

THE EMERGENT PROPERTIES OF QUORUM SENSING: CONSEQUENCES TO BACTERIA OF AUTOINDUCER SIGNALING IN THEIR NATURAL ENVIRONMENT

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21

The discovery that *Vibrio fischeri* cells regulate the production of bioluminescence through the use of a cell density-sensing mechanism first termed autoinduction has led to the identification of a common theme among many different species of bacteria, namely, the coordinate control of a set of genes in response to the presence of a quorum of like bacteria (Fuqua et al., 1994). This control occurs through the synthesis of one or more autoinducer (AI) molecules, whose accumulation and recognition by a specific protein receptor/regulator present inside the bacterial cell are responsible for the autoinduction phenomenon. Although this mechanism for sensing the density of sibling cells in a particular environment is apparently not used by all bacteria, there is increasing evidence for its widespread adoption among bacteria found in a variety of biological niches (chapter 19 of this volume; Fuqua et al., 1994). In addition, a number of the AI-producing species carry two or more AI synthase/regulator gene pairs (Kuo et al.,

1994; Pesci et al., 1997). In some of these bacteria, one pair is controlled by another (Pesci et al., 1997), while there is at least a single example in which two autoinduction systems appear to encode redundant regulatory functions (Bassler et al., 1994). Finally, the sets of genes modulated by AI systems are diverse in function, and the mode of regulation, whether positive or negative, also varies.

The best-studied form of AI signaling is the communication between conspecific, or genetically identical, bacterial cells. In the case of many gram-negative bacteria like *V. fischeri*, the signaling molecule is an acyl derivative of homoserine lactone (HSL) [e.g., the *N*-(3-oxohexanoyl)-HSL (3-oxo-C6-HSL), produced by *V. fischeri* (Eberhard et al., 1981)] that freely diffuses through the cell's membranes (Kaplan and Greenberg, 1985). When such an AI is produced by a population of cells in an enclosed space, it accumulates externally, giving rise to an equivalent level (typically in the nanomolar to micromolar range) within the cells. Intracellularly, the AI is believed to bind a receptor protein, which assumes a conformation that stimulates changes in gene expression (Hanzelka and Greenberg, 1995; Schaefer et al., 1996; Sitnikov et al., 1995). An analogous, but distinct, type of cell-density signaling results from the secretion of short

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peptides into the external medium by bacteria such as *Enterococcus faecalis*, *Bacillus subtilis*, and *Staphylococcus aureus* (Kleerebezem et al., 1997). Both HSL-type and peptide-type cues allow bacteria to coordinate expression of genes that are useful and/or required only when a sufficiently large population of cells is present, such as genes that regulate swarming motility (activated by an HSL derivative in *Serratia liquefaciens*) or conjugation (signaled by peptides in *E. faecalis*). For the purposes of this chapter, we will use the term AI only to refer to molecules that are derivatives of HSL.

Recent studies have demonstrated that communication through the use of AI molecules is not limited to recognition among cells of the same species. Although there is often considerable specificity in the recognition of AIs by their receptor proteins, there are examples of cross-species, as well as some cross-genus, regulation by AIs (Bassler et al., 1997; Greenberg et al., 1979; McKenney et al., 1995). Furthermore, many of the genes regulated by these quorum-sensing systems encode important virulence determinants, and evidence is accumulating that AIs can play a significant role in the signaling that occurs between bacteria and their eukaryotic hosts (DiMango et al., 1995; Dunphy et al., 1997; Visick et al., 1996).

The regulation of gene expression by a variety of AIs and their cognate receptors is reviewed at length in other chapters of this volume. However, while the molecular biology and biochemical mechanisms underlying autoinduction have been well studied in culture, the functioning of this signaling mechanism under natural biological conditions has been more difficult to assess (Fig. 1). In this chapter, we focus on what is currently known about the emergent properties of quorum sensing, the properties of a biological system whose existence cannot be predicted simply through a complete description of the components making up an organism (Campbell, 1993). The classic example of emergent properties comes from studies of sickle cell anemia, in which morphologically deformed cells syn-

thesized a human hemoglobin molecule with an altered affinity for oxygen and led biochemists to interpret this condition to be a genetic defect. Only after studies by population biologists, who demonstrated that this trait was maintained in certain human populations, and by ecologists, who linked the distribution of these populations with the co-occurrence of malaria, was it revealed that the "defective" hemoglobin played a crucial protective role against this endemic disease. Thus, only by discovering the presence of the emergent properties, through studies at the population and ecological levels, can we begin to assign an importance to their existence. It seems particularly relevant to address these levels when considering quorum sensing, a mechanism of signaling that, by definition, operates among a population of individual bacterial cells and within a specific subset of environments.

In this chapter we discuss three areas concerning the emergent properties of quorum sensing: (i) the potential consequences to a bacterium of possessing an extracellular signaling system, (ii) the use of AIs in the interactions of quorum-sensing bacteria with genetically distinct organisms, and (iii) some new frontiers in our understanding of AIs in the bacterial cell's extracellular environment.

CONSEQUENCES OF AN EXTRACELLULAR SIGNALING SYSTEM

By their nature, AIs are external signals and, as such, are not without risks as a method of constructive signaling between one bacterium and another. Some of these consequences, their negative and positive effects, and the possible defenses erected by the producing bacterium are outlined in Table 1. For example, alkaline conditions (such as are characteristic of seawater, pH 8.5) potentiate the breakdown of acyl HSL derivatives (Eberhard et al., 1981) and thus could cause attenuation of the cue. While a fast turnover would result in a waste of cellular resources dedicated to AI production for bacteria present in such an en-

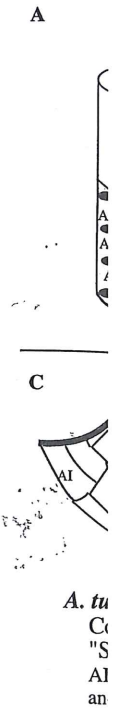


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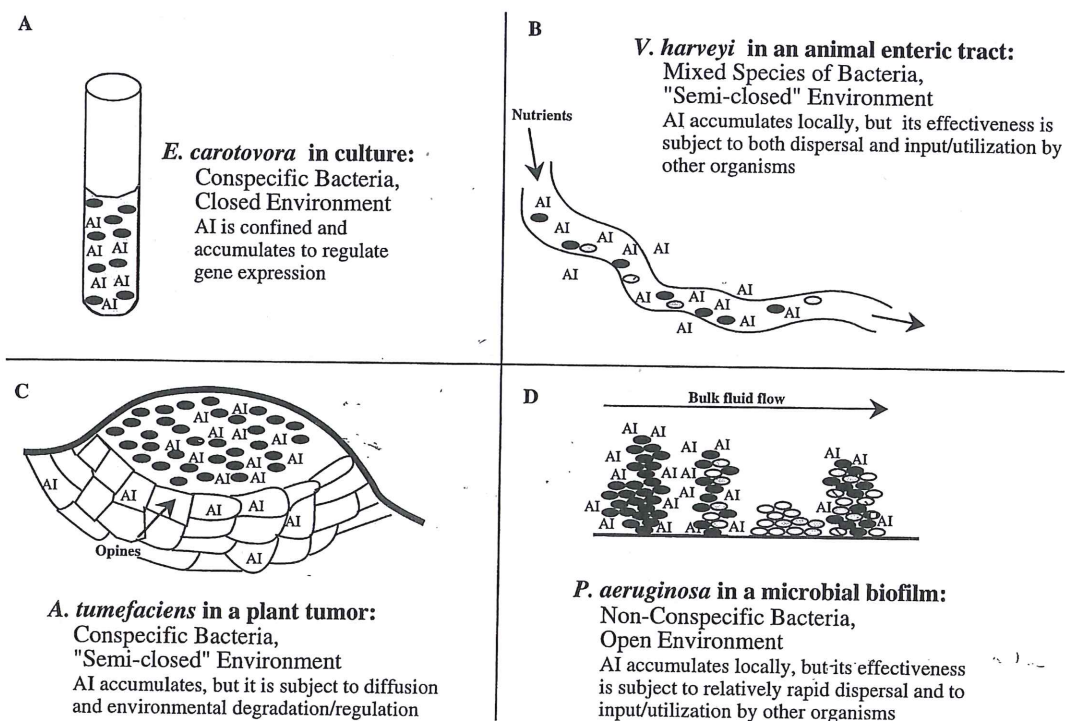


