CHAPTER 1

Quorum sensing and regulation of *Pseudomonas aeruginosa* infections

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INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative microorganism that thrives in many environments, from soil and water to animals and people. It is an opportunistic pathogen that can cause respiratory infections, urinary tract infections, gastrointestinal infections, keratitis, otitis media, and bacteremia. *P. aeruginosa* is the fourth most common nosocomial pathogen, accounting for approximately 10% of hospital-acquired infections (www.cdc.gov). Immunocompromised patients, such as those undergoing cancer treatment or those infected with AIDS, burn patients, or cystic fibrosis (CF) patients, are susceptible to *P. aeruginosa* infections. These infections are difficult to treat by using conventional antibiotic therapies, and hence result in significant morbidity and mortality in such patients. The recalcitrant nature of *P. aeruginosa* infections is thought to be due to the organism's intrinsic antibiotic resistance mechanisms and its ability to form communities of bacteria encased in an exopolysaccharide matrix; such communities are known as biofilms.

P. aeruginosa possesses an impressive arsenal of virulence factors to initiate infection and persist in the host. These include secreted factors, such as elastase, proteases, phospholipase C, hydrogen cyanide, exotoxin A, and exoenzyme S, as well as cell-associated factors, such as lipopolysaccharide (LPS), flagella, and pili. The expression of these factors is tightly regulated. Many factors are expressed in a cell-density-dependent manner known as quorum sensing (QS). Quorum sensing, or cell-to-cell communication, is a means by which bacteria can monitor cell density and coordinate population behavior. The behavior was first identified in *Vibrio fischeri* as a mechanism to induce bioluminescence (20). In *P. aeruginosa*, this

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mechanism consists of a regulatory protein and a cognate molecule, termed an autoinducer. At low cell densities, a small amount of autoinducer is present in the extracellular milieu. As cell densities increase, the autoinducer concentration rises in the extracellular environment and a threshold intracellular concentration is exceeded. At this critical concentration, the autoinducer binds to the regulatory protein and this complex acts to induce or repress expression of target genes. The cell-density-dependent regulation of virulence factor production has been suggested as a protective means to prevent host response to invading bacteria before sufficient bacterial numbers have accumulated (15). *P. aeruginosa* possesses two well-studied QS systems, the *las* and *rhl* systems, which have been shown to be important in its pathogenesis. The following sections describe QS in *P. aeruginosa* and its contribution to *P. aeruginosa* virulence, and discuss how QS may represent an attractive target to develop new antimicrobial therapies.

P. AERUGINOSA QUORUM SENSING SYSTEMS

In 1991, Gambello and Iglewski reported that a protein encoded by a gene termed *lasR* could complement elastase production in an elastase-deficient *P. aeruginosa* strain (24). Homology searches showed that LasR belongs to the LuxR family of QS transcriptional regulators (2). Subsequently, two complete QS systems, *las* and *rhl*, have been identified and well studied in *P. aeruginosa*. Each system consists of a transcriptional regulatory protein (LasR in the *las* system and RhlR in the *rhl* system) and cognate autoinducer signal molecules (*N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C_{12}-HSL) in the *las* system, and *N*-butyryl homoserine lactone (C_4-HSL) in the *rhl* system). LasR and RhlR are members of the LuxR family of transcriptional regulators and share 31% and 23% identity, respectively, with the transcriptional activator LuxR in *V. fischeri* (20, 38). Each protein contains an autoinducer binding site and a DNA binding region. Both LasR and RhlR form multimers upon binding to their respective autoinducer (35, 36). This transcriptional regulator protein : autoinducer complex modulates target gene expression, presumably by binding to conserved DNA elements, termed *las* boxes, located upstream of the translational start site of QS-regulated genes (91). The autoinducers are synthesized from *S*-adenolsylmethioinine (SAM), which contributes the homoserine lactone ring and the available cellular acyl–acyl carrier protein (ACP) pool that is used to form the acyl side chain (31). The *lasI* gene encodes the synthase that directs the synthesis of 3-oxo-C_{12}-HSL; *rhlI* encodes the synthase required for C_4-HSL production. The acyl side-chain length has

been demonstrated to be important in the specificity of the autoinducer molecule for its cognate transcriptional regulatory protein (49). The length of the acyl side chain is believed to contribute to the differences in transport of the autoinducer molecules from the cell. Diffusion studies have found that, whereas C_4-HSL, with a relatively short acyl side chain, is able to diffuse freely across the cell membrane, 3-oxo-C_{12}-HSL, which has a much longer acyl side chain and is more hydrophobic, is actively pumped from the cell by the multidrug efflux pump MexAB-OprM (51).

