Quorum-sensing: The phenomenon of microbial communication

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Bacterial cells exhibit several density-dependent phenotypes. Such a biological phenomenon is referred to as quorum-sensing. Quorum-sensing is achieved through the production of an autoinducer by the organism, which upon acquiring a threshold concentration, a direct reflection of population density, is able to activate the genes, bringing into effect the concerned phenotypes. While this cell-to-cell communication has played an important role in many diverse community-based functions, it also helps the establishment of a population in changing environment according to the requirement. This sensing can be put to numerous uses such as in controlling plant and animal diseases, regulation of the production of useful/toxic fermentation products, etc. in the future.

Keywords: Autoinducer, bioassay, homoserine lactone, quorum-sensing.

SINCE the discovery of prokaryotic life, it was thought that these simple organisms have a unicellular existence and that their sole aim is to divide and produce more of their kind. The widely held view was that these unicellular organisms are solitary and respond to external stimuli isolatedly. This concept has undergone a radical change in recent times. It is now clear that a large number of developmental processes and other forms of behaviour not directly related to vegetative growth, generally ascribed to a multicellular organization, are critical elements in the biology of microorganisms as well. Several genetic and biochemical studies in the 1960s and 70s, provided compelling evidence for an 'organized social behaviour' employing sophisticated communication systems to coordinate the activities of individuals within a population. Once considered to be a rare phenomenon, restricted to a few scattered examples, it is now increasingly apparent that an extensive range of microorganisms have the ability to perceive and respond to the presence of neighbouring populations. The term 'quorum-sensing' has been employed to describe such density-dependent phenomenon. Such a system is accomplished by the extracellular accumulation of small, self-generated chemical signalling moieties that induce a concerted effort on behalf of a population to produce the desired phenotypic effect[1].

The term quorum-sensing was first used in a review by Fuqua et al.[2], which essentially reflected the minimum threshold level of individual cell mass required to initiate a concerted population response. The signal molecule used for communication was dubbed as 'autoinducer', owing to its origin inside the bacterial cell. The desired response can be arrived at by attainment of quorum employing the autoinducer and the process was labelled as 'autoinduction'. In other words, the whole circuit relies on the intracellular production and export of a low-molecular-mass signalling molecule, the extracellular concentration of which grows with the population density of the producing organism. The signalling molecule can be sensed and reimported into these cells, thus allowing the whole population to respond to changing environment/requirement once a critical concentration (corresponding to a particular cell density) has been achieved.

Several classes of microbially-derived signalling molecules have now been identified. Broadly, these can be divided into two main categories (i) amino acids and short peptide derivatives, commonly utilized by Gram-positive bacteria[3,4], and (ii) fatty acid derivatives, called homoserine lactones (HSLs) frequently utilized by Gram-negative members[5,6]. Whatever may be the nature of the signal molecule, the whole network functions by its reentry into the cell either via diffusion or an active transport[6]. The signalling mechanism involves subsequent interaction of the signal with an intracellular effector that will induce the pathway for the concerned phenotype.

Bioluminescence – the *Lux* system

The first incidence of such a biological phenomenon came to light with the discovery of luminescence produced by certain marine bacteria such as *Vibrio fischeri* and *V. harveyi*. These bacteria, when free-living in sea water (i.e. at low cell density) are non-luminescent. However, when grown to high cell densities in the laboratory, a *V. fischeri* culture bioluminisces with a blue-green light. Interestingly, this bacterium commonly forms symbiotic relationships with some fishes (such as the Japanese pinecone fish *Monocentris japonica*) and squid species (such as *Euprymna scolopes*)[7]. These marine animals carry a specialized organ called the light organ, in which bacteria like *V. fischeri* are housed. *E. scolopes* may express bio-
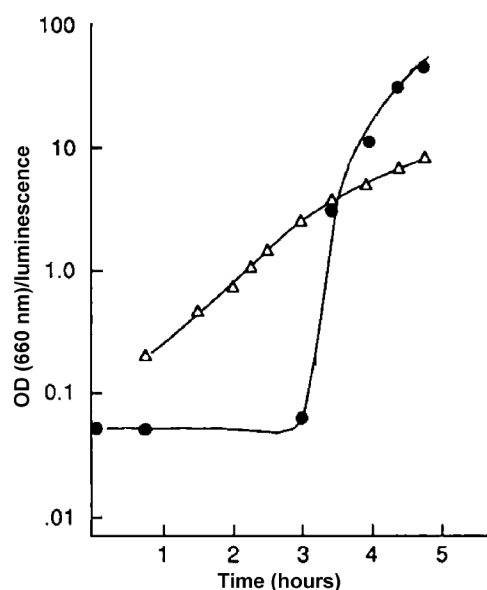
luminescent appearance in dark environments due to the maintenance of a high-density *V. fischeri* population (10^{10}–10^{11} cells ml^{-1}) in the light organ. This bioluminescent phenotype is exploited by the squid in order to perform a behavioural phenomenon called counter-illumination. At night, the squid camouflages itself from predators residing below it by controlling the intensity of light that it projects downwards, thus eliminating a visible shadow created by moonlight. This is a case of perfect symbiosis, as in return *E. scolopes* provides the *V. fischeri* population with nutrients. The presence of luminescent-competent *V. fischeri* cells in the light organ of juvenile squid is crucial for the correct development of this organ[7].

