REVIEW ARTICLES

Quorum sensing in Gram-negative bacteria

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Abstract Bacteria can communicate with each other by means of signal molecules to coordinate the behavior of the entire community, and the mechanism is referred to as quorum sensing (QS). Signal systems enable bacteria to sense the size of their densities by monitoring the concentration of the signal molecules. Among Gram-negative bacteria *N*-acyl-L-homoserine lactone (acyl-HSL)-dependent quorum sensing systems are particularly widespread. These systems are used to coordinate expression of phenotypes that are fundamental to the interaction of bacteria with each other and with their environment and particularly higher organisms, covering a variety of functions ranging from pathogenic to symbiotic interactions. The detailed knowledge of these bacterial communication systems has opened completely new perspectives for controlling undesired microbial activities.

Keywords: quorum sensing, bacterial communication, *N*-acyl-homoserine lactone, bacterial biofilms.

Biofilms are ubiquitous in nature[1]. The first paper to report on biofilms dates back to 1923 when the fouling of ships' hulls by microorganisms was reported to be a serious problem for navy vessels[2]. In 1933 Henrici stated that drinking water bacteria are not free floating but rather grow upon submerged surfaces[3]. In most cases biofilms form at the interface between a solid surface and an aqueous phase. Bacteria must sense the presence of a surface and in response activate specific genetic programs ensuring the step-wise development of the mature biofilm. The introduction of laser scanning confocal microscopy brought a revolutionary progress in the observing technique and according to the prevailing conceptual model, bacterial biofilms consist of microcolonies as the basic unit. Biofilm development is believed to proceed through a temporal series of stages[4]. This hypothesis has gained momentum from the isolation of mutants that appear to be arrested in certain stages of this development[4~7]. In the initial phase, bacteria attach to a surface, aggregate to each other and then proliferate to form microcolonies. These microcolonies are hydrated structures in which bacterial cells are enmeshed in a matrix of self-produced slime, commonly referred to as exopolymeric substances (EPS). With time, as substrate availability becomes limiting due to increased diffusion distances, growth will decrease and biofilm development will reach a steady-state. Such mature biofilms typically consist of "towers" and "mushrooms" of cells enmeshed copious amounts of EPS separated by channels and interstitial voids to allow convective flow to transport nutrients to interior parts of the biofilm and remove waste products (Fig. 1). One environment that contains a large number of bacteria in close proximity is bacterial biofilms. The dense and diffusion-limited biofilm matrix provides ideal conditions for accumulation of signal molecules and a protected environment for bacteria to induce quorum sensing-regulated virulence factors and launch an attack on the host. The involvement of quorum sensing in biofilm development has neither been satisfactorily demonstrated nor discarded. Its role in the regulation of biofilm formation was originally reported for *P. aeruginosa*[8]. In this study the authors reported that a *lasI* mutant of *P. aeruginosa* only formed flat and undifferentiated biofilms when compared with the wild type, which formed characteristic microcolonies separated by water channels. This led the authors to suggest that the *las* quorum sensing system is required for development of the characteristic biofilm architecture. More and more evidence accumulates that QS is an important mechanism controlling and coordinating the bacterial behavior such as production of virulence factors, function like multi-

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cellular organisms and formation of biofilms to develop an overwhelming infection or a chronic and persistent infection in higher organisms[9~16]. While the bacterial number is low, the level of signal molecules is also diluted and the bacterial cells keep relatively silent. However, after the bacterial population increased to a certain size and the signal level reaches a critical threshold, QS will be activated to coordinate the actions of all bacterial cells toward a common task like the phenomenon seen in primitive multicellular orgasms. It would be therefore important to understand the concept and mechanism of QS, the contribution of QS to infections, the effects of QS on biofilm formation, and the methods to monitor and interfere with QS. In this review, we will address those key points according to our current understanding and research findings.

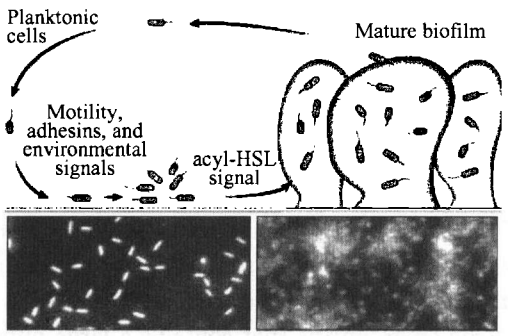

Fig. 1. Bacteria can live in both planktonic and biofilm modes. The figure shows how the planktonic bacterial cells form biofilm and also the programmed planktonic cells release from the biofilm. QS controls the behavior of the biofilm cells. The confocal microscopic images on the bottom show the planktonic bacterial cells (left) and the mature biofilm bacterial cells (right). The figure is cited from Center of Biofilm Engineering, Montana State University.