The *las* and *rhl* systems are organized in a hierarchical manner in which the *las* system regulates expression of the *rhl* system (Figure 1.1). The LasR-3-oxo-C_{12}-HSL complex was shown to exert both transcriptional control, through activation of *rhlR* transcription, and post-translational control, thought to be mediated by the competitive binding of 3-oxo-C_{12}-HSL to RhlR when the C_4-HSL concentration is low, of the *rhl* system (53). The *las* system also regulates production of elastase (*lasB*), LasA protease (*lasA*), exotoxin A (*toxA*), alkaline proteases (*apr*), and a type two secretion pathway (*xcpR*, *xcpP*) (3, 23, 24, 48, 50, 68, 86). Expression of *rsaL*, which has been demonstrated to repress *lasI* transcription and alter elastase production when over expressed on a plasmid in *P. aeruginosa*, is also activated by the *las* QS system (14, 28, 70, 89). The *rhl* system has been shown to regulate production of rhamnolipids (*rhlA*, *rhlB*), pyocyanin, and lectin-binding protein (*lecA*), and to be required for maximal activation of LasA protease (*lasA*) and alkaline proteases (*apr*) (50). The *rhl* system has also been reported to regulate expression of the stationary-phase factor RpoS (*rpoS*) (37), although a conflicting report stated that *rpoS* regulates QS (93). A more recent analysis proposed a complex model of *rpoS* regulation in which *rpoS* can activate the *las* system, which activates the *rhl* system, which then activates *rpoS* transcription (69).

A third LuxR homolog in *P. aeruginosa*, with 29% identity to LasR and 32% identity to RhlR, was identified and shown to act as a repressor of quorum sensing (5). This transcriptional regulator, termed *qscR* for quorum-sensing-control repressor, has been shown to repress transcription of *lasI*, *rhlI*, *hcnAB*, *lasB*, *pqsH*, and two clusters of phenazine genes (*phzA1* and *phzA2*) (5, 40). QscR has been demonstrated to interact directly with both LasR and RhlR to form heterodimers in the absence of autoinducer molecules and can associate with 3-oxo-C_{12}-HSL and C_4-HSL (40). Further study is needed to define the role of QscR in the QS-regulon.

A third signal molecule, 2-heptyl-3-hydroxy-4-quinolone or the *Pseudomonas* quinolone signal (PQS), has also been described (54). Interestingly, the chemical structure of PQS is not an acylated homoserine