Further studies on *V. fischeri* revealed that the bacterium grows very fast, directly entering the exponential phase, but the luminescence increases only at about mid-log phase of its growth[8] (Figure 1). The sudden increase in luminescence was attributed to the transcriptional regulation of the enzyme, luciferase, which in turn corresponded to a threshold density of cells. This whole circuit is based on the bacterial assessment of its population density by means of release of chemical signalling molecules or autoinducers. The autoinducer then establishes a communication between the cells, that gets reflected in the expression of a particular gene, in this case, the luciferase gene (*lux*). For a long time, bioluminescene expressed by *V. fischeri* remained a model system to study density-dependent expression of a gene function[8].
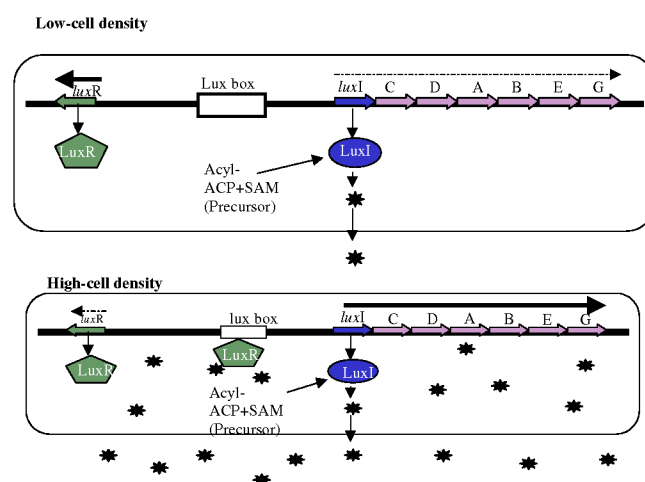
Molecular basis of bioluminescence regulation

The *lux* gene organization, regulation, function and molecular characterization of the luminescence system of *V.*

fischeri MJ1 became possible in 1983, through the cloning of a 9-kb fragment of its DNA that encodes all the functions required for autoinducible luminescence in the heterologous host *Escherichia coli*[9]. The bioluminescence gene cluster of *V. fischeri* consists of eight *lux* genes (*luxA–E*, *luxG*, *luxI* and *luxR*), which are arranged in two bi-directionally transcribed operons separated by about 218 bp[9] (Figure 2). This structure is referred to as *lux* regulon. One unit contains *luxR*, and the other unit, which is activated by the LuxR protein along with the autoinducer, contains the *luxICDABEG* operon[10]. The products of both the *luxI* and *luxR* genes function as regulators of bioluminescence[10]. The *luxI* gene is the only *V. fischeri* gene required for synthesis of the autoinducer, 3-oxo-hexanoylhomoserine lactone (3-oxo-C6-HSL) or OOHL in *E. coli*[9]. The *luxA* and *luxB* genes encode subunits of the heterodimeric luciferase enzyme. Luciferase catalyses the oxidation of an aldehyde and reduced flavin mononucleotide, and the products of this reaction are a long-chain fatty acid, water and flavin mononucleotide. Emission of blue-green light, with a maximum intensity at 490 nm, accompanying the oxidation reaction has led to this reaction being referred to as bioluminescence. The source of

Figure 2. The *Vibrio fischeri* LuxI/LuxR quorum sensing system circuit. There are six luciferase structural genes (*luxCDABEG*) and two regulatory genes (*luxR* and *luxI*) required for quorum-sensing-controlled light emission in *V. fischeri*. The genes are arranged in two adjacent but divergently transcribed units. The LuxI protein is responsible for synthesis of the HSL autoinducer N-(3-oxohexanoyl)-homoserine lactone (OHHL ✳). At low population density, *luxI* and *luxR* are transcribed at a low level and there is insufficient accumulation of pheromone signal (OHHL) to elicit the LuxR-dependent transcription of the *lux* operon for visible luminescence. As the cell population density increases, concentration of the autoinducer also increases both intra- and extracellularly. At a critical autoinducer concentration, the LuxR protein binds to the autoinducer and this complex binds to the *lux* promoter to activate transcription of the operon. This action results in an exponential increase in autoinducer synthesis via the increase in transcription of *luxI* and an exponential increase in light production via increase in transcription from *lux* operon. The LuxR-autoinducer complex also binds at the *luxR* promoter, but in this case the complex represses the transcription of *luxR*. This negative action compensates for the positive action at the *lux* promoter.

Figure 1. Development of luminescence (●) compared with growth (△) (measured by optical density), along time course. Luminescence was measured *in vitro* photometrically.

energy required for the emission of light is derived primarily from the oxidation of the substrates. Different luminescent bacteria may show differences in the luminescence spectrum and the colour of the emitted light due to sensitizer proteins that cause shift in wavelength[11]. While *luxC, D, E* encode products that form a multienzyme complex responsible for the synthesis of the aldehyde substrate utilized by the luciferase[12], *luxG* encodes a probable flavin reductase[13], and is followed by a transcriptional termination site[14]. A second acylhomoserine lactone synthase gene, *ainS*, has also been identified in *V. fischeri*[15].

The initial stage of bioluminescence induction involves an interaction between OOHL, often equated with a pheromone, and the transcriptional regulator protein, LuxR. *V. fischeri* cells express *luxI* at a basal level when present in low population densities, so the concentration of OOHL in the medium remains low. However, as the population density increases within the confines of a light organ, the concentration of OOHL in the environment also increases. As the critical concentration of OOHL is achieved (corresponding to a particular cell density or 'quorum' of the bacteria), OOHL diffuses back into the cell and binds to LuxR[16]. Once the autoinducer is bound to the N-terminal regulatory domain, multimer formation by LuxR is enhanced and the C-terminal domain activates transcription from both the *lux* operons[17]. LuxR functions probably by the OOHL-mediated induction of a conformational change.

