Self perception in bacteria: quorum sensing with acylated homoserine lactones

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A variety of Gram-negative bacteria produce membrane permeant, acylated homoserine lactone (HL) pheromones that act as cell density cues. Synthesis and response to these factors requires proteins homologous to the LuxI acylhomoserine lactone synthase and the LuxR transcription factor from *Vibrio fischeri*. Recent genetic and biochemical studies have begun to provide a mechanistic understanding of acyl HL dependent gene regulation. Examination of the role of acyl HLs in diverse bacteria positions LuxR–LuxI type systems within an increasingly broad regulatory context and suggests that, in some bacteria, they comprise a global regulatory circuit.

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Abbreviations

ACP	acyl carrier protein
HL	homoserine lactone
MTA	5-methylthioadenosine
SAM	*S*-adenosylmethionine

Introduction

In animals and plants, membrane-permeant signal molecules, including steroid and brassenosteroid hormones, nitric oxide and methyl jasmonate, are well established as important regulators of development, cell differentiation and metabolism. Considering the widespread occurrence of such diffusible signals in eukaryotic organisms, it is not entirely surprising that prokaryotes also employ membrane-permeant signal molecules. Over the past few years, it has become clear that a diverse collection of Gram-negative bacteria produce acylated homoserine lactone (HL) derivatives that function in cell–cell communication. Bacteria utilize acyl HLs to monitor the density of cells within their own population in a process called quorum sensing [1–4]. Each cell in the population produces a low basal level of the diffusible acyl HL via the activity of an acyl HL synthase, usually a member of the LuxI family of proteins. As the bacterial population density rises, the concentration of the acyl HL also increases. At a sufficiently high population density, in essence a bacterial quorum, the accumulated acyl HL interacts with a receptor protein, usually a member of the LuxR family of transcription regulators, that acts to control a specific constellation of acyl HL dependent genes. In different bacteria, the basic mechanism of acyl HL regulation is conserved but the target genes are extremely varied. The first acyl HL quorum sensor to be fully described was that of the marine bacterium *Vibrio fischeri* that regulates bioluminescence (*lux*) gene expression (for a review see [5]); however, acyl HL quorum sensors in other bacteria regulate a range of functions including virulence genes in *Pseudomonas aeruginosa*, conjugal transfer in *Agrobacterium tumefaciens*, swarming motility in *Serratia liquefaciens*, and antibiotic production in *Erwinia caratovora* [1,6]. New acyl HL quorum sensors with different regulatory targets are reported monthly and the past five years have seen the list of bacteria known to employ these regulatory systems grow from certain bioluminescent marine vibrios to a large group of phylogenetically diverse bacteria.

The purpose of this review is to highlight several topics within the area of acyl HL quorum sensing that have seen particularly rapid progress during the past two years (see [2–4] for more comprehensive reviews). We will discuss the biochemical basis of acyl HL synthesis and perception as well as the global nature of at least a subset of these regulatory systems.