FIGURE 1 Schematic diagram of representative quorum-sensing bacteria in various biological niches: in a laboratory pure culture (A); in the enteric tract of an animal host (B); in a plant tumor (C); and in a biofilm composed of pure and mixed colonies (D). In each panel the quorum-sensing bacteria are depicted as black ovals. The predicted location and relative concentration of autoinducer molecules (AI) are indicated in each panel. Secretion of opines by the plant cells is indicated in panel C.

vironment, it would also allow a more rapid sensing of changes in cell density. Another potential difficulty is that other organisms could secrete similar compounds that function either in competition or as a defense mechanism, thus creating a confusing mixture of "false-positive" or even "false-negative" signals that would lead to unproductive responses. Finally, the AI signal might be recognized and utilized by other organisms. Those organisms that do not invest in creating their own signal, but profit by its production by others, might be considered "cheaters," whose success could lead to a selective disadvantage for producers of the cue. Other organisms may use the AI cue as a means of detecting the presence of the producing bacterium; in some cases, the presence of the external compound may result

in unwanted competition or even predation. AI-excreting bacteria probably face one or more of these challenges and, at least in some cases, may have evolved counteractive strategies to defend against them (Table 1).

One feature of AI systems that may serve as a defense against improper signaling is the specificity of the AI signal recognition process. In some organisms, the receptor protein has a high degree of specificity for its cognate AI and generally will not modulate gene expression in the presence of a different acyl HSL derivative. For example, the *Pseudomonas aeruginosa* receptor protein LasR, together with *N*-3-(oxododecanoyl)-HSL (3-oxo-C12-HSL), regulates the *las* genes by recognizing and binding to upstream regulatory sequences (chapter 10 of this volume; Gray et

TABLE 1 Consequences of using quorum sensing in the natural environment

	Consequences		
	Loss of the signal	Susceptibility to a "false" signal	"Interception" of the signal
Causes	By diffusion into environment By abiotic degradation By competitive removal	Through the secretion of a similar or identical molecule by an unrelated bacterial species	By a signal receptor of an unrelated organism
Negative effects	Creates a continuous energy cost to the cell	Leads to inappropriate gene induction or inhibition	Attracts and/or promotes unwanted competition or predation ("cheater") Initiates host defenses
Positive effects	Prevents unnecessary gene induction Allows a rapid sensing of changes in the environment	Allows input from different organisms in the same niche	Serves to coordinate consortial metabolism among bacterial species Serves to coordinate host and bacterial development Interferes with host defenses
Defenses	Ensure that positive autoregulation occurs only under specific ambient conditions Decrease the threshold concentration of AI required for response	Increase specificity of receptor Increase threshold concentration of AI required for response Use multiple AIs and receptors in overlapping signal pathways	Create community-level responses that are of general selective value

al., 1994; Passador et al., 1993). Interestingly, although the 3-oxo-C12-HSL/LasR complex is also able to recognize similar sequences upstream of the luminescence (*lux*) genes of *V. fischeri* and activate gene expression, LasR in the presence of the *V. fischeri* AI 3-oxo-C6-HSL can activate neither *las* nor *lux* transcription (Gray et al., 1994). Similarly, while the *V. fischeri* regulator LuxR in the presence of the *Pseudomonas* AI (3-oxo-C12-HSL) (Pearson et al., 1994) cannot activate either *lux* or *las* transcription, the LuxR/3-oxo-C6-HSL couple can (Gray et al., 1994). Thus, despite the fact that the two autoinducers are structurally similar, their respective receptor proteins have a high degree of specificity for the native signaling molecule. Recent results suggest that both the length and substituents of the acyl chain are responsible for this specificity (Eberhard et al., 1986; McClean et al., 1997; Passador et al., 1996; Schaefer et al., 1996; Zhang et al., 1993).

Other receptor proteins appear to be less specific in the recognition of their cognate AIs. It is possible that this lack of specificity occurs only in the artificial conditions of the test tube, since the biologically relevant concentration of AIs in nature has not been well investigated. Alternatively, the lack of specificity may indicate an adaptation of the organism to accept signaling input from nonconspecific bacteria. For example, a *swrI* mutant of *S. liquefaciens*, which is defective for the synthesis of its own two AIs (*N*-butanoyl-L-HSL [C4-HSL] and *N*-hexanoyl-L-HSL [C6-HSL]), is unable to achieve swarming motility (Eberl et al., 1996). Restoration of swarming motility requires either the addition of synthetic C4-HSL (150 nM) or the addition of C6-HSL (900 nM). However, swarming motility can also be restored by the addition of a 5- to 50-fold higher concentration of certain other AIs, such as *N*-3-oxobutanoyl-HSL (6 μ M) or 3-oxo-C6-HSL (9 μ M) (Eberl et al., 1996). Thus, higher concentrations of a nonnative signaling molecule are capable of activating gene expression through the native AI regulator, although it is not known whether these concentrations would be pres-

ent in the natural environment of *S. liquefaciens*.

In addition, the threshold of detection of the same native AI may be different for different organisms. For example, the closely related species *Vibrio logei* and *V. fischeri* both produce and respond to 3-oxo-C6-HSL (Greenberg, personal communication); however, the induction of AI-regulated genes in *V. logei* requires higher levels of VAI-1 than those required for induction of their homologs in *V. fischeri* (Boettcher and Ruby, 1990). These differences in minimal effective dosages may reflect the levels of AI achieved in the niches typically occupied by these organisms.

Another mechanism by which bacteria achieve a greater signal specificity may be the encoding of multiple, interacting AI systems within a single organism. *V. fischeri* cells carry two AI systems, one of which is the well-studied prototype LuxR/3-oxo-C6-HSL, which is involved in the control of bioluminescence, and the other is the more recently described *N*-(octanoyl-L/-HSL)-synthase, AinS (Gilson et al., 1995; Kuo et al., 1994). While C8-HSL is capable of stimulating luminescence in the presence of LuxR, its natural role appears to be one of competitive inhibition (Kuo et al., 1996). A mutant defective in *ainS* induces luminescence at a lower cell density, and, once induced, luminescence production increases more rapidly in this mutant (Kuo et al., 1996). The addition of C8-HSL (75 nM) either to the *ainS* mutant or to the wild-type parent causes a delayed onset of luminescence (Kuo et al., 1996). These results suggest that the inhibition by C8-HSL may provide a level of "fine-tuning" that prevents premature gene induction under environmental conditions that are inappropriate for luminescence. Such a mechanism would also be useful in the hypothetical situation in which other organisms emit a signal that falsely simulates the presence of a quorum; the secretion of a second, inhibitory, signaling molecule that competes for the same receptor protein may function as a defense mechanism to decrease the possibility of false signaling, as well as premature gene expression. C8-HSL could

also have a distinct role in the control of other genes, but such a function has not yet been established.