The *lux* regulon is subjected to a tight regulation. *luxI*, together with *luxR* and the stretch of 218 base pairs separating the two operons constitutes the primary regulatory circuit. Expression of *luxR* is regulated by two regulatory proteins, LuxR and CAP[18]. A 20 bp region of dyad symmetry, the *lux* box, centred at −42.5 from the *luxI* transcription start site has been implicated as a LuxR binding site[2,19]. Induction of transcription from *luxICDABEG* operon increases the cellular levels of mRNA transcripts required both for bioluminescence and OOHL synthesis, a process referred to as autoinduction. With increase in the concentration of OOHL molecules, more of it diffuses into the cell and is able to activate more LuxR protein within the *V. fischeri* population. Thus, autoinduction ensures that bioluminescence and signalling molecule production continues.

As the autoinduction mechanism is not initiated until a population has achieved a particular cell density, individual *V. fischeri* cells avoid expending considerable amount of energy required for light emission as they are not host-associated. However, when OOHL is abundant, activated LuxR represses the transcription of *luxR*[20]. The mechanism of this autorepression is unknown, but it is dependent upon the presence of a *lux* box-type element located within *luxD*. The function of this repression is proposed to self-limit the autoinduction of bioluminescence[18].

Thus, three components are necessary to sense cell density: (i) a signal (a LuxI homologue), (ii) a means of rec-ognizing the signal (a LuxR homologue), and (iii) accumulation of the signal. Signal accumulation results either from increase in cell number in space with limited flowthrough or, theoretically, by enclosing the cells in a smaller space. The two bacterial groups, Gram-negative and Gram-positive, differ in the nature of the signal molecule. We shall discuss this aspect later in the article.

Phenotypes employing quorum-sensing systems

As this phenomenon is being examined in detail, many diverse and unrelated phenotypes throughout the microbial world that fall under its fold are being revealed. Many diverse microorganisms, both Gram-negative and Gram-positive use quorum-sensing systems to regulate phenotypes ranging from mating to virulence against the host, antibiotics and production of other metabolites, and scores of others[5] (Table 1). The common feature between these diverse phenotypes is the success of a microbial function based on appropriate population size, cross-communication to ascertain its own community or even differentiate self from non-self. For example, *Streptococcus pneumoniae* employs a peptide-mediated quorum-sensing system for establishing competence for genetic transformation[5]. In *Bacillus subtilis*, on the other hand, high cell density contributes to the regulation of at least two different developmental processes – development of genetic competence and sporulation. The mating system in *Enterococcus faecalis* has a peptide-pheromone-based quorum-sensing system[3]. In *Myxococcus xanthus*, the phenotypes affected are its unusual mode of cooperative feeding and the formation of a fruiting body[5]. Since the list is too long, we shall describe some of the well worked-out examples.
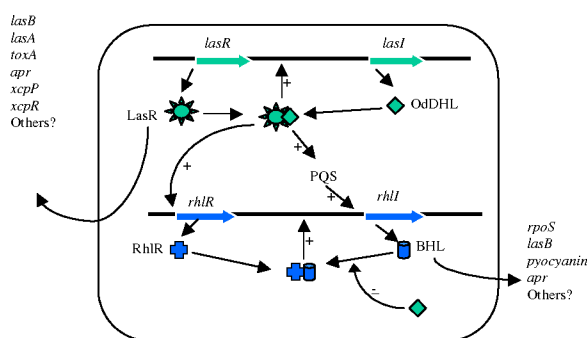
Virulence in some Gram-negative bacteria

Besides the classical example of bioluminescence exhibited by *Vibrio* species, virulence of many Gram-negative members is regulated by quorum-sensing[1]. In recent years, a number of studies have been conducted on *Pseudomonas aeruginosa,* an opportunistic human pathogen that produces and secretes multiple extracellular virulence factors that cause extensive host tissue damage. Production of virulence factors is under the hierarchial control of two pairs of LuxI/LuxR homologues, LasI/LasR[21] and RhlI/RhlR[22] (Figure 3). Both LasI and RhlI are autoinducer synthases that catalyse the formation of HSLs, N-(3-oxododecanoyl)-homoserine lactone (OdDHL) and N-(butanoyl)-homoserine lactone (BHL)[23] respectively.

At high cell density, LasR binds to its cognate HSL autoinducer, and together they bind at promoter elements immediately preceding the genes encoding a number of secreted virulence factors–elastase (*lasB*), a protease (*lasA*), endotoxin A (*toxA*) and alkaline phosphatase (*aprA*)[1]. LasR bound to autoinducer activates *lasI* expression,

