Quorum Sensing Systems in Microbes:

Quorum-Sensing Systems in *Pseudomonas*

Introduction

Quorum sensing (QS) or cell-to-cell communication is a mechanism used by bacteria to control a broad range of activities in bacteria. The modulation of gene expression by quorum sensing causes phenotypic changes in bacteria leading to their better adjustment to environmental conditions and stress during growth. Quorum sensing involves the production, secretion, and response to small diffusible signaling molecules also known as autoinducers. Bacteria produce signaling molecules at a basal level during the stationary phase of their growth, and with the increase in cell density, the concentration of the signaling molecule in the environmental medium increases; and on reaching a threshold level, it induces phenotypic effects by regulating quorum-sensing-dependent target gene expression. Quorum sensing is involved mainly in the regulation of virulence, development of genetic competence, transfer of conjugative plasmids, sporulation, biofilm formation, antimicrobial peptide synthesis, and symbiosis. There are two groups of signal molecules involved in bacterial quorum sensing. One is the peptide derivatives typically used by Gram-positive bacteria, while the fatty acid derivatives are utilized by the Gram-negative bacteria. Most bacteria utilize two general mechanisms for detecting and responding to quorum-sensing signals and in modulating the target gene expression. In the acyl-homoserine lactone (AHL)-dependent quorum-sensing systems, the quorum-sensing signal is detected by a cytosolic transcription factor, whereas the quorum-sensing signal autoinducing peptide (AIP) is detected by a membrane-associated two component response regulatory system. In AHL-mediated quorum sensing, AHL synthase encoded by LuxI homologue synthesizes AHL molecules using S-adenosylmethionine (SAM) and acyl chains derived from the common fatty acid biosynthesis pathway. The short-chain AHL signal passively diffuses across bacterial membranes and accumulates in the environment,

and the long chain AHL signals require active transportation mechanisms for their efflux. The bacteria produce signaling molecules at a basal level during the stationary phase of their growth. With an increase in bacterial population, the concentration of AHL signal reaches a threshold level, resulting in signal accumulation and recognition by the cognate receptors. The signal reception involves R protein which belongs to the LuxR family of transcriptional regulators and acts as a receptor for the AHLs synthesized by the LuxI proteins. The R-AHL complex is a dimer binds to conserved palindromic sequences of the quorum-controlled promoters, including the promoter of the luxI-type gene, and boosts AHL production (autoinduction) and expression of other genes in the quorum-sensing regulon. Thus, the R-AHL complex is involved in autoinduction and control of quorum-sensing regulons. The AHL degradation enzyme and the cognate regulatory transcription factor(s) are involved in signal decay. The LuxI-LuxR system was first discovered in *Vibrio fischeri* during the investigation of the phenomenon of bioluminescence. Now, the LuxI/LuxR system has become the model system upon that the other quorum-sensing systems have been based. Homologous LuxI/LuxR systems have been identified in many Gram-negative bacteria, each capable of producing specific AHLs. In the opportunistic pathogens, such as *P. aeruginosa* and *Serratia marcescens*, these signaling mechanisms control the expression of the virulence factors. *Pseudomonas aeruginosa* contains two systems homologous to LuxI/LuxR. LasI/LasR has been shown to control biofilm formation and the production of extracellular enzymes, as well as transcription of another quorum-sensing system, RhlI/RhlR, adding an additional level of control through AHL signaling.

Pseudomonads are ubiquitous Gram-negative bacteria capable of surviving in several environmental niches. The genus comprises of important plant pathogens (e.g., *P. syringae*) and human opportunistic pathogens (*P. aeruginosa*). Some of them are able to colonize plant-related niches, such as the rhizosphere (e.g., *P. aeruginosa, P. fluorescens, P. putida, P. aureofaciens, and P. chlororaphis*), where they can act as biocontrol agents through the production of traits that directly influence plant disease resistance and growth. Therefore,

it is important to study the quorum-sensing systems in the genus *Pseudomonas* as it comprises of opportunistic pathogens, plant pathogens, biocontrol agents, and industrially relevant organisms.

