling molecules as part of a prophylactic or therapeutic QQ strategy might be highly desirable and may require a macromolecular solution rather than a small-molecule antagonist approach.

Microbial QS systems represent an excellent target for an antibody-based antivirulence therapy given the evolutionary conservation and extracellular distribution of QS signaling molecules; thus, researchers have utilized hapten−carrier protein conjugates to elicit hapten-specific immune responses either to use this approach as an active vaccination strategy or to generate monoclonal antibodies that could ultimately be used to prevent or treat bacterial infections.[103−106] An additional hurdle to overcome in the generation of QQ antibodies was the inherent disadvantageous chemical nature of bacterial QS molecules, i.e., their hydrolytic instability, hydrophobicity, and lack of charge and aromaticity (AHLs and DPD) in addition to the low molecular weight, which imposed significant difficulties on the generation of quorum quenching antibodies that recognize their respective QS molecules with high specificity and affinity.

In the past few years, a number of reports have described the generation and evaluation of quorum quenching antibodies in vitro and in vivo. Janda and co-workers have pioneered this immunotherapeutic approach for QQ with the generation of an anti-AHL antibody, RS2−1G9, elicited against the

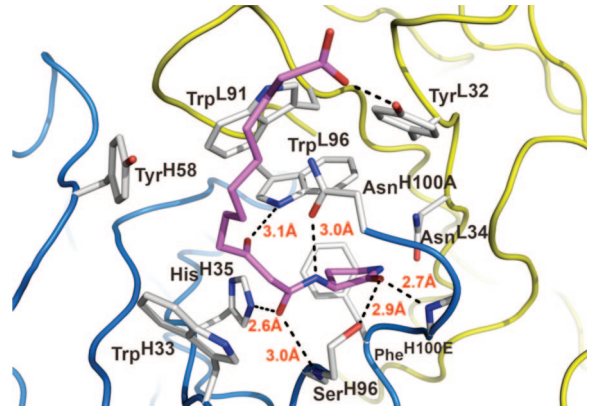


Figure 7. Antibody combining site of RS2−1G9. Hydrogen bonds are shown as broken lines. Only Fab side chains that contact hapten **1** (pink) are displayed. All of the hapten's functional groups are satisfied, except for the ring amide. Reproduced with permission from ref 107.

synthetic 3-oxo-AHL analogue RS2 (Figure 5) that efficiently suppressed QS signaling in vitro in *P. aeruginosa* via sequestration of 3-oxo-C_{12}-HSL.[105] The underlying rationale for using an AHL analogue as hapten in their experiments was founded upon the well-described hydrolytic instability of the AHLs, which has also been discussed in this review.[24,25] The RS2 hapten features a lactam-containing moiety in place of the homoserine lactone ring, which resulted in increased stability of the molecule while maintaining the 5-membered ring structure. In biochemical studies RS2−1G9 demonstrated excellent specificity and affinity for 3-oxo-C_{12}-HSL while recognizing related short-chain 3-oxo-AHLs not at all or with lower affinity. Though these data might be surprising at first, the structural elucidation of RS2−1G9 in complex with the RS2 by Wilson and co-workers offered an explanation of the observed preferred recognition of 3-oxo-C_{12}-HSL.[107]

The crystal structure of Fab RS2−1G9 in complex with the RS2 hapten analogue revealed the complete encapsulation of the ring moiety in the antibody combining site (Figure 6). This mode of recognition provides an elegant immunological solution for tight binding to an aliphatic, lipid-like ligand with a small headgroup lacking typical haptenic features, such as aromaticity or charge, which are often incorporated into hapten design to generate high-affinity antibodies. A short and narrow tunnel exiting at the protein surface harbors a portion of the acyl chain and would not allow for entry of the headgroup, implying that conformational changes, most likely via an induced fit mechanism, must occur upon ligand binding and release. A high degree of discrimination of RS2−1G9 against closely related AHLs is conferred by six hydrogen bonds to the ligand (Figure 7).

