Chapter 20

Microbial Communication: Bacteria/Bacteria and Bacteria/Host

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20.1 INTRODUCTION

Bacteria were the first organisms to evolve on Earth, and were present approximately 2 billion years before the first eukaryotes appeared. Thus, bacteria were critical to the development of the biosphere that enabled the evolution of higher life forms. Since all eukaryotic organisms evolved in the presence of bacteria, they are intimately associated with bacteria. The range of these associations can vary from: (1) bacteria living on the surfaces of the host (saprophytes); (2) those that benefit the host; or (3) those that are detrimental or pathogenic to the host. Since both bacteria and hosts need to sense their environment and the presence of the other, bacteria and their hosts have evolved complex mechanisms of signaling between and among themselves and each other, as a means of communication.

Our current knowledge regarding bacterial physiology and development is based primarily on *in vitro* experiments using pure, single microbial cultures. However, this fails to accurately represent the complexity that bacteria face in their natural environments since they rarely exist in isolation. In nature, most bacteria are members of complex micro, macro- or even megacommunities that predominantly exist as surface-associated biofilms composed of cells embedded in a complex matrix composed of self-synthesized extracellular polysaccharides and DNA known as a biofilm (see Chapters 6 and 19). Often, the survival of a given bacterial species is dependent on the ability of the individual bacterial cells within that population to communicate among themselves, and/or between themselves and other organisms. These other organisms include unrelated bacteria, organisms that share the same ecological niche or eukaryotic hosts, including plants, nematodes, insects, animals and humans.

The environments that bacteria inhabit are complex and subject to rapid change. In order to be successful, bacteria must be able to sense and respond rapidly to these changes by altering the expression of specific genes and metabolic pathways. This ultimately affects the behavior of the bacteria. It is now well recognized that most bacteria produce signals that allow communication between cells. Fundamentally, cells communicate by emitting specific chemical signals, into a particular

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environment inhabited by other organisms. When, or if, the concentration of the signal reaches a level where other cells are able to perceive it, known as the threshold concentration, the gene expression of all the organisms present becomes modified. This cell–cell communication is important for coordinating gene expression within a single population of bacteria (intraspecies signaling), between bacterial populations (interspecies signaling) and between bacteria and other organisms (interkingdom signaling) (Figure 20.1).

Communication signals consist of a wide variety of chemical structures. The primary requirements for these signals are that they are small, they can be released from cells either by passive diffusion or active transport, and that other cells possess the ability to recognize them and alter behavioral patterns in response to their presence. Because these signals alter bacterial behaviors, they have been referred to as bacterial pheromones. Scientists are now beginning to appreciate the world of bacteria–bacteria and bacteria–host signaling. This chapter discusses the current understanding of bacterial signaling using examples of communication systems, including: signaling in Gram-negative bacteria via quorum sensing with *N*-acyl homoserine lactones; signaling in Gram-positive bacteria via γ-butyryl lactones and small peptide signals; and signaling via autoinducer-2 (AI-2), autoinducer-3 (AI-3) and bacterial muropeptides. In addition, this chapter will touch upon several additional areas of communication such as bacterial eavesdropping, bacterial signal interference (quorum quenching) and interkingdom signaling.

20.2 SIGNALING VIA QUORUM SENSING IN GRAM-NEGATIVE BACTERIA

Quorum sensing is the regulation of gene expression in response to levels of diffusible signal molecules, which usually correlate with population density, i.e., a sufficient number of cells or a quorum must be present in order for gene expression to occur. Specifically, quorum sensing bacteria produce and release chemical signal molecules or autoinducers that control gene expression of the whole bacterial population. Both Gram-positive and Gram-negative bacteria use quorum sensing systems, but each tends to utilize different chemical signals to control target gene expression.

20.2.1 *N*-acyl Homoserine Lactones (AHLs)

In Gram-negative bacteria, the best-studied diffusible signals are the *N*-acyl homoserine lactones (AHL) (Table 20.1). To date, over 50 bacterial species have been shown to produce AHL signals (Scott *et al.*, 2006). This signal class consists of a conserved homoserine lactone ring moiety connected to a fatty acyl side chain (Dong and Zhang, 2005). The specificity of AHL signals is

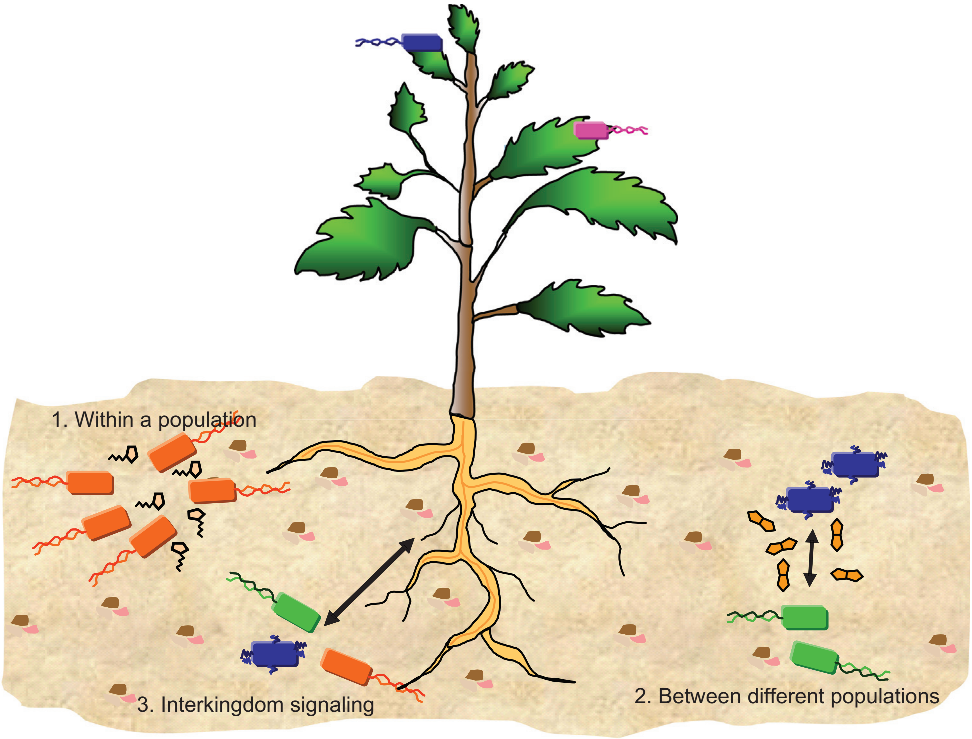
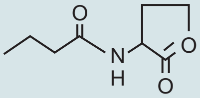
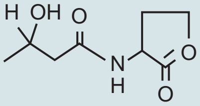
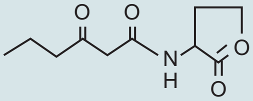
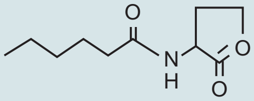
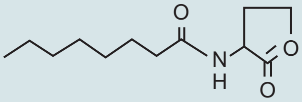
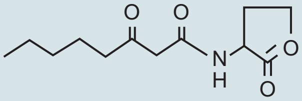
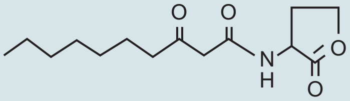
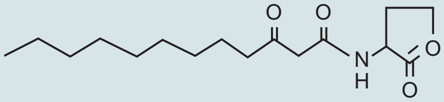
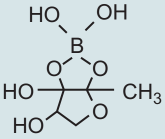
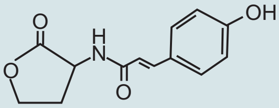
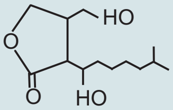
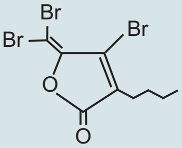
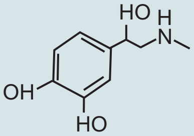