Table 1. Some examples of cell–cell communication systems in bacteria

Genus	Phenotype	Signal molecule(s)	Reference
Aeromonas hydrophila	Extracellular protease, biofilm formation	C4-HSL	55
Aeromonas salmonicida	Extracellular protease	C4-HSL	55
Agrobacterium tumefaciens	Conjugation	3-oxo-C8-HSL	56
Bacillus	Competence, development	Peptides	25
Chromobacterium violaceum	Antibiotics, violacein, exoenzymes, cyanide	C6- HSL	38
Erwinia caratovora subsp. *caratovora*	Carbapenem antibiotic, exoenzymes	3-oxo-C6-HSL	42
Erwinia stewartii	Exopolysaccharide	3-oxo-C6-HSL	57
Escherichia coli	Cell division	?	58
Lactococcus and other lactic acid bacteria	Bacteriocin production	Peptides	59
Myxococcus	Development	Peptides, amino acids, others ??	60
Nitrosomonas europaea	Emergence from lag phase	3-oxo-C6-HSL	28
Pseudomonas aeruginosa	Multiple exoenzymes, Xcp, RhlR, biofilm formation, cyanide, RpoS, lectin, pyocyanin, rhamnolipid	3-oxo-C12-HSL C4-HSL	23
Pseudomonas aureofaciens	Phenazine antibiotic	C6-HSL	29, 49
Ralstonia solanacearum	?	C8-HSL	40
Rhizobium elti	Restriction of nodule number	?	61
Rhodobacter sphaeroides	Community escape	7-cis-C14-HSL	62
Serratia liquifaciens	Swarming, protease	C4-HSL	43
Streptococcus	Competence, virulence	Peptides	3
Streptomyces	Antibiotic production	γ-Butyrolactone	45
Vibrio(Photobacterium) fischeri	Bioluminescence	3-oxo-C10-HSL	41
Xenorhabdus nematophilus	Virulence, bacterial lipase	3-hydroxy-C4-HSL or an antagonist	63
Yersinia enteroliticola	?	C6-HSL	64
Yersinia pseudotuberculosis	?	3-oxo-C6-HSL, C8-HSL	65

Figure 3. Model of hierarchical quorum-sensing in *Pseudomonas aeruginosa*. The Las and Rhl quorum-sensing systems control the production of multiple virulence factors. The LasI protein produces the homoserine lactone signalling molecule N-(3-oxododecanoyl)-homoserine lactone (OdDHL), and the RhlI protein synthesizes N-(butanoyl)-homoserine lactone (BHL). The LasR protein binds the LasI-dependent autoinducer when this signal molecule has accumulated to a critical threshold level. The Las–autoinducer complex then stimulates the production of various virulence factors. The LasR–autoinducer complex also induces transcription of *rhlR* to initiate the second quorum-sensing circuit. RhlR bound to BHL activates the transcription of a subset of LasR-activated virulence genes that are not regulated by LasR. The *Pseudomonas* quinolone signal (PQS) is an additional regulatory link between the Las and Rhl quorum-sensing circuits. (+) indicates transcriptional activation and (–) indicates transcriptional repression of the concerned genes.

which establishes a positive feedback loop. The LasR–autoinducer complex also activates the expression of the second quorum-sensing system of *P. aeruginosa* by in-

ducing *rhlR*[23]. RhlR thus produced binds to RhlI and this complex induces the expression of two genes that are also under the control of the LasI/LasR system, *lasB* and *aprA*. RhlR–autoinducer complex also activates a second class of specific target genes. These genes include *rpoS*, which encodes the stationary phase sigma factor (σ^s) and *rhlAB*, which encodes rhamnosyltransferase and is involved in the synthesis of biosurfactant/hemolysin rhamnolipid, genes involved in pyocyanin antibiotic synthesis, *lecA* gene which encodes a cytotoxic lectin, and the *rhlI* gene[1,22]. Again, similar to LasI/LasR, activation of *rhlI* establishes an autoregulatory loop.

While on one hand, the LasR–autoinducer complex activates *rhlR* expression to initiate the second signalling cascade, the LasR-dependent autoinducer, OdDHL also prevents binding of the RhlI-dependent autoinducer, BHL to its cognate regulator[21]. This second level of control of RhlI/RhlR autoinduction by the LasI/LasR system ensures that the two systems initiate their cascades sequentially and in the appropriate order.

A third autoinducer has been recently demonstrated to be involved in quorum-sensing in *P. aeruginosa*. The signal, 2-heptyl-3-hydroxy-4-quinolone or PQS[24] (*Pseudomonas* quinolone signal) is unique because it does not fall under the category of homoserine lactones. PQS partially controls the expression of elastase gene *lasB* in conjunction with the Las and Rhl quorum-sensing systems. Expression of PQS requires LasR, and PQS in turn induces transcrip-

tion of rhlI. Thus, PQS serves as an additional link bet-ween the Las and Rhl circuits. It is believed that PQS ini-tiates the Rhl cascade by allowing production of RhlI-directed autoinducer only after the establishment of LasI/LasR signalling cascade.

Quorum-sensing is also employed by plant-associated bacteria for their pathogenic as well as symbiotic life-styles (see Table 1).

Quorum-sensing in some Gram-positive bacteria

A number of Gram-positive bacteria are also known to employ quorum-sensing systems. The nature of the signal molecules used in these systems differs from those of Gram-negative organisms[3,25]. Quorum-sensing is used to regu-late the development of bacterial competence in *Bacillus subtilis* and *Streptococcus pneumoniae*, conjugation in *Enterococcus faecalis*, and virulence in *Staphylococcus aureus*[25,26]. In pneuomococci, five genes have been im-plicated in the peptide-mediated regulatory circuit, viz., *com ABCDE* for competence development[27]. The peptide signal required for development of the competent state is called CSP (competence stimulating peptide). CSP is a 17-amino acid peptide that is produced from a 41-amino acid precursor peptide called ComC[27]. The ComAB-ATP binding cassette (ABC) transporter processes and secretes CSP extracellularly[26]. ComC expression is normally maintained at a basal level, allowing production of peptide in proportion to cell numbers. ComD acts as a membrane-bound receptor/kinase and acts through a response regulator, ComE, to transmit a signal reflecting the extracellular abundance of CSP to responder genes. High levels of CSP induce autophosphorylation of ComD, which leads to subsequent transfer of the phosphoryl group to ComE[26]. Phospho-ComE activates transcription of the *comX* gene. ComX is an alternative sigma factor that initiates the transcription of competence-specific operons involved in DNA uptake and recombination by recognizing a *com*-box (also referred to as *cin*-box) consensus sequence (TACGAATA) in their promoter regions[27].