1 Bacterial quorum sensing

Bacteria can act in flocks. Under appropriate conditions and when their numbers have reached a critical level ($\geqslant$ approximately 10^7 CFU · mL^{-1} in liquid medium) and acyl-HSL signal concentration has increased to 10 nmol · L^{-1} [17,18], bacteria can modify and coordinate their behavior to act uniformly as a multicellular group. This takes place when the number of bacteria has increased to a threshold value (i.e. a quorum has been reached), then the entire bacterial population responds via the signal molecules and achieve what the individual cells could not. Hence, the population of bacterial cells exhibits a new characteristic dependent on their cell density. Most Gram-negative bacteria can sense, integrate and process information from the environment via intercellular communication after a critical population is achieved. The phenomenon was originally termed "autoinduction", because of the presence of a positive feed-back loop in the control of gene expression. Now it is most frequently referred to as quorum sensing (QS), "a symphony of bacterial voices" or simply bacterial communication (Fig. 2)[19~23].

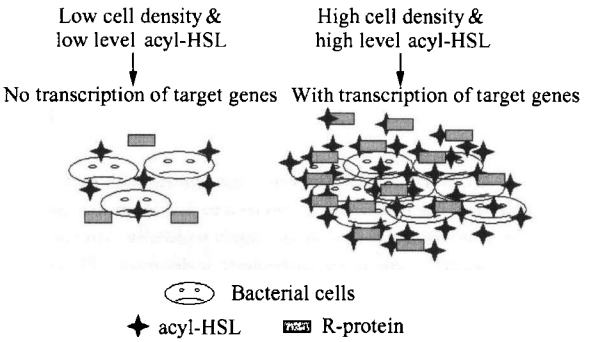

Fig. 2. AHL mediated QS in Gram-negative organisms involves two regulatory components: the transcriptional activator protein (R-protein) and the acyl-HSL molecules produced by the acyl-HSL synthase. Accumulation of acyl-HSL molecules occurs in a cell-density-dependent manner until a threshold level is reached, i.e. a quorate has been reached. At this time the acyl-HSL molecules bind to R-protein and activate the R-protein, then initiate the transcription of different target genes. The R-protein consists of two domains: the N terminus of the protein that interacts with acyl-HSL and the C terminus that is involved in DNA binding.

Since the 1970s, bacterial communication has been studied extensively on the bioluminescent, marine symbiotic *Vibrio fischeri* and knowledge generated with this system has formed a consensus for how QS systems in general operate. In the last decade, it was elucidated that *V. fischeri* did not emit any light when its cells were free living (planktonic state) at low cell densities. As the bacterial cells multiply inside the light organ of the squid, the extracellular signal molecule accumulates. When a certain critical concentration has been reached (in parallel with the cell density) the cells collectively switch on bacterial genes involved in the generation of bioluminescence[24,25]. The regulatory region of the luminescence was found during the 1980s, which consists of two genes: *luxR* which encodes a transcriptional activator, and *luxI* which encodes a protein required for acyl-HSL synthesis. The region between *luxR* and *luxI* contains the regulated *lux* promoter elements. It is considered that *luxI* is positively autoregulated. Basal levels of luminescence operon transcription lead to low rates of acyl-HSL production, and high density of cells is needed for activation of the luminescence genes[25]. The acyl-HSL molecules are

synthesized from precursors by a protein *luxI*. When the bacteria reach a certain population size, the concentration of acyl-HSLs exceeds a threshold level, the acyl-HSL molecules bind to *luxR*, a specific protein, and *luxR* and *luxI* interact with each other to initiate the transcription of different target genes, e.g. expression of luminescence. Hence, it can be termed "cell density-dependent gene expression" (Fig. 2)[19].

Bacterial communication within and between bacterial species is regulated by QS systems via signal molecules that modulate cell density-dependent gene expression, appear to be common to a wide variety of pathogenic and non-pathogenic bacterial species (Table 1)[11]. Up to now, it has been reported that both Gram-negative and Gram-positive bacteria possess QS system. Such bacterial cell-cell communication mediated by specific tiny diffusible chemicals, known as signals or pheromones, are secreted by bacteria into environment. The signals used by Gram-positive bacteria are small peptides, whereas most Gram-negative bacteria employ *N*-acyl homoserine lactone (acyl-HSL) as signaling molecules[21,26]. Except for acyl-HSL signals, there are some other types of signal molecules that have been found in Gram-negative bacteria[27~30], but acyl-HSL dependent QS is the focus of the present review.