FIGURE 20.1 Examples of cell–cell signaling interactions in nature. (1) Signaling within a single bacterial population. Signals such as AHLs (Gram-negative) and γ-butyrolactones (Gram-positive) are commonly used in bacterial communication within a population. (2) Signaling also occurs between unrelated bacteria. The AHLs have been shown also to participate in communication between different bacteria, as has the universal Autoinducer AI-2. (3) It is increasingly being recognized that signaling occurs between bacteria and eukaryotic hosts such as plants.

TABLE 20.1 Structures of Bacterial Communication Signals

***N*-Acyl homoserine lactone (AHL) signals, common names, and representative organisms that produce them**		
Pseudomonas	**C4-HSL**	
Vibrio harveyi	**4-OH-C4-HSL**	
Erwinia carotovora, Pantoea stewartii	**3-Oxo-C6-HSL**	
Pseudomonas	**C6-HSL**	
Ralstonia solanacearum	**C8-HSL**	
Agrobacterium	**3-Oxo-C8-HSL**	
Pseudomonas	**3-Oxo-C10-HSL**	
Pseudomonas	**C12-HSL**	
Other types of communication signals		
An A1-2 autoinducer		
p-Coumaroyl-HSL		
γ-butyrolactone (produced by *Streptomyces* spp.)		
Halogenated furanone		
Epinephrine		

determined by the length of the fatty acyl side chain as well as the types and number of side chain modifications. AHL-mediated regulation of gene expression is one of the best known examples of quorum sensing.

An excellent example of a quorum sensing system is that of the bioluminescent marine bacterium *Vibrio fischeri* (now classified as *Aliivibrio fischeri* by some). This bacterium exists in a symbiotic association with the marine squid *Euprymna scolopes* (Information Box 20.1). This tiny nocturnal squid contains specialized organs called light organelles that are colonized only by *V. fischeri*, which is ubiquitous in the ocean at low cell densities. Immature *E. scolopes* have cilated "arms" that collect sea water and pass it over the empty light organelles. When *V. fischeri* comes in contact with the light organelles, it colonizes the organs and is supplied nutrients by the host. Colonization by *V. fischeri* induces the loss of the squid's ciliated "arms" by apoptosis (programmed cell death), and also causes the bacteria to lose their flagella and reduce their cell size, indicating a true symbiosis. The eukaryotic squid host provides the prokaryotic bacterium with a nutrient-rich environment in which to live. In return the bacterium produces bioluminescence, or light. The benefit of bioluminescence for the squid may be several-fold. As one example, it may serve as an anti-predation strategy in which light production enables the squid to counter-illuminate itself using the light from *V. fischeri.* This counter-illumination is aimed downwards and enables the squid to avoid casting a shadow beneath it on nights when light from the stars and moon penetrates the seawater, thus allowing the squid to be invisible to predators beneath it. Alternatively, it may enable the squid to locate each other in the darkness of the oceans depths.

The occurrence of bioluminescence is correlated with the cell-population density of the bacteria in the host. As the population of bacterial cells increases it produces and releases an AHL signal into its extracellular environment, which is the eukaryotic squid's light organ. Due to the physical boundaries of the organ, the concentration of the AHL increases, and hence acts as a signal which communicates to the bacteria that they are inside the host as opposed to outside in the seawater. The AHL also initiates a signaling cascade that results in the emission of light. The squid can flush the light organelles until the bacterial population size and signal concentration is below the threshold required for bioluminescence. Hence, the squid controls the level of bioluminescence (Information Box 20.1)

The simplest molecular model for quorum sensing regulation involves two proteins. The first is an AHL synthase (I protein) encoded by a gene commonly referred to as an *I* gene (*luxI*, *phzI*, *traI*, *lasI*, etc.), which converts cellular precursors into one or more AHL signals. The second is an AHL-responsive regulatory protein (R protein), encoded by a gene referred to as an *R* gene (*luxR*, *phzR*, *traR*, *lasR*, etc.), required for the activation (or in some cases, the repression) of specific genes. At low cell densities, the AHL signal either diffuses out of the cell following a concentration gradient, or is actively transported out of the cell. As cell density increases, the concentration of AHL signal accumulates within the cell. Upon reaching a threshold concentration, the AHL interacts with the R protein resulting in dimerization of the R protein. This causes the R protein dimer to bind to a specific sequence in the promoter of the quorum sensing-regulated gene(s). This binding of the R protein results in enhanced recruitment of RNA polymerase that activates gene expression (Information Box 20.1).

Many Gram-negative bacteria have been shown to utilize quorum sensing to regulate the expression of diverse traits. In all cases, increasing cell numbers result in increased AHL signal concentration. This in turn results in interaction with the R protein that alters the binding affinity of the R protein for a specific sequence located within the promoter regions for genes under quorum sensing control (Dunlap, 1999; Zhu and Winans, 1999; Qin *et al*., 2000). Evidence that production of AHL signals is required for quorum sensing expression has been shown for many Gram-negative bacteria. An early example was the demonstration that inactivation of the *V. fischeri luxI* gene results in no light production *in vitro* unless exogenous AHL is supplied. Many Gram-negative plant-associated soil bacteria also contain quorum sensing regulatory systems. The first example that AHLs were required for bacterial gene expression on plant roots was demonstrated when a *phzI* AHL mutant of *Pseudomonas chlororaphis* had a 1000-fold reduction in expression of the quorum sensing regulated phenazine genes on wheat roots (Wood and Pierson, 1996, see below). Other evidence that AHLs are important includes the discovery that concentrations of C_4-HSL and 3-oxo-C_{12}-HSL, two AHL signals produced by the opportunistic human pathogen *Pseudomonas aeruginosa*, can be detected in sputum samples of infected patients (Erickson *et al*., 2002).