Due to the highly diverse nature of phenotypes governed by cell–cell signalling cascade, it is difficult to describe all of them. Some of the important phenotypes are enlisted in Table 1, along with the nature of the signal molecule and the relevant reference from where the details can be ascertained. This list is by no means complete, as more and more phenotypes are being classified under the quo-rum-sensing pathway.

Nature of signal molecule

As described earlier, two general classes of signal mole-cules, exemplified by Gram-negative and Gram-positive bacteria, have been reported. These are: acyl homoserine lactones (AHLs) and peptide pheromones respectively.

Acyl HSL molecules

Diversity: Since their discovery, the family of N-acyl homoserine lactones seems to be almost the universal signal factor in Gram-negative bacteria. Earlier school of thought associated the production of AHLs by bacteria, with pathogenic or symbiotic nature. For example, oppor-tunistic pathogens such as *P. aeruginosa* and plant pathogens such as *Erwinia* produce AHLs. Besides the biolumines-cence caused by symbiotic strains of *Vibrio*, other symbi-otic strains such as *Rhizobium* were also found to be AHL producers (Table 1). In many of these organisms, AHLs induce the synthesis of compounds interacting with the host organism, such as toxins, antibiotics or exoenzymes. However, the observation that N-acyl homoserine lactones are also formed by certain planktonic phototropic bacteria indicated that the phenomenon was much more generalized and widespread and so also the phenotypes. Furthermore, AHLs have been found recently in natural microbial habi-tats, e.g. biofilm, microbial mats and algal blooms[28].

A relatively small but growing number of Gram-nega-tive bacteria have been discovered to have genes similar to either *luxR* or *luxI*. More often than not, a protein ho-mologous to LuxR functions in concert with a protein homologous to LuxI. Although there are certain highly conserved regions, the overall levels of sequence similarity are frequently rather low, often no higher than 18–25% for LuxR homologues and 28–35% for LuxI homologues. The AHLs known so far are the products of a *luxI*-type gene (AHL synthase) for the induction of bacterial species-specific reactions. AHLs formed by *luxI* bind to the prod-uct of the adjacent *luxR* gene, which in turn activates the transcription of genes responsible for the specific response. A variety of seemingly phylogenetically unrelated bacte-ria produce the same AHL, whereas some other bacteria form more than one type of AHL. How these compounds interplay in natural habitats is still incompletely known[29].

Although AHL signal molecules from various bacteria are related in structure, they can differ in nature of the acyl side chain moiety (Table 2). Depending on the parti-cular autoinducer, the acyl group varies from 4 to 14 carbons in length, possesses a hydroxyl group, a carbonyl group, or without a substitution on the third carbon, and is either fully saturated or contains a single carbon–carbon double bond. The overwhelming majority of microbial acyl HSLs identified so far have an even number of carbons in their acyl side chain. Many of the individual acyl HSL species are synthesized by representatives of different bacterial genera. Likewise, many bacterial species can produce more than one type of acyl HSL. The type of acyl HSL produced by a particular species can be strain-dependent. This, in turn, may reflect the differing habitats in which individual strains reside and respond to.

It was originally believed that acyl-HSL molecules were freely diffusible through cellular membranes. How-ever, this view is beginning to change. Welch *et al.*[30] offered

Table 2. Diversity exhibited by AHL molecules

Structure	Name
	N-butanoyl-L-homoserine lactone (C4-HSL)
	N-(3-hydroxybutanoyl)-L-homoserine lactone (3-hydroxy-C4-HSL)
	N-hexanoyl-L-homoserine lactone (C6-HSL)
	N-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL)
	N-octanoyl-L-homoserine lactone (C8-HSL)
	N-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C8-HSL)
	N-decanoyl-L-homoserine lactone (C10-HSL)
	N-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL)
	N-dodecanoyl-L-homoserine lactone (C12-HSL)
	N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL)
	N-tetradecanoyl-L-homoserine lactone (C14-HSL)
	N-(3-oxotetradecanoyl)-L-homoserine lactone (3-oxo-C14-HSL)
	N-(7-cis-tetradecanoyl)-L-homoserine lactone (7-cis-C14-HSL)
	N-(3R-hydroxy-7-cis-tetradecanoyl)-L-homoserine lactone (3-hydroxy-7-cis-C14-HSL)

evidence that the activation of CarR by a range of acyl HSLs was somewhat dependent on the ability of these ligands to avoid aggregation in the cellular membrane of *E. carotovora* subsp. *carotovora*. Another study identified an active efflux pump necessary for the effective translocation of 12-carbon acyl HSL in *P. aeruginosa*[31].