Table 1. acyl-HSL signals mediated quorum sensing in Gram-negative bacteria

Organism	Major signal molecule	Regulatory proteins	Phenotype
Aeromonas hydrophila	C_4-HSL	AhyI/AhyR	Exoprotease production
Aeromonas salmonicida	C_4-HSL	AsaI/AsaR	Extracellular protease
Agrobacterium tumefaciens	3-oxo-C_8-HSL	TraI/TraR	Ti plasmid conjugation
Burkholderia cepacia	C_8-HSL	CepI/R	Protease, siderophores
Chromobacterium violaceum	C_6-HSL	CviI/CviR	Exoenzymes, antibiotics, cyanide, violacein
Erwinia carotovora subsp. carotovora	3-oxo-C_6-HSL	ExpI/ExpR	Exoenzymes
		CarI/CarR	Carbapenem antibiotics
Erwinia chrysanthemi	3-oxo-C_6-HSL	ExpI/ExpR	Pectate lyases
Erwinia stewartii	3-oxo-C_6-HSL	EsaI/EsaR	Exopolysaccharide, virulence factors
Pseudomonas aureofaciens	C_6-HSL	PhzI/PhzR	Phenazine antibiotics
Pseudomonas aeruginosa	3-Oxo-C_{12}-HSL	LasI/LasR	Multiple extracellular enzymes, RhlR, Xcp, biofilm formation
	C_4-HSL	RhlI/RhlR	Multiple extracellular enzymes, rhamnolipid, RpoS, secondary metabolites
Rhizobium leguminosarum	C_6-HSL C_8-HSL 3-Hydroxy-7-*cis*-C_{14}-HSL	RhiI/RhiR	RhiABC rhizosphere-expressed genes, nodulation
Rhodobacter sphaeroides	7-*cis*-C_{14}-HSL	CerI/CerR	Dispersal from bacterial aggregates
Serratia liquifaciens	C_4-HSL	SwrI/SwrR	Extracellular protease, swarming
Vibrio fischeri	3-oxo-C_6-HSL	LuxI/LuxR	Bioluminescence
Vibrio harveyi	3-hydroxy-C_4-HSL	LuxLM/LuxN	Bioluminescence

QS controls a variety of phenotypes, such as the production of bioluminescence in *Vibrio fischeri*[24,25], Ti plasmid transfer in *Agrobacterium tumefaciens*[31,32], swarming surface motility in *Serratia liquefaciens*[13], biofilm formation[8,15], biosurfactants synthesis[33,34], production of multiple extracellular enzymes in *P. aeruginosa*[11,35], and antibiotic biosynthesis in both *Erwinia carotovora* and *P. aureofaciens*[36~38]. Biosurfactants, exoenzymes and other virulence factors are often employed during the process of colonization and invasion of higher organisms by pathogens. QS is thought to afford bacteria with a mechanism to minimize host responses by delaying the production of tissue-damaging virulence factors until sufficient bacteria have been amassed to overwhelm host defense mechanisms and to establish a successful infection. But actually, QS brings us bifunctional effects: pathogenic bacteria rely on QS to coordinate the infectious process, and beneficial bacterial species tie to QS to carry out their activities. For instance, bacterial response of nitrogen fixation uses QS to optimize nodule formation on plant roots[39,40]. Many bacterial species produce chemicals that kill or inhibit the growth of other disease-causing microorganisms, and can therefore be used as "biocontrol" agents[36,38,41]. The biocontrol as well as nitrogen fixation is, however, only produced when the population size of the bacterial cells reaches a critical

threshold density, so-called cell density-dependent gene expression.

By now more than a dozen of LuxI and LuxR homologues have been discovered in several bacterial species: *Erwinia carotovora* (ExpI/ExpR, CarI/CarR) produces 3-oxo-C_6-HSL, *Pseudomonas aeruginosa* (LasI/LasR, RhlI/RhlR) produces 3-oxo-C_{12}-HSL and C_4-HSL, *Yersinia enterocolitica* (YenI/YenR, YesI/YesR) produces C_6-HSL, *Agrobacterium tumefaciens* (TraI/TraR) produces 3-oxo-C_8-HSL, and *Rhizobium leguminosarum* (RhiI/RhiR) produces C_6-HSL and C_8-HSL[42,43] (Table 1). Acyl-HSL molecules are thought to possess a similar basic structure, i.e. a homoserine lactone ring with a variable side acyl chain that ranges from 4- to 14-carbon and either an oxo, a hydroxy, or no substitution at the third carbon[19]. Short and medium-chain acyl-HSL molecules are thought to pass freely through the bacterial membrane[19,25]. However, long-chain molecules seem to require the mexA-mexB-oprM-encoded efflux pump inside bacteria[44].

2 The quorum sensing systems in *P. aeruginosa*

P. aeruginosa holds impressive weaponry of cell-associated and extracellular virulent determinants which are closely associated with its pathogenicity. *P. aeruginosa* QS has been studied since the 1980s. Whooley et al. first reported that gene expression of *P. aeruginosa* was controlled by a cell density-dependent manner[45]. There are at least two complete QS *las* and *rhl* systems in this pathogen, which consist of two cognate signal-generating synthetases (LasI/RhlI) and transcriptional regulators (LasR/RhlR) that are homologues with LuxI and LuxR family[34,46] (Fig. 3).