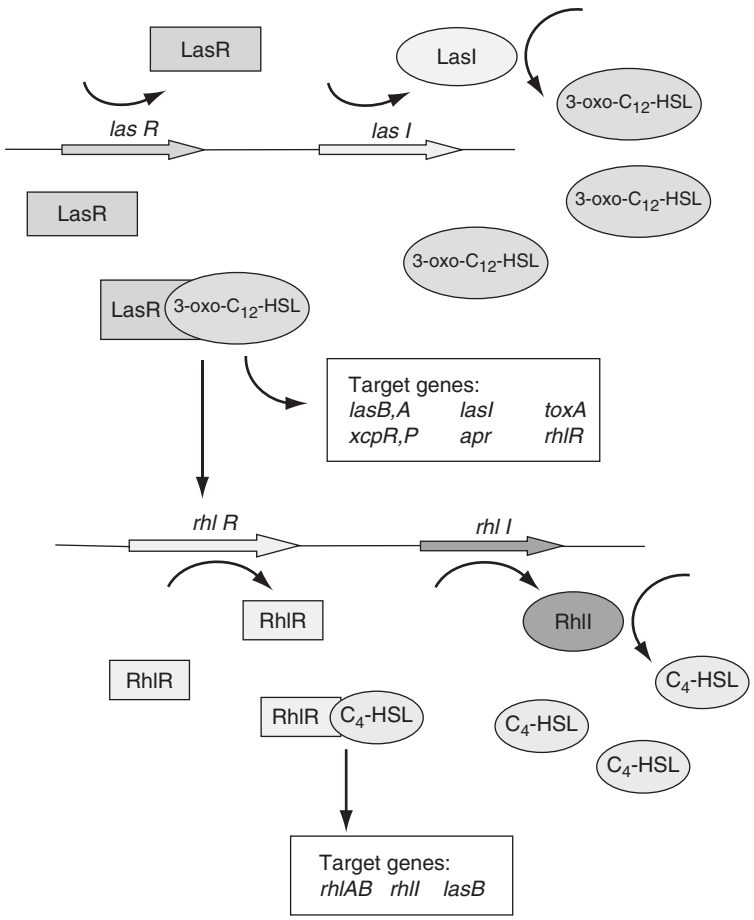

Figure 1.1. The *las* and *rhl* QS systems in *P. aeruginosa*. Both the *las* and *rhl* systems are composed of a transcriptional regulator protein (LasR, RhlR) and an autoinducer synthase (LasI, RhlI). The LasI synthase produces 3-oxo-C_{12}-HSL, and the RhlI synthase produces C_4-HSL. The autoinducer then binds to its respective cognate protein. The LasR:3-oxo-C_{12}-HSL complex activates transcription of genes involved in the production of several virulence factors, including *lasB*, *aprA*, and *toxA*, as well as upregulating transcription of *lasI* and of *rhlR*. The RhlR:C_4-HSL complex activates transcription of several genes involved in virulence, including *rhlAB*. (see also color plate section)

lactone molecule but rather a quinolone molecule. This signal has been proposed to act as a link between the *las* and *rhl* systems (45, 54). A recent study identified several genes, including *pqsABCD* and *pqsH*, in *P. aeruginosa* PAO1 that are required for PQS biosynthesis (22). LasR is required for

PQS synthesis whereas RhlR is required for PQS bioactivity, although the precise mechanism is not clear (22). Interestingly, the *rhl* system has been shown to repress synthesis of PQS (44). The balance of 3-oxo-C_{12}-HSL and C_4-HSL concentrations appears to play a key role in the production of PQS (44). A more thorough discussion of PQS and its place in the QS system is presented elsewhere in this volume (see Chapter 2).

EXTENDING THE *P. AERUGINOSA* QS REALM

Recent efforts to map the QS regulon have elucidated a large number of genes that are regulated by the *las* and *rhl* QS systems. A recent study used transposon mutagenesis to identify QS regulated genes (92). A promoterless-*lacZ* cassette was inserted randomly into the chromosome of a *lasIrhlI P. aeruginosa* PAO1 mutant. The mutant library was screened for *lacZ* induction upon exposure to 3-oxo-C_{12}-HSL alone, C_4-HSL alone, or both autoinducer molecules. Of 7,000 mutants screened, 270 mutants produced a greater than 2-fold stimulation of β-galactosidase activity in response to exogenous autoinducer(s). Forty-seven of these mutants that reproducibly exhibited a greater than 5-fold β-galactosidase activity were mapped to determine the locus of disruption. A total of 39 unique genes were identified. These included some previously known QS-regulated genes, such as *rhlB* and *rhlI*, and other genes involved in known QS-regulated processes, such as phenazine synthesis, cyanide synthesis, and pyoveridine synthesis. Seven putative operons were discovered; 14 of the 39 genes identified as QS-regulated possessed putative upstream *las*-box sequences. Interestingly, these mutations could be classified into four distinct classes based upon the timing of activation and requirement for 3-oxo-C_{12}-HSL alone, C_4-HSL alone, or both autoinducer molecules for maximal induction. The authors hypothesized that based upon their results approximately 3%–4% of *P. aeruginosa* genes are controlled by quorum sensing.

