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Pseudomonas aeruginosa Quorum Sensing

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Abstract

Pseudomonas aeruginosa, like many bacteria, uses chemical signals to communicate between cells in a process called quorum sensing (QS). QS allows groups of bacteria to sense population density and, in response to changing cell densities, to coordinate behaviors. The *P. aeruginosa* QS system consists of two complete circuits that involve acyl-homoserine lactone signals and a third system that uses quinolone signals. Together, these three QS circuits regulate the expression of hundreds of genes, many of which code for virulence factors. *P. aeruginosa* has become a model for studying the molecular biology of QS and the ecology and evolution of group behaviors in bacteria. In this chapter, we recount the history of discovery of QS systems in *P. aeruginosa*, discuss how QS relates to virulence and the ecology of this bacterium, and explore strategies to inhibit QS. Finally, we discuss future directions for research in *P. aeruginosa* QS.

Keywords

Acyl-homoserine lactone; cooperation; sociomicrobiology; quinolone; LasR; RhlR; PqsR; quorum inhibition

History and Introduction to *P. aeruginosa* Quorum Sensing

A constellation of discoveries in the early 1990s opened up the very active *P. aeruginosa* quorum sensing (QS) research field. Investigations of luminescence gene regulation in a marine bacterium, *Vibrio fischeri*, had revealed a regulatory circuit requiring two genes, *luxI*, which codes for an enzyme that catalyzes the synthesis of a so-called autoinducer, 3-oxo-hexanoyl-homoserine lactone (3OC6-HSL), and *luxR*, which codes for a 3OC6-HSL-dependent transcriptional activator of the luminescence genes (Eberhard et al., 1981; Engebrecht et al., 1983). In 1991, the *Pseudomonas* research group of Barbara Iglewski reported the discovery of a gene coding for a transcription factor that controlled expression of several virulence genes (Gambello and Iglewski, 1991). The closest relative to the product of this gene, called *lasR*, was the product of the *V. fischeri luxR* gene. This discovery triggered a collaboration between the Iglewski group and the Greenberg *V. fischeri* research

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group. Because the *V. fischeri luxR* and *luxI* genes are adjacent, the Iglewski laboratory set out to sequence DNA flanking *lasR*, and indeed an adjacent gene coded for a LuxI homolog (Passador et al., 1993). Greenberg and colleagues began a search for a 3OC6-HSL-like molecule. Through the collaboration it was clear that LasR did not respond to 3OC6-HSL itself. Their efforts were spurred by a 1992 report by the group of Gordon Stewart that a variety of proteobacteria produced 3OC6-HSL. The list of bacteria included *P. aeruginosa* (Bainton et al., 1992). This led Greenberg and Iglewski to hypothesize that the *P. aeruginosa* LasI was responsible for the production of a molecule that was similar to but not 3OC6-HSL, something that could be mistaken for 3OC6-HSL. A year later they reported that the LasI-LasR signal was 3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL) (Pearson et al., 1994). The *P. aeruginosa* inducer had a fatty acyl tail six carbons longer than the *V. fischeri* inducer. At about the same time, studies of the plant pathogen *Agrobacterium tumefaciens* showed a LuxR-LuxI-like system called TraR-TraI, which produced and responded to 3-oxooctanoyl-homoserine lactone (3OC8-HSL) (Hwang et al., 1994; Zhang et al., 1993).

It was these foundational discoveries that led to publication of an important minireview, “Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators” and the birth of an active QS research field (Fuqua et al., 1994). The minireview posited that LuxR-LuxI-type systems enabled bacteria to sense their own population densities, and when they reached a bacterial quorum, they could respond by activating genes that were useful only when they were at sufficient density. This hypothesis generalized an existing view about control of luminescence in *V. fischeri* (Hastings and Nealson, 1977). Likely because *P. aeruginosa* is an important human pathogen and LasR controls several virulence genes, QS in this species has received much attention, more attention than QS in any other species, and *P. aeruginosa* has become a model for QS research. Studies of *P. aeruginosa* QS have revealed a wealth of information about general molecular and biochemical underpinnings of this type of regulatory apparatus, about bacterial sociality, and about the role of QS in virulence of this important pathogen.

This initial discovery of *lasR-lasI* led to a more complete picture of *P. aeruginosa* QS. In 1995, the Greenberg and Iglewski collaboration reported that *P. aeruginosa* produced a second acylhomoserine lactone (AHL), butanoyl-homoserine lactone (C4-HSL) (Pearson et al., 1995). That same year two groups discovered that this was due to a second set of *luxI-luxR* homologs that controlled production of several extracellular factors including the genes for rhamnolipid synthesis (Brint and Ohman, 1995; Ochsner et al., 1994). These QS genes were called *rhlR* and *rhlI*. The RhlR-RhlI circuit was subsequently found to be regulated by LasR-LasI, resulting in a hierarchy of AHL QS circuits in *P. aeruginosa* (Pesci et al., 1997). We note here that this QS hierarchy was found in the commonly used strain PAO1. As we will discuss in subsequent sections of this chapter, there is conservation of the genetic elements of QS among the many sequenced *P. aeruginosa* genomes, but there is plasticity in the hierarchy and, to some extent, the genes controlled by QS.

The *P. aeruginosa* QS signal chemistry was primarily the product of James Pearson, a graduate student working towards a Master’s degree with Greenberg. At the time they knew there was a third non-acyl-homoserine lactone signal whose production depended on the LasR-LasI circuit, but it had not been identified. Pearson moved to the Iglewski laboratory

to continue towards his PhD and the collaboration continued. He enlisted the help of a postdoctoral fellow, Everett Pesci to work on this third signal, which was identified and named the Pseudomonas Quinolone Signal (PQS) (Pesci et al., 1999). Together, these three signals were shown to be important for virulence in murine acute infection models (Smith and Iglewski, 2003), as described later in this chapter.

An additional AHL-responsive transcription factor in the *P. aeruginosa* QS circuit was revealed with the complete genome sequence of *P. aeruginosa* PAO1. A third *luxR*-like gene was found in the genome. This gene is called *qscR*, and it codes for a 3OC12-HSL responsive transcription factor (Chugani et al., 2001). QscR mutants are hypervirulent in a fruit fly infection model. A final piece from the first decade of *P. aeruginosa* QS research came in 1999 with an unbiased Tn5-*lac* screen for QS activated genes. This report revealed that QS-activated genes were spread around the chromosome, showed many genes not previously known to be controlled by QS, and indicated that there were more QS activated genes to be discovered (Whiteley et al., 1999). It was also notable in that it described a method for random transposon mutagenesis of *P. aeruginosa*. At that time there was some consensus in the field that this was not achievable.

In summary, the decade of the 1990s (actually 1992–2001) provided a richness of seminal discoveries leading to our general understanding of the quorum sensing circuits in *P. aeruginosa*. The diagram in Figure 1 is meant to capture the essence of these circuits.

The ensuing twenty years has provided a wealth of information. A simple PubMed search for *Pseudomonas aeruginosa* quorum sensing shows more than 2,500 results. In this chapter we do not endeavor to exhaustively cover the research field. The Introduction, including the information to follow, is meant to provide background for the sections to follow.

A first question is what genes are controlled by QS? The advent of transcriptomic technologies, first *P. aeruginosa* PAO1 microarrays, and then RNA-seq technology allowed development of a more thorough catalog of QS controlled genes. We learned that hundreds of genes were activated by QS, and that the timing of gene activation was complex (Schuster et al., 2003; Wagner et al., 2003). The Las, Rhl and PQS system are embedded in the general regulatory circuits of the cell and many quorum-activated genes are AND logic gated. That is, an adequate concentration of signal (a proxy for cell density) is required but not sufficient for expression of many QS-controlled genes (Schuster et al., 2004; Schuster et al., 2003; Wagner et al., 2003). The quorum regulon contains an overrepresentation of genes coding for secreted products or for enzymes involved in the synthesis of secreted products (Schuster et al., 2003). This finding was consistent with the idea that *V. fischeri* luminescence is a public good, shared by all members of the population. Excreted or secreted products can also be shared amongst the population (Fuqua et al., 1994).