In summary, there are multiple means by which bacteria may defend against false signaling in the environment. They have evolved receptors that are fairly specific to their cognate AIs and that are responsive to a certain minimal concentration of signal. The presence of multiple AI systems in a single bacterium may also serve as a defense. However, while cell-to-cell communication through co-occurring AI signals in nature can lead to nonproductive signaling, in some cases stimulation of autoinduction by the presence of other species of bacteria may represent an effective mechanism for a bacterium to recognize and respond to additional biological characteristics of its environment.

QUORUM SENSING WITHIN AND BETWEEN POPULATIONS

The natural environments in which AI-producing bacteria are found are diverse in character and in many cases contain multiple bacterial taxa (Fig. 1). In other cases, such as certain well-studied bacteria-host associations (Ruby, 1996), the bacterial population is present as a pure or monospecific culture for at least a portion of its life cycle. In addition, new AI-producing strains from other environments are currently being identified using global screening methods (Shaw et al., 1997; Taylor et al., 1996). Thus, when determining the role of AIs in nature, the synergistic effects of the surrounding environment, including other bacteria and/or host tissue, must be considered. In the following sections, we discuss the influence that other organisms may have on AI-mediated gene expression in a quorum-inducible bacterium, as well as the consequences that this consortial or group behavior of bacteria may exert on other organisms.

Quorum Sensing between Bacterial Taxa

One of the earliest studies to consider the role of AIs in the natural environment demonstrated that autoinduction of bioluminescence

in the marine organism *Vibrio harveyi* could be stimulated by other marine bacteria (Greenberg et al., 1979). Since that time, it has been established that *V. harveyi* encodes two quorum-sensing systems for the control of bioluminescence, sensor system 1 and sensor system 2 (chapter 17 of this volume). The two systems converge to stimulate regulation of the *lux* genes through a single regulatory protein (Showalter et al., 1990). Sensor system 1 produces a typical AI/receptor complex, while the nature of the second quorum-sensing signal, believed not to be an HSL derivative, remains undetermined (Bassler et al., 1997).

Bassler et al. (1997) have clarified the original *V. harveyi* study and added depth to our understanding of the interactions of different bacteria with each other. Although the two sensor systems initially appeared to be redundant in the control of bioluminescence, it now seems likely that the presence of two systems allows *V. harveyi* to respond to input signals from either conspecific or nonconspecific bacteria. The majority of the other species of microbes that showed the ability to stimulate bioluminescence of *V. harveyi* did so by acting through system 2. These microbes included the closely related *Vibrio* species *V. cholerae*, *V. anguillarum*, and *V. natriegens*, as well as the more distantly related *Yersinia enterocolitica*. Although we do not yet know whether the AI activities produced by these bacteria are identical to that of *V. harveyi*, there are examples of two unrelated bacteria making identical AIs (Fuqua et al., 1996). Only one of the organisms that was examined signaled *V. harveyi* luminescence via system 1 (Bassler et al., 1997). These data indicate that the quorum-sensing mechanism of *V. harveyi* regulates bioluminescence genes by integrating conspecific input (obtained specifically through system 1) with input from other organisms (through system 2).

This ability to receive and respond to a signal from other organisms may be an important factor in communication in certain environments, such as the enteric tracts of fishes, which are typically colonized by a diverse population of bacteria, often including *V.*

harveyi (Fig. 1) (Ruby and Morin, 1979). The potential for external control of luminescence suggests that light emission is useful when *V. harveyi* is present in a group of cells, even of nonconspecific bacteria. It is not clear why the expression of bioluminescence genes might be important to *V. harveyi* and others in such an environment, but a survival advantage may be linked to the high affinity that luciferase has for oxygen (Lloyd et al., 1985). The ability to lower the ambient oxygen tension, and thereby decrease potential oxidative stress or competition from aerobes, may confer a survival advantage on the facultatively anaerobic portion of the population, including *V. harveyi* (Nealson and Hastings, 1979). An alternative explanation that has been suggested is that the fecal pellets produced by marine animals, if bioluminescent, may be preferentially consumed by other organisms, thus returning the excreted fecal cohort to an enteric environment where they can continue to propagate (Andrews et al., 1984; Nealson and Hastings, 1979).

In addition to the level of bioluminescence, the production of poly-3-hydroxybutyrate, a fatty acid storage product, is also induced in *V. harveyi* by the accumulation of AI (β -hydroxy-C4-HSL), although the significance of this connection between luminescence and carbon metabolism is unclear (Sun et al., 1994). Additional sets of *V. harveyi* genes may be controlled by one or both of the sensing systems, and the expression of these other genes may be important under specific environmental conditions. The identification of additional genes controlled by either of these sensing systems, and the determination of the environmental conditions optimal for their expression, will help elucidate whether the systems are truly redundant or whether the different signals result in distinct physiological responses. This work has revealed an example of a bacterium that has evolved a regulatory network based on signaling input from other species of apparently cooperative bacteria, resulting in a community-level communication process.

Interspecific use of AIs is a mechanism for *Burkholderia cepacia* (Lalor et al., 1995). *B. cepacia* in patients with cystic fibrosis in these cases to co-colonizing addition of C12-HSL to their producting a sevenfold thesis and metalloprotease excretion small elevation (threefold) in also obtained of spent media cultures, suggest encode its quorum-sensing (McKenney et al., 1994). *B. cepacia* colonization of such as *P. aeruginosa* presence of (McKenney et al., 1994). In one case, *B. cepacia* a "cheater" in an environment organism's cell production (Table 1).

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Quorum Sensing in Bacterial-Host Interactions
Many quorum-sensing high-cell-density

Interspecies communication through the use of AIs has been proposed as a possible mechanism by which the pathogenicity of *Burkholderia cepacia* is enhanced (McKenney et al., 1995). *B. cepacia* is associated with fatalities in patients with cystic fibrosis, but in most of these cases this organism was found to be co-colonizing tissue with *P. aeruginosa*. The addition of the *P. aeruginosa* AI (3-oxo-C12-HSL) to cultures of *B. cepacia* potentiated their production of virulence factors, including a sevenfold increase in siderophore synthesis and more than a twofold increase in protease excretion (McKenney et al., 1995). A small elevation in virulence factor production (threefold in the case of siderophores) was also obtained by the addition of a concentrate of spent medium obtained from *B. cepacia* cultures, suggesting that *B. cepacia* may also encode its own quorum-sensing system (McKenney et al., 1995). The supernumerary colonization of lungs by the opportunistic *B. cepacia*, following infection by other microbes such as *P. aeruginosa*, may be aided by the presence of 3-oxo-C12-HSL molecules (McKenney et al., 1995). If this is in fact the case, *B. cepacia* would represent an example of a "cheater" organism that exploits a micro-environment through the use of another organism's cellular investment in signal production (Table 1).

We have described two examples of AI signaling that may occur between distantly related bacteria that are present in the same niche. While the biological relevance has yet to be confirmed, it is likely that, with the widespread nature of quorum-sensing systems, such interactions are occurring in the environment. The development of specific signaling molecules that can be used to sense not only conspecific bacteria but also certain noncon-specific bacteria present in specific niches is a clever mechanism for community-level regulation of gene expression.