The sequencing of the *P. aeruginosa* PAO1 genome allowed the development of *P. aeruginosa* high-density oligonucleotide microarrays (80). The advent of these arrays led to subsequent global analyses of QS regulation in *P. aeruginosa* PAO1. The ability to probe the expression of all known annotated genes under various experimental conditions significantly expanded the QS regulon. One microarray experiment mapped the *las* and/or *rhl* regulon by using RNA isolated from a *lasIrhlI* mutant grown in the absence or presence of 3-oxo-C_{12}-HSL and C_4-HSL (89). In this study, 616 genes were identified as QS-regulated ($p \leq 0.05$ based on three

biological replicates per condition), with 394 genes being QS-activated and 222 genes being QS-repressed (89). These included 32 of the 52 previously known QS-regulated genes. Many of the 616 genes (34%) encoded proteins of unclassified or unknown function. The remainders were grouped into 24 of the 26 functional categories used to annotate *P. aeruginosa* gene products (www.pseudomonas.com), with genes encoding membrane proteins or putative enzymes representing the next two largest functional categories of QS-regulated genes. Not surprisingly, more than 50 of the 616 genes encoded known or putative virulence factors involved in attachment, colonization, dissemination, and destruction of host tissues. Of note, the microarrays revealed a link between QS and expression of type III secretion genes. Only 7% (43 genes) possessed upstream *las* boxes, suggesting that many of the genes identified are indirectly QS-regulated. Interestingly, 37 of the 616 QS-regulated genes encode known or putative transcriptional regulators. Therefore these genes may regulate the expression of many QS-regulated genes that do not possess upstream *las* boxes. A recent analysis has further characterized the recognition sites of LasR:3-oxo-C_{12}-HSL binding and found that this complex is able to recognize and activate genes that do not possess a putative *las* box and is unable to activate other genes with putative *las* boxes (71). For example, although PA1897 possesses a putative *las* box and has previously been shown to require the *las* system for activation, the *las*-dependence activation of PA1897 is apparently due to its direct regulation by *qscR* (PA1898), which is regulated by LasR. A more complex picture of the QS regulon, consisting of multiple layers of regulation, emerged (Figure 1.2).

Concurrently, another group also performed a transcriptome analysis to identify QS-regulated genes by using both a *lasIrhlI* signal mutant and a *lasRrhlR* receptor mutant (70). Transcripts were deemed to be QS-regulated if a fold-change difference of greater than or equal to 2.5, derived from comparison of transcript levels by using a *lasIrhlI* mutant grown in the absence or presence of exogenous autoinducer and the *lasRrhlR* mutant as compared with the wild-type, was observed. Transcripts identified as QS-regulated from both sets of experiments were compared; those transcripts that overlapped and had consistent regulatory expression patterns above background level were used to define a list of QS-regulated genes. By this criterion, 315 genes were identified as QS-activated and 38 genes were identified as QS-repressed. More than 87 possible operons were discovered. Importantly, a kinetic analysis of QS regulation suggested that the concentration of LasR was critical in the timing of activation or repression of QS-regulated genes.

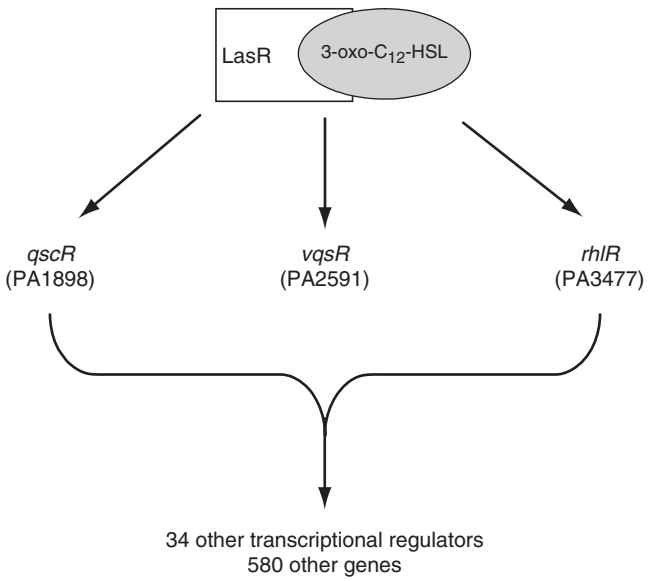

Figure 1.2. Increasing complexity of the QS regulon. Microarray analysis has revealed that conservatively more than 600 genes are part of the QS regulon. Of these genes, three transcriptional regulators, *rhlR*, *vqsR*, and *qscR*, possess putative *las* boxes that suggest these genes are directly activated by the LasR:3-oxo-C_{12}-HSL complex. These regulators may regulate expression of other QS-regulated genes that encode transcriptional regulators and genes that encode proteins representing other functional categories. The QS regulon is hypothesized to have multiple layers via several transcriptional regulatory circuits.