By now the genomes of hundreds of *P. aeruginosa* isolates have been sequenced and there is a high degree of conservation of the QS regulatory circuits (Pseudomonas.com). There are examples of genomes with deletions including the *lasR-lasI* genes, and point mutations in *lasR* are not uncommon (Groleau et al., 2021), but generally speaking all elements of the QS

regulatory circuits are highly conserved in the species, although as mentioned above there are important strain-to-strain differences in the way the circuits control gene expression.

Because it will become important in the context of other sections of this review, we provide here some basic information about the biochemistry and molecular biology of AHL QS with particular attention to *P. aeruginosa*, and information about PQS signaling. The two *P. aeruginosa* AHL signals are synthesized by the typical LuxI-like AHL synthases, LasI and RhlI. In fact, RhlI was one of the first AHL synthases to be purified and studied *in vitro*. We know from these early studies that the substrates for C4-HSL synthesis are *S*-adenosylmethionine (SAM) and butyryl-ACP (Parsek et al., 1999). The energy for synthesis is in the activated butyric acid. There is an enzyme-bound butyryl-SAM intermediate synthesized prior to release of C4-HSL and the side product methylthioadenosine. This is thought to represent the general reaction mechanism for LuxI-type AHL synthases. We note that some AHL synthases use acyl-CoAs as the activated acid substrate rather than acyl-ACPs (Lindemann et al., 2011; Schaefer et al., 2008), but both RhlI and LasI are of the acyl-ACP type.

In general, LuxR-like transcription factors are activators, although a few repressors have been described (Fuqua et al., 1994; Whitehead et al., 2001). They are homodimers, and each monomer consists of two domains: an N-terminal AHL binding domain, and a C-terminal DNA-binding domain, which recognizes sequences in the promoter regions of QS regulated genes. Some *P. aeruginosa* QS-activated genes rely on LasR alone, others on RhlR alone, and some for example *lasB*, the gene coding for the protease elastase, have both a LasR and a RhlR binding site in their promoter region (Brint and Ohman, 1995; Gambello and Iglewski, 1991; Pearson et al., 1997). In the case of *lasB*, LasR is the dominant transcription activator (Pearson et al., 1997). The orphan 3OC12-HSL receptor QscR appears to control just a single set of genes to which it is linked (Ding et al., 2018). It is not clear how QscR mutations enhance virulence (at least in fruit flies), but the genes activated by QscR may affect 3OC12-HSL levels (Ding et al., 2018).

Finally, by way of introduction we will briefly review the complicated relationship of PQS to the LasR and RhlR QS systems. PQS production involves the *pqsABCDE* operon, and an unlinked gene, *pqsH* (Gallagher et al., 2002). With the exception of *pqsE*, the gene products are required for synthesis of PQS and several other related compounds. An adjacent and divergently transcribed gene, *pqsR* (also called *mvfR*), codes for a quinolone-dependent transcriptional activator of the *pqsA-E* operon (Cao et al., 2001; Deziel et al., 2004; McGrath et al., 2004). That is, synthesis of PQS and related compounds is positively autoregulated, a feature shared with many AHL QS circuits including the *lasR-lasI* circuit. PQS synthesis is induced by LasR and repressed by RhlR (Wade et al., 2005). PQS also modulates RhlR activity (Jensen et al., 2006). Recently, the Bassler laboratory has sought to understand the role of the *pqsE* gene product in QS. The *pqsE* gene is not required for PQS synthesis, but they and others have shown that the gene product affects RhlR activity (Farrow et al., 2008; Mukherjee et al., 2018; Taylor et al., 2021), and they have reported that PqsE is responsible for production of a small as yet unidentified molecule that can serve as a ligand for RhlR (Mukherjee et al., 2018), and/or that PqsE physically interacts with RhlR and affects its activity (Taylor et al., 2021). Finally, there is an interesting

relationship between PQS and *P. aeruginosa* membrane vesicles (Mashburn-Warren et al., 2009). Membrane vesicle formation is impaired in a *pqsA* mutant, and in PQS producing cells, membrane vesicles preferentially contain PQS.

QS and Virulence of *P. aeruginosa*

As discussed above, the *P. aeruginosa* QS transcription factors were discovered in part because they regulate virulence factors – elastase in the case of LasR, rhamnolipids for RhlR. Elastase is a secreted protease and rhamnolipids are secreted biosurfactant molecules that can be toxic to eukaryotic cells. In addition to elastase and rhamnolipids, QS regulators also activate genes encoding the production of hydrogen cyanide synthase, phenazines, and alkaline protease, among others (Schuster et al., 2003; Wagner et al., 2003). This observation naturally led to the hypothesis that QS receptors would have a role in the virulence of *P. aeruginosa*. Even before the QS circuitry was fully understood, work from the Iglewski and Prince laboratories demonstrated a deficiency in acute virulence of *lasR* deletion mutants of the widely-studied strain PAO1 in a mouse pneumonia model of infection (Tang et al., 1996). This result has been recapitulated in a variety of model systems, including murine burn wounds (Rumbaugh et al., 1999a; Rumbaugh et al., 1999c), in fruit flies (D'Argenio et al., 2001), in plants such as *Arabidopsis* (Rahme et al., 1995) and *Ocimum basilicum* (sweet basil) (Walker et al., 2004), and in the nematode *Caenorhabditis elegans* (Darby et al., 1999; Tan et al., 1999).

Subsequent work has showed the Las and Rhl systems have non-overlapping contributions to virulence. For example, a study in fruit flies identified RhlR as a key element in counteracting cellular immunity (Limmer et al., 2011) and the Bassler group found, using an approach of QS inhibition, that RhlR and not LasR was the important target for an inhibitor to modulate pathogenesis in *C. elegans* (O'Loughlin et al., 2013). Deletion of the gene encoding the third AHL receptor of *P. aeruginosa*, QscR, resulted in heightened virulence in a fruit fly model (Chugani et al., 2001), and it has subsequently been shown that QscR negatively regulates Las QS (Ding et al., 2018). The Pseudomonas quinolone signal, PQS, is also involved in *P. aeruginosa* virulence. Deletion of *pqsR* or inhibition of PQS synthesis decreases the production of virulence factors such as elastase and phenazines (Cao et al., 2001; Deziel et al., 2005; Rampioni et al., 2010), and *pqsR* is required for full virulence in several model infections, including mice, plants, and nematodes (Cao et al., 2001; Rahme et al., 1997).

The initial identification of QS transcription factors as important in acute virulence of *P. aeruginosa* was tempered by a series of reports that *lasR* mutations were common in a variety of chronic infections, including burn and diabetic wounds and cystic fibrosis (CF) (Rumbaugh et al., 1999b; Rumbaugh et al., 1999c; Turner et al., 2014; Wilder et al., 2009). People with CF often acquire *P. aeruginosa* lung infections and even after repeated courses of antibiotic treatment can harbor descendants of the founding colonizer (Smith et al., 2006). Sequential isolates over years of chronic CF lung infections have enabled studies of pathoadaptive evolution. We now understand *P. aeruginosa* can lives in functionally isolated, distinct populations in aggregates stratified throughout the CF lung, and this isolation may drive diversification (Jorth et al., 2015). *lasR* mutations are among the pathoadaptive

mutations common in *P. aeruginosa* isolates from lungs of chronically infected CF patients (Smith et al., 2006).