Quorum Sensing in a Bacterial-Host Symbiosis

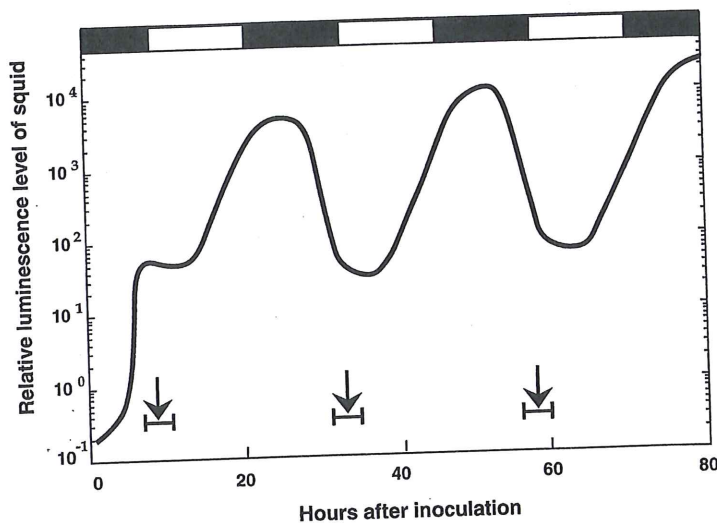
Many quorum-sensing bacteria are found in high-cell-density niches inside eukaryotic

hosts. *V. fischeri* cells, unlike those of *V. harveyi*, are known to occur in dense, mono-specific populations in nature where an environmental importance for bioluminescence has been established. Certain species of marine squids and fishes possess specialized light-emitting organs that contain as many as 10^{10} *V. fischeri* cells. This bacterial culture produces the light that is used in predatory and/or antipredatory behaviors of the host (Nealson and Hastings, 1979). The symbiosis between *V. fischeri* and the Hawaiian sepiolid squid *Euprymna scolopes* has been established as a model system for studying the signaling interactions between bacteria and their hosts (reviewed by Ruby, 1996).

Newly hatched *E. scolopes* juveniles are rapidly colonized by symbiosis-competent *V. fischeri* cells that are present in the surrounding seawater. Colonization is accompanied by the onset of the AI-controlled (chapter 18 of this volume) luminescence activity within 8 to 10 h of the initiation of the association (Fig. 2). The amount of light emitted per cell, or specific luminescence, also increases over 100-fold (Ruby and Asato, 1993). The maximal level of colonization in the juvenile squid is between 10^5 and 10^6 *V. fischeri* cells and occurs extracellularly in a series of crypts that have a total volume of about 750 pl (Montgomery and McFall-Ngai, 1993). The result is an effective concentration of greater than 10^9 bacteria per ml, a level that is 10-fold higher than that necessary to fully induce luminescence in culture (Boettcher and Ruby, 1990).

In addition to providing an environment suitable for both rapid colonization and the production of bioluminescence by *V. fischeri* cells, the squid host plays an active role in controlling the level of their bioluminescence emission. This is achieved by a combination of at least three mechanisms. The first and simplest is the presence of several accessory tissues, including a muscle-controlled ink sac that the animal can use as a moveable shutter to either conceal or reveal the bioluminescence of its symbiotic partner (Fig. 3) (McFall-Ngai and Montgomery, 1990).

FIGURE 2 Diagrammatic representation of the daily rhythm of luminescence and symbiont cell expulsion by the squid *E. scolopes*. The thick black line represents the level of luminescence emitted by the juvenile squid and is roughly proportional to the level of colonization by *V. fischeri* cells. This luminescence begins during the first 12 h after inoculation and subsequently cycles up during the night (black boxes across top strip) and down during the day (white boxes). The onset of the drop in luminescence corresponds to the peak of bacterial cell expulsion from the light organ crypts into the surrounding seawater during the periods indicated by the arrows. Modified from Boettcher et al. (1996) and Lee and Ruby (1994).



The other two light-modulating mechanisms are linked to a diurnal phenomenon that illustrates the host's ability to control both the number of cells in the bacterial population and the level of luminescence of these cells. Each morning, over 90% of the population of bacterial symbionts is expelled from the light organ crypts, with a concomitant drop in bioluminescence (Fig. 2) (Boettcher et al., 1996; Lee and Ruby, 1994). Luminescence subsequently increases to a maximal level by nightfall, owing in part to the regrowth of the remaining bacteria in the crypts. This cyclic pattern of bioluminescence is not observed in squid that are held in either constant dim illumination or constant dark conditions, demonstrating that the primary cue for the control of bioluminescence is external to both the bacterium and the squid (Boettcher et al., 1996) and is linked to the environmental light conditions.

In addition, regardless of the number of symbiont cells present, the host is also able to directly modulate the specific activity of light emission by the bacteria (Boettcher et al., 1996). Boettcher et al. (1996) compared the average level of luminescence produced by

bacterial cells in the intact juvenile squid light organ (their "actual luminescence"), with that of these same cells immediately after their release from the light organ (their "potential luminescence"), at various times during the normal day/night cycle. There was a significant similarity between the actual and potential levels of luminescence (per cell) during the "peak" period of luminescence (that is, near the onset of nightfall) (Fig. 2). However, during daylight or "non-peak" periods, the actual luminescence level was about 10-fold below that of the potential luminescence. These data suggested that the animal host actively represses the bioluminescence of its symbiotic partner during a significant portion of each 24-h cycle. One mechanism for repression may be through the control of the rate that oxygen, a substrate for the bioluminescence reaction, is provided by the host to the symbiont population (Boettcher et al., 1996). Another hypothetical mechanism is that luminescence is repressed in part through the stimulation of synthesis of C8-HSL, which may competitively inhibit LuxR stimulation of *lux* gene expression at certain times of the day. In any case, the multiple levels of control