Autoinducer biosynthesis: Homoserine and related compounds are found in most bacteria as intermediates of the methionine–lysine–threonine biosynthetic pathway. One of the intermediates of the methionine/homocysteine pathway is S-adenosylmethionine (SAM). This and acyl–acyl carrier protein (acyl-ACP) function as subsrates for the LuxI-type enzymes[32] (Figure 4). Acyl-ACP is generated as an intermediate in fatty-acid biosynthesis. The LuxI proteins couple a specific acyl-ACP to SAM via amide bond formation between the acyl side chain of the acyl-ACP and the amino group of the homocysteine moiety of SAM. The subsequent lactonization of the ligated intermediate in the reaction, along with the release of methylthioadenosine, results in the formation of the HSL. The specificity for the interaction of a particular LuxI-type protein with the correct acyl-ACP is encoded in the acyl side chain moiety of the acyl-ACP[33]. This specificity provides a means for one LuxI-like protein to produce only one type of autoinducer. However, a few cases are known in which a LuxI-like protein synthesizes more than one type of HSL.
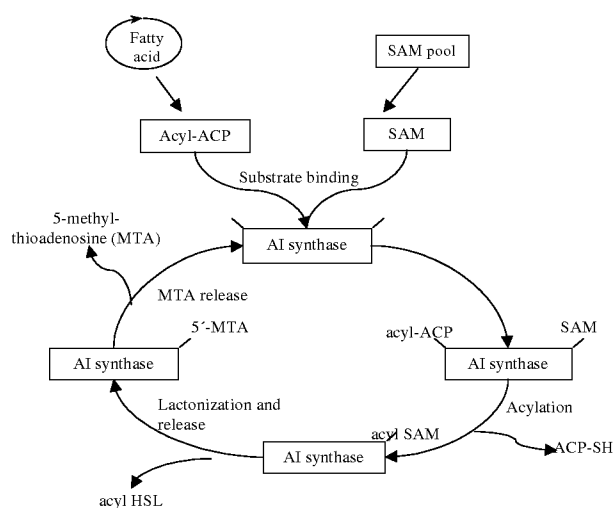
Detection through bioassays: Density-dependent expression of a phenotype has become a key regulatory component in responses as diverse as pathogenesis, symbiosis, antibiotic production, motility, genetic competence, conjuga-

tion, biofilm formation, and several others. Novel cell–cell signalling systems are continually being discovered, necessitating easy and reliable methods to detect and identify the cognate signal molecule. Numerous bioassays and sensor systems have been developed that allow facile detection, characterization and quantitation of microbial acyl HSLs[34–40]. Detection of acyl HSLs has been facilitated by the development of a variety of bioassay strains. Such strains contain an easily assayable reporter gene and lack all AHL synthases, such that reporter activity requires exogenous AHLs. Various reporter genes have been employed, including *lacZ*, *gfp*, *lux* and the production of an endogenous pigment. The commonly used bioassays and their basis have been compiled in Table 3.
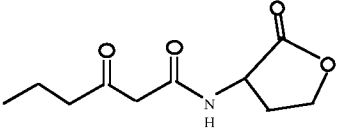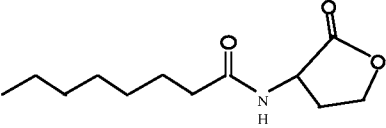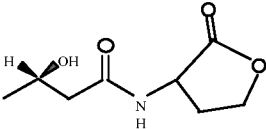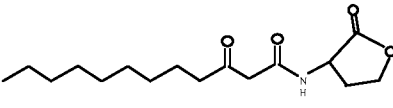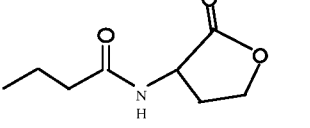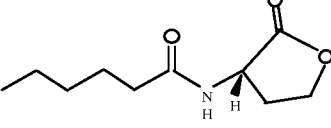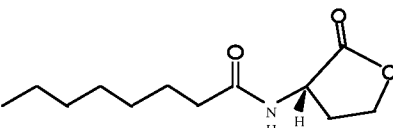
Monitoring through T-streaks or assays of conditioned media (Table 3), AHL biosensors greatly facilitate the characterization of quorum-sensing signal molecule(s) produced by a given organism. Other compounds produced by the target organism may give false-negative results in these assays because of bactericidal or bacteristatic effects on the biosensor. The extraction of AHLs from spent culture medium using organic solvents[41,42] can overcome this problem and also allow for concentration of any AHL present. Where possible, transformation of target organisms with biosensor plasmids can also circumvent the problem of antimicrobial activity[43]. Furthermore, the assay of reporter gene expression throughout growth in these transformed strains enables any cell-density dependent production to be determined. Broad-host-range vectors such as pSB403, based upon pRK415, are best suited to this type of study[35].

AHL biosensors have also been used effectively to screen for recombinant clones of AHL synthase genes in *E. coli*. Genomic libraries prepared from organisms activating the biosensor can be introduced into an *E. coli* strain containing an AHL reporter plasmid, and the resulting transformants can be screened for reporter activation[35]. Alternatively, patched libraries can be screened with biosensor overlays.

Chemical detection: AHLs can be purified by fractionating concentrated supernatant extracts using high-pressure liquid chromatography (HPLC)[32] or thin-layer chromatography (TLC)[34]. Separation of supernatant with organic molecules (e.g. dichloromethane, ethyl acetate) is made on the basis of differences in mass and polarity[32]. Using TLC overlay procedures[34], fractionation and detection is possible in 24 h. Spots of pigmentation or bioluminescence can be imaged and compared with known standards on the basis of R_f. HPLC is an effective method for the fractionation and preparation of AHLs for structural analysis. Biosensors can be used to identify active HPLC fractions, which can then be subjected to mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. The structure of the predicted molecule can then be confirmed by chemical synthesis[44].