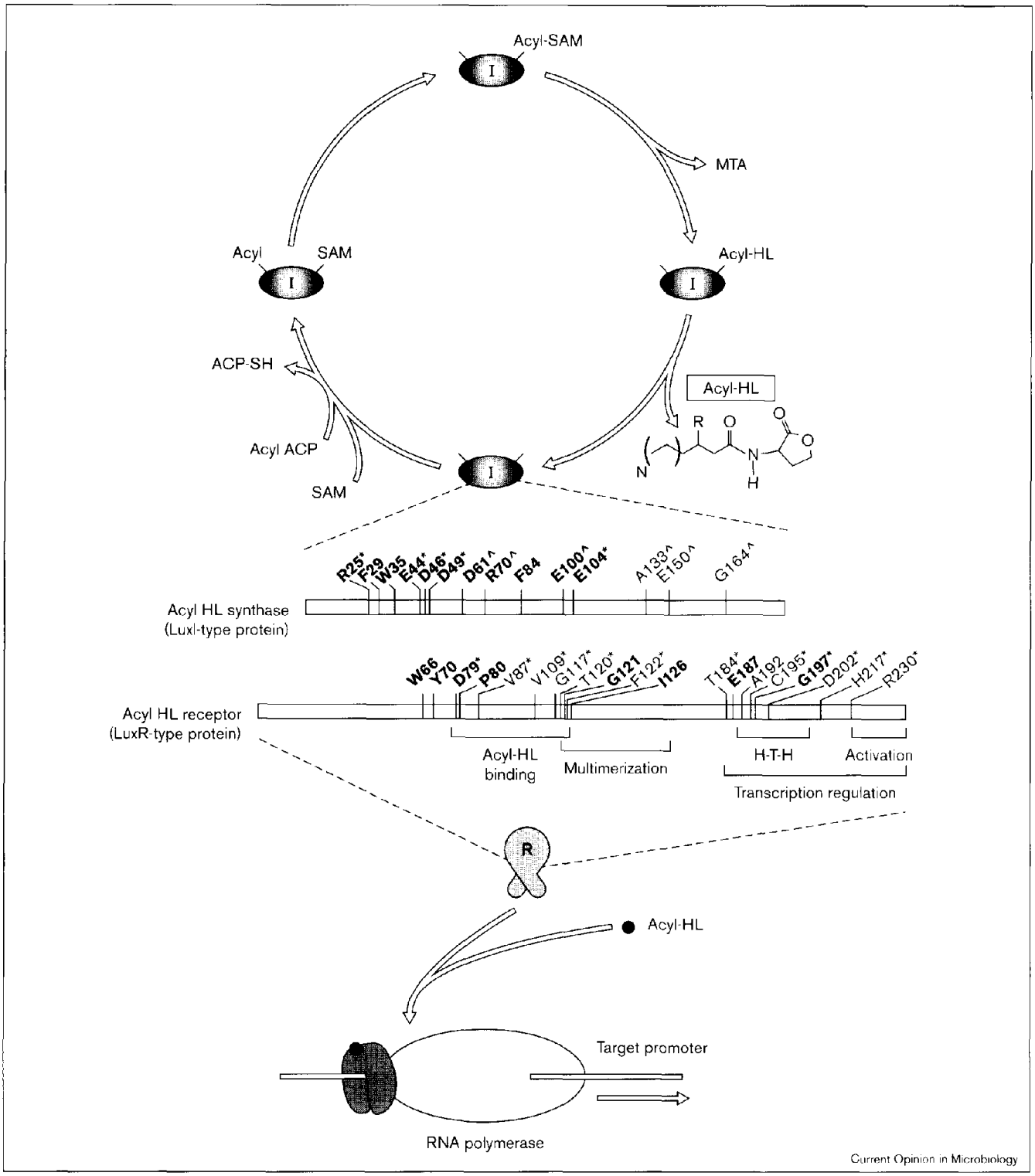
Enzymology of acyl HL synthesis

Acyl HLs have the general structure of an acyl chain of variable length and chemistry conjugated via an amide linkage to a lactonized homoserine moiety (Figure 1). Genetic studies of *luxI* and related genes clearly indicated a role in synthesis of the acyl HL signal. Only recently, however, has it been been established that LuxI-type proteins are in fact acyl HL synthases that recruit their substrates from normal metabolic precursors.

Studies of LuxI and its homologs TraI from *A. tumefaciens* and RhlI from *P. aeruginosa* show that these proteins catalyze the ligation of *S*-adenosylmethionine (SAM) and a fatty acyl chain derived from acyl–acyl carrier protein (ACP) conjugates ([7,8••,9••]; MR Parsek, EP Greenberg, unpublished data). A catalytic model has been proposed that entails the nucleophilic attack of the C1 position of the acyl–ACP conjugate by the amino nitrogen of SAM, generating an amide linkage (Figure 1). The lactonization of SAM, subsequent to, or concominant with, amide bond formation, results in ring formation and release of the acyl HL, as well as the formation of the side product 5-methylthioadenosine (MTA).