As a leading member of the *las* QS system, the *lasR* gene encodes a 239-amino-acid homolog of the prototypical QS transcriptional activator LuxR of *Vibrio fischeri*[19,46]. The LasR protein like other LuxR homologs has two highly conserved regions. One is the putative acyl-HSL-binding region (36% identity to the LuxR autoinducer binding region) that constitutes the two-thirds of the protein at N-terminus, and the other is the putative DNA-binding region (53% identity to the LuxR DNA-binding region) which consists of a helix-turn-helix motif located at the C-terminal of the protein[46]. The *lasR* gene is transcribed as a monocistronic operon[46],

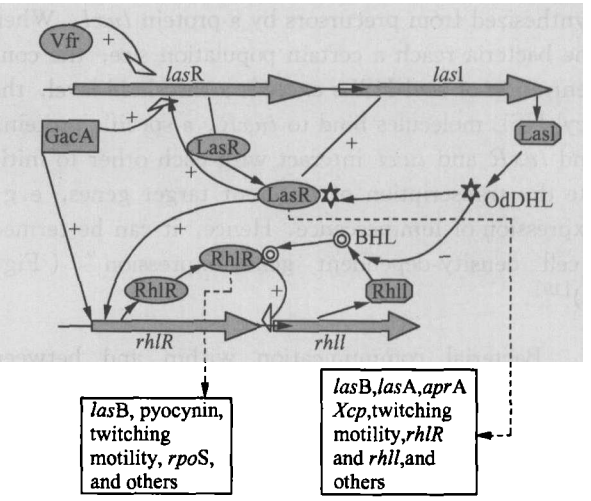

Fig. 3. The QS circuitry of *P. aeruginosa*. Expression of the *lasR* gene is subject to at least two levels of control: the global regulators Vfr and GacA and the *las* QS system, which regulates expression of both *lasR* and *lasI*. The latter creates an auto-induction feedback loop. Regulation of the *rhl* system is similar to *las* in that GacA affects expression of *rhlR*, and the *rhlR* and *rhlI* genes are controlled to some degree by the *las* system. Interestingly, the *las* QS system was shown to elicit an additional level of control over the *rhl* system; the *las* signal molecule, OdDHL, can act post-translationally to block RhlR activation by BHL. The *las* and *rhl* QS systems regulate expression of numerous genes that contribute to the virulence of *P. aeruginosa*.

which is directly followed on the *P. aeruginosa* chromosome by the *lasI* gene[47]. The *lasI* gene encodes LasI, a 201-amino-acid homolog (35% identity, 56% similarity) of the prototypical *V. fischeri* LuxI autoinducer synthase protein[19,47].

The second *P. aeruginosa* LuxR homolog was discovered by Ochsner et al. The gene was named *rhlR* because of its regulation of rhamnolipid production which functions as a biosurfactants. RhlR is 31% identical to LasR and 23% identical to LuxR and contains a typical helix-turn-helix motif in its carboxyl region[33].

The *lasI* and *rhlI* products are *N*-3-oxododecanoyl homoserine lactone (3-oxo-C_{12}-HSL or OdOHL)[48], and *N*-butyryl homoserine lactone (C_4-HSL or PAI-2)[49,50], respectively. In spite of the similarities between two transcriptional activators LasR and RhlR as well as between the two synthases LasI and RhlI, 3-oxo-C_{12}-HSL is only capable of low-level RhlR activation; whereas LasR is not activated by C4-HSL. The R proteins show a high specificity with their cognate acyl-HSL signals[51].

The *las* QS system has been shown to modulate

expression of *lasB* (elastase)[47,51], *lasA* (Staphylolytic protease)[52], *aprA* (alkaline protease)[52], *xcp* (secretion pathway)[53], twitching motility[54], and of *lasI* itself to create an autoinduction feedback loop[55]. The *las* system positively regulates the expression of both *rhlR* and *rhlI*[56,57]. The *rhl* QS system modulates expression of *rhlI* itself[56], as well as *rhlAB* (rhamnolipid biosynthesis)[34,51], *lasB*[49,51,58], twitching motility[54] and *rpoS*[56] (Fig. 3). The two systems do not operate independently as the *las* system positively regulates expression of both *rhlR* and *rhlI*. Thus, the two quorum sensing systems of *P. aeruginosa* are hierarchically arranged with the *las* system being on top of the signaling cascade[56,57]. Intertwined in this quorum sensing hierarchy is the quinolone signal (PQS) system which provides a link between the *las* and *rhl* quorum sensing systems[30] in which the PQS cooperatively regulates expression of *rhlI* and *lasB* with C_4-HSL[59]. The signal molecule, 2-heptyl-3-hydroxy-4-quinolone is designated as the *Pseudomonas* quinoline signal (PQS). This unique signal controls *lasB* expression that encodes for the major extracellular enzyme, elastase. A recent report suggests that PQS acts as a link between the las and rhl QS systems and that this signal is not involved in sensing cell density[59]. However, in comparison to *las* and *rhl* QS systems, PQS has not been well studied.

2.1 Determination of quorum sensing activity

Bacteria with QS systems can be identified by measuring production of acyl-HSL molecules. Most of the methods used currently for determining QS activities have been designed for *in vitro* studies. These methods include thin-layer chromatography (TLC)[60,61], high-performance liquid chromatography (HPLC)[62], radiolabel assay[63], *Chromobacterium violaceum* agar plate assay[60,64] and bioluminescence-based acyl-HSL sensor systems[65,66].