Quorum Sensing in *Pseudomonas aeruginosa*

P. aeruginosa is an opportunistic human pathogen responsible for microbial keratitis, burn wound, and pulmonary infections in cystic fibrosis and immunocompromised patients. It is a highly environmentally adaptable pathogen having a large dynamic genome of which 10 % codes for regulatory elements including a complex multi-signal QS system. Quorum sensing plays a key role in regulating a majority of genes related to various physiological processes, virulence factor production, motility, biofilm formation, and the expression of antibiotic efflux pumps, while the QS signal molecules are involved in the host–pathogen interactions. In *P. aeruginosa*, the QS circuit is integrated by two complete sets of LuxI-LuxR systems, LasI-LasR and RhlI-RhlR, and one incomplete system, QscR which has no cognate "I" protein. LasR responds to N-3- oxododecanoyl homoserine lactone (3-oxo-C12- HSL) synthesized by LasI, while RhlR responds to N-butyryl homoserine lactone (C4-HSL) produced by RhlI, and QscR responds to 3- oxo-C12-HSL, sharing it with LasR. Through this QS circuit, the LasI-LasR system controls expression of many extracellular virulence factors, the RhlI-RhlR system regulates production of rhamnolipid and secondary metabolites. These QS systems are well regulated in a hierarchical cascade in which LasR activates expression of the RhlI-RhlR system and LasI-producing 3-oxoC12-HSL activates QscR, which autoactivates its own expression. **Thus, *P. aeruginosa* has two acylHSL synthases and three receptors. The LasI synthase produces 3-oxo-C12-HSL, for which there are two receptors,** LasR and QscR. The RhlI synthase produces C4-HSL, for which the receptor is RhlR. Integrated into the acylHSL quorum-sensing circuits is a third signal, 2-heptyl-3-hydroxy-4-quinolone, known as the *Pseudomonas* quinolone signal (PQS). Transcriptome analyses have shown that quinolone signaling directly or indirectly controls the expression of at least 90 genes. The acyl-HSL and PQS signaling systems influence each other; the las system activates synthesis of PQS, which in turn activates rhlI

expression. In addition, LasR, RhlR, and QscR influence expression of genes that can potentially alter intracellular levels of the PQS biosynthesis precursor anthranilate. Together these quorum-sensing systems regulate hundreds of *P. aeruginosa* genes. Different elements of the *P. aeruginosa* quorum-sensing circuit also influence each other at multiple levels; for example, LasR-3-oxo-C12-HSL activates rhlR and rhlI transcription, and QscR influences expression of a subset of las- and rhl-controlled genes. In fact, the regulons of LasR, RhlR, and QscR are partially overlapping. The primary system is the Las system, which encodes the proteins LasI and LasR. The LasI protein catalyzes the production of the AHL molecule N-3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL). The 3-oxo-C12- HSL molecule docks with the DNA-binding transcription regulator LasR, which allows LasR to bind to the promoters of QS-regulated genes to control virulence factor such as *lasB* (elastase), lasA (protease), *aprA* (alkaline protease), *toxA* (exotoxin A), *hcnABC* (hydrogen cyanide synthase), and lasI. The Las circuit induces a positive feedback loop to produce more AHL and also induces a secondary QS circuit, the Rhl system. The Rhl system consists of RhlI, which synthesizes N-butyryl-L-homoserine lactone (C4-HSL), and the receptor RhlR. As with the Las system, C4-HSL accumulates to a sufficient concentration and binds to RhlR. The Rhl system induces expression of *rhlAB* (rhamnolipid synthesis genes), rhlI, lasB, rpoS (the stationary phase sigma factor), *lecA* (type 1 lectin), *lecB* (type II lectin), hcnABC, and genes involved in pyocyanin production. The additional *P. aeruginosa* gene that codes for a homologue of LasR and RhlR, QscR, is an orphan quorum-sensing signal receptor. QscR mutants are hypervirulent, and a number of genes controlled by the other AHL-based QS systems are repressed by QscR. There are several possible mechanisms for QscR repression of LasR- or RhlR-activated genes. The QscR protein forms homomultimers and also heteromultimers with LasR and RhlR. The heteromultimer formation could interfere with the activity of LasR and RhlR. QscR might also bind to the AHLs and compete with LasR and RhlR for these signals. QscR can also function by direct binding as a homomultimer to specific promoters and function in an acyl-HSL-independent manner, or it could utilize the signal produced by LasI or RhlI. It has been shown that QscR does not bind to LasR-dependent promoters. QscR can repress