Although this basic mechanism is likely to be correct, there remain a number of catalytic details that are unclear; for example, acyl transferases that interact with ACP-linked

Figure 1



Structure and function of LuxR-type (R) and LuxI-type (I) proteins. Proteins are depicted in linear form. Amino acid residues conserved in all members of the protein family, or implicated from mutational analysis, are marked by vertical lines. Conserved amino acid residues are in bold and positions where mutations have been found to abolish activity (*) are indicated. For LuxI-type proteins, positions where inactivating mutations have been isolated in LuxI or RhlI, but not both, (^) are indicated. The upper portion of the figure depicts a proposed model for acyl HL synthesis by LuxI-type proteins. The lower portion of the figure illustrates transcriptional stimulation by LuxR-type transcriptional activators. H–T–H, helix–turn–helix. SH, sulfhydryl group on 5′phosphopantethiene prosthetic group of ACP.

substrates often form transient covalent linkages with the acyl chains via ester or thioester bonds at cysteine or serine residues. The existence of a conserved cysteine residue and a conserved serine residue (Cys68 and Ser104 in LuxI) prompted the proposal that acyl HL synthesis might involve a transient acylation mechanism [2,8••,10]. Directed mutational analyses of the LuxI, TraI and RhlI acyl HL synthases, however, have revealed that the conserved cysteine (as well as several nonconserved cysteines in each protein) is dispensable for activity ([11,12]; C Fuqua, unpublished data). A mutation in the conserved serine in TraI abolishes activity, but this residue is not essential in LuxI and RhlI, suggesting an indirect role in TraI function. Although these results demonstrate that transient acylation does not occur at these residues, it remains possible that a covalent linkage occurs with another residue in the protein.

Mutational analyses of LuxI and RhlI have indicated a role for conserved charged residues clustered in the amino-terminal halves (residues 25–104 in LuxI; see Figure 1) of these proteins in acyl HL synthesis [11,12]. Seven out of the ten residues that are absolutely conserved among all LuxI-type proteins are charged (Arg25, Glu44, Asp46, Asp49, Arg70, Glu100, and Arg104 in LuxI), and mutations in any of these residues can lead to a loss of activity. Many other enzymes that utilize SAM as a substrate, namely, the methyl transferases, also employ charged residues within defined SAM-binding sites [13,14]; however, there are no discernable SAM-binding sites in the amino acid sequences of acyl HL synthases. Mutations in the three nonpolar conserved residues (Pro29, Trp35 and Pro84 in LuxI) significantly reduce, but do not abolish, activity [11,12]. Although a region in the carboxy-terminal half of LuxI was implicated in catalysis (residues 133–164; see Figure 1), mutations within the corresponding region of RhlI do not disrupt activity [12].

Putative acyl HL synthases, AinS and LuxM, that share no homology with LuxI have been identified in *V. fischeri* and *V. harveyi*, respectively. These two proteins share a region of strong sequence conservation with each other and are required for acyl HL synthesis [15,16••], and thus appear to represent a second family of acyl HL synthases. Preliminary *in vitro* studies suggest that AinS will synthesize its cognate acyl HL (*N*-octanoyl homoserine lactone) using SAM and octanoyl-ACP (BH Hanzelka, PV Dunlap, EP Greenberg, unpublished data). Further comparisons between these proteins and LuxI-type acyl HL synthases should help to establish general enzymatic requirements for acyl HL synthesis.

Acyl HL perception and transcriptional activation by LuxR-type proteins

Genetic analyses of LuxR suggest that it is composed of two functional modules or domains: an amino-terminal domain (residues 20–156) with an acyl HL binding region (residues 79–120) and a carboxy-terminal transcription regulation domain (residues 160–250), which includes a helix–turn–helix DNA-binding motif (residues 190–210; see Figure 1). Mutations in key residues within the amino-terminal acyl HL binding domain abolish or severely reduce responsiveness to *N*-3-oxohexanoyl HL (Figure 1; see [17,18]). Furthermore, the amino-terminal half of the protein (residues 1–193) is sufficient to sequester radiolabeled *N*-3-oxohexanoyl HL when expressed in *Escherichia coli* and deletions or missense mutations resulting in acyl HL nonresponsive LuxR alleles abolish this binding [19].