This finding that *lasR* mutations are common in clinical isolates was paradoxical, as laboratory strains with *lasR* deletions have a QS-null phenotype and do not exhibit RhlR or PqsR activity (Lee and Zhang, 2015) and, as discussed above, QS is important for acute virulence. In the late 2000s, a body of work began to emerge suggesting that, in some circumstances, LasR was not necessary for QS in some *P. aeruginosa* isolates. The Deziel group offered evidence that RhlR was active in stationary phase in the absence of LasR (Dekimpe and Deziel, 2009) and evidence from CF isolates suggested that RhlR QS was maintained even in the absence of LasR: most *lasR* mutant isolates produced C4-HSL, and *P. aeruginosa* with *rhlR* mutations are only rarely isolated from CF lungs (Bjarnsholt et al., 2010). Isolates from chronic infections have since been shown to engage in RhlR QS in a LasR-independent manner (Chen et al., 2019; Cruz et al., 2020; Feltner et al., 2016), suggesting that RhlR is the central AHL QS virulence regulator for *P. aeruginosa*, even in chronic infections.

A phenomenon related to the production of virulence factors is the role of quorum sensing in the development of *P. aeruginosa* biofilms. The Greenberg and Iglewski groups working with the biofilm group of the late Bill Costerton reported in 1998 that *P. aeruginosa* PAO1 LasI-null mutants formed thin, flat, undifferentiated biofilms compared to the wildtype, which formed characteristic mushroom-shaped structures (Davies et al., 1998). This was a notable publication in that 1) it established a genetic link to biofilm structure in bacteria. This was not a given prior to this publication. There was an idea that physical forces alone could explain the formation of the mushroom-like structures. 2) It was the first report describing the use of GFP in confocal microscopy investigations of biofilm development. This technique is now a standard approach in biofilm studies because it allows excellent imaging of live biofilms over time. This work created some controversy in the years shortly after its publication. We now understand that formation of characteristic mushroom-like structures in dependent on several conditions in addition to QS, and although initial transcriptomics experiments did not link QS to control of genes that might influence biofilm development, we later learned that QS in strains PAO1 and PA14 regulates genes coding for the production of the extracellular polysaccharides Psl and Pel (Gilbert et al., 2009; Sakuragi and Kolter, 2007; Ueda and Wood, 2009). We also learned that rhamnolipids play an important role in biofilm development (Pamp and Tolker-Nielsen, 2007). In the 30-plus years since the original paper linking QS to biofilm production there have been countless publications linking these two traits in many different bacterial species.

Ecological and Evolutionary Considerations

The emergence of LasR mutants in environments such as the CF lung led to the question of what the selective advantage of these variants might be in the environment. Experiments performed with laboratory strains have enabled resolution of some of the evolutionary forces acting to shape QS signaling in a variety of environments. Pleiotropic regulation through QS means selection against a single regulator can have a multitude of downstream effects. Experimental evolution experiments and targeted studies of QS-mediated fitness have shown

that under specific conditions QS regulators are targets of selection. As discussed earlier in this chapter, *P. aeruginosa* QS controls many secreted virulence factors that can be considered public goods (West et al., 2006). Public goods are those, which are shared among the population and whose fitness benefits do not accrue to the individual producing the good, but instead to the population at large. Public goods production is therefore vulnerable to cheating by non-producers, who gain the benefit of public goods without incurring a cost of production. In the case of *P. aeruginosa* QS, the cost savings can be substantial, as the QS regulon comprises roughly 5% of the genome (Schuster et al., 2003).

Two foundational studies described this phenomenon of cheater emergence in cooperating populations when *P. aeruginosa* was allowed to grow in a minimal medium with protein serving as the sole source of carbon and energy (Diggle et al., 2007; Sandoz et al., 2007). In these experiments, growth on casein or bovine serum albumin requires secretion of the QS-controlled extracellular protease LasB elastase, which digests the protein so that small polypeptides and amino acids can be taken up by *P. aeruginosa*. Cells producing the protease are cooperators. LasR mutants reproducibly emerge in these evolution experiments, as inactivation of LasR provides a fitness benefit.

In serial passage on casein as a sole carbon and energy source, cheaters usually come to a population equilibrium with cooperators, reaching roughly 30% of the population (Dandekar et al., 2012; Sandoz et al., 2007). Why don't cheats completely overrun the cooperators, resulting in a population collapse? This stable existence of cooperation can be explained mathematically and according to the kin selection hypothesis (Griffin and West, 2003). According to Hamilton's Rule, when the relatedness of the individuals multiplied by the benefit is greater than the cost of goods ($rb>c$) stable cooperation can evolve (Hamilton, 1964a, b). What might this mean mechanistically? Several mechanisms have been established to prolong or preserve *P. aeruginosa* QS-mediated population fitness in the presence of strong social selection for cheats. The nature of QS as a facultative cooperation strategy intrinsically isolates the benefits of cooperative behavior to scenarios where cooperating cells are numerous, thereby isolating costly secretion to cell densities above a certain threshold of actively communicating cells – a feature referred to as density dependence (Figure 2A). QS also activates production of some cell-associated factors that can be considered private goods, such as the periplasmic enzyme nucleoside hydrolase (Nuh) which confers growth on adenosine. Growth in conditions where both public goods (LasB) and private goods (Nuh) regulated by QS influence fitness provides a "metabolic incentive" to cooperate, thereby reducing the selective pressure that favors LasR mutant cheats (Dandekar et al., 2012). However, if the cost of cooperation is raised – for instance by providing casein as the sole carbon, energy, and nitrogen source – the balance is tipped in favor of cheaters and the cooperators are overrun by these LasR mutants (Dandekar et al., 2012). The cost of cooperation depends on the environment. Because QS activation of many genes is co-regulated by other factors, *P. aeruginosa* can exhibit what is called "metabolic prudence". For example, rhamnolipids, which are carbon-rich secreted products, will be produced at high cell densities when population growth is slowed by nitrogen limitation, where carbon and energy sources are in excess. (Xavier et al., 2011). In this environmental condition Hamilton's cost, C is negligible. If there is any benefit of rhamnolipid production rb will be greater than c.

Multiple studies have demonstrated that RhlR-regulated factors, especially synthesis of hydrogen cyanide via the products of the *hcnABC* operon, are involved in the constraint of cheaters in the population (Wang et al., 2015; Yan et al., 2019). Here, cooperators with active QS systems produce cyanide which can intoxicate all cells in the population, yet these cooperators also use QS to conditionally activate a cyanide-insensitive terminal oxidase encoded by *cioAB* (Figure 2B) (Yan et al., 2019). This provides QS cells with a private cyanide resistance. Other QS-regulated secreted products of *P. aeruginosa*, including phenazines (Castaneda-Tamez et al., 2018), have also been implicated in the restraint of cheaters, and the stress response can also contribute to cheater restraint (Garcia-Contreras et al., 2014).

The observation from clinical isolates that RhlR can activate gene expression in the absence of LasR led to studies to understand how such a rewiring of QS might occur. In the well-studied strain PAO1, LasR mutants can evolve the ability to grow on casein and this evolved trait relies on a null mutation in a non-social gene, the transcriptional regulator *mexT* (Kostylev et al., 2019; Oshri et al., 2018). A *lasR, mexT* mutant regains some QS-regulated phenotypes and these traits are RhlR-dependent. It remains unclear how this laboratory evolution relates to *lasR* mutations that occur during chronic CF infections.