Figure 4. Putative biosynthetic scheme for acyl-HSL synthesis by LuxI-type protein. SAM binds to the active site on LuxI, and the hexanoyl group is transferred from the appropriately charged ACP. The hexanoyl group forms an amide bond with the amino group of SAM, 5′-methylthioadenosine is released, and a lactonization reaction results in the synthesis of acyl HSL.

Table 3. Autoinducer bioassay strains and acyl homoserine lactone molecules detected by each strain

Relevant characteristics	Reporter strain	Assay	Structure	AHLs detected	Reference
V. fischeri ES114*lux* regulon with inactivated *luxI* in pBR322; Ap[R]	*E. coli* VJS533 (pHV200I⁻)	VAI		3-Oxo hexanoyl-HSL, hexanoyl-HSL[a], 3-oxo octanoyl-HSL, ocatnoyl-HSL[a]	22
traI::lacZ and *traR* on separate plasmids; pTi is cured; Cb[R], Km[R]	*A. tumefaciens* NT1 (pJM749, pSVB33-23)	AAI		3-Oxooctanoyl-HSLs, octanoyl-HSL, and other acyl-HSLs[a]	44
Unknown mutation resulting in reduced autoinducer production	*V. harveyi* D1	HAI		3-Hydroxy, butanoyl-HSL, 3-hydroxy valeryl-HSL	39
lasB::lacZ translational fusion and ptac-*lasR*; Ap[R]	*E. coli* MG-4 (pKDT17)	PAI-1		2-Hydroxy, 3-oxo, and unsubstituted acyl-HSLs with side chain lengths of 8-14 carbons	22, 49
rhlA::lacZ translational fusion and ptac-*rhlR* in pSW205; Ap[R]	*E. coli* XL1 Blue (pECP61.5)	PAI-2		Butanoyl-HSL, hexanoyl-HSL[a]	22
cviI::Tn5 xylE (inactivated *cviI*, an autoinducer synthase required for violacein production); Hg[R], Km[R], Cm[R]	*C. violaceum* CV0 blu	CAI		Hexanoyl-HSL, butanoyl-HSL, 3-oxohexanoyl-HSL[a], octanoyl-HSL[a], acyl-HSLs with longer side chains can be detected by screening for inhibition of hexanoyl-HSL-mediated violacein production	55
Inactivated *solI*, p395B contains *aidA::lacZ* fusion, Nx[R], Sp[R], Tc[R]	*R. solanacearum* AW1–AI 8 (p395B)	OHL		Unsubstituted acyl-HSLs with acyl side chains of 8 carbons or longer	40

[a]Indicates weak activation of the reporter.
Resistance to ampicillin (Ap[R]), nalidixic acid (Nx[R]), tetracycline (Tc[R]), kanamycin (Km[R]), carbenicillin (Cb[R]), spectinomycin (Sp[R]), and mercury (Hg[R]).

The peptide pheromone

To date, acyl-HSL production has not been shown for any Gram-positive bacterium, although the antibiotic-producing filamentous *Streptomyces* uses acylated-lactones (called γ-butyrolactones) as signals[45]. Aside from this specialized group, Gram-positive quorum-sensing systems typically make use of small post-transcriptionally processed peptide signal molecules[25] (Figure 5). These peptides are usually secreted by ATP-binding cassette (ABC) transporters. Some peptides interact with membrane-bound sensor kinases that transduce a signal across the membrane. Others are transported into the cell by oligopeptide permeases, where they then interact with intracellular receptors. While certain species of *Streptococcus* and *Enterococcus* produce unmodified linear peptides, some species of *Staphylococcus* and *Enterococcus* synthesize and release cyclic peptides[46]. As a generalization, both the linear and the cyclic peptide signals are synthesized as an unprocessed translation product and are subseque-

ntly processed and secreted into the external environment. While the Gram-negative bacteria use LuxR-type proteins for autoinduction, Gram-positive bacteria use two-component adaptive response proteins for the detection of the autoinducers[47]. The signalling mechanism is a phosphorylation/dephosphorylation cascade[3,46]. The secreted peptide autoinducer increases its concentration as a function of the cell population density. Two-component sensor kinases are the detectors for the secreted peptide signals. Interaction with the peptide ligand initiates a series of phosphorylation events that culminate in the phosphorylation of cognate response regulator protein. Phosphorylation of the response regulator activates it, allowing it to bind to the target DNA and alter the transcription of the quorum-sensing transcription controlled gene(s).

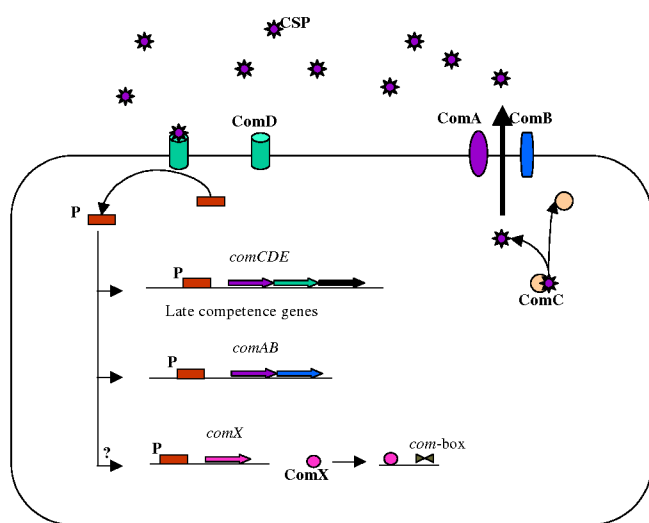## Microbial cross-communication: Environmental implications