the activation of selected LasR- and RhlR-dependent quorum-sensing responsive genes. This could be the result of competition for signal, competition for binding sites on the regulatory DNA, or heterodimer formation. Thus, it is clear that the qscR gene codes for an orphan AHL transcription factor. Unlike lasR and rhlR, which are linked to *lasI and rhlI,* genes that code for the production of acyl-HSL signals, there is no I gene linked to qscR. However, the DNA binding activity of QscR is dependent on the presence of a long-chain acyl-HSL. The 3-oxoC12-HSL produced by LasI and to which LasR responds is an effective ligand for QscR. Like LasR, QscR requires 3-oxo-C12-HSL to fold into an active conformation, but unlike LasR signal binding to QscR is not irreversible. However, binding of purified QscR to DNA is dependent on added acyl-HSL. That two 3-oxo-C12-HSLresponsive transcription factors differ fundamentally in their ability to exist in the absence of the signal. This would allow for a very rapid response of the QscR regulon to sudden decreases in environmental levels of 3-oxo-C12-HSL where the LasR regulon may respond more slowly. Like other transcriptional activators in the LuxR family that have been studied, QscR requires the presence of an acyl-HSL in the culture growth medium for folding in an active state. However, unlike LasR, which also responds to 3-oxo-C12- HSL, purified QscR requires exogenous addition of 3-oxo-C12-HSL for binding to target DNA. QscR has a broader signal specificity than does LasR, and QscR may even respond to 3-oxo-C10- HSL, C10, and C12 better than it does to 3-OxoC12 HSL. This suggests that QscR might respond to signals produced by other bacteria that coexist with *P. aeruginosa.* This also shows the possibility that qscR and the genes surrounding it may be relatively recent acquisitions in the *P. aeruginosa* genome.

The *Pseudomonas* quinolone signal (PQS) is a third *P. aeruginosa* QS signal that is dependent on the balanced production of 3-oxo-C12-HSL and C4-HSL. The PQS molecule (2-heptyl-3- hydroxy-4-quinolone) plays a significant role in the transcription of Rhl-dependent *P. aeruginosa* virulence genes encoding the production of pyocyanin and rhamnolipid. PQS production is intimately linked to the QS hierarchy, with its production and bioactivity requiring both the las and rhl QS systems. Additionally, LasR has been