Recently highly sensitive acyl-HSL monitor strains were developed. The monitor bacteria have been equipped with a *gfp*-based acyl-HSL sensor systems, components of the *Vibrio fischeri* quorum sensor encoded by *luxR*-P_{luxI} have been fused to modified versions of *gfp*mut3* gene encoding unstable green fluorescent protein (GFP). This system is highly sensitive to as little as 1 nmol of 3-oxo-C_6-HSL in the environment and highly promiscuous in its acceptance of acyl-HSL signal molecules[65]. The short half-life of the GFP enables on-line monitoring of gene expression and therefore becomes a useful tool for non destructive QS investigation *in vitro* and *in vivo*. Using such monitor strains, we have successfully detected the acyl-HSL molecules in mouse lung tissues infected with *P. aeruginosa* and that was the first direct evidence we found that acyl-HSLs exist in the tissues with a concomitant bacterial infection (Plate I)[67]. Since then, more articles regarding *in vivo* determination of acyl-HSLs have been reported[66,68,69]. The *gfp*-based acyl-HSL sensor has been exchanged with P*lasB-gfp* (ASV), and the correspondent *P. aeruginosa* monitor strain is capable of responding to the two *P. aeruginosa* signals[70].

2.2 Pathogenesis of quorum sensing in lung infection with *P. aeruginosa*

Reports that show acyl-HSL-mediated QS to be involved in the pathogenesis of *P. aeruginosa* in lung, corneal and burn wound infections are accumulating. The first evidence showed that the *lasR* gene product played an important role in a neonatal mouse model of acute *P. aeruginosa* lung infection[71]. In comparison with PAO1 wild type, a *lasR* mutant was essentially avirulent, producing no mortality and low incidence of bacteremia[71]. Another neonatal mouse model was also utilized by Pearson et al. to examine the involvement of QS in pulmonary infection. In comparison with PAO1, three *P. aeruginosa* mutants (defective in the *lasI* or the *rhlI* or the *lasI* and *rhlI* genes respectively) showed a significant reduction in their lethality and ability to cause pneumonia and bacteremia. The most avirulent strain was the one carrying mutations in both the *lasI* and *rhlI* genes. The pathogenicity of this strain was significantly increased after the mutations were complemented by utilizing a plasmid that carried the *lasI* and *rhlI* gene[72]. These investigations strongly suggest that QS is crucial for *P. aeruginosa* pathogenesis in pulmonary infection.

The role of acyl-HSL-mediated QS in producing cytotoxicity in *P. aeruginosa* was also investigated. For that purpose, large doses of bacteria were applied to cultured lung epithelial cells or instilled into the lungs of animals. The results suggested that there are multiple mechanisms for the induction of cytotoxicity, pathology, and mortality *in vivo*. The results also showed that QS might inversely correlate with virulence in the *P. aeruginosa* strain producing 3-oxo-C_{12}-HSL signals. This strain appeared to attract more polymorphnuclear leukocytes *in vivo* and were conse-

quently cleared more rapidly[73].

The influence of the *P. aeruginosa las* QS system in the lung infections associated with cystic fibrosis (CF) has been partially examined by Storey et al.[74]. Their study was to determine if genes regulated by the *las* QS system were coordinately regulated by the *P. aeruginosa* cell densities during the lung infections associated with CF, and if there was a relationship between the expression of lasR and some *P. aeruginosa* virulence factors during these infections. The RNAs from the bacterial populations of 23 CF patients' sputa over a period of 3 years were analyzed with specific probes to *lasA*, *lasB*, *toxA* and *algD* encoded messengers. The results revealed close correlations between the transcript accumulations originating from the three genes, indicating that the expression of *lasA*, *lasB*, and *toxA* might be coordinately regulated during CF lung infections[74]. Storey et al. also found that the LasR transcription was correlated to LasA, LasB, ToxA, and AlgD transcript accumulations, suggesting that the *las* QS system may control the expression of some virulence factors in the CF patient's lungs[74]. It must be emphasized that there is no genetic evidence for quorum sensing control of *algD* transcription so far[75].

A piece of new evidence of acyl-HSL-mediated QS involved in the pathogenesis of *P. aeruginosa* was released recently. The authors demonstrated that in an adult mouse acute-pneumonia model, deletion of the *lasI* gene or both the *lasI* and *rhlI* genes greatly diminished the ability of *P. aeruginosa* to colonize in the lung. They also examined the effects of 3-oxo-C_{12}-HSL injected into the skin of mice. 3-oxo-C_{12}-HSL stimulated a significant induction of mRNAs for the cytokines IL-1α and IL-6, and they also demonstrated that 3-oxo-C_{12}-HSL activated T cells to produce the inflammatory cytokine interferon gamma (IFN-γ)[76]. The correlation between *P. aeruginosa* acyl-HSL signals and IL-8 had been described by DiMango et al. The *P. aeruginosa* 3-oxo-C_{12}-HSL was found to stimulate the production of IL-8 from respiratory epithelial cells in a dose-dependent manner[77]. The 3-oxo-C_{12}-HSL from *P. aeruginosa* can induce an imbalance of Th1/Th2 response *in vitro*, i. e. suppressing IL-12 and TNF-α synthesis and increasing IgG1 and IgE production[78], which are known to be harmful to CF patients[79]. However, the above limited findings in the effects of 3-oxo-C_{12}-HSL on immune responses are contradictory, which might be caused by different experimental conditions used.