This gene regulation mechanism was originally named quorum sensing because it was believed that it enabled a bacterium to determine its own population size or "quorum" (Fuqua *et al*., 2001). It is now recognized that a single bacterial cell will activate quorum sensing-regulated genes in the presence of sufficient AHL signal. Thus, it is the concentration of AHL, not the number of bacteria *per se*, that determines gene expression patterns (Dulla and Lindow, 2008). This has important implications regarding the effect of AHL signaling on bacterial behavior in single or mixed species populations.

Information Box 20.1 Quorum Sensing in a Marine Squid

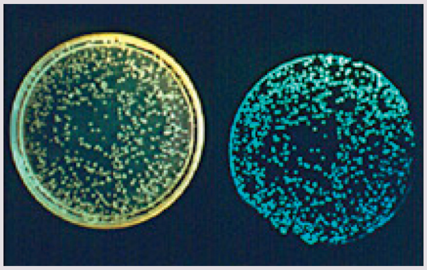
Quorum sensing was first discovered in the late 1960s during studies on a light-producing marine squid, *Euprymna scolopes* (see top left figure). This tiny nocturnal squid contains specialized organs called light organelles that are colonized by a single luminescent bacterium, *Vibrio fischeri*. The ability of *Vibrio fischeri* to luminesce is contained on an operon (the *lux* operon) that encodes enzymatic machinery that results in the release of photons of light (see signaling pathway figure below right). The first gene in the operon, *luxI*, encodes for an AHL synthase (LuxI) that converts cellular precursors into the AHL signal C6-HSL. Upstream of the *lux* operon is *luxR*, which encodes the transcriptional protein (LuxR) required for activation of high levels of expression of the *lux* operon. In the absence of AHL signal, LuxR is inactive.

At low cell densities, the AHL signals generated by LuxI diffuse passively out of the cell following a concentration gradient. Thus, the *lux* operon is not expressed.

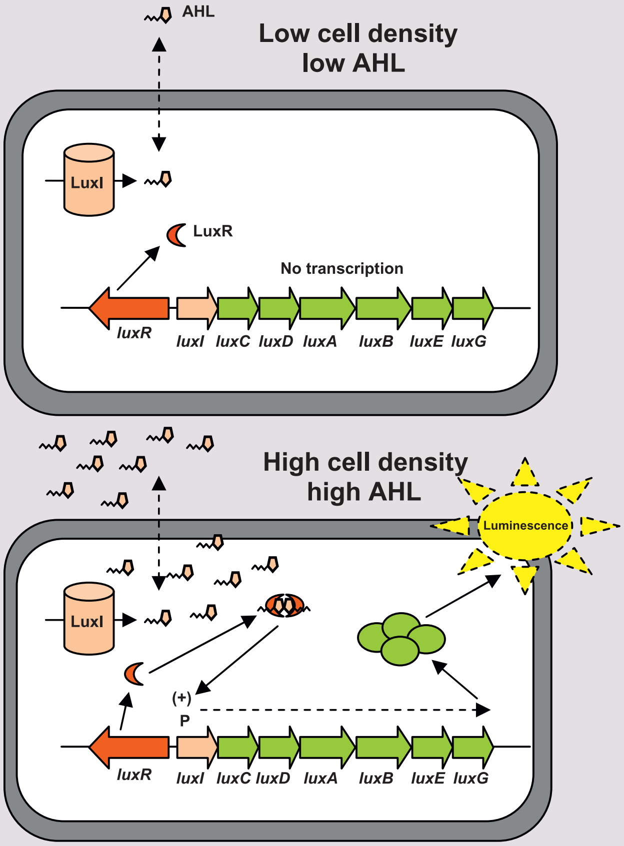
As the bacterial population size increases, the number of C6-HSL signals accumulates in the light organelle and thus within each bacterial cell. When a sufficient concentration of C6-HSL is reached within the bacterial cell, it interacts with the LuxR protein, causing LuxR to dimerize. Dimerization of LuxR allows it to bind to the *lux* operon promoter region and increase expression of the *lux* operon. Note that expression of the operon results in increased levels of LuxI, resulting in even more C6-HSL signal production and ensuring a rapid onset of light production.

Interestingly, the squid can control the amount of light produced by *V. fischeri* in the light organelles either by covering the light organelle with its black ink sac or by reducing the *V. fischeri* population in the light organelle by flushing out excess bacteria with seawater.

Euprymna scolopes, a bioluminescent squid. From the National Science Foundation, 2005a.



Colonies of *Vibrio fischeri*. (Left) Photo taken under light source. (Right) Photo taken in the dark showing the bacteria luminescing.



From the National Science Foundation, 2005b.

20.2.2 Interspecies Cross-Signaling

Quorum sensing was originally termed "autoinduction" as it was first identified and studied in single species bacterial communities. More recently, it is recognized that communication via AHL signals occurs between related and unrelated bacterial populations, as well as between bacteria and their eukaryotic hosts. The ability of AHLs to serve as communication signals between species of bacteria (referred to as interspecies signaling, cross-talk or cross-communication) is now widely recognized. One of the first demonstrations of cross-communication utilized the beneficial root-colonizing "rhizosphere" bacterium *Pseudomonas chlororaphis* strain 30-84 (Pierson *et al.*, 1998). *P. chlororaphis* produces three pigmented antibiotics called phenazines. Phenazines are nitrogen-containing broad-spectrum compounds synthesized by the products of the phenazine operon (*phzXYFABCDO*) (Mavrodi *et al.*, 1998). One of the phenazines produced by *P. chlororaphis* is colored bright orange. Phenazine production is regulated, in part, by the PhzR/PhzI quorum sensing system. PhzI is an AHL synthase that produces the AHL C_6-HSL (Table 20.1), and PhzR is the transcriptional regulator that responds to the AHL signal.