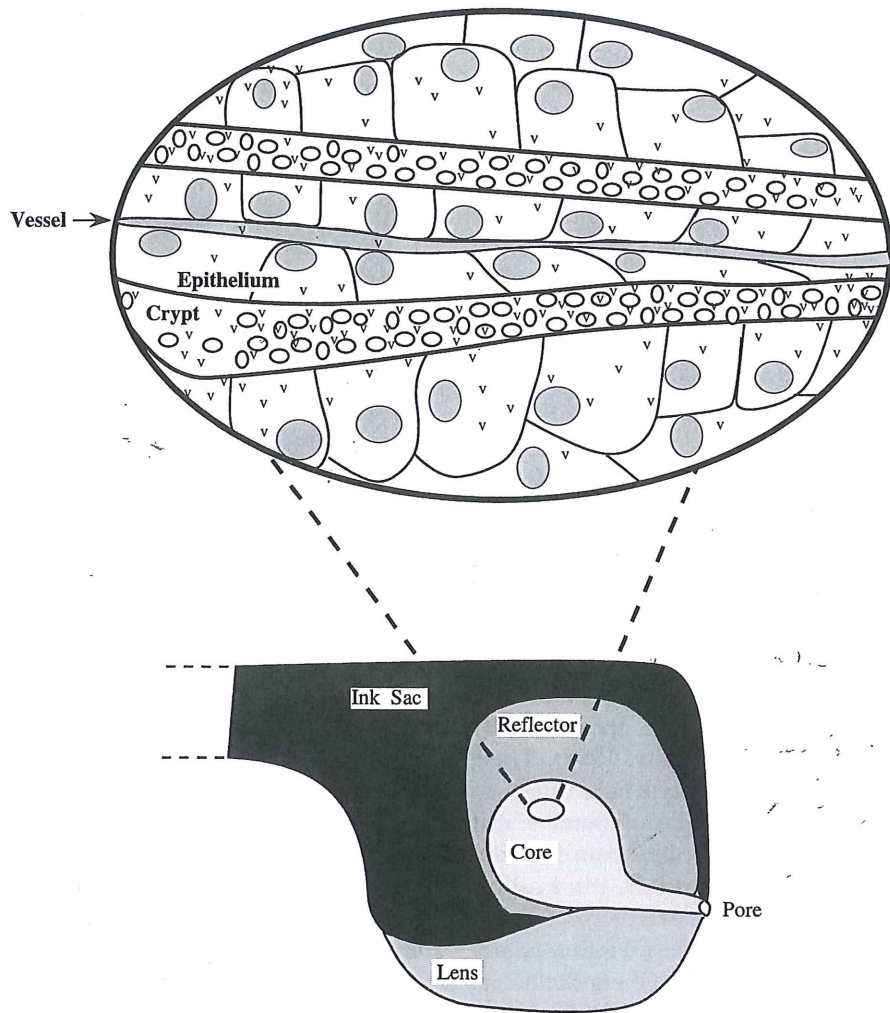


FIGURE 3 Structure of the light organ of an adult squid. One half of an adult *E. scolopes* light organ is depicted in the lower drawing. The central core is composed of a series of crypt spaces in which the bioluminescent bacteria are located, flanked by host epithelial cells. The core is surrounded by three tissues: the reflector, lens, and ink sac. The crypts of the light organ communicate with the surrounding seawater through a lateral pore. A small segment of the core is enlarged to show the location of the symbiotic *V. fischeri* (indicated by small white ovals) in the crypt spaces. The arrangement of epithelial cells and the vascularization of the tissue are also indicated. The small v's represent 3-oxo-C6-HSL and C8-HSL and their diffusion into the host tissue (Boettcher and Ruby, 1995), although there are as yet no data to confirm the presence of C8-HSL in the light organ. Modified from McFall-Ngai and Montgomery, 1990.

exerted by both the squid and the bacterium strongly suggest that the ability to modulate bioluminescence is of considerable importance to these organisms.

Interestingly, when grown in culture, bioluminescent strains of *V. fischeri* isolated from the light organ of *E. scolopes* emit only low levels of light that are not visible to the human

eye (measured at 0.8 quantum per s per cell with a sensitive photometer), but are 1,000-fold brighter immediately after release from the light organ, achieving a level of about 800 quanta per s per cell (Boettcher and Ruby, 1990). This low level of in-culture luminescence is not seen in other *V. fischeri* strains isolated from the light organs of other symbiotic squid or fish associations. For example, MJ-1, a strain of *V. fischeri* isolated from the fish *Monocentris japonicum* (Ruby and Neilson, 1976), produces over 1,000 times higher luminescence in culture than do *E. scolopes* isolates such as strain ES114 (Boettcher and Ruby, 1990). Studies of strain ES114 in culture have revealed that these *V. fischeri* cells are underproducers of 3-oxo-C6-HSL (Gray and Greenberg, 1992). The amount of this AI produced in culture by ES114 is about 10-fold less than that of *V. fischeri* cells isolated from the related squid *Euprymna morsei*, although the concentrations of AI present in the respective squid associations are approximately the same (Boettcher and Ruby, 1995). These data suggest that the *E. scolopes* light organ association provides a special environment that promotes a sufficiently high concentration of *V. fischeri* cells to result in the maximal induction of luminescence even in these AI-underproducer isolates. Whether this induction involves the presence of conditions or molecules other than 3-oxo-C6-HSL remains to be determined. (Studies describing the amount and diffusion of 3-oxo-C6-HSL in the squid light organ are discussed further later in this chapter.)

Mutants of *V. fischeri* that are defective for either of the luminescence regulators, LuxR or LuxI, do not show an increase in specific activity of luminescence during colonization of the squid light organ and, in fact, produce no detectable light at all in the early stages of the association, despite the relatively high level of residual luminescence observed in these strains in laboratory culture (Fig. 4) (Dunlap and Kuo, 1992; Visick et al., 1996). These data support the hypothesis that the mechanism of induction of bioluminescence in the

squid has the same requirement for the presence of the *luxR* and *luxI* regulatory genes as is seen in culture. In addition, these data suggest that C8-HSL, despite having some ability to initiate luminescence (Kuo et al., 1996), is not, by itself, sufficient for symbiotic induction of luminescence (Visick et al., 1996).

In addition to the diminished capacity to luminesce in the squid, both *luxI* and *luxR* mutants exhibit between a 3- and 10-fold decrease in the level of colonization of the juvenile light organ within 48 h of the initiation of the association (Visick et al., 1996). Curiously, a similar colonization defect is seen for a mutant lacking the AI-regulated gene, *luxA*, which encodes a subunit of bacterial luciferase, the enzyme that catalyzes the luminescence reaction. It is unclear at this time whether the colonization deficiency of all three *lux* mutant strains is due to an inability of these dim or dark cells to survive certain conditions in the light organ or to a recognition of the luminescence defect by the squid, leading to their expulsion in favor of light-emitting strains.

In summary, the squid-*V. fischeri* symbiosis has proved to be an effective model system for studying autoinduction at the community level and has revealed that AI-controlled genes and their products are subject to multiple levels of control in nature. Luminescence induction inside the host is higher than the maximal level obtained in culture and is dependent on the same regulatory control genes, *luxR* and *luxI*. The absence either of these quorum-sensing components or of luciferase itself results in a defect in the colonization of the juvenile squid, further supporting the importance of luminescence in the symbiotic association. Continued investigation of the role of bioluminescence should yield a better understanding of the consequences of AI-controlled gene regulation in the natural environment provided by the host light organ.

Quorum Sensing during Bacterial Virulence

It is rapidly becoming clear that both AI/receptor regulatory pairs and the genes that they

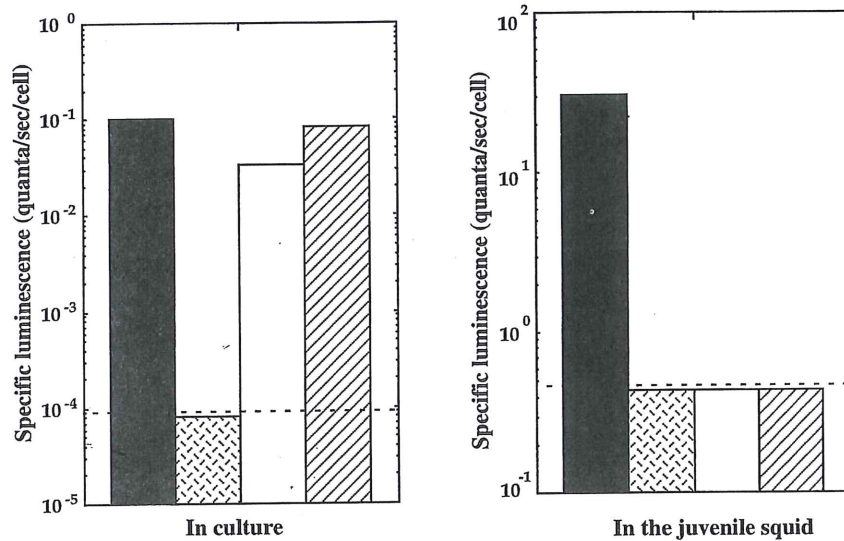


FIGURE 4 Specific luminescence of wild-type and *lux* mutants of *V. fischeri*, both in culture and in the juvenile squid light organ. The maximum specific activities of luminescence of the parent strain (solid box) and the *luxA* (hatched box), *luxI* (open box), and *luxR* (striped box) mutants are plotted. The horizontal dashed line at the bottom of the graph represents the lower limit of luminescence detection by the photometers used in each experiment.

control play an important role in the interactions of bacteria with a number of higher organisms. There are several examples of plant and animal pathogens whose virulence is attenuated by mutations in their AI synthase or receptor genes (Beck von Bodman and Farrand, 1995; Tang et al., 1996). While the vast majority of host-associated bacteria are commensal or beneficial, the examination of a number of pathogenic associations has provided an important source of information on the role of quorum sensing in the ecology of bacteria that colonize host tissues.