The DNA-binding domains of LuxR-type proteins share sequence similarity with a much larger group of transcription factors (the so-called LuxR or FixJ superfamily; see [20,21]). LuxR molecules consisting of amino-terminal deletion polypeptides (residues 156–250 and 162–250) show acyl HL independent activation of *lux* genes [22]. These results suggested that in the absence of acyl HL, the amino-terminal half of the protein blocks the function of the DNA-binding domain. Interaction with the acyl HL abolishes this inhibition and allows transcriptional activation. Similar amino-terminal inhibitory domains have been described for FixJ and NarL, two component-type response regulators of the LuxR superfamily that do not interact with acyl HLs but are instead phosphorylated at a conserved aspartate residue [21,23]. The recent three-dimensional structure of NarL reveals that the amino-terminal domain of this protein in the inactive, nonphosphorylated state effectively blocks residues within the helix–turn–helix motif, presumably preventing DNA binding [24•].

In addition, the observation of dominant-negative effects when inactive alleles of both LuxR and the TraR protein from *A. tumefaciens* are coexpressed with the wild-type proteins suggest that transcriptional activation of target promoters requires formation of multimers [25,26]. It remains unclear how the inhibitory effect of the amino-terminal acyl HL binding domain is integrated with multimerization. Perhaps binding of the pheromone elaborates a sequestered dimerization site and, in turn, dimerization facilitates DNA binding. The mechanistic integration of these two processes is possibly reflected by the observation that amino acid substitutions that result in acyl HL independent *luxR* proteins have been isolated across the length of the protein (residues 9–236; see [27,28]).

Transcriptional activation of the *lux* operon by LuxR requires a *cis*-acting DNA element, a 20 base pair inverted repeat centered at about –40, now called the *lux* box [1,29]. Similar *lux* box-like sequences are found upstream of at least some promoters regulated by LuxR-type proteins in other bacteria [1,30–33]. Specific DNA binding by LuxR-type proteins has been reported. Using a truncated, acyl HL independent allele of LuxR, Stevens *et al.* [34,35] demonstrated specific binding to the *lux* regulatory

DNA; however, binding to the *lux* box and *lux* promoter, as assessed by DNase I protection analysis, was only observed in the presence of RNA polymerase. It was therefore impossible to determine which of the two proteins was responsible for the observed protection. It is unclear whether this apparent synergism reflects the mechanism of activation by LuxR or is an artifact of using the acyl HL independent protein. More recently, the full length LasR protein from *P. aeruginosa*, purified as a glutathione-*S*-transferase (GST) fusion, was reported to bind to *lux* box-like sequences upstream of the *lasB* promoter sequences in the absence of RNA polymerase [36]. Further studies are required to confirm this report.

There are several acyl HL regulatory systems, thus far exclusively from the plant pathogenic *Erwinia* species, in which the LuxR-type protein acts as a transcriptional repressor rather than an activator ([37]; S Beck von Bodman, personal communication). As yet, little is known about LuxR-type repressors, although their mechanism of action is likely to deviate substantially from those employed by LuxR-type transcriptional activators.

Global gene regulation by acyl HL quorum sensors

The discovery of acyl HL based quorum sensing in diverse bacteria has provided several examples of acyl HLs that regulate multiple genes of vastly different function within the same bacterium. The most extensively studied of these is found in the opportunistic plant and animal pathogen *P. aeruginosa* (Figure 2), where recent genetic analyses have revealed a broad range of target genes. The LasR transcriptional regulator and *N*-3-oxododecanoyl HL (synthesized by the LasI protein) were identified by the role they play in regulating the expression of the *lasB* gene, which encodes an elastase involved in virulence [38,39]. LasR also affects the expression of the *lasA* gene (a second elastase), the *apr* alkaline protease, and may indirectly influence exotoxin A (ETA) activity (Figure 2; [40,41]). In addition, LasR controls the expression of the gene for a second LuxR-type protein called RhlR [42••,43••]. The RhlR protein was identified as a regulator of genes required for rhamnolipid biosynthesis (*rhlAB*) and requires the activity of the RhlI acyl HL synthase to generate its inducing ligand *N*-butyryl HL [44–47]. *P. aeruginosa* mutants deficient in *N*-butyryl HL synthesis do not produce the secondary metabolites pyocyanin and hydrogen cyanide, and also manifest defects in elastase gene expression [44,48]. Furthermore, expression of *xcp* genes encoding proteins of the outer membrane component of the general secretion pathway is activated by LasR and RhlR [49•]. Finally, there is a report that exogenous *N*-3-oxohexanoyl HL promotes phenazine antibiotic synthesis, although the mechanism for this is unclear [50].