To test the hypothesis that *P. chlororaphis* could cross-communicate with other members of the wheat rhizosphere community, 800 culturable bacterial strains from the rhizosphere (the zone surrounding the plant root; Chapter 19) of wheat plants from different U.S. geographic regions were utilized. These were spotted individually onto a lawn of a *phzI* mutant of strain 30-84 that did not produce orange phenazines because it could not produce the C_6-HSL quorum sensing signal. Hence, the *phzI* mutant lawn appeared white. Approximately 8% of the library strains restored phenazine production to the *phzI* mutant as indicated by restoration of orange pigmentation in the lawn, a phenomenon termed positive cross-communication (Figure 20.2).

Of even greater importance, cross-communication between various rhizosphere strains was demonstrated *in situ* on wheat roots using a *phzI*$^-$, *phzB::inaZ* reporter of strain 30-84 (Pierson *et al.*, 1998). This nomenclature indicates that the reporter does not produce phenazine as it is defective in the PhzI AHL synthase, and that it has a reporter gene encoding ice nucleation activity inserted within the genomic *phzB* biosynthetic gene (*phzB::inaZ*). For further explanation of reporter genes, see Section 13.6.3. Thus, this reporter expresses ice nucleation activity only when the phenazine biosynthesis operon has been induced. However, since the reporter's *phzI* gene is defective, it does not express the phenazine operon, and so has a 1000-fold decrease in ice nucleation

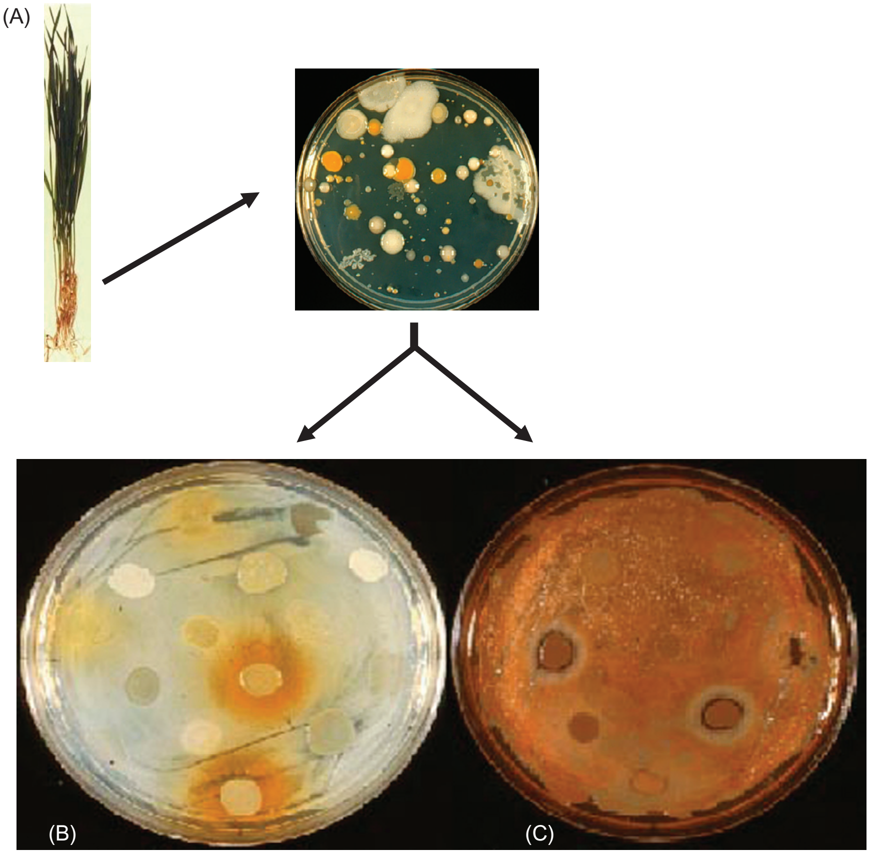


FIGURE 20.2 Positive and negative cross-talk by rhizosphere bacteria and *Pseudomonas chloroaphis*. (A) Collection of wheat rhizosphere strains. (B) Positive cross-talk on a lawn of a *phzI* mutant of *P. chloroaphis* unable to produce phenazines. The spots are rhizosphere isolates that restore various levels of phenazine production via AHL signals. (C) Negative cross-talk on a lawn of wild-type *P. chloroaphis*. The spots are rhizosphere isolates that inhibit phenazine production by producing diffusible signals (Morello *et al.*, 2004). Photos courtesy L.S. Pierson III.

activity as compared to the wild-type *phzI*$^+$ strain. When the *phzI*$^-$, *phzB::inaZ* reporter was grown with several of the 800 unrelated wheat rhizosphere strains, ice nucleation activity by the reporter strain was restored to wild-type levels on roots in soil, demonstrating that quorum sensing was required for phenazine production on roots, and that communication occurred between different bacterial populations via AHL signals! Thus, different bacteria can communicate via AHL signals in a natural environment (the wheat rhizosphere).

A large number of diverse Gram-negative bacteria have been shown to utilize quorum sensing as a key regulatory mechanism. Additionally, many currently nonculturable bacteria also appear to produce quorum sensing signals or related compounds as detected by several different quorum sensing reporter bacteria. This widespread occurrence of quorum sensing in bacteria indicates that quorum sensing plays important roles in bacterial ecology. It is difficult to obtain direct evidence for many of the ecological roles of quorum sensing. Some good examples include the demonstration that quorum sensing defective mutants of bacterial pathogens have reduced ability to persist in the host and to cause disease, and that mutants of beneficial bacteria such as *P. chlororaphis* defective in quorum sensing are impaired in their ability to persist and prevent disease. More recently, quorum sensing has been shown to be critical to the ability of several bacteria to form biofilms on surfaces and tissues (see Lazar, 2011 for a review).

Some of the many possible ecological roles of quorum sensing include: (1) coordination of gene expression within a single bacterial population; (2) coordination of gene expression and bacterial behavior among multiple populations; (3) avoidance of host defense responses; and (4) direct signaling between the bacterium and the host organism (Section 20.4.5).