Intra-patient diversity of QS alleles underscores the heterogenous nature of naturally evolving infection populations. Lung infections in CF patients are initiated by a single infecting clone, and acquisition of new strains is not common (Marvig et al., 2015). However, sequencing efforts focused on understanding within-patient diversity of *P. aeruginosa* have confirmed what phenotypic surveys have long predicted. Even with a common infecting ancestor, infections ultimately come to comprise heterogenous populations under positive selection (Jorth et al., 2015; Markussen et al., 2014). LasR mutants are routinely cultured from CF patients at frequencies which can exceed 50% of all *P. aeruginosa* isolates (Kohler et al., 2009; Wilder et al., 2009). A longitudinal study in a CF patient found *lasR* mutations emerge along with mutations in a small number of other genes during chronic infection (Smith et al., 2006). Genome sequencing of 474 *P. aeruginosa* isolates from 34 individuals with CF found *lasR* was among 52 genes associated with pathoadaptation to diseased lungs (Marvig et al., 2015). Interestingly, despite overlap in LasR and RhlR regulatory targets, RhlR mutants do not appear to be selected and are rarely isolated from CF infections. Attenuated virulence phenotypes observed in established infection isolates provides additional support for the notion of intra-host diversification. In a survey of 135 CF lung infection isolates from 8 adult CF patients, mutations in *lasR*, *lasI*, and *rhlI*, were found to be principally responsible for the loss of many virulence phenotypes (Wilder et al., 2009). In a larger survey, 580 of 2583 *P. aeruginosa* isolates from the lungs of children with CF harbored nonsynonymous *lasR* polymorphisms as compared to the reference PAO1 sequence (Feltner et al., 2016). Of these 580 isolates, more than half harbored missense mutations, in addition to others spanning the gamut of loss-of-function mutation types. Mutation in *lasR* is not a phenomenon restricted to the lungs of people with CF. Such mutants have been described in the airways of intubated patients (Denervaud et al., 2004). A recent study extended the common theme of pathoadaptive *lasR* mutation to burn wounds, where *P. aeruginosa* isolates were followed for 42 days in a porcine chronic wound model; again, *lasR* mutations were implicated in adaptation of multiple

independently evolving infection populations (Vanderwoude et al., 2020). Together, this body of evidence suggests selection acts strongly on *lasR* as a common mode of adaptation where *P. aeruginosa* QS may be tuned to suit the environment of a chronic infection. Mutation of QS regulators like LasR signifies perhaps the strongest selective forces acting to alter QS regulation, but evolutionary adaptation of QS regulon content and amplitude of expression may actually be more common. Transcriptome analysis of PAO1 and seven *P. aeruginosa* isolates from soil, water, and CF infections found QS maintained a core regulon of 42 genes, but the overall size of QS regulons and level of regulation varied widely between the isolates (Chugani et al., 2012). QS adaptation that is independent of the LasR allele is clearly possible, with several avenues for ancillary regulation of the *las* and *rhl* systems including: alternative sigma factors (RpoS, RpoN), transcriptional repressor and post-translational anti-activator proteins (RsaL, QslA, QteE), and the stringent response (RelA), among other regulators (Asfahl and Schuster, 2017; Juhas et al., 2005; Schuster and Greenberg, 2006; Schuster et al., 2004).

Less is known about how pathoadaptive selection may target the *P. aeruginosa* PQS system. Strains with mutations in *pqsR* show negative frequency dependence when co-cultured with their wild-type parent in casein minimal media, yet these mutants also retain QS-mediated phenotypes and grow well on their own (Wilder et al., 2011). However, a LasR-null cystic fibrosis isolate, called E80, showed a different QS phenotype when passaged in minimal casein medium (Chen et al., 2019). With this strain, the authors found isolates deficient in QS phenotypes evolved quickly and enriched in the population; six protease-negative isolates were studied further. None produced PQS and four harbored a mutation in *pqsR*. Together these reports suggest that there may be strain-specific variability in the selective pressures for mutation of QS regulators other than *lasR*.

The suite of *P. aeruginosa* QS signals provides an additional, distinct set of targets for selection separate from those related to signal reception and response. The short-chained C4-HSL signal may freely diffuse across the membrane, as can the longer-chained 3OC12-HSL, but an efflux pump called MexAB-OprM might provide an additional layer of regulation of 3OC12-HSL (Pearson et al., 1999) that could be tuned by selection. The PQS signal, 2-heptyl-3-hydroxy-4quinolone, on the other hand is not membrane permeable. Its release may involve efflux pumps and it can be found in membrane vesicles derived from *P. aeruginosa* cells (Mashburn-Warren et al., 2009).

Once beyond the cell membrane, released signals face a number of potential fates. Lactonase enzymes that effectively neutralize all AHLs through lactone ring hydrolysis can be produced by conspecific soil bacteria such as *Bacillus thuringiensis*, providing a mechanism to silence *P. aeruginosa* signaling (Dong et al., 2000). Animal cells, including human cells, produce AHL-degrading lactonases, also. These are paroxinases or PON enzymes (Chun et al., 2004; Ozer et al., 2005). Furthermore, conspecific soil bacteria like *Variovorax* (Leadbetter and Greenberg, 2000) produce AHL-degrading acylases, which cleave fatty acid tails off AHLs. AHLs and PQS signals may face distinct selective forces owing to their disparate chemistry. For example, AHLs are pH sensitive and under alkaline conditions the homoserine lactone ring is cleaved. Different AHLs are degraded at high pH at different rates (Yates et al., 2002).

When a released signal can be received and integrated by related and non-related individuals alike, discerning the contributions of specific selective forces may become difficult. AHL signaling in *P. aeruginosa* may clearly follow the definition of a signal between clone mates in one context, but other organisms may have a different response to the same AHL. The potential for *P. aeruginosa* signals to serve as cues for other bacteria becomes even more apparent upon consideration of the selectivity of AHL receptors for ligands with variable sidechains (Wellington and Greenberg, 2019). While all signal-receptor pairs fall on a continuum with strict selectivity on one end and crosstalk on the other, our understanding of how the ecological context of *P. aeruginosa* growth can affect signaling requires more investigation.

Quorum Sensing Inhibition Strategies

As we have already alluded to, due to its established role in virulence, QS may be an alternative to traditional antibiotic targets. This section will discuss potential targets for QS inhibition (QSI): signal detection, signal production, and the signals themselves, as well as the sources of these QS inhibitors (Figure 3). Natural products are one primary source of QS inhibitors. Bacteria and eukaryotes have multiple strategies for disabling the QS systems of competing or invading bacteria. The first QS inhibitors described were furanones produced by the macro-alga *Delisea pulchra* (Givskov et al., 1996). These furanones attracted attention due to their structural similarity to AHLs. Since this initial discovery, the majority of efforts to identify QS inhibitors have focused on isolating or synthesizing AHL mimics, which have a number of advantages (Shin et al., 2019). First, AHL-like compounds could theoretically inhibit either the signal receptor, the synthase, or both. Second, AHLs have favorable diffusion characteristics, which is an important consideration given the challenge of penetrating the Gram-negative outer membrane. Finally, AHL-like compounds are potentially specific for QS, thereby preventing off-target effects on the host or the host's microbiome. A third source of QS inhibitors is large, unbiased chemical libraries used in high throughput screening (Borlee et al., 2010; Christensen et al., 2013; Müh et al., 2006; Starkey et al., 2014). Each of these sources have been mined for inhibitors with a variety of mechanisms.

Inhibiting AHL detection.

Because LasR sits at the top of the regulatory hierarchy in laboratory strains of *P. aeruginosa*, most efforts to identify QS inhibitors have focused on blocking LasR activity. Halogenated furanones inspired by the *D. pulchra* secondary metabolites have been shown to competitively inhibit the *V. fischeri* receptor LuxR, resulting in destabilization and decreased half-life of the receptor (Manefield et al., 2002). By using a fluorescent reporter of LasR transcriptional activation in *P. aeruginosa*, furanones were also shown to suppress QS activity in a mouse lung infection. They also improved lung pathology, increased bacterial clearance, and prolonged survival this mouse model of lung infection (Wu et al., 2004). Complicating the interpretation of these data, the most widely studied of these furanones, C-30, inhibits the growth of *P. aeruginosa* in a non-QS based manner at concentrations necessary to inhibit QS (Gerdt and Blackwell, 2014). Thus, the observed effects *in vivo* may be due to the furanones' non-QS activity. There have also been efforts to identify novel

LasR inhibitors through screening of large chemical libraries (Borlee et al., 2010; Müh et al., 2006). The first of such efforts was a particularly noteworthy screen of approximately 200,000 compounds conducted against *P. aeruginosa* carrying a fluorescent reporter of LasR activity (Müh et al., 2006). While the library was diverse in chemical structure, the two most potent inhibitors identified in the screen are structurally similar to AHLs. Consistent with their inhibition of LasR activity in the fluorescent reporter, the hit compounds decreased the production of elastase and of the phenazine pyocyanin without inhibiting *P. aeruginosa* growth *in vitro*. More recently, another research group has improved the activity of the most potent inhibitor from this screen, V-06–018, 10-fold. They also demonstrated that the compound is LasR-selective and functions, at least in part, by stabilizing an inactive form of the receptor (Manson et al., 2020). A separate high throughput screen of 16,000 compounds identified LasR antagonists that are structurally dissimilar from the native 3OC12-HSL signal, providing new scaffolds that could be an alternative to AHL-mimics (Borlee et al., 2010).