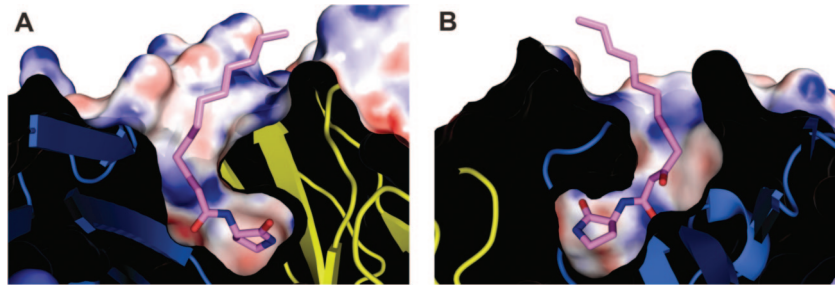


Figure 6. High shape complementarity of the hapten in the binding site of RS2−1G9. The light and heavy chains of the Fab are colored in yellow and blue, respectively. A slice through the center of the binding site is shown. The electrostatic potential is mapped onto the surface, ranging from −30 kT/e (red) to +30 kT/e (blue). Reproduced with permission from ref 107.

We direct particular attention to the hydrogen bond formed between the tryptophan L96 and the oxygen of the γ-keto group. Data from competition ELISA experiments showed exquisite discrimination of the antibody between 3-oxo-AHLs and AHLs without the additional keto group.[105]

The authors surmised that this Trp^{L96}-oxygen hydrogen bond is the molecular manifestation of this discriminatory ability of RS2−1G9 between the different AHLs. Conversely, cross-reactivity of RS2−1G9 toward the native 3-oxo-C_{12}-HSL is likely to originate from conservation of these hydrogen bonds, as well as an additional potential hydrogen bond to the oxygen of the lactone ring. The detailed molecular delineation of this quorum-quenching antibody in complex with its ligand clearly illustrates how the immune system has evolved to potently bind and discriminate a low molecular weight lipophilic compound with a small headgroup, such as the *N*-acyl homoserine lactam, with nanomolar affinity.

As discussed above, AHLs not only influence bacterial behavior but also induce biochemical changes and exert cytotoxicity in mammalian cells. Ulevitch and co-workers demonstrated that 3-oxo-C_{12}-HSL induces phosphorylation of protein kinases, p38 and eIF2α, in mammalian cells. These two proteins represent two key biochemical markers of the cytotoxic effects induced by *N*-(3-oxo-acyl) homoserine lactones.[100] The phosphorylation events occurred in a wide variety of cell types, including primary murine alveolar macrophages and human bronchial epithelial cells, and absolutely require structural integrity of the lactone ring motif and its natural stereochemistry.[100,108] This led to the hypothesis that these effects of 3-oxo-C_{12}-HSL on host cells might play a critical role in establishing and maintaining persistent airway infections caused by *P. aeruginosa*. As a logical progression, the anti-AHL mAb RS2−1G9 was evaluated for its ability to prevent these AHL-induced cytotoxic, immunosuppressant, and biochemical effects.[109] In a concentration-dependent manner, RS2−1G9 prevented cell death in primary bone marrow-derived murine macrophages (BMDM) in the presence of 3-oxo-C_{12}-HSL, whereas an isotype control antibody (elicited against a different AHL hapten) did not offer any protection. Taken together, mAb RS2−1G9 was shown to readily sequester bacterial 3-oxo-C_{12}-HSL signal molecules, interfering with their biological effects in prokaryotic and eukaryotic in vitro systems.[105,109]

A different approach to the discovery of anti-AHL quorum quenching mAbs was described by Janda and co-workers, who screened a panel of existing catalytic antibodies for their ability to hydrolyze 3-oxo-C_{12}-HSL and, thus, quench the autoinducer.[110] The catalytic antibodies were generated using a reactive immunization strategy with a squaric monoester monoamide hapten.[111] One of the catalytic antibodies, namely, XYD-11G2, was found to hydrolyze 3-oxo-C_{12}-HSL and to prevent virulence factor production in *P. aeruginosa*.[110] Recently, the promising concept of using catalytic quorum quenching antibodies was developed further by Spring and co-workers as they reported on the design and synthesis of transition-state mimics for AHL lactone hydrolysis.[112] The molecules were validated as lactonase inhibitors and could be used to generate catalytic anti-AHL antibodies de novo either using classical hybridoma methodology or phage display technology.