Although both microarray studies revealed that many genes (*c.*6%–10% of the genome) representing diverse functional groups in *P. aeruginosa* were QS-regulated, there was only approximately 50% agreement between the data sets as to the identity of these genes. Interestingly, researchers discovered that by altering the medium formulation (i.e. by using more minimal media or rich media) and oxygen availability used to cultivate *P. aeruginosa* PAO1, the detection of QS-regulated transcripts obtained by microarray analysis varied (89). For example, the level of expression of *lasR* and *rhlR* was lower in the absence of oxygen; this decrease in expression may have contributed to the inability to detect transcripts for certain QS-regulated genes under anaerobic conditions (89). Therefore, the discrepancies between both studies may reflect not only different microarray analysis tools and criteria to define QS-regulated transcripts but also the different experimental conditions used by each group.

In a third microarray analysis of the *las* and *rhl* QS regulon, the authors reported yet another set of QS-regulated genes (28). This study explored the QS regulon in cells grown planktonically, as the previous studies did, and also in cells grown as a biofilm. Taking advantage of the previous array results, the authors compared data sets to form a cohesive list of genes defined as the "general QS regulon." The authors noted that all of these genes, with the exception of one gene (PA0144), were expressed in *P. aeruginosa* biofilms. This is not surprising: it has been demonstrated that *lasI* is critical for mature biofilm formation in *P. aeruginosa* (13). Another study found that during *P. aeruginosa* biofilm development in a once-flow-through biofilm system, transcripts for 56 out of 72 previously identified QS-regulated virulence genes were detected by using microarray analysis (90). Of these, 32 genes (57%) respond to 3-oxo-C_{12}-HSL alone. Interestingly, some of these genes were detected only at day 1 (immature biofilm) or only at day 4 (mature biofilm). These data suggest that different components of the QS system are important during specific phases of biofilm development and maintenance. Interestingly, several genes that have been identified as QS-regulated, including *rhlA*, *rpoS*, *pslB* (PA2232), and PA5057–5059, have recently been shown to be important in biofilm development and maintenance (12, 30, 32, 55).

Recently, a fourth LuxR-transcriptional regulator that has been identified as QS-regulated by using microarrays (28, 70, 89) was found to be required for autoinducer synthesis and extracellular virulence factor production, as well as full pathogenicity in a *Caenorhabditis elegans* infection model (34). The gene, termed *vqsR*, was discovered by using transposon mutagenesis screening in the *P. aeruginosa* CF clinical isolate TB. *vqsR* possesses a putative *las* box upstream of its annotated translational start site, suggesting that it is directly regulated by the LasR–3-oxo-C_{12}-HSL complex (34, 89). Microarray analysis of the *vqsR* mutant compared with the wild type revealed that several genes that have been previously identified as QS-regulated, including *rhlA* and genes involved in the denitrification pathway, are also regulated by *vqsR* when grown in the presence of H_2O_2 or human serum. Interestingly, *vqsR* regulated expression of several genes that have been previously determined to be iron-regulated (8, 34). Iron plays a key role in *P. aeruginosa* pathogenesis (88). These data suggest that *vqsR* may be the link between QS and iron regulation that had been previously put forward by other studies (2, 92).

Several genes have been identified that affect activation of the QS regulon. These include the response regulator *gacA*, the CRP-homolog *vfr*, the transcriptional regulator *mvaT*, the sigma factor *rpoN*, the stationary

phase sigma factor *rpoS*, and *relA*, which is involved in the stringent response (1, 17, 64, 69, 87, 93). Polyphosphate kinase (PPK) has been shown to be required for maximal 3-oxo-C_{12}-HSL and C_4-HSL synthesis and elastase and rhamnolipid production (63). The regulatory protein *RsmA*, has also been demonstrated to influence homoserine lactone autoinducer production, rhamnolipid production, and swarming (29). Undoubtedly, the QS regulon is subject to global regulation at several levels; this regulation may reflect the importance of tight regulation of QS in response to environmental cues as well as to the metabolic state of the cell. Further study is needed to elucidate the influence of these factors on QS regulation. However, it is clear that more than just cell density (a "quorum") regulates the QS regulon in *P. aeruginosa*.