As described, there is a widespread occurrence of cell–cell signalling among different bacterial species. Also, many organisms utilize the same species of molecule to regulate different phenotypes. Thus, one would predict that some form of interspecies communication would be likely in environments where different autoinducer-pro-

Figure 5. Model of quorum-sensing in *Streptococcus pneumoniae*. Induction of genetic competence is regulated by a CSP-mediated quorum-sensing system. Quorum-sensing involves the expression of early gene products encoded by two genetic loci, *comAB* and *comCDE*. Genes in the operon *comAB*, encode an ATP-binding cassette transporter (ComA) and an accessory protein to ComA (ComB). These secretory proteins are involved in the processing and export of the CSP. The loci, *comCDE*, respectively encode the precursor to the CSP, a histidine kinase that acts as a CSP receptor, and a response regulator that activates both *comAB* and *comCDE* operons. Quorum-sensing signals initiate competence through activity of ComX, a global transcription modulator, which was shown to act as an alternate sigma factor. This sigma factor initiates the transcription of competence-specific operons involved in DNA uptake and recombination by recognizing a *com*-box (also referred to as *cin*-box) consensus sequence (TACGAATA) in their promoter regions.

ducing bacterial species inhabit a common locale. Results from numerous studies have shown that various LuxR homologues can interact with non-cognate acyl HSL molecules[30,44,48,49]. Depending on the LuxR homologue and the acyl HSLs in question, such interactions can result in the activation of the specific transcriptional regulator. Conversely, when assayed in the presence of the cognate acyl HSL, other species of acyl HSL have been found to essentially block activation of the LuxR homologue, presumably by competing for the ligand-binding site on the protein.

It seems likely that in the environment, one bacterial community could produce acyl HSLs not to inhibit the quorum-sensing phenotypes expressed by another community but to regulate physiological processes of its own. One reason why bacteria like *Xanthomonas* spp. use non-acyl HSL-based quorum-sensing systems could be to gain a competitive advantage over their neighbouring bacteria by avoiding such interference and crosstalk. It is important to remember that a bacterial species could also respond to the presence of foreign acyl HSLs by utilizing the signalling molecules to up- or down-regulate competitively advantageous phenotypes. For example, this phenomenon could be used for expression of competitor-inhibitory antibiotics. A study led by Wood *et al.*[49] demonstrated that phenazine biosynthesis can be stimulated in one population of *P. aureofaciens* by acyl HSLs produced by a distinct population of the same organism. Similarly, TraR of *A. tumefaciens* can also respond to signals (cognate and non-cognate) produced by other microorganisms that occupy its habitat[48]. Thus, the development of specific signalling molecules used to sense not only conspecific bacteria but also certain nonconspecific bacteria present in certain specific niches, is a clever mechanism for community-level regulation of gene expression.

Many of the recognized acyl HSL-producing microorganisms are renowned for their capacity to associate with higher organisms either in a pathogenic or symbiotic relationship. Higher organisms have also evolved mechanisms that enable them to detect and respond to acyl HSL messaging systems in order to prevent or limit infection[50,51]. For example, the macroalga *Delisea pulchra* produces compounds, commonly known as furanones, which have the ability to specifically interfere with acyl HSL-mediated quorum-sensing systems[50].

While serving as prokaryotic cell-to-cell signals, some acyl HSLs may act as virulence factors per se. In particular, one of the *P. aeruginosa*-produced molecules, OdDHL, could act as a potential modulatory agent of the mammalian immune systems[52]. Interspecies communication through the use of autoinducers has been inculcated as a possible mechanism by which the pathogenicity of certain virulent bacteria such as *Burkholderia cepacia* is enhanced[53]. Thus, when determining the role of autoinducers in nature, the synergistic effects of the surrounding environment, including other bacteria and/or host must be considered.

Exploiting quorum-sensing

A great number of bacteria employ quorum-sensing for regulation of various phenotypes as a part of their pathogenic or symbiotic lifestyles. As such, the ability to block or promote these systems provides a powerful tool to solve many problems and enhance productivity. Genetically modified plants can be used to produce AHLs to manipulate plant–bacterium associations[1]. Quorum-sensing may also provide a much needed strategy to control multiple-drug-resistant strains of bacteria that employ quorum-sensing for virulence and pathogenicity and for control of trigger of host-cell response, for novel antimicrobial targets and immunomodulatory agents[54]. Therefore, it is important to take a look at the methods employed, which can enhance/degrade this signalling cascade[1].

Concluding remarks

It is only recently that the complexity and scope of quorum-sensing-specific bacterial regulation has been appreciated by many in the scientific community. Far from being singular entities, it is now apparent that bacteria exist in multifaceted communities and are constantly communicating with each other. However, our current understanding of the extent and significance of bacterial intercellular communication is still in its infancy. The importance of this field of study is being appreciated as it is already showing signs of becoming a promising solution to man's most troublesome problems such as biofilm formation on surgical equipments and other aspects of pathogenicity[1].

While the list of bacteria that utilize quorum-sensing systems is ever growing, it is unlikely to be complete. It is therefore certain that our current comprehension of quorum-sensing is severely limited and that the true extent of bacterial cell–cell communication in the environment and its significance await further and exciting discoveries.

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