shown to regulate PQS production, and the provision of exogenous PQS induces expression of *lasB* (coding for elastase), rhlI, and rhlR implying that PQS activity constitutes a regulatory link between the las and rhl quorum-sensing systems. It is possible that PQS upregulates the rhl quorum-sensing system in late stationary-phase cultures. The structural genes required for PQS have been identified (*pqsABCDH*) along with a transcriptional regulator (*pqsR)* and a response effector (pqsE). The transcription of *pqsH* is regulated by the las QS system, linking QS and PQS regulation. Mutations in the PQS genes result in a loss of PQS synthesis and a corresponding loss of pyocyanin production. A mutation in the *pqsE* gene also results in a loss of pyocyanin even though PQS synthesis remains intact. This suggests that pqsE is not required for PQS biosynthesis and may have a role in the cellular response to PQS. PQS is synthesized via a "head-to-head" condensation of anthranilate and "-keto dodecanoate and requires the products of the *pqsA, pqsB, pqsC, and pqsD* genes, which also generate over 50 other 2-alkyl-4-quinolones (AHQs) including 2-heptyl-4(1H)-quinolone (HHQ). The function of the last gene in the pqs operon (*pqsE*) is not known, but while pqsE mutants produce parental levels of AHQs, they do not exhibit any PQS-associated phenotypes; consequently, *PqsE* is considered to facilitate the response to PQS. The immediate precursor of PQS is HHQ, and its conversion to PQS depends on the action of *PqsH*, a putative monooxygenase that is LasR regulated so linking the AHL and AHQ regulatory systems. Expression of the pqsABCDE operon and hence AHQ production are controlled by the LasR-type regulator PqsR (MvfR), which binds directly to the pqsA promoter. As PqsR binding is enhanced in the presence of PQS, it implies that PQS acts as a PqsR coinducer. The pqsR gene is itself positively regulated by lasR and negatively regulated by rhlR, establishing a further link between AHL-dependent quorum sensing and AHQ biosynthesis and hence AHQ signaling. Among the many different AHQs produced by *P. aeruginosa*, two of the major compounds are PQS and its precursor, HHQ, although similar concentrations of 2-nonyl-4- quinolone (HNQ), 2-nonenyl-4-quinolone, and 2- heptyl-4-quinolone-N-oxide (HQNO) have been reported to be present in culture supernatants. PQS regulates the production of virulence determinants including elastase,

rhamnolipids, the galactophilic lectin, *LecA*, and pyocyanin and influences biofilm development. Thus, PQS signaling plays an important role in *P. aeruginosa* pathogenesis.