Tateda and co-workers evaluated the effects of an active immunization with 3-oxo-C_{12}-HSL−carrier protein conjugate on acute *P. aeruginosa* lung infections in mice.[103] The immunization with a 3-oxo-C_{12}-HSL−BSA conjugate elicited specific anti-AHL antibody titers in the animal. Immunized mice were challenged intranasally with *P. aeruginosa*, and their increased survival compared to mock-vaccinated animals strongly suggests a protective effect of the vaccine. Interestingly, bacterial numbers in the lungs were found not to be different between control and immunized groups, indicating that enhanced bacterial clearance was not the underlying mechanism of protection. However, levels of the pro-inflammatory cytokine tumor necrosis factor (TNF)-α in the immunized mice were significantly lower than those of control mice. These data indicate that neutralizing anti-3-oxo-C_{12} antibodies elicited via active immunization can assume a protective role in acute *P. aeruginosa* infection, most likely via blockade of excessive pro-inflammatory host responses.

A target for QQ immunotherapy in Gram-positive bacteria that recently has emerged is accessory gene regulator (*agr*)-controlled virulence gene expression in *S. aureus*, originally identified by Novick and co-workers.[113] Subsequently, *agr* systems have been identified in many staphylococci and they all appear to utilize cyclic oligopeptides, termed autoinducing peptides (AIPs), as specific signaling molecules.[114,115] A highly conserved cysteine residue forms a thiolactone bond with the carboxyl group of the C-terminal amino acid, resulting in the cyclic AIP. Genes regulated by *agr* encode cell surface proteins, such as protein A, and secreted proteins, including proteases, hemolysins, and other toxins. The *agr* system contributes to *S. aureus* pathogenesis by orchestrating the temporal cell density-dependent expression of these virulence genes.[114] Interestingly, QS signaling interference was observed when AIPs from different *S. aureus* strains were evaluated for the QS signaling properties in other *S. aureus*. On the basis of these findings, *S. aureus* strains can be divided into 4 distinct subgroups, with the AIPs of any one subgroup activating their own RNAIII transcription but inhibiting the RNAIII transcription in the other *agr* subgroups.[116] These findings have led to the discovery of peptidic *agr* antagonists as well as an immunotherapeutic approach interfering with the AIP-based *S. aureus* QS system in vitro and in vivo.[117−119]

In analogy to their autoinducer analogue hapten approach pursed in the generation of anti-AHL mAbs, Janda and co-workers synthesized a hydrolytically more stable AIP 4 hapten, coined AP4, in which the critical cysteine residue was replaced by a serine amino acid forming a lactone bond, thus granting more hydrolytic stability.[106] Immunization with AP4−carrier protein conjugates resulted in the generation of anti-AIP4 mAbs and specifically the isolation of mAb AP4−24H11 was further characterized. First, the QQ properties of AP4−24H11 were evaluated in vitro. Protein A and α-hemolysin, encoded by the *spa* and *hla* genes, respectively, are two important virulence factors regulated by *agr*.[120−122] Administration of mAb AP4−24H11 reduced α-hemolysin expression significantly in the prototypical *agr* group IV strain, RN4850a, as well as in NRS168, a clinically isolated *agr* group IV *S. aureus* strain.[118] Protein A production was increased as a result of the inhibition of the *agr* signaling network in *S. aureus*. Notably, other regulatory systems in *S. aureus*, such as *sar* (staphylococcal accessory regulator) and *sae* (staphylococcal accessory protein effector), were not affected, indicating that the mAb-mediated inhibition is specific for *agr* QS signaling.