AI-production mutants of the plant pathogens *Erwinia carotovora* and *Erwinia stewartii*, bacteria that cause soft rot and Stewart's wilt, respectively, are avirulent (chapter 7 of this volume; Beck von Bodman and Farrand, 1995; Pirhonen et al., 1993). The avirulence is presumably due to the inactivity of the AI-controlled genes, encoding either cell wall-degrading enzymes (in the case of *E. carotovora*) or heteropolysaccharide capsule production

(in *E. stewartii*). When the mutant bacteria are preincubated with their species-specific AI and inoculated onto plant leaves, virulence is at least partially restored, but this restoration is dependent upon additional application of the AI to the leaf. In both cases, continued treatment of the plant leaves with added AI was required to sustain the spread of the bacterial infection. The addition of the AI alone to mock-inoculated leaf tissue did not induce any disease symptoms (Beck von Bodman and Farrand, 1995), suggesting that it is not the AI itself that is responsible. Interestingly, in the case of *E. stewartii*, external application of AI on the leaves was insufficient for the mutant to elicit all of the disease symptoms that the wild-type strain produced. These latter results may simply indicate either that the AI fails to persist on the leaves or that the levels of AI added were not comparable to those resulting from a natural infection and thus were insufficient to completely complement the virulence defect.

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It is interesting that restoration of virulence to the AI mutants of both of the *Erwinia* species required exogenous addition of AI, even though the mutants had been preincubated with AI. Thus, AI is required not only for *Erwinia* species to initiate the infection, but also for the infection to persist. This is indicative of a need for the continual communication of the bacteria with each other during the infection. Neither study (Beck von Bodman and Farrand, 1995; Pirhonen et al., 1993) reported the concentration of AI at the application site or the minimal level required for successful virulence. Thus, it appears that the study of plant pathogenesis could benefit from an investigation of the rate at which AIs can penetrate plant cells and a description of the concentration and stability of AI present in a natural infection.

A mutant strain of the opportunistic human pathogen *P. aeruginosa*, defective for the 3-oxo-C12-HSL receptor LasR, has a decreased ability to bind host epithelial cells and is significantly impaired in virulence when tested in a mouse infection model (Tang et al., 1996). These defects in virulence may result from an underexpression of the genes that LasR regulates (chapter 10 of this volume), whose products include the virulence factors elastase, endotoxin A, and alkaline protease, as well as those involved in the general secretion pathway (Chapon-Hervé et al., 1997). The relative consequences on virulence due to the direct and indirect effects of a mutation in *lasR* await further evaluation.

A role for AIs in virulence has also been demonstrated in the insect pathogen *Xenorhabdus nematophilus*, which forms a cooperative symbiosis with the nematode *Steinernema carpocapsae* (Forst and Neilson, 1996). *S. carpocapsae* invades susceptible insect larvae and delivers a fatal inoculation of *X. nematophilus*. Direct injection of a wild-type *X. nematophilus* strain into insects causes a 50% mortality within 9.6 h, while insects injected with an AI mutant (defective for the production of *N*- β -hydroxybutanoyl HSL [β -hydroxy-C4-HSL]) survive for over 2 weeks (Dunphy et al.,

1997). Infections carried out in the presence of 1 μ g of added AI decrease the time necessary to achieve 50% mortality both for the wild-type strain (to 8.6 h) and for the AI mutant (to less than 2 days) (Dunphy et al., 1997). Although the results are highly suggestive of a direct role for AI in the virulence of this bacterium, it should be noted that the experimental injection of the bacterium and AI is not the natural avenue of infection and that the concentration of AI present in the natural infection is as yet unknown. Future studies that ask whether the AI-synthesis mutation has an effect not only on infection of the insect but also on the bacterium's ability to form the natural cooperative association with the nematode will yield useful information regarding the roles of AI in the cooperative and pathogenic phases of the ecology of *X. nematophilus*.

NEW FRONTIERS IN AI FUNCTIONS

The environment includes not only other bacteria and host cells, but also other chemical cues. We discuss in this section what is currently known about the consequences of AI diffusion into a bacterium's surroundings, the influence of other molecules on AI-regulatory circuits, as well as the potential conflicts posed by AI mimicry in the environment.

Effect of AI Diffusion into the Surrounding Environment

While much is known about the activity of AIs inside the cell, other characteristics, such as the diffusion of these molecules through the bacterial cell envelope, across abiotic surroundings (water and soil) and into other cells are only beginning to be studied. As yet, the ability of acyl HSL derivatives to freely diffuse through the bacterial envelope has been experimentally demonstrated only for 3-oxo-C6-HSL (Kaplan and Greenberg, 1985). Although it is likely that all acyl HSL derivatives can permeate the cell membrane, the diversity of acyl adjuncts suggests that the rates of diffusion may be different for different molecules. Similarly, the nature of the bacterial (or host) membrane may present a barrier of dif-

ferential effectiveness. For example, the concentration of C4-HSL required to achieve induction of a reporter construct in *P. aeruginosa* is only one-tenth that required for the activation of the same construct in *Escherichia coli* (Pearson et al., 1997). While these results may be due simply to a higher expression of LasR in *E. coli*, the authors speculate that C4-HSL may pass more freely through the *P. aeruginosa* membrane, although they acknowledge that these results could also be explained by the absence of other specific factors in *E. coli*. Clearly, the rates of diffusion of various acyl HSL derivatives through different bacterial membranes could have a profound influence on both cell-cell communication and gene induction, because the relative levels of these molecules appear to be a significant factor in at least some systems (Passador et al., 1996; Schaefer et al., 1996).

Once the AI leaves the cell, the nature of its environment becomes important. Recent studies have shown that AI signaling between cells is able to occur within the rhizosphere of wheat seedlings. In co-inoculation studies, the level of expression of AI-controlled genes in an AI-synthase mutant strain increased with increasing ratios of complementing wild-type to mutant cells in the mixed population (Wood et al., 1997). Studies of this type provide biologically relevant information about the bacterial communication in natural settings.

The consequence of AI diffusion through eukaryotic tissues must also be considered, because most of the AI-producing bacteria identified to date are known to interact with the cells of specific eukaryotic host species (chapter 19 of this volume). In principle, the chemical properties of AIs should make their diffusion into host cells impossible to prevent. However, in only one bacteria-host association do we have data on the amount and flux of an AI in host tissue. The amount of 3-oxo-C6-HSL autoinducer present in an adult *E. scolopes* light organ has been estimated to be 100 to 200 pg of extractable AI activity, based on a luminescence bioassay (Boettcher and

Ruby, 1995). This level would result in an effective concentration of at least 100 nM of 3-oxo-C6-HSL in the whole light organ. Further experiments showed that this AI diffuses into and through the epithelial cells surrounding the crypt space of the light organ where the bacteria are housed (Fig. 3). The epithelium is surrounded by two other tissues, the reflector and lens (McFall-Ngai and Montgomery, 1990). Because of the chemical and structural nature of these tissues, AIs are less likely to penetrate them freely. Thus, these tissues may effectively confine the AI, promoting the high level of luminescence induction characteristic of the bacterial symbionts (Ruby and Asato, 1993). It is less clear how AI molecules would accumulate within the light organ of a juvenile squid, in which the reflector and lens are less developed (Montgomery and McFall-Ngai, 1993). Similarly, the possible effect that the vascularization of the crypt epithelium (Fig. 3) might have on the flux of AI out of the crypts and into the rest of the animal is unknown. The biochemical consequences of the AI molecules diffusing into the epithelial cells of the adult squid remains to be defined, although, by itself, AI is incapable of initiating the program of host tissue apoptosis (Doino and McFall-Ngai, 1995) that normally ensues after colonization of the nascent juvenile light organ (Montgomery and McFall-Ngai, 1994).