Finally, there have been numerous efforts to design synthetic inhibitors of LasR based on the structure of 3OC12-HSL. Due to the labile nature of AHLs, one particular focus of these efforts has been to replace the lactone core with more stable heterocycles (McInnis and Blackwell, 2011). Other efforts have focused on designing covalent inhibitors based on the LasR crystal structure (Amara et al., 2009). Although these inhibitors are moderately active against LasR expressed in *Escherichia coli*, they are an order of magnitude less active against LasR in *P. aeruginosa*. More recent efforts to design covalent inhibitors based on non-native LasR agonists have resulted in more potent inhibitors that are capable of disrupting pyocyanin production and biofilm formation in *P. aeruginosa* (O'Brien et al., 2015).

A growing body of evidence suggests RhlR may be a more relevant target than LasR, particularly for chronic *P. aeruginosa* lung infections in patients with cystic fibrosis. As discussed earlier in this chapter, *lasR* mutations are common in clinical isolates. Because in our laboratory strain *lasR* mutants are also defective in RhlR QS, this observation initially called the utility of QSI into question. It has since been demonstrated that even though LasR may be inactive, several clinical isolates maintain QS function through RhlR (Feltner et al., 2016). Further, under some *in vitro* growth conditions RhlR is not subordinate to LasR (Soto-Aceves et al., 2021), and in some infection models, RhlR is primarily responsible for virulence (O'Loughlin et al., 2013). Given these findings, attention has turned toward identifying RhlR-selective inhibitors (Eibergen et al., 2015).

Beginning with the structure of a known inhibitor of the *Chromobacterium violaceum* AHL receptor CviR, a small library of approximately 30 compounds was screened for the ability to decrease pyocyanin production in *P. aeruginosa*, with the hypothesis that such a compound would function via QS inhibition (O'Loughlin et al., 2013). Based on fluorescent reporters of receptor activity in *E. coli*, the most potent hit from this effort, mBTL, was found to partially inhibit both LasR and RhlR, but microarray analysis demonstrated that RhlR is the relevant target in *P. aeruginosa*. mBTL inhibits pyocyanin production and biofilm formation in *P. aeruginosa* and protects lung epithelial cells and *C. elegans* from killing by *P. aeruginosa* infection. A more recent study identified a potent

RhlR-specific modulator, S4, a RhlR agonist. Interestingly, these modulators revealed an inverse relationship between pyocyanin and rhamnolipids that was not predicted by genetic approaches: both mBTL and S4 repress pyocyanin while increasing rhamnolipids. In the case of S4, this is likely due to disruption of the cross-regulation between the Rhl and PQS systems (Welsh et al., 2015). Thus, in addition to potential therapeutic applications, these small molecule modulators also serve as tools to better understand the complex QS circuitry in *P. aeruginosa*.

Inhibition of AHL production.

Due to the relative challenge of measuring AHL synthase activity, less attention has been given to identifying inhibitors of the LasI and RhlI synthases. A cell-free high throughput screen against the *Burkholderia mallei* synthase BmaI1 revealed non-competitive inhibitors of BmaI1 that were also inhibitory against other AHL synthases (Christensen et al., 2013). Researchers have also sought to identify plant extracts or plant-derived compounds with anti-synthase activity. In one study, researchers screened for the ability to inhibit LasI or RhlI expressed in *E. coli* and by doing so, identified two inhibitors of RhlI that partially block pyocyanin production in *P. aeruginosa* (Chang et al., 2014).

More targeted approaches have also been taken. One study of LasI inhibition focused on whether thiazolidinedione-type molecules, a class of synthetic compounds with a wide spectrum of activity, might also have anti-QS activity (Lidor et al., 2015). This study identified a compound with strong anti-biofilm activity that also partially inhibited 3OC12-HSL production and swarming motility in *P. aeruginosa*. *In silico* docking of the compound predicted high affinity for LasI, with site-directed mutagenesis confirming this interaction. A separate study evaluated a series of RhlR-activating signal mimetics for RhlI modulatory activity, revealing multiple compounds with moderate activity against purified RhlI (Shin et al., 2019). Such studies demonstrate the chemical plausibility of inhibiting an AHL synthase and serve as a starting point for the development of more potent compounds.

Targeting PQS signaling.

Given the large number of enzymes involved in synthesizing PQS, there are numerous potential targets for inhibiting PQS production. Early efforts focused on PqsA, an anthranilate-CoA synthetase and the first PQS biosynthetic enzyme. Substrate analogs such as methyl anthranilate have been shown to inhibit PQS and elastase production (Calfee et al., 2001). Additional anthranilate analogs have been demonstrated to decrease the expression of PQS-regulated genes as well as to restrict *P. aeruginosa* dissemination and improve survival in a mouse model of infection (Lesic et al., 2007). Anthranilate is also a precursor for tryptophan biosynthesis and these compounds, therefore, also inhibit tryptophan biosynthesis in *P. aeruginosa*, confounding interpretations of their impact in infection models. Many additional studies have focused on developing inhibitors against PqsA and other PQS biosynthetic enzymes (Maura et al., 2017; Sahner et al., 2015; Storz et al., 2012; Weidel et al., 2013).

The PQS receptor, PqsR (MvfR), has proven to be a highly effective target (Grossman et al., 2020; Starkey et al., 2014). A high throughput screen of approximately 285,000

compounds identified several compounds that inhibit the ability of PqsR to activate gene expression, resulting in decreased production of PQS and related quinolones, without affecting bacterial growth (Starkey et al., 2014). Optimization of these hit compounds resulted in inhibitors with nanomolar affinity for PqsR, with several lines of evidence supporting direct engagement with PqsR as the mechanism of action in whole *P. aeruginosa* cells. Further study demonstrated that the lead compound, M64, is a competitive inhibitor of PqsR that prevents its binding to DNA (Kitao et al., 2018). M64 decreased *P. aeruginosa* cytotoxicity against macrophages and improved mouse survival in both lung and burn infection models. Though improvements in survival were evident early during the model infections, bacterial CFU were unaffected until after many days of treatment. M64 also reduced macrophage accumulation at the infection sites, consistent with a role in disarming *P. aeruginosa* virulence. Finally, M64 was demonstrated to inhibit *P. aeruginosa* persistence in infected mice (Starkey et al., 2014). Persister cells, or cells that are phenotypically antibiotic tolerant, have been implicated in antibiotic treatment failures and are believed to serve as a reservoir for chronic and relapsing infections. Given their promising *in vivo* efficacy and ability to prevent persistence, Spero Therapeutics, Inc has further developed these compounds (Zahler, 2016; Zahler et al., 2016).

Targeting the signal.