In a possible explanation for the alarmingly broad regulatory net of acyl HLs in *P. aeruginosa*, recent reports suggest that RhlR and *N*-butyryl HL are required for expression of the *rpoS* stationary-phase sigma factor gene (Figure 2; [42••,51]). In *E. coli* there are a wide variety of genes that are known to be part of the RpoS regulon [52], and it is assumed that the *P. aeruginosa rpoS* gene product has a similarly wide regulatory influence. Alterations in specific functions in a *las* or *rhl* mutant background may reflect direct control via the quorum-sensing cascade, or indirect control by RpoS. Clearly, in addition to the range of functions that have been shown to be definitively within the Las–Rhl signal cascade, the effect of placing *rpoS* under acyl HL control should greatly broaden the Las–Rhl regulon.

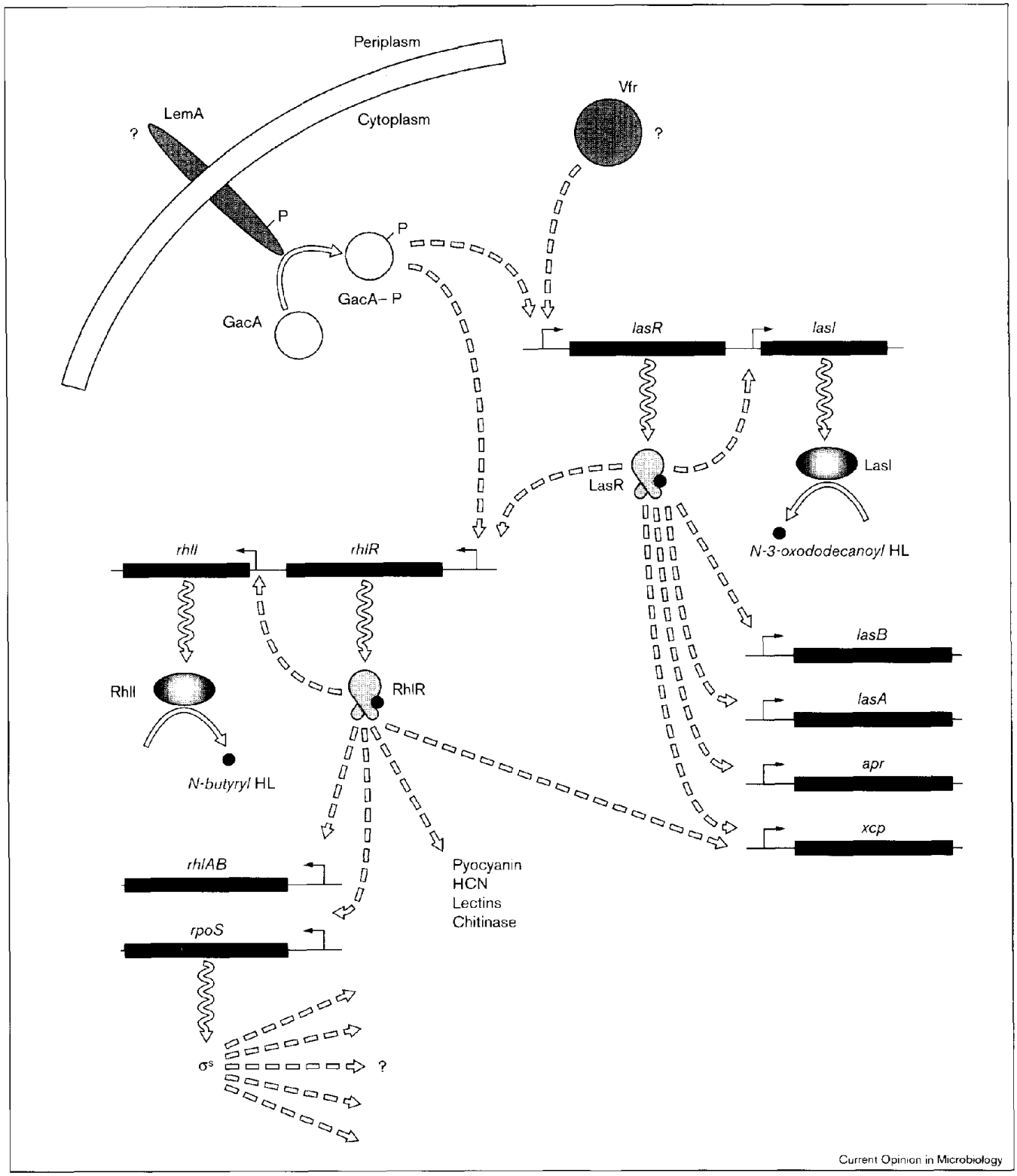
Although population density (acyl HL) and nutritional status of the cell (RpoS) are not obligately linked, they do overlap. It is not entirely surprising that bacteria have evolved mechanisms for integrating responses to both. An interesting example where both conditions are likely is in natural bacterial surface films. Indeed, recent work directly demonstrates the production of acyl HL signals by naturally occurring biofilms [53]. The importance of acyl HL quorum sensing in biofilm metabolism is an area of active investigation. Studies with *P. aeruginosa* mutants suggest that *lasI* is essential for proper biofilm development [54].