20.2.2.1 Additional Quorum Sensing Signals

Another class of bacterial signal molecules was identified from the photosynthetic soil bacterium *Rhodopseudomonas palustris* (Schaefer *et al.*, 2008). Similar to the better studied AHL signals, this signal molecule contains a homoserine lactone (HSL) ring. However, in contrast to the fatty acid lipid side chain joined to the HSL in AHLs, this signal utilizes p-coumaroyl, a major monomer component of plant lignin. This signal was named pC-HSL to distinguish it from the classical AHL signals (Table 20.1). Since bacteria do not synthesize p-coumaroyl, it must come from an exogenous source, presumably a lignin-containing plant host. When pC-HSL reaches a threshold concentration in *R. palustris*, several genes involved in bacterial chemotaxis become activated as well as many others for which the function is currently unknown. A fascinating interaction dependent on the levels of pC-HSL quorum sensing signal occurs between *Emiliania huxleyi*, an environmentally important marine plant-like microalga involved in algal blooms, and *Phaeobacter gallaeciensis*, an α-proteobacterium that colonizes the alga and gains nutrients and a surface to colonize (Seyedsayamdost *et al.*, 2011). As the algal population lives in relatively nutrient-poor marine seawater, it benefits from this association with *P. gallaeciensis*, which produces the growth hormone phenylacetate acid that stimulates algal growth. In addition, *P. gallaeciensis* also produces a broad-spectrum antibiotic called tropodithietic acid that prevents parasitic pathogens from attacking *E. huxleyi.* However, as the algal cells age, lignin in the algal cell wall breaks down, resulting in accumulation of p-coumaric acid, which is in turn converted by *P. gallaeciensis* into pC-HSL. When the level of pC-HSL reaches a threshold concentration, *P. gallaeciensis* activates quorum sensing regulated genes that produce novel algaecides known as roseobacticides. The production of roseobacticides converts *P. gallaeciensis* from a beneficial to an opportunistic pathogen of *E. huxleyi*, causing algal death and release of *P. gallaeciensis* to colonize younger algal cells.

The discovery of this new class of pC-HSL signals has expanded greatly our ideas of potential HSL-based bacterial signals—from molecules synthesized completely from bacterial components to molecules synthesized from bacteria- and plant-derived compounds. This class of signal could integrate the need for a cell density-based quorum of bacteria with a requirement for the presence of a plant host. The identification of this new signal opens up the possibility that there are probably many more novel types of signals yet to be discovered.

20.2.3 Quorum Sensing in *Agrobacterium tumefaciens*, a Ubiquitous Plant Pathogen

Agrobacterium tumefaciens is commonly found in soil and is a plant pathogenic bacterium with an extremely wide host range (>140 plant genera). *A. tumefaciens* causes crown gall disease, so named because the symptoms usually occur at the soil surface or “crown” of the plant (Figure 20.3). Typical disease symptoms include the development of galls, tumor-like growths due to excessive plant cell division at the site of infection. The disease is often easily identified in a variety of dicotyledonous plants, particularly stone fruits, roses and grapes. The ability of *A. tumefaciens* to cause crown gall disease depends on genes necessary for tumor induction that are found within a large 180-kb plasmid called the Ti plasmid (Figure 20.4). This plasmid contains virulence (*vir*) genes required for the processing and transfer of a specific region of the Ti plasmid, known as T-DNA, to the plant. The *vir* genes themselves consist of about 35-kb

FIGURE 20.3 The symptoms of crown gall disease on grapevines caused by *Agrobacterium tumefaciens.* © Queen's Printer for Ontario, 2003. Reproduced with permission.

of DNA, and are essential for tumor formation although they themselves are not transferred into the plant. The induction of the *vir* genes occurs following exposure to signal molecules synthesized by the plant in response to wounding. This explains why crops that rely on root cuttings are particularly susceptible to crown gall disease. One of the signal molecules has been identified as the phenolic compound acetosyringone. This molecule plus sugar monomers, which are precursors of the plant cell wall, are sensed by *Agrobacterium* through the *virA* and *virG* genes, which control the expression of all other *vir* genes. The *virA* gene produces a protein located in the cell wall that appears to sense the phenolic compound directly. This protein has a cytoplasmic domain that becomes activated and in turn activates the cytoplasmic VirG protein, which subsequently activates all other *vir* genes.

Following transfer of the T-DNA from the bacterium to the plant, the T-DNA is targeted to the plant nucleus where it is integrated into a plant chromosome where it codes for the synthesis of two plant growth regulators, auxin and cytokinin, as well as for a group of amino acid derivatives known as "opines." It is fascinating that the promoters that drive the expression of these genes are closely related to eukaryotic promoters. The constitutive synthesis of these plant growth hormones gives rise to the symptoms of crown gall disease. The Ti plasmid in the *A. tumefaciens* on the roots also contains genes that allow the bacterium to utilize the unusual opine amino acids now being produced by the plant cells as a food source. In essence, the bacterium "engineers" the plant to produce a novel food source and to undergo multiple rounds of cell division to increase the plant surface area for bacterial colonization and opine production.

So how does quorum sensing fit into *A. tumefaciens* infection? During the initial rapid growth of the bacterium inside wounded plant tissues, some bacterial cells inadvertently lose the Ti plasmid and their ability to utilize opines. The Ti plasmid contains a quorum sensing system comprised of *traR* and *traI*. This quorum sensing-based strategy is used to ensure that the plasmid is maintained in the population. This is based on maintaining rapid rates of plasmid transfer via conjugation to any cells that may have lost the plasmid (Figure 20.5). Thus, activation of plasmid conjugation by opine-induced quorum sensing control serves to ensure that all members of the *A. tumefaciens* community contain a copy of the Ti plasmid and are able to utilize opines for growth.

20.3 SIGNALING IN GRAM-POSITIVE BACTERIA

Gram-positive bacteria do not utilize the AHL-mediated quorum sensing communication systems found in Gram-negative bacteria. One possible reason is that Gram-positive bacteria lack a porous outer membrane, and instead contain a thick peptidoglycan layer which may restrict diffusion of AHL signals through the cell wall. Instead, some Gram-positive bacteria utilize γ-butyrolactones, molecules that have some structurally similarity to AHLs, to regulate specific gene expression in a cell density-dependent manner. However, the majority of Gram-positive bacteria utilize small peptides as their primary communication molecules.