A final QSI strategy is to target the signal itself through degradation or sequestration, also known as “quorum quenching”. Because the signals are extracellular, these strategies do not face the challenge of permeating the *P. aeruginosa* cell wall. Numerous bacteria and eukaryotes produce AHL degrading enzymes, with a variety of mechanisms (Lin et al., 2003). The first quorum quenching enzyme discovered is a lactonase, AiiA, that is produced by *Bacillus* sp. 240B1 (Dong et al., 2001). AiiA, and other lactonases, hydrolyze the ester bond of the homoserine lactone ring of AHLs. AiiA is active against the *P. aeruginosa* AHLs, and since its discovery, multiple lactonases have been identified from a variety of species (Dong et al., 2018; Migiyama et al., 2013). These lactonases have been used to demonstrate the potential therapeutic utility of AHL signal degradation. Purified lactonase inhibits *P. aeruginosa* biofilm formation and decreases elastase and pyocyanin production without impacting growth (Dong et al., 2018). Lactonases can also decrease swarming and improve *C. elegans* survival during *P. aeruginosa* infection. As a proof of concept, *P. aeruginosa* expressing the lactonase AiiM was shown to have decreased cytotoxicity against human lung epithelial cells and decreased virulence in a mouse model of acute pneumonia (Migiyama et al., 2013). While lactonase expression decreased *P. aeruginosa* dissemination, improved lung pathology, and resulted in better survival rates in the mouse infection model, it did not decrease the amount of *P. aeruginosa* recovered from the lungs of infected mice. We note here that humans produce enzymes with lactonase activity. There are three genes, *PON1, PON2*, and *PON3*, which code for paroxinases (Camps et al., 2009). These enzymes each have potent lactonase activity (Chun et al., 2004; Ozer et al., 2005) and transgenic expression of PON1 can protect fruit flies against lethal *P. aeruginosa* infection (Stoltz et al., 2008). It is not inconceivable that a strategy could be developed to stimulate PON production in relevant host environments to help control *P. aeruginosa* infections.

AHLs can also be degraded by acylases, which release the lactone core from the fatty acid (Lin et al., 2003). Acylases have been shown to have effects similar to lactonases. *P. aeruginosa* expressing the acylase AiiD has impaired pyocyanin and elastase production as well as decreased swarming. Further, nematodes survive better when infected with *P. aeruginosa* expressing AiiD (Lin et al., 2003). *P. aeruginosa* encodes its own acylase, PvdQ, which degrades 3OC12-HSL. Overexpression of *pvdQ* has also been shown to reduce *P. aeruginosa* virulence and treatment of *P. aeruginosa* with pure PvdQ reduces pathogenicity and improves survival in a *C. elegans* infection model (Papaioannou et al., 2009).

A final strategy in quorum quenching is antibody interference. Antibodies have been designed and optimized against 3OC12-HSL (Kaufmann et al., 2006). One such antibody was demonstrated to inhibit LasR activity in *P. aeruginosa* (Kaufmann et al., 2006) and to protect macrophages from *P. aeruginosa* cytotoxicity (Kaufmann et al., 2008).

Resistance to QSI.

Anti-virulence compounds may disarm a bacterium without impacting bacterial growth. Such strategies can prevent the damage caused by bacteria during an infection and may improve clearance of the infection by the host immune system. However, because growth is not inhibited there is theoretically less selective pressure for the bacteria to develop resistance. The selective pressure toward resistance ultimately depends on the mechanism of anti-virulence and the roles of the virulence factors during infection (Allen et al., 2014). The social nature of QS provides two additional barriers to the development of resistance (Figure 4): (1) a single QSI-resistant mutant is unlikely to produce sufficient signal to activate QS and (2) if QSI-resistant bacteria do activate their QS regulon, they are likely to be outcompeted by QSI-sensitive bacteria which would benefit from the public goods produced by QSI-resistant bacteria without incurring the cost of their production. Each of these barriers has been demonstrated by using genetic mutants that mimic QSI-sensitive and -resistant strains. In media that require QS-produced exoproducts for growth, QSI-resistant bacteria are unable to invade QSI-sensitive strains, even in structured environments (Gerdt and Blackwell, 2014; Mellbye and Schuster, 2011). Because the metabolic cost of signal production is lower than the cost of producing QS-regulated public goods, synthase inhibition may be more prone to the development of resistance than inhibition of receptors (Allen et al., 2014).

Cross resistance with other antibiotics must also be considered. Studies have shown that resistance to the furanone C-30 rapidly arises *in vitro* due to mutations that increase efflux activity (Maeda et al., 2012). One study provided evidence that resistance to C-30 through mutations in the drug efflux regulator *mexR* is enhanced when oxidative stress from hydrogen peroxide is increased, which might occur in a host environment (Garcia-Contreras et al., 2014). This is a common mechanism of antibiotic resistance, and multiple clinical isolates of *P. aeruginosa* have mutations that would make them resistant to C-30 through this mechanism. The ease with which C-30 resistance developed may be due to its non-QSI growth inhibitory effects (Gerdt and Blackwell, 2014), but researchers have nevertheless sought to develop QS inhibitors that are not susceptible to common resistance mechanisms such as efflux (Moore et al., 2015).

No specific QS inhibitor has made it to the clinic. Althoguh azithromycin (AZM) is not bacteriostatic or bacteriocidal for *P. aeruginosa*, in laboratory cultures, this antibiotic has been shown to decrease signal production, delay biofilm formation, and decrease the production of *P. aeruginosa* QS-regulated products such as elastase and pyocyanin through an unknown mechanism (Gillis and Iglewski, 2004; Hoffmann et al., 2007; Tateda et al., 2001). Further, AZM treatment resulted in lower levels of QS products in a CF mouse model of chronic *P. aeruginosa* infection and reduced the severity of lung pathology in this model. However, AZM treatment also reduced bacterial burden in treated mice (Hoffmann et al., 2007) and may, therefore, reduce QS products and disease through a mechanism other than direct QSI. Touted as a QS inhibitor, AZM has been tested in clinical trials where it resulted in modest reductions in *P. aeruginosa* ventilator-associated pneumonia (van Delden et al., 2012). In a separate trial, AZM decreased QS-gene expression in tracheal aspirates from intubated patients colonized by *P. aeruginosa*, but also decreased bacterial burden in these patients such that bacterial densities were twice as large in the untreated group (Köhler et al., 2010). AZM also improves clinical outcomes in CF patients infected with *P. aeruginosa* (Equi et al., 2002; Saiman et al., 2003; Wolter et al., 2002). The efficacy of AZM in these trials may be due to QSI, but may also be due to “off-target” effects. Thus, the clinical efficacy of QSI awaits further research.

A Look Ahead

It is obvious that one big question pertains to whether QS can be a useful therapeutic target for *P. aeruginosa* infections. In addition, it is a good bet that investigations of strains established as laboratory models will continue to provide information about the evolution of bacterial communication and cooperation. Initial work focused on the LasR system as a potential therapeutic target. As further fundamental investigations continued, findings indicated this might have been a mistake and perhaps RhlR is a better therapeutic target. This idea deserves to be pursued with additional studies. Of note, RhlR has rather unique characteristics as an AHL receptor. It shows significantly more specificity for its cognate AHL, C4-HSL, than do other known LuxR homologs, and significantly less sensitivity for its cognate AHL. The differences in sensitivity are on the order of at least two logs (Wellington and Greenberg, 2019). Is there a trade-off between sensitivity and selectivity in LuxR homologs? Studies of RhlR may help answer this question. Does the exquisite selectivity of RhlR make it a more inviting target for drug development? Does the selectivity make it possible to develop inhibitors that target this receptor and no others? Might the more relaxed specificity of LasR and QscR have significance in multispecies microbial communites?

It is also evident that we cannot develop a clear picture of the role of QS in the biology of *P. aeruginosa* by studying a handful of strains alone. Although elements of QS are quite conserved in this species, the ways in which QS is employed appear to be quite variable. To date we know very little about QS in the complex microbial environments in which *P. aeruginosa* is often found. Questions about the role of QS in these communities are just beginning to be asked (Abisado et al., 2018; An et al., 2006; Groleau et al., 2021; Smith et al., 2017). What competitive advantage does QS provide *P. aeruginosa*? What sort of interspecies interactions might occur, either beneficial or detrimental to *P. aeruginosa* or

other species. As discussed in this chapter, there are strong hints about such interactions already.