In our animal studies, the effects of QS on the lung infection were compared between *P. aeruginosa* wild-type PAO1 and its *lasI* and *rhlI* double mutant. The results showed that the bacteria without complete and intact QS systems were cleared significantly faster and they induced more severe lung pathology in acute phase, but significantly milder pathology in chronic phase compared to the wild type, indicating that in the absence of a functional QS system, the host immune response can oppose the pathogen faster and more powerful; furthermore, the infection with double-mutant strain induced a Th1 dominated immune response compared to the wild type induced Th2 response[80], which in case of CF patients has been demonstrated to correlate with a better prognosis[81].

In addition to the studies associated with pulmonary infections, the acyl-HSL-mediated QS was also thought to contribute to *P. aeruginosa* pathogenesis in ocular infection[82], and burned wound infections[83].

3 Bacterial biofilm

In clinical microbiology, the biofilm mode of bacterial growth has attracted particular attention as many persistent and chronic bacterial infections, including periodontitis, otitis media, biliary tract infection, and endocarditis are now believed to be intrinsically linked to the formation of biofilms. Another well-recognized medical problem involving biofilms is the colonization of medical implants by pathogenic bacteria[10]. Bacterial biofilm infections are particularly problematic as sessile bacteria can withstand host immune responses and are drastically more tolerant to antibiotics and biocides than cells grown in suspension[10,84]. The investigation of the regulation of biofilm formation has made some progress. Recently a new gene *migA* in *P. aeruginosa*, encoding a putative glycosyl-transferase and regulated by *rhl* QS system, has been demonstrated to be highly induced in CF lung by Yang et al. Their data suggested that the *migA* up-regulation in the CF lung environment is part of the adaptive response which offers *P. aeruginosa* a survival advantage since the *migA* gene indeed affects the structure of lipopolysaccharide in *P. aeruginosa*[85]. It was confirmed that *P. aeruginosa* QS systems play an important role in the process of biofilm formation in the *in vitro* laboratory environ-

ment. De Kievit et al. investigated the role of the acyl-HSL-mediated QS systems during the early stages of static biofilm formation when cells were adhering to a surface and forming microcolonies. Their studies revealed a marked difference in biofilm formation between the PAO1 and the QS mutants when glucose, but not citrate, was used as the sole carbon source[86]. During an 8-day biofilm development, *lasI* expression was found to progressively decrease over time. Inversely, *rhlI* expression remained steady throughout biofilm development but occurred in a lower percentage of cells. Spatial analysis revealed that *lasI* and *rhlI* were maximally expressed in cells located at the substratum and that expression decreased with increasing biofilm thickness. Singh et al. found that the ratio of the two signals produced by the biofilm mode of growth of *P. aeruginosa* was changed when compared to planktonic mode of growth[87]. However, Geisenberger et al. analyzed the acyl-HSLs produced by sequential *P. aeruginosa* isolates from chronically infected patients with CF by thin-layer chromatography. Their results demonstrated that both the amounts and the types of molecules synthesized by clinical isolates from patients who were monitored over periods of up to 11 years did not change significantly during chronic colonization[69]. *P. aeruginosa* PAO1 and the QS mutants were also studied by Shih et al. to determine the variations in biofilm formation and antibiotic resistance[16]. The maximal biofilm formation in PAO1 began immediately and a plateau phase was reached after 24 hours, whereas the QS mutants showed a lag of 36 ~ 48 hours. After 72 hours, the cell density of the PAO1 biofilms was 10 times greater than that of the mutants. Fluorescent micrographs revealed that the PAO1 biofilms were much thicker than those of the QS-deficient mutants[16]. Bacterial cells in PAO1 biofilms were slightly affected by kanamycin, even at 100 mg/L, whereas those in *rhlI* biofilms were susceptible to kanamycin of 100 mg/L but not to lower concentrations (10 and 50 mg/L). In contrast, cells in *lasI* and double mutant biofilms were susceptible to kanamycin at all three concentrations[16]. The above findings may have important implications for the design of the prevention and eradication strategies of biofilm infection.