The first evidence that AIs may provide a signal to eukaryotic cells has been obtained during experiments with the *P. aeruginosa* autoinducer 3-oxo-C12-HSL. The addition of synthetic 3-oxo-C12-HSL was shown to be sufficient to elicit interleukin-8 production in a respiratory epithelial cell line (DiMango et al., 1995), a response that is characteristic of infection of these cells with *P. aeruginosa*. Because interleukin-8 is a chemoattractant for neutrophils (Huber et al., 1991), it appears unlikely that this effect would benefit the infecting *P. aeruginosa* cells; rather, AI production in this case may be an inadvertent signal that alerts the host to the pathogen's presence. Thus, this phenomenon might be an

example of a host adaptation to take advantage of a chemical cue excreted by the bacterium and to utilize it for an entirely different purpose.

A more extensive study of the role of 3-oxo-C12-HSL in eliciting an immune response has yielded data that support a role for this molecule in immunomodulation (Telford et al., 1998). Levels of IL-12 and tumor necrosis factor- α are decreased by the addition of 3-oxo-C12-HSL, although the latter effect required approximately 40 μ M AI, a level that may or may not be achieved in the host tissue (Telford et al., 1998). These data suggest that the AI produced and secreted by *P. aeruginosa* may by itself function as a virulence determinant by modulating the host immune response.

AIs have also been shown to influence host development in a bacterial-plant symbiosis, suggesting that they may be used as host signals in cooperative bacterial associations as well. Inoculation of bean seedlings with an AI-synthase mutant, *raiI*, of *Rhizobium etli* resulted in a twofold increase in nodule formation in the plant relative to the number produced by inoculation with the wild-type strain (Rosemeyer et al., 1998). The same effect was not seen with a *raiR* mutant defective for the putative receptor protein, suggesting that the AI is functioning with another receptor, perhaps in the host. *R. etli* produces seven AIs, two of which are differentially expressed by the *raiI* and *raiR* mutants (lower in the *raiI* mutant). These data implicate a role for one or both of these AIs, or for genes downstream of these regulatory factors, in the restriction of host nodule number (Rosemeyer et al., 1998).

Taken together, these studies provide evidence that AIs may function not only to signal cell density and coordinate gene expression in the bacterium, but also to provide a cue that is of significance to the host (Table 1). Clearly, much remains to be learned about the consequences of AIs in nature; for instance, the turnover rates of AIs in any natural environment, including host tissues, have yet to be measured. It will also be exciting to discover

the identity of target molecules in or on the eukaryotic cells that recognize and respond to the presence of AI or of the targets of the genes that they may control. Whether the targets of AI in eukaryotic cells are the same in hosts for pathogenic bacteria as in hosts for symbiotic bacteria is clearly another important and useful line of inquiry.

Selective Advantage of AI Regulatory Circuits

The results of many genetic studies have shown that quorum sensing is an effective mechanism for coordinating the regulation of a set of genes that may be useful only when a high density of cells is present. What has been less well documented is how AI-regulated genes may confer a selective advantage on the bacterium that encodes them. Such a selective advantage must manifest itself by an increased fitness or competitiveness in the organism's natural environment. Earlier, we described the importance of luminescence induction to the maintenance of a symbiotic light organ population. Below we discuss other organisms for which evidence is emerging for the use of AI-controlled genes to confer a selective advantage.

In the pathogenic association between *Agrobacterium tumefaciens* and its plant host, it is clear that the signaling process follows a two-way path: the T-DNA that is transferred from the bacterium into the plant cell encodes proteins that cause plant cells to produce and secrete a class of compounds called opines; these opines are in turn recognized by the bacterium, resulting in an induction of both the opine-catabolizing genes and the AI-regulated genes required for conjugation and transfer of plasmid DNA (chapter 8 of this volume; Desaux et al., 1992; Fuqua and Winans, 1994; Piper et al., 1993; Zhang et al., 1993). In nature, the ability to utilize opines as a source of nutrients may confer a selective advantage on the bacteria. It has been shown that in a mixed infection, bacteria that have the ability to catabolize opines can successfully outcompete bacteria that cannot utilize them, even though

both bacterial strains colonized the plant roots with the same kinetics when inoculated individually (Savka and Farrand, 1997). In addition, the soil environment of plants that have been engineered to produce a particular opine become enriched for bacteria that can catabolize that compound (Oger et al., 1997). Because the genes encoding the advantageous opine catabolism enzymes are carried by the conjugative plasmid, the AI-controlled conjugation behavior is therefore beneficial to the community of conjugation-competent recipients.

AIs are also important in biofilms. When compared to liquid cultures, cells in biofilms are known to have an increased resistance to such stresses as antibiotics and starvation (Costerton et al., 1995). Researchers have determined that the recovery of ammonia-oxidizing bacteria from ammonia starvation in liquid culture is significantly enhanced (i.e., the lag phase is significantly decreased) by the addition of 3-oxo-C6-HSL (Batchelor et al., 1997). They speculate that the higher cell densities achieved in a biofilm result in a greater concentration of AI, which in turn may induce genes whose products promote a more rapid recovery from ammonia starvation (Batchelor et al., 1997). The demonstration of the presence of AIs in naturally occurring biofilms (McLean et al., 1997) further supports this hypothesis.

Additional evidence of a role for AIs in the development of biofilms has been found using an AI-synthase mutant of *P. aeruginosa* (defective for *lasI*). The *lasI* mutant produced a biofilm that was thinner and contained denser layers of cells than that achieved by the wild-type strain (Davies et al., 1998), a phenotype that was rescued by the topical addition of the *P. aeruginosa* AI 3-oxo-C12-HSL. Unlike the biofilms produced by the wild-type strain, those of the *lasI* mutant were susceptible to the addition of detergent. Growth of the *lasI* biofilm in the presence of 3-oxo-C12-HSL restored the detergent-resistant phenotype displayed by the wild-type strain (Davies et al., 1998). These data provide the first direct ev-

idence of a role for AIs not only in the structural development of a biofilm, but also in the establishment of at least one of the resistance qualities known to be characteristic of cells in biofilms.

Dunlap (1997) has speculated that AI-mediated control of antibiotic production by *E. carotovora* may provide a selective advantage for this organism in its natural environment of a plant infection. The production of cell wall-degrading exoenzymes and the consequent release of nutrients could result in competition from other soil bacteria. To minimize this, *E. carotovora* has adapted the strategy of placing antibiotic biosynthetic genes under the same regulatory system, thereby eliminating competition from other soil bacteria for the nutrients released during the infection (Dunlap, 1997). Antibiotic production is also under the control of AIs in *Pseudomonas aureofaciens* and *Pseudomonas fluorescens*, where these molecules may also bestow a competitive advantage over other bacteria (Cook et al., 1995; Pierson et al., 1994; Wood and Pierson, 1996). Although it is difficult to distinguish whether the functions of AI-controlled genes are necessary, or merely advantageous, it seems likely that examples of key ecological functions for these molecules will be plentiful, given the widespread nature of the AI signal among gram-negative bacteria.