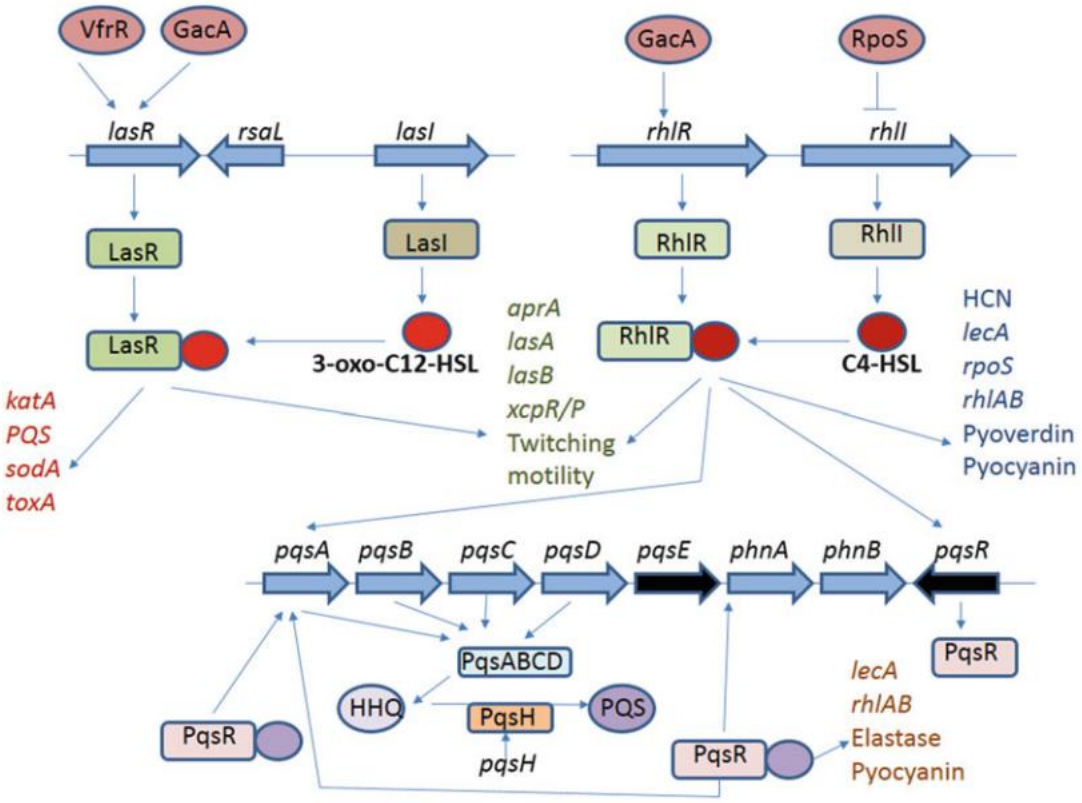

Fig. 2 Regulation of the LasI/R, RhlI/R, and PQS quorum-sensing systems in *Pseudomonas aeruginosa*

PQS is also produced in the lungs of cystic fibrosis patients infected with *P. aeruginosa* and is required for virulence in eukaryotes. It can also induce apoptosis and decrease viability of eukaryotic cells. The QS signaling systems of *P. aeruginosa* are complex in nature and are well regulated in a hierarchical cascade targeting the expression of genes required for growth, survival, virulence, and biofilm formation.

Quorum-Sensing Regulatory Network in *P. aeruginosa*

Quorum sensing controls a significant proportion of the virulence factors used by *P. aeruginosa* for establishing infection. Additional genes can also influence the QS response. The QS regulator (QscR) represses 3-oxo-C12-HSL-regulated virulence factors and prevents the premature activation of QS cascade within a host and in environments where it is not required. This inhibitory effect is controlled by the global activator protein GacA. RsaL, the product of a gene found between lasI and lasR, negatively regulates the Las QS circuit. The product of the *vrf* gene is a cAMP receptor homologue and is required for the transcription of lasR. RsmA avoids early activation and downregulates production of QS-regulated phenotypes – protease, elastase, and proteolytic activities – and the production of a cytotoxic lectin, hydrogen cyanide, and pyocyanin. Overexpression of RsmA results in reduction in the expression of the AHL synthase genes lasI and rhlI. The RpoN is a negative transcriptional regulator of the lasIR and rhlIR but positively regulates the expression of rhlI in minimal media. The catabolite repressor homologue Vfr directly induces lasR transcription. The stringent response protein RelA, which synthesizes guanosine tetraphosphate (ppGpp) under amino acid starvation conditions on overexpression, causes early activation of several QS-controlled processes and lasR and rhlR expression. The GacA/GacS two-component regulatory system posttranscriptionally regulates QS through RsmZ and RsmA. In the absence of RsmZ, RsmA represses the synthesis of acyl-HSL signals. RsmA also regulates the production of virulence factors. The anaerobic regulator ANR activates expression of the quorum-controlled hydrogen cyanide biosynthetic genes hcnABC; ANR appears to be an important factor in the co-regulation of quorum-controlled genes under oxygen-limiting conditions. The rsaL gene, which is directly activated by LasR-3OC12-HSL, encodes an 11-kDa protein which inhibits QS by repressing lasI. The stationary-phase sigma factor RpoS can affect the expression of 40 % of QS-regulated genes. The transcriptional regulator VqsR, which is activated by LasR-3-oxo-C12- HSL, is required for AHL production and the expression of QS-controlled genes. Further, the genes required for the synthesis of a direct precursor of

PQS (*pqsABCD and phnAB*) are activated by the transcriptional regulator MvfR, and it itself is under the control of LasR-3-oxoC12-HSL. MvfR regulates rhl-dependent genes without affecting the production of AHLs or the expression of lasR or rhlR. MvfR/PQS and rhl QS are parallel pathways that converge at the promoters of their target genes. All these suggest that QS in *P. aeruginosa* is highly complex and the QS gene expression is integrated in a highly interconnected network of other regulatory systems.