LasR and *N*-3-oxododecanoyl HL stand at the head of an impressive and rapidly expanding list of quorum-dependent functions in *P. aeruginosa*; however, there are several reasons not to consider LasR as a master switch. Recent studies suggest that *lasR* gene expression is itself regulated by the Vfr protein (virulence factor regulator, a homolog of the *E. coli* catabolite repressor protein) in response to an as yet unidentified signal [55,56]. Furthermore, it has also been proposed that *rhlR* and perhaps *lasR* are under the control of the GacA protein, a two-component response regulator and, in fact, a member of the LuxR/FixJ superfamily [57]. GacA and its sensor kinase partner LemA are known to regulate the expression of a variety of virulence genes in pathogenic pseudomonads in response to an unidentified environmental signal [58]. GacA and LemA also control the expression of the *phzI* acyl HL synthase that directly regulates phenazine antibiotic synthesis in the pseudomonad *P. aureofaciens* ([59]; S Chancey, LS Pierson, personal communication); therefore, in at least two pseudomonads, GacA–LemA regulation influences the function of a LuxR–LuxI type quorum sensor. Future research should address the ways in which LuxR–LuxI systems are integrated into the general regulatory circuits of a bacterial cell.

Conclusion

Over recent years, acyl HL dependent gene regulation has received increasing recognition as an important form of cell–cell communication in Gram-negative bacteria. Often, the genes controlled by LuxR–LuxI type quorum sensors are involved in important microbial processes,

Figure 2



Quorum sensing and global regulation in *P. aeruginosa*. A proposed regulatory cascade incorporating the *las* and *rhl* quorum sensors. Dashed arrows indicate activation of target promoters. Solid open arrows indicate enzymatic activity: kinase activity for LemA and acyl HL synthesis by LasI and RhlI. Squiggles indicate translation products. Although the response regulator GacA is shown in association with its cognate sensor kinase LemA, there is as yet no experimental evidence to support this speculation. Unidentified environmental signals to which GacA (LemA) and Vfr respond are indicated as question marks, as are those genes transcribed by *P. aeruginosa* σ^S RNA polymerase.

including microbial–host interactions such as pathogenesis and symbiosis. As our understanding of the biochemical mechanisms by which bacteria synthesize and respond to acyl HLs increases, human intervention designed to manipulate this regulation and to harness or mollify quorum-dependent gene products will become possible. Furthermore, the observation that acyl HL regulation can act in a global fashion suggests that a wide range of functions may be targeted with a single strategy.

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