INFLUENCE OF QS ON *P. AERUGINOSA* VIRULENCE IN PLANT AND ANIMAL MODELS

To determine the role of QS in pathogenesis, several models of infection have been developed to identify virulence genes in *P. aeruginosa* in addition to the use of mammals. These include *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Galleria melonella*, *Drosophila melanogaster*, and *Dictyostelium discoideum* (10, 19, 26, 33, 42, 43, 46, 56, 59, 60, 61, 62, 81). These latter systems are attractive because they allow for rapid screening of many mutants without subjecting large numbers of mammals to infection and subsequent death. Importantly, data from these experiments have shown that a number of QS-regulated *P. aeruginosa* virulence factors appear to be conserved across host species. In the *A. thaliana* model, *gacA*, *toxA*, *plcS*, and *dsbA*, as well as several genes of unknown function, were found to be important in plant pathogenesis. The *C. elegans* model identified genes that were important in two modes of killing, slow and fast killing. These two modes were found to be dependent on the strain of *P. aeruginosa* used as well as on media conditions. From studies of *C. elegans*, numerous virulence genes were identified including *gacA*, *lasR*, *rhlR*, *vqsR*, *pqsR*, and *np20*, as well as genes with no previously described functions. A paralytic mode of killing was determined to be due to production of hydrogen cyanide, a toxic virulence factor that is QS-regulated (21). By using the *D. discoideum* model, components of the *rhl* QS (*rhlR*, *rhlI*, and *rhlA*) in addition to *nfxC* were determined to be required for virulence (9). In a *G. melonella* model, genes involved in type III secretion, including *exoU* and *exoT*, were found to be important in killing as well as *gacA*, *lasR*, and *pscD*. A *Drosophila melanogaster* model revealed that genes required for twitching

Table 1.1. *Genes in the QS regulon found to be important in pathogenesis of* P. aeruginosa *in various host infection models*

Host species	Genes	References
Arabidopsis thaliana	*gacA*, toxA*	62
Caenorhabditis elegans	*hcnA, pqsCDEH, pqsR, phnA, lasR*, rhlR, rhlI*, gacA*, gacS, phzB*, mexA, vqsR, PA0745, PA3032*	10, 22, 34, 42
Dictyostelium discoideum	*rhlR, lasRrhlR, rhlI*, lasIrhlI*, rhlA, pscJ*	9, 59
Drosophila melanogaster	*relA, pilGHIJKL*	11, 19
Galleria mellonella	*phzB*, gacA*, lasR**	46, 60
Medicago sativa seedling	*rhlR*	72

*Genes also required for virulence in a thermal injury mouse model. Note that not all genes have been tested in the thermal injury mouse model.

motility (*pilGHIJKL, chpABCDE*), amino acid, nucleotide, and central metabolism (*pyrF, pgm, cca*), and a gene of unknown function (PA5441), were important in killing (11, 57). Both *rhlR* and *algT* have been identified as important in an alfalfa seedling (*Medicago sativa*) model of infection (72). Many of the genes found to influence pathogenesis are part of the QS regulon, either as a global regulator of QS activation (e.g. *gacAS*), QS transcriptional regulators (e.g. *lasR, rhlR, vqsR, pqsR*), or genes involved in QS autoinducer synthesis (e.g. *lasI, rhlI, pqsCDEH*) or production of extracellular secreted factors (e.g. *hcnA, rhlA*) (Table 1.1).

Research using animal models of both acute and chronic infection has supported the premise that QS significantly contributes to *P. aeruginosa* pathogenesis (15, 58, 66, 83) (Table 1.2). In a mouse model of acute pulmonary infection, *lasR, lasI, rhlI,* and *lasIrhlI* mutants were significantly attenuated in virulence (52, 83). Analysis of the *lasI* and *rhlI* mutants revealed that a *rhlI* mutant caused pneumonia in 15% of mice, as opposed to a *lasI* mutant, which caused pneumonia in 30% of mice (52). This suggests that, whereas both *lasI* and *rhlI* contribute to infection, *rhlI* regulates specific factor(s) that stimulate airway inflammation and resultant pneumonia. In addition, pili were demonstrated to contribute to pathogenesis in the same model of acute infection (82). Several QS genes have also been demonstrated to be important in the rat model of acute pneumonia. A *lasR* mutant was avirulent in the rat model (41). When a *lasIrhlI* mutant was