AI-2-Mediated QS in *P. aeruginosa*

P. aeruginosa is unique from the other bacteria because it is does not make its own signaling molecule autoinducer-2 (AI-2). However, there is an increase in the expression of its virulence factor in response to AI-2 produced by other microflora. Although the *P. aeruginosa* QS circuit is AHL signaling based, it is capable of sensing AI- 2 and is therefore susceptible to AI-2-mediated QS inhibition. *P. aeruginosa* was able to sense and respond to AI-2 produced by the normal microflora of cystic fibrosis patients, which led to increased virulence factor expression and infection.

Interkingdom Signaling in *P. aeruginosa*

The 3-oxo-C12-HSL and PQS of *P. aeruginosa* are capable of modulating inflammatory and immune responses in mammals. The QS signal 3-oxo-C12-HSL exerts immune-suppressive or anti-inflammatory effects at concentrations below 10 μM, whereas pro-inflammatory or pro-apoptotic effects are found at much higher concentrations. The host environment modulates QS in *P. aeruginosa* either through nonenzymatic or enzymatic destruction of AHLs through lactonolysis. The mammalian paraoxonases (PON1, PON2, PON3) are a unique family of calcium-dependent hydrolases and are known to possess enzymatic activities toward a broad range of substrates including the AHLs.

P. aeruginosa QS as a Therapeutic Target

The *P. aeruginosa* QS circuits make attractive targets for novel antimicrobials because QS controls virulence factor production and no homologs to known QS components exist in

humans. This is especially critical in the treatment of persistent infections in cystic fibrosis patients given the resistance of many *P. aeruginosa* isolates to available antibiotics. Small molecule inhibitors of *P. aeruginosa* QS have been extensively reviewed. Because LasR sits at the top of the *P. aeruginosa* QS cascade, identifying LasR inhibitors has been a major focus. Competitive inhibitors have been reported that contain modifications to the native 3OC12HSL ligand. In this realm, alterations to both the head group and the acyl tail have led to molecules that out-compete 3OC12HSL for binding to LasR. Importantly, some of these modifications, such as substitution of the lactone ring for a thiolactone ring, are useful because of their increased stability under biological conditions.

Second, natural products have been isolated that inhibit QS by antagonizing LasR. These inhibitors include furanones and patulin , which have been further modified to increase their efficacy. Finally, high-throughput screens of small molecule libraries have revealed additional scaffolds for the design of inhibitors. A potential complication in targeting LasR is that, some clinical *P. aeruginosa* isolates possess defective LasR proteins. Nonetheless, establishment of the initial infection is known to be LasI/LasR-dependent, suggesting that, at a minimum, LasR inhibitors could be used as prophylactics.

Other factors involved in *P. aeruginosa* QS have also been targeted for drug discovery. For example, inhibitors of LasR have been successfully modified to act as competitive inhibitors of RhlR and in some cases these molecules have proven to be potent inhibitors of both LasR and RhlR.

Natural products have been isolated that inhibit RhlR. A final approach is to target regulators that affect both the LasI/LasR and RhlI/RhlR QS systems. For example, a small molecule library was screened for agonists and antagonists of QscR, which, as described, influences both the LasI/LasR and RhlI/RhlR systems. By agonizing QscR, it could be possible to diminish the overall QS response and thus prevent or delay expression of virulence factors. Interestingly, some non-natural AHLs that target QscR also inhibit LasR, raising the intriguing possibility of a compound that can act broadly to target all of the *P. aeruginosa* QS systems

Conclusion

P. aeruginosa is the most common nosocomial pathogen and is associated with chronic lung disease in cystic fibrosis (CF) patients. The major virulence signaling systems in *P. aeruginosa* are the AHL systems Las and Rhl, which together control the expression of multiple virulence factors in response to cell density. The third group of signaling system, the PQS, connects virulence factor production with adaptation and survival as a strategy to eliminate competition when survival depends on iron availability. Understanding the molecular mechanisms of regulation of QS and the complete signaling integration network in *Pseudomonas* will help in identifying the various drug targets to interrupt pathogenic activities by designing novel antimicrobials.