The most important question for any potential antivirulence therapy, such as the use of a QQ antibody, is whether such

antibodies have therapeutic potential in vivo. Thus, Janda and co-workers evaluated AP4−24H11 in two independent murine *S. aureus* infection models. First, they investigated whether AP4−24H11 was able to mitigate *S. aureus*-induced injury in a murine subcutaneous infection model.[117,119,123] Whereas untreated mice developed dermonecrosis, skin injury was abrogated in mice that received the higher-dose AP4−24H11. This is encouraging in vivo evidence that an anti-AIP immunotherapy might be a promising avenue for the prophylaxis and treatment of *S. aureus* infections.

Most importantly, Janda and co-workers also evaluated the effectiveness of a passive immunization approach using AP4−24H11 in a lethal *S. aureus* challenge mouse model.[117] All of the mice receiving AP4−24H11 survived through the 8-day observation period. In contrast, only one of the control mice survived longer than 24 h. These data further validated the pursuit of an immunopharmacotherapeutic approach for preventing and treating *S. aureus* infections.

Taken together, these reports provide the foundation for the development of tailor-made QQ proteins, namely, antibodies that could be used in prophylactic and therapeutic clinical settings. Additionally, a QQ vaccine or component in a multivalent vaccine might offer significant benefits by suppressing virulence factor expression rather than targeting the bacteria directly.

4. Active Uptake of Quorum Sensing Signals

A third and less studied area of QQ by macromolecules is the scavenging or processing of QS molecules of one species by competing organisms. Xavier and Bassler performed an elegant study in which they showed that *E. coli* is able to modulate QS in *V. harveyi* through uptake of AI-2;[124] internal phosphorylation of AI-2 in *E. coli* by LsrK leads to derepression of *lsrR* expression, resulting in an accelerated internalization of AI-2. The authors expected to see interference with *V. harveyi* QS by *E. coli* when the two species would be cultured together. Indeed, when wild-type *V. harveyi* was cocultured with wild-type *E. coli*, production of bioluminescence by *V. harveyi* at high cell density diminished to 18%, while coincubation with an *E. coli* mutant containing a constitutively derepressed LsrK resulted in even greater reduction of luminescence (>90%). In sharp contrast, an *lsrK* deletion strain that is not able to phosphorylate AI-2 and transport the autoinducer did not interfere with induction of bioluminescence in *V. harveyi*. Although these interesting results may not have immediate relevance in vivo, as *E. coli* and *V. harveyi* do not ordinarily share the same habitat, the closely related human pathogen *Vibrio cholerae* does encounter *E. coli* regularly.[125] It remains to be seen, however, whether potential interference with *V. cholerae* QS by *E. coli* will have an effect on *V. cholerae* virulence and colonization in vivo.

In a related study by Bentley and co-workers,[126] intraspecies communication was targeted in cocultures of *E. coli*, *S. typhimurium*, and *V. harveyi*, resulting in diminished QS activity in the mixed populations. The authors showed that addition of purified LsrK results in phosphorylation of DPD, preventing its diffusion through the cell membrane. Addition of LsrK and ATP to cultures of *E. coli*, *S. typhimurium*, and *V. harveyi*, as well as its addition to cocultures of these bacteria was shown to quench both interspecies and intraspecies communication. However, the utility of LsrK as a multispecies QS quencher and concomitant effects in animal infection models have not yet been examined.

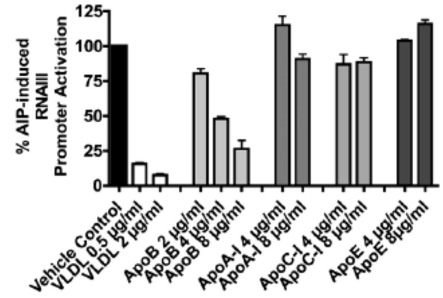


Figure 8. Apolipoprotein B inhibits RNAIII promoter activation.[129] Reproduced with permission from ref 129.