4 Approaches to inhibiting quorum sensing

The excitation for the discovery of QS systems is that it not only led us to realize how bacteria can talk and survive, but also deepened our understanding of the switches that turn on the disease-causing machinery of virulent bacteria. But quorum sensing is also a mechanism by which bacteria expose part of their genetic repertoire for other organisms, prokaryotes as well as eukaryotes. The central role of QS systems in expression of host-associated phenotypes including virulence factor production and biofilm development and the fact that they function by means of low molecular weight external substances make them ideal drug targets[75]. The concept differs from the traditional antibiotics. These new anti-pathogenic agents neither kill bacteria nor inhibit their reproduction but rather interfere with their ability to communicate.

One way to disrupt QS is to inhibit acyl-HSL synthesis by introducing appropriate or synthetic autoinducer precursor antagonists. This strategy prevents native acyl-HSLs binding to LuxR homolog, subsequently switches off the expression of virulent genes[37,88~90]. Branny et al. have suggested that the overproduction of the *P. aeruginosa* DksA homolog would inhibit quorum sensing-dependent virulence factor production by down regulating the transcription of the synthase gene *rhlI*[91].

A second approach is to use blockers or analogues that function to displace the cognate signal from its receptor. The discovery of halogenated furanone compounds from the Australian macro alga which have structural similarity to acyl-HSLs have accelerated the research in this particular field[70,92~94]. It has been reported that intercellular signaling antagonists produced by *D. pulchra* have potential utility in the control of *Vibrio harveyi* prawn infections. In the *Penaeus monodon* pathogen *V. harveyi*, expression of luminescence is regulated by QS mechanism, and the virulence and luminescence are co-regulated. Luminescence and toxin productions in this strain were both inhibited by the signal antagonist at concentrations that had no impact on growth[94]. In mice challenged intratracheally with the bacteria equipped with *gfp*-based acyl-HSL sensor systems, acyl-HSL and acyl-HSL-antagonistic materials were given intravenously to compare the effects[75]. Acyl-HSL injection induced GFP expression successfully (activation of the QS sensor); injection of both acyl-HSL and antagonist led to disappearance of the GFP signal (blocking the QS sensor); increasing acyl-HSL dose, GFP appeared again[75]. These experiments revealed important information about the mode of action of the furanone compounds. First, the compounds signifi-

cantly repress QS regulated gene expression *in vivo*; second, the effect of the furanones is concentration dependent; and third, the compounds are turned over within the duration of the experiment. This tells us that the QS inhibitory effect of a single furanone injection lasts approximately six hours in the present animal model.

Furthermore, in the animals infected with *P. aeruginosa* in lung, treatment with acyl-HSL-antagonist significantly prolonged the surviving time in a lethal bacterial infection, or speeded up the bacterial clearance, suggesting a promising therapeutic effect (Hong Wu et al. unpublished data).

Another possibility could be to inhibit or interfere with the generation of acyl-HSLs by providing inappropriate amino donor or acyl-ACP, which are required for the synthesis of acyl-HSLs[95]. A knockout mutation in the polyphosphate kinase (PPK) gene, encoding PPK responsible for the synthesis of inorganic polyphosphate from ATP, is defective in QS system and reduces significantly the productions of elastase and rhamnolipid which are controlled by the QS. The mutation makes *P. aeruginosa* bacterial cells unable to form thick and differentiated biofilm[96]. The conservation of PPK among many bacterial pathogens and its absence in eukaryotes suggest that PPK might be an attractive target for antimicrobial drugs[96]. Some common antibiotics, e.g. erythromycin or azithromycin have been shown to repress the acyl-HSLs synthesis in subminimal growth inhibitory concentrations[97,98]. The productions of *P. aeruginosa* hemagglutinins (including lectins), protease, hemolysin and acyl-HSLs are suppressed simultaneously by erythromycin at subminimal growth inhibitory concentrations. The antibiotic-treated bacteria also show reduced virulence to mice, endorsing clinical observations that indicate the efficiency of low-dose erythromycin treatment of persistent drug-resistant *P. aeruginosa* infections[97,98]. Tateda et al. reported that azithromycin of 2 μg/mL inhibited the QS circuitry of *P. aeruginosa* PAO1, and proposed that azithromycin interfered with the synthesis of autoinducers by an unknown mechanism and subsequently reduced the production of elastase and rhamnolipid[98]. The ideal approach to disrupting QS may modify or degrade acyl-HSLs. Usually acyl-HSLs are chemically stable at natural or acidic pH in aqueous solution[99], thus acyl-HSLs are enslaved to alkaline condition to be hydrolysed[100]. A new report from Leadbetter indicates that *Variovorax paradoxus* degrades and grows on acyl-HSLs as the sole energy and nitrogen sources. The bacteria degrade acyl-HSLs by cleavage and partial mineralization of the acyl-HSL ring. This study provides clues about the metabolic pathway of acyl-HSL degradation by *V. paradoxus*[101].

QS plays a crucial role in bacteria to deal with numerous challenges in their living circumstances. For pathogenic bacteria, the coordination and timing of the attack is extremely important to establishing the infection during which the chronic, biofilm phase greatly protect the bacteria from the host immune response and antibiotic treatment. Blocking or interfering with QS can lead to a significant attenuation of bacterial virulence without killing or influencing the growth of bacteria, suggesting QS might be a better target to create novel antibiotics in order to overcome the increasing antibiotic resistant problem.