Production of AI Antagonists in the Environment

Because of the ubiquitous distribution of AI-producing bacteria in the natural environment, it would not be surprising if other organisms have evolved competitor molecules as a defense against microbial colonization. This evolutionary strategy appears to be functioning in the seaweed *Delisea pulchra*, which produces compounds called furanones that are structurally similar to AIs and appear to be involved in the inhibition of bacterial growth at the growing tip of the organism (Steinberg et al., 1997). While the basis for this inhibition is still under investigation, it may be related to the loss of the colonizing organism's swarming

motility. Both *S. liquefaciens* and *Proteus mirabilis* are able to swim either as individual cells or in a multicellular swarm. The addition of either of two furanones (designated 1 and 2) to *S. liquefaciens* cells inhibits the swarming but not the swimming behavior of these cells (Givskov et al., 1996). This effect can be overcome by the addition of 0.2 μM *N*-butanoyl-L-HSL (C4-HSL), one of the known *S. liquefaciens* AIs. *P. mirabilis* swarming can also be inhibited by the addition of another furanone (designated 4), but in this case, the ability to swarm is not restored by either C4-HSL or an extract from *P. mirabilis* cells (Gram et al., 1996). In addition, the ability of *P. mirabilis* cells to come into close contact, which is required for effective consolidation and swarming, is abolished by a crude preparation extracted from *D. pulchra* cells but not by addition of any of the individual furanones tested, suggesting that an additional inhibitor may be present in this alga that has a further effect on bacterial behavior (Gram et al., 1996).

Although direct proof that furanones are used specifically to inhibit bacterial growth awaits further investigation, it is not unlikely that eukaryotic cells have developed mechanisms for controlling bacterial colonization of their surfaces by interference with AI signaling. The work with *D. pulchra* appears to be the first description of a eukaryotic organism creating a substance(s) that antagonizes a bacterial signaling system by mimicking an AI. Further studies will help delineate the array of eukaryotic defenses against bacteria that may occur via AI molecules or AI antagonists.

CONCLUDING REMARKS

Quorum sensing is only one of a number of known bacterial-sensing systems. Individual bacterial cells have the ability to coordinate their own gene expression using sigma factors, antisigma factors, and other global protein response regulators. By themselves they can sense changes in the environment and relay that information through methylation and phosphorylation relay pathways. They are able

to produce surface or exported proteins and to use those proteins to bind to other bacteria and even to eukaryotic hosts. Specific communication with eukaryotic hosts can proceed through pathways stimulated by attachment, or even through the insertion of proteins into their hosts. Given all of these other mechanisms, one can ask: why have quorum-sensing systems evolved, and why are they so broadly distributed in gram-negative bacteria?

With perhaps one exception (Puskas et al., 1997), AIs are found in bacteria that are known to exist in high cell densities in nature (chapter 19 of this volume). Although microbiologists generally think about selection at the cellular level, it is important to remember that AI systems typically function at the population or community level and may in some cases represent a mechanism for survival of the group rather than of the individual. Perhaps AIs are unique in providing a low-energy mechanism by which to signal across distances and through host tissue. In these natural settings, the accumulation of an AI may be easier to accomplish than the probability of achieving direct cell-to-cell contact. Regardless of the reason, it is clear not only that AIs are an effective method of promoting bacterial unity in the coordination of gene expression, but also that the existence of these chemical signals in the environment will surely translate into a reciprocal response by other bacteria and eukaryotic cells to achieve interorganismal communication. As the field of quorum sensing continues to grow and mature in the coming years, the development of our knowledge of the biological emerging properties of this signaling strategy will be an increasingly important area of discovery.

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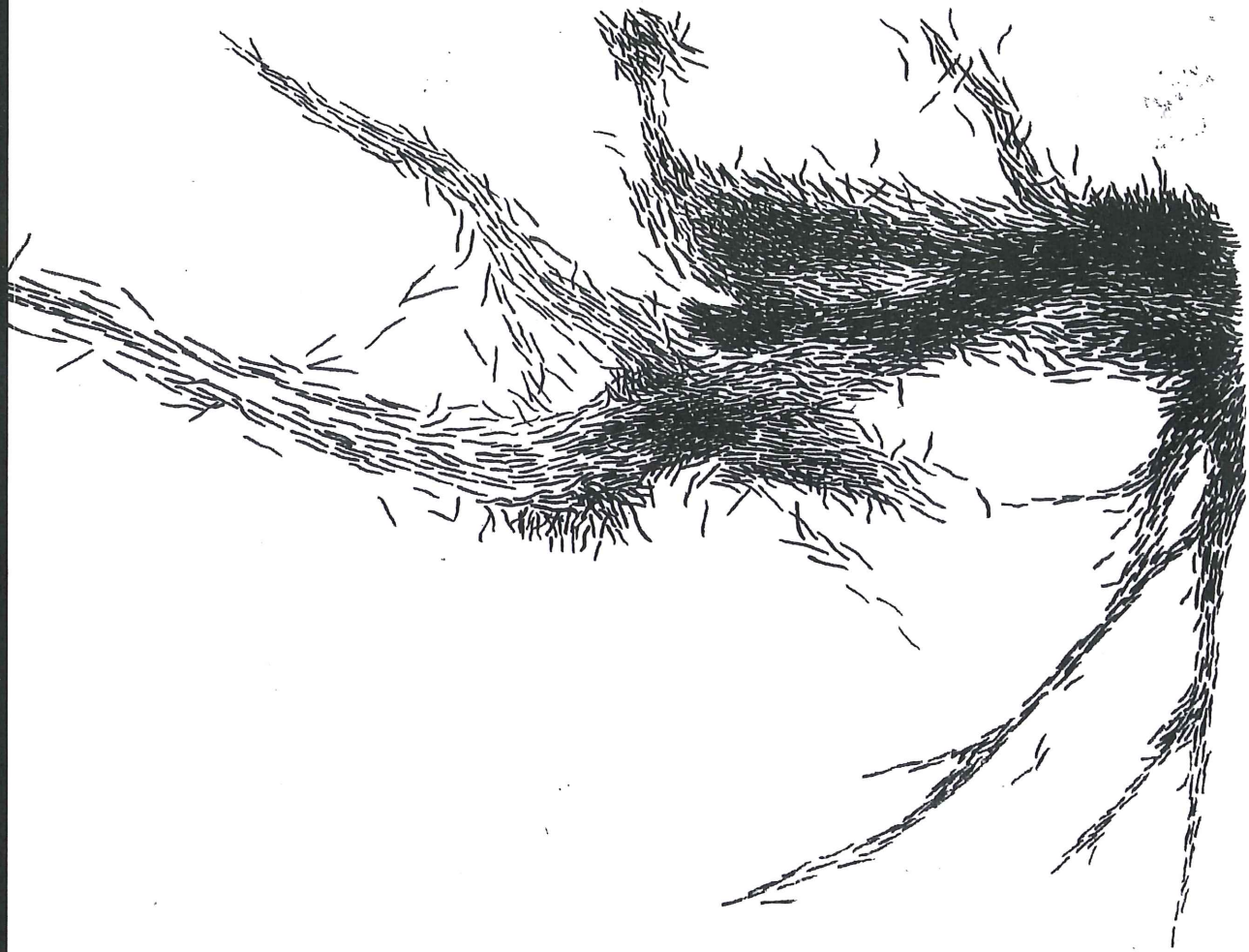
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Cell-Cell Signaling in Bacteria



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