20.3.1 Gamma-Butyrolactones

The first bacterial communication signals discovered in the 1960s were the gamma-butyrolactones (γ-butyrolactones) produced by *Streptomyces* spp. These organisms are Gram-positive soil bacteria (actinomycetes) that undergo cellular differentiation and are known to produce many secondary metabolites. In fact, many antibiotics in use today are derived from *Streptomyces* spp. (see Section 19.4). A handful of γ-butyrolactones have been purified from different *Streptomyces* species (Table 20.1). These signaling compounds superficially resemble AHLs, and, analogously to AHLs, they differ in their stereochemistry, the length of their fatty acid side chains and side branch number. Both γ-butyrolactones and AHLs are biologically active at

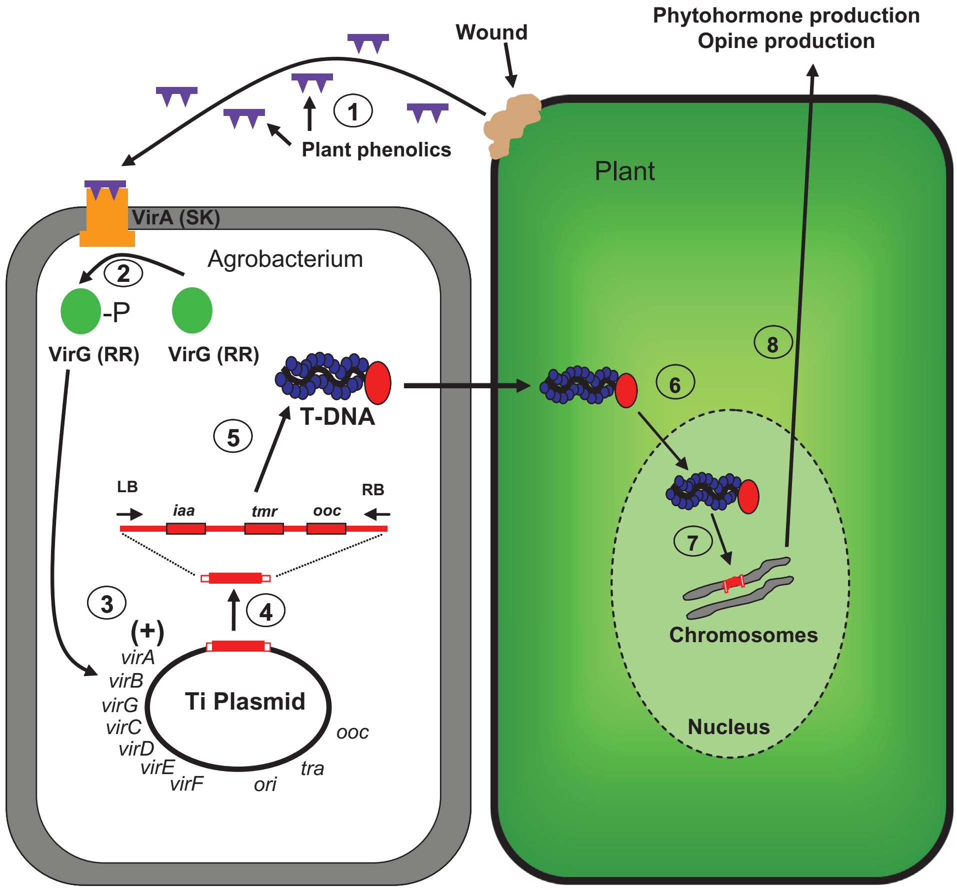


FIGURE 20.4 The plant—*A. tumefaciens* interaction that results in crown gall formation. In soil, *A. tumefaciens* is attracted to plant roots due to their release of root exudates. Wounded plant roots release additional phenolic compounds such as acetosyringone (step 1). Acetosyringone is recognized by a two-component regulatory system (VirA/VirG) encoded by the Ti plasmid as a signal that a plant wound is present (step 2). This recognition induces the expression of a complex region of the Ti plasmid called the *vir* region (for virulence) (step 3). The *vir* region encodes several proteins that interact with a 25 kb T-DNA region on the Ti plasmid. Some of the *vir* gene products are responsible for excising a copy of the T-DNA (step 4) while others encode a type four secretory system (T4SS) that is involved in transferring the copy of the T-DNA across the bacterial and plant cell walls and into the plant cell cytoplasm (steps 5 and 6). The end result is the random insertion of the T-DNA into one of the plant chromosomes (step 7). The integrated T-DNA contains genes that encode for the production of plant hormones and genes that encode enzymes for the production of opines, unusual amino acid derivatives, by the plant cells (step 8). The T-DNA-directed production of opines provides a unique carbon and nitrogen food source for the growth of *A. tumefaciens*. The T-DNA-directed production of growth hormones results in uncontrolled cell division, and the development of the symptoms typical of *A. tumefaciens* infection, plant galls.

extremely low concentrations at the nano- to micromolar level. However, despite these similarities, Gram-negative AHL receptors do not respond to γ-butyrolactones and vice versa, indicating that each sensory system is specific to its own signal type. Hence, AHLs and γ-butyrolactones represent different "languages" used by bacteria. Recall that most AHLs activate target gene expression by altering the affinity of a transcriptional regulatory protein that binds to a promoter region and recruits RNA polymerase to stimulate gene expression. In contrast, γ-butyrolactones usually act by alleviating repression of gene expression. In other words, they cause a repressor protein to dissociate from the promoter region of the target gene(s), which results in subsequent gene expression.

The compound A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) made by the soil bacterium *Streptomyces griseus* was the first γ-butyrolactone identified, and is the best known example of this type of cell density-dependent signaling. The A-factor stimulates aerial mycelium formation and production of the antibiotic streptomycin by regulating the expression of the transcriptional activator AdpA (Figure 20.6).