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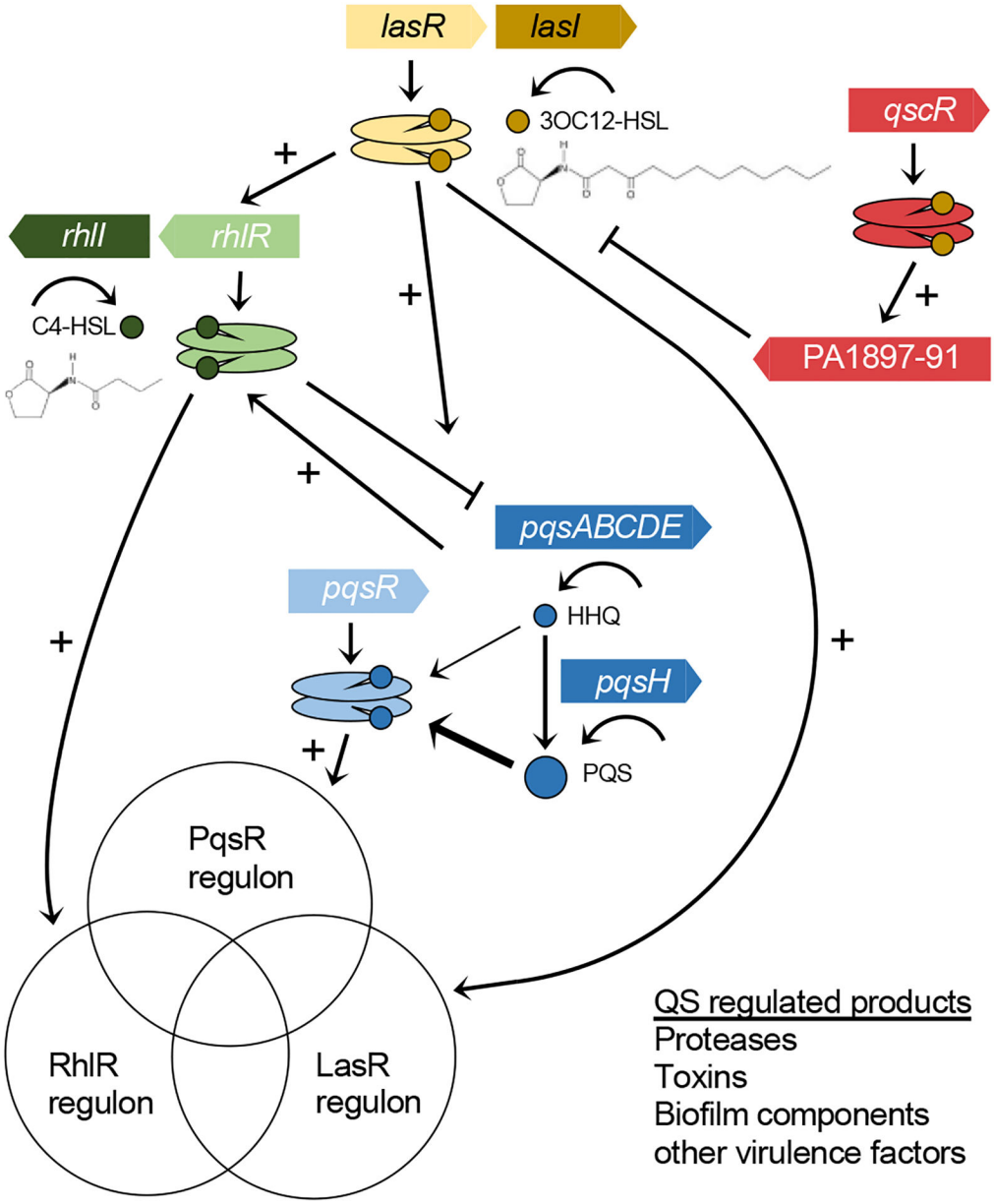

Figure 1. The *Pseudomonas aeruginosa* QS circuitry:

Together, the Las, Rhl and PQS systems of *P. aeruginosa* regulate hundreds of genes in response to increasing cell density. Some virulence factors such as LasB elastase or phenazine biosynthesis are directly regulated by multiple QS systems. Other factors such as rhamnolipids are strictly regulated by a single QS system, in this case the Rhl system. Another regulator, QscR, binds the LasI-generated 3OC12-HSL signal and activates a single linked operon, PA1897–91. Unlike the AHL QS systems Las and Rhl, the PQS system receptor PqsR responds to both the low affinity signal HHQ and the high affinity signal PQS. HHQ is converted to PQS by the *pqsH* gene product. The regulatory systems interact with each other as shown by the connecting lines.

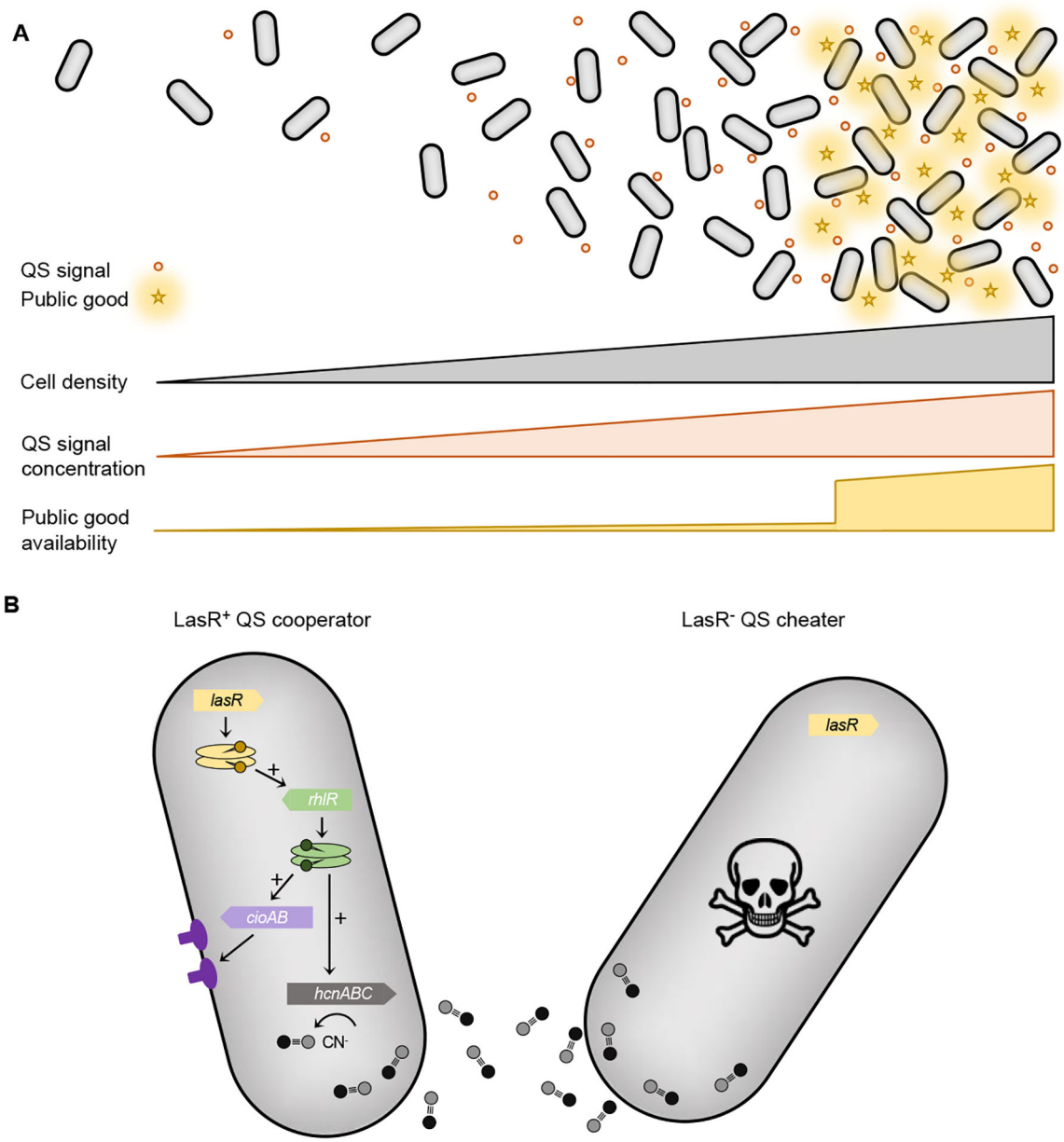

Figure 2. Evolutionary considerations of QS-regulated secretions:

Several features of *P. aeruginosa* that prevent exploitation by QS cheats have been described. These include, amongst others, **A)** Density dependence, or the nature of QS as a mechanism to isolate facultative cooperative behaviors to periods when more cooperators are present, and **B)** Policing through QS-regulated hydrogen cyanide production. In addition to QS-regulated production of the toxic metabolite hydrogen cyanide (CN^- anion shown), cooperators also conditionally regulate the cyanide insensitive terminal oxidase encoded by *cioAB*. Respiration is impaired by cyanide in LasR mutant cheaters.