The ability to synthesize AI-2 is found in a large number of phylogenetically distinct bacteria. This by itself, however, does not demonstrate that the same bacteria utilize AI-2 as a QS molecule. To discern whether AI-2 is used for intra- or interspecies signaling in a given species, the recognition of this signaling molecule needs to be described at a molecular level. In other words, without identification of an AI-2 receptor for an AI-2 producer, evidence for a role of this molecule as a QS signal is incomplete. Until recently, no AI-2 receptors in species other than *V. harveyi*, *S. typhimurium*, and *Sinorhizobium meliloti*[127] were identified. The groups of Xavier and Miller, however, have gathered evidence that AI-2 receptors are present in very distinct bacterial families, such as *Enterobacteriaceae*, *Bacilliciaceae*, and *Rhizobiaceae*.[128]

Presence of an AI-2 receptor in a bacterium, however, is by itself not sufficient evidence for a role of the protein in relaying a cell density signal. Alternative potential roles for such a protein include the uptake of AI-2 as a food source and the scavenging of AI-2 to interfere with its role of signaling molecule in competing species. The studies by the groups of Bassler and Bentley (vide supra), in which LsrK essentially plays such a role, may support this hypothesis.

A different example of cross-species QQ through signal scavenging was discovered by Gresham and co-workers. In their search for components of the innate immune system that could interfere with bacterial signaling, they hypothesized that serum proteins could bind AIPs.[129] Indeed, the *S. aureus* QS molecule AIP1 was shown to bind to apolipoprotein B (ApoB), and addition of this protein to a culture of *S. aureus* resulted in inhibition of QS, as measured by degree of AIP-induced RNAIII promoter activation (Figure 8).

This inhibitory activity was specific for ApoB, as ApoA, ApoC, and ApoE were shown to be inactive in this assay. Furthermore, mice deficient in ApoB were more susceptible to infection by *S. aureus* that contained an active *agr* signaling system, while *agr* deletion mutants were unable to infect these mice.

In an earlier study by Gresham and co-workers, a different mechanism of AIP inactivation was discovered. The authors studied innate defense mechanisms against *S. aureus* infections in a murine skin model and found that AIP1 is oxidized by phagocytes.[130] Specifically, NADPH oxidase was found to catalyze oxidation of the methionine residue in AIP1 (Figure 1) through generation of HOCl and $ONOO^-$. The two different studies by this group suggest that our innate immune system is able to employ widely varying methods to quench QS with use of proteins.

The studies described in this section indicate that quenching of QS through scavenging of signals by macromolecules has biological relevance, although the overall importance and scope in determining the outcome of relationships between

species occupying the same habitat has yet to be determined. Compared with the production of enzymes that hydrolyze QS signals, it appears to be energetically unfavorable for an organism to synthesize a macromolecule that can only bind one signaling molecule at a time, whereas an enzyme can catalyze the hydrolysis of a large variety of signaling molecules. However, as can be seen in the case of apolipoprotein B, scavenging of a QS molecule by a binding protein can be an effective defense strategy—although the primary function of apolipoproteins is to bind lipids, and as such a secondary role in providing protection against bacterial infection would not constitute an additional energy expenditure.

5. Conclusions

In this review, many different macromolecules that can inhibit bacterial QS with varying degrees of potency are described. However, whether biological relevance (as natural quencher of QS) can be attributed to most, some, or even any of these proteins is still by large an unanswered question. Many of these proteins, however, were shown to effectively inhibit QS, and the strong efficacy of several enzymes and antibodies was demonstrated in vivo. These studies point to a promising role for macromolecules as quenchers of bacterial virulence and/or biofilm formation in clinical settings. To evaluate the potential of such macromolecules as therapeutic agents, however, several important questions will have to be addressed in future studies, such as whether the macromolecules (especially those that are based on bacterial enzymes) can elicit an immune response in humans, and whether degradation of AHLs would affect the commensal gut microflora of humans to the extent that this may cause harm to the host. Also, to what rate should QQ enzymes accelerate the hydrolysis of autoinducers to be effective, given that most autoinducers undergo aqueous hydrolysis also in the absence of hydrolases. Regardless of the potential therapeutical value of QQ macromolecules, however, they will undoubtedly function as powerful molecular tools to study the importance and robustness of intercellular signaling in bacterial populations.

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7. Note Added after ASAP Publication

After this paper was published online November 18, 2010, a correction was made to the third paragraph in section 2.1.2. The revised version was published December 27, 2010.

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