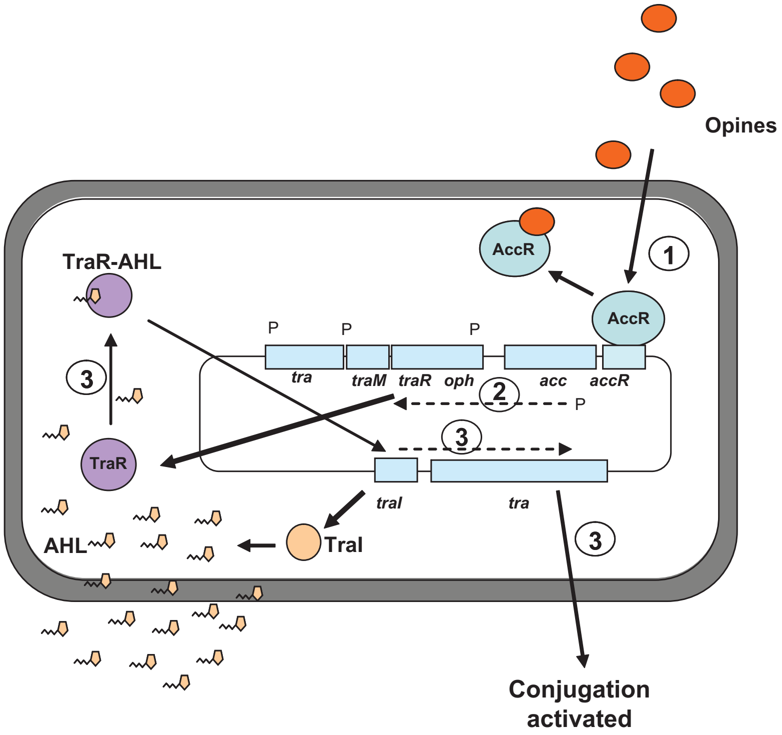


FIGURE 20.5 Quorum sensing control of *A. tumefaciens* infection. *A. tumefaciens* needs to maintain the Ti plasmid during the plant infection process. Therefore, the Ti plasmid contains genes (*tra* genes for transfer) involved in conjugation and transfer of the Ti plasmid back into *A. tumefaciens* cells that may have lost the plasmid. Conjugation is regulated by the TraR/TraI quorum sensing system, a classic quorum sensing regulatory system in which TraR is stimulated by the AHL C8-HSL signal produced by TraI. Uniquely, the TraR/TraI system is only active in the presence of plant-provided opines. In the absence of opines, a repressor protein, AccR, binds within the opine promoter regions blocking the expression of the genes required for uptake and catabolism of opines. When opines are present, however, they bind to AccR (step 1), causing it to dissociate from the promoters and allowing expression of the opine uptake and catabolic regions (step 2). The *traR* promoter region also contains an AccR-binding sequence (step 2). The consequence of this is that TraR is produced only when opines are present. When present, TraR recognizes the *A. tumefaciens* AHL signal and activates bacterial conjugation (step 3).

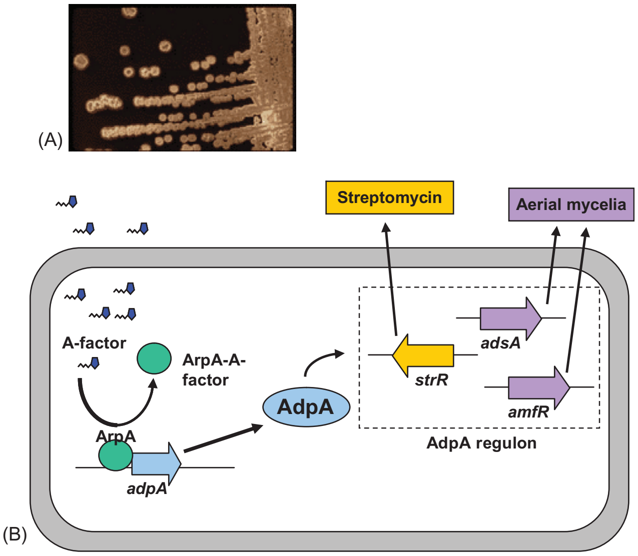


FIGURE 20.6 Quorum sensing in *Streptomyces griseus*. (A) *S. griseus* colonies on an agar plate. (B) Mode of action of A-factor. When A-factor levels are low, the ArpA repressor protein is bound to the promoter region of *adpA* blocking its expression. Once A-factor has accumulated to a threshold concentration within the cell, usually at the mid-exponential phase of growth, it binds to the promoter-bound ArpA. This alters the conformation of ArpA resulting in dissociation from the *adpA* promoter and AdpA production. AdpA subsequently induces the expression of a number of genes (the Adp regulon) including *strR*, which encodes an activator of streptomycin production, and *amfR* and *adsA*, which encode activators for aerial mycelium formation.

20.3.2 Peptide Signaling

Most Gram-positive bacteria utilize a variation of a quorum sensing system that incorporates a two-component regulatory system (see Information Box 20.2) to form a combined system that has been termed a three-component quorum sensing system. This combined system consists of a cell membrane-localized histidine kinase (HSK) sensor protein and a cytoplasmic response-regulator protein (RR), coupled to an autoinducing peptide (AIP) secreted by the producing cell (Lyon and Novick, 2004).

The ubiquity of this type of signaling is exemplified by the Gram-positive foodborne pathogen *Staphylococcus aureus*. This microbe's genome contains approximately 17 putative two-component systems (Rasmussen *et al*., 2000), all of which are believed to be involved in bacteria–bacteria or bacteria–environment signaling! Perhaps the best-studied three-component quorum sensing system is the regulation of exotoxin production by *S. aureus*. These heat-stable exotoxins, including toxic shock syndrome toxin 1 [TSST-1], cause illness in animals and humans (Diggle *et al*., 2003). As shown in Figure 20.7, *S. aureus* utilizes a cell-density sensing mechanism to activate virulence gene (exotoxin) expression, while simultaneously repressing surface factors to avoid host detection.

A second example of Gram-positive cell–cell signaling includes a group of bacteria known as probiotics.

Information Box 20.2 Two-component Regulatory Systems

Two-component regulatory systems comprise a sensor protein and a response-regulator (RR) protein. The sensor protein is normally located within the cell's outer membrane and can detect changes in the external environment surrounding the cell. The sensor protein then communicates these changes to the response-regulator protein inside the cell. The response-regulator protein in turn regulates the expression of key genes to allow an appropriate response to the external stimulus. Communication between the sensor protein and the response-regulator protein is via phosphorylation—dephosphorylation reactions.

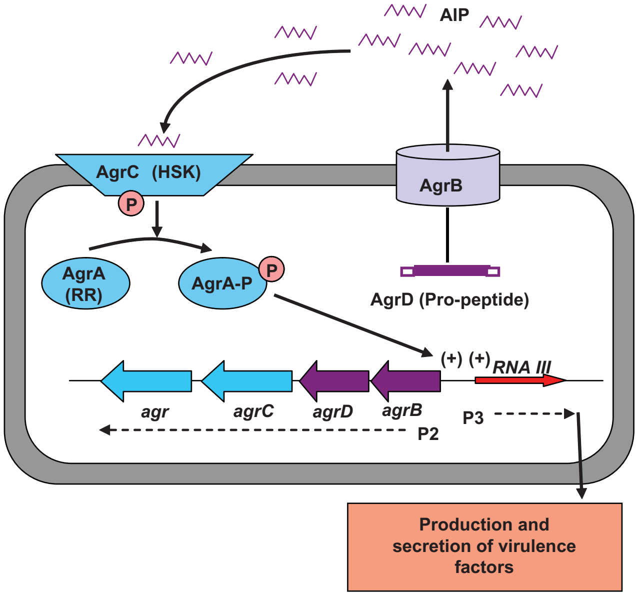


FIGURE 20.7 Regulation of *S. aureus* exotoxin synthesis utilizes an auto-inducing peptide (AIP) signal. The *agr* locus encodes four proteins (AgrA, AgrB, AgrC and AgrD). The AgrD protein is an AIP that is processed and secreted by AgrB, a membrane-associated protease. As the bacterial cell density increases, the AIP concentration accumulates. When AIP reaches a critical level, it binds to the AgrC/AgrA two-component regulatory system. AgrC is a cell membrane-localized histidine sensor kinase (HSK) that, when bound to AIP, transfers a phosphate group to AgrA, a cytoplasmically-localized response regulator (RR). Phosphorylated AgrA (AgrA-PO_4) activates transcription from a promoter (P3) that expresses a small noncoding regulatory RNA (RNA III). RNA III is involved in the activation of a number of virulence genes, including those involved in production and secretion of several exoproteins, enterotoxins, exfoliatins, hemolysins, leukocidins and lipases. Additionally, AgrA-PO_4 serves to repress the expression of several bacterial surface proteins. Because cell surface components are often the triggers for host defense responses, the repression of expression of these surface proteins might assist the bacterium in evading recognition by the host.