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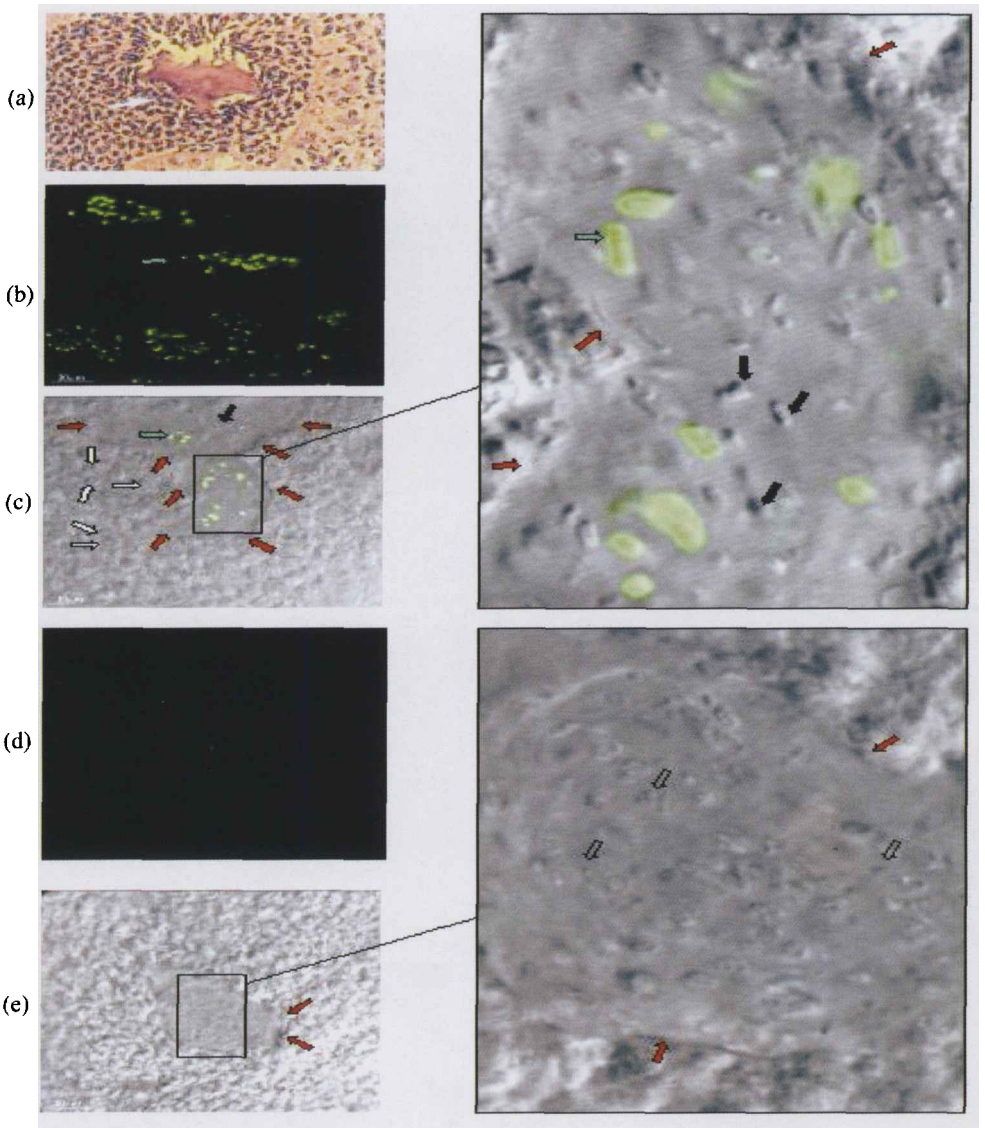

Plate Ⅰ. *In vivo* detection of AHL production in mouse lung tissues.

(a) Frozen section of the mouse lung tissues containing mixed inocula of *P. aeruginosa* PAO579 and *E. coli* JB357-*gfp*(ASV) were stained with hematoxylin and eosin. The white arrow points at an alginate bead surrounded by numerous PMNs in small bronchia three days post-challenge. (b) CSLM of the mouse lung tissues containing alginate beads three days post-challenge. Lung tissues with mixed inocula of *P. aeruginosa* PAO579 and *E. coli* JB357- *gfp*(ASV). (c) Epi- fluorescence superimposed on reflection image (computer generated) showing the presence of multiple green fluorescent monitor cells (green arrows) and non-fluorescent cells (black arrows). The borderline of the alginate bead is clearly seen (red arrows) surrounded by PMNs (white arrows). (d) Lung tissues inoculated with *E. coli* JB357-*gfp*(ASV) alone. (e) Epi-fluorescence superimposed on reflection image (computer generated) showing the absence of green fluorescent monitor cells (open arrows).