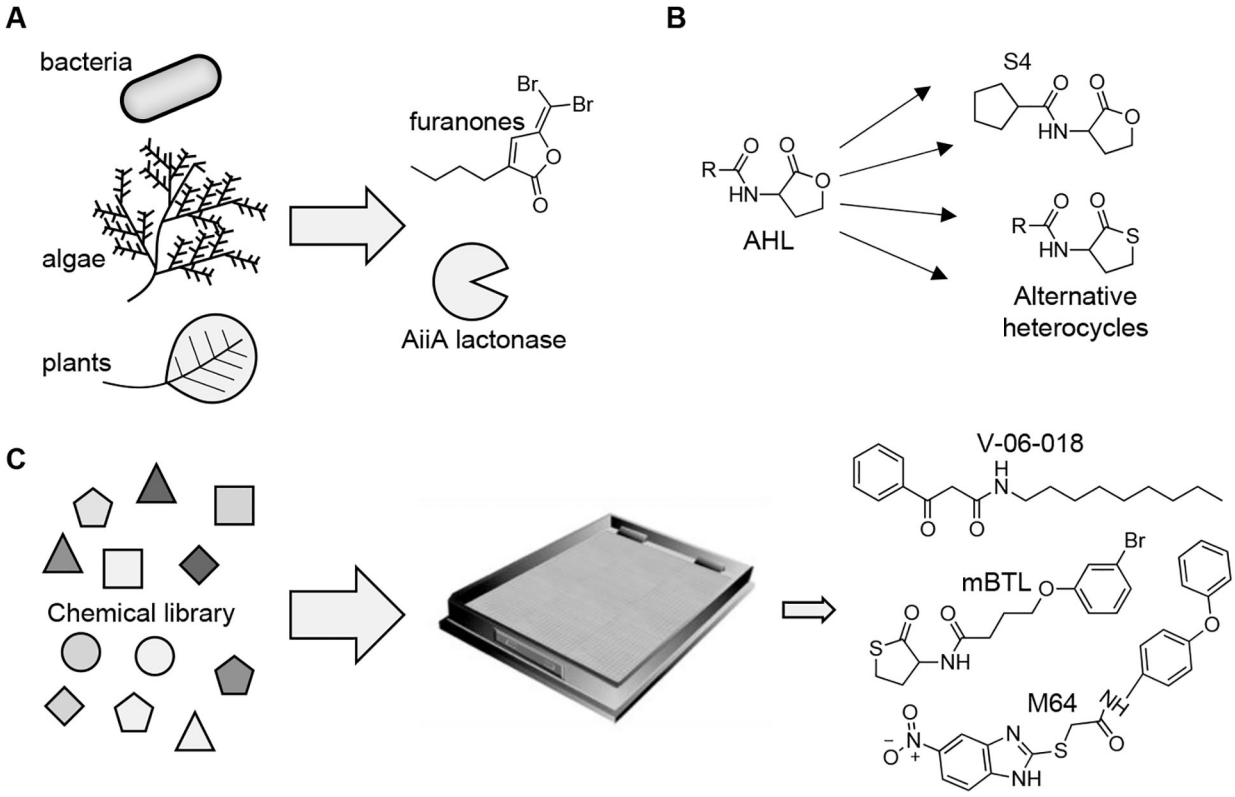

Figure 3. Sources of QS inhibitors:

Researchers have exploited multiple sources to identify inhibitors of QS. These include **A)** small molecules and proteins produced by various organisms, **B)** small molecules inspired by the AHL chemical structure, and **C)** unbiased screens of large chemical libraries. Example chemical structures are shown for each source.

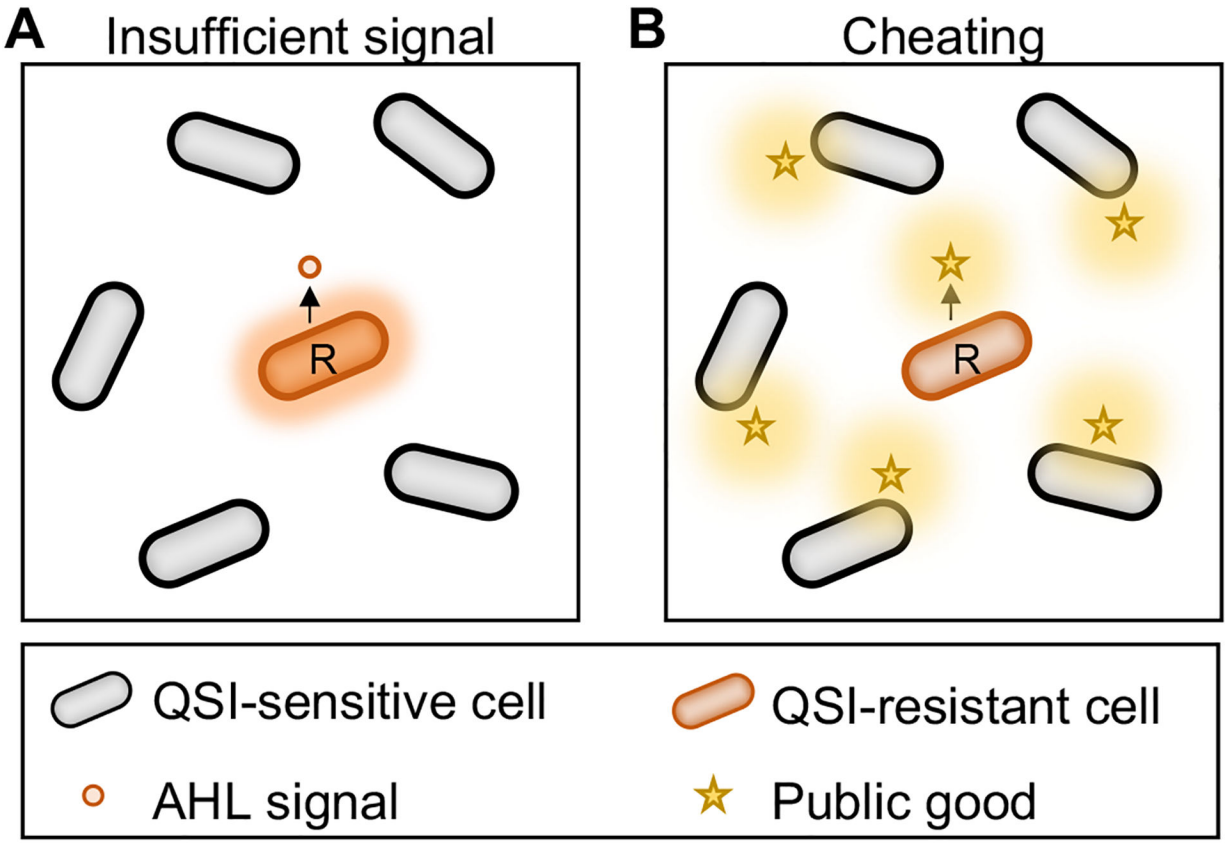

Figure 4. Barriers to QSI resistance:

Anti-virulence drugs may impose less selective pressure than traditional antibiotics if they disarm a bacterium without impairing growth. The social nature of QS imposes two additional barriers to the development of QSI resistance: A) a single resistant cell is unlikely to produce sufficient signal to activate a quorum and B) QSI-resistant cells might act as cooperators and be at a disadvantage compared to QSI-sensitive cells which could cheat on public goods produced by the resistant bacteria