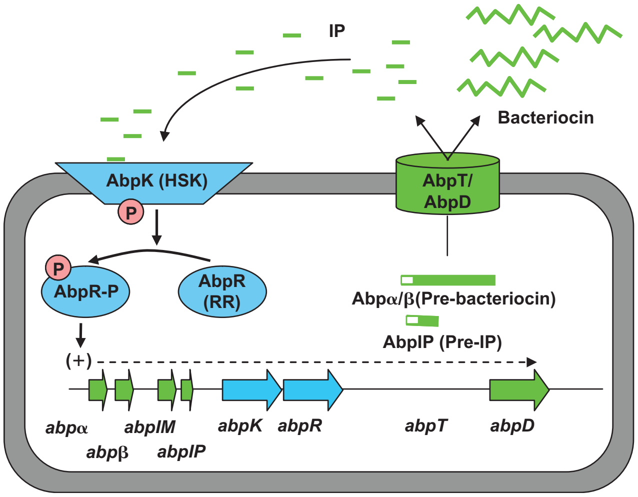


FIGURE 20.8 Probiotic production is regulated by an auto-inducing peptide in *L. salivarius*. Each *L. salivarius* cell produces a small amount of a pre-inducing peptide (AbpIP) that is processed and secreted into the environment by the ABC transporter complex AbpT/AbpD as inducing peptide (IP). As the numbers of *L. salivarius* increase, the level of IP increases until it is recognized by the HSK AbpK that, in turn, phosphorylates AbpR. Phosphorylated AbpR up-regulates production of the pre-bacteriocin genes (*abp118α* and *abp118β*). These encode enzymes that synthesize the pre-bacteriocin that is subsequently processed and secreted by the AbpT/AbpD complex and that then inhibits harmful bacteria.

Probiotics are intestinal bacteria that exert positive effects on the health of the human or animal host by interfering with the ability of deleterious bacteria to colonize (Guarner and Schaafsma, 1998). Probiotic bacteria inhibit colonization via the production of extracellular peptides known as bacteriocins (Riley and Wertz, 2002). *Lactobacillus salivarius* UCC118 is an example of a well-studied probiotic that colonizes the human intestine and produces a broad-spectrum bacteriocin effective against a number of foodborne and medically important bacterial pathogens (Flynn *et al.*, 2002). *L. salivarius* utilizes a three-component regulatory system to control bacteriocin production (Figure 20.8).

20.4 OTHER TYPES OF SIGNALING

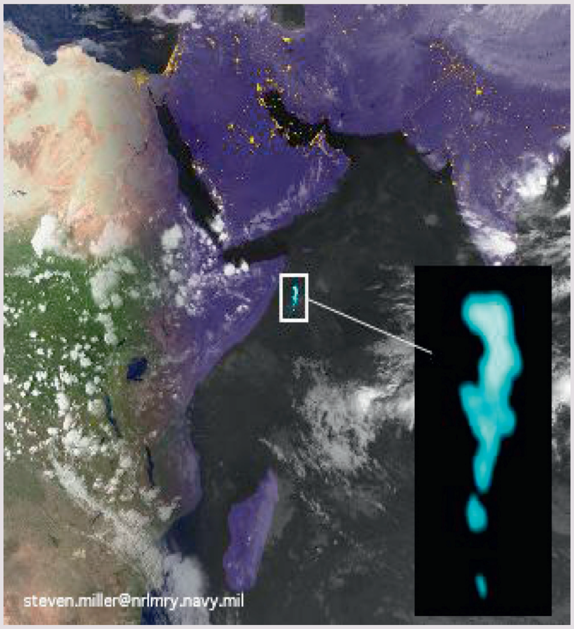
20.4.1 Universal Signals Autoinducer-2 and Autoinducer-3

In 1997 a novel type of universal bacterial signal was reported that is quite different from AHL (Gram-negative) and peptide (Gram-positive) signals (Xavier and Bassler, 2003). This new class of signal, termed

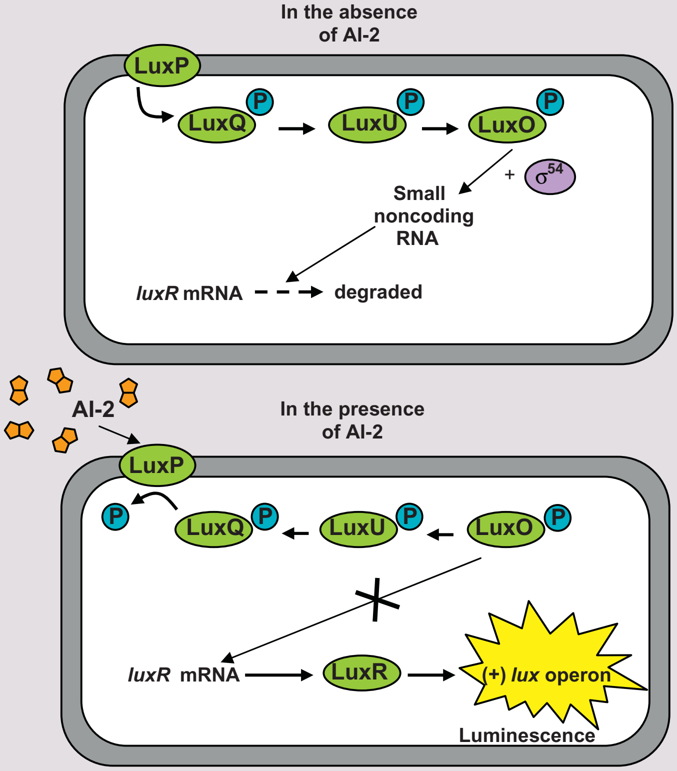
Autoinducer 2 (AI-2), is a family of related furanosylborate diester molecules that is produced by over 55 Gram-positive and Gram-negative bacteria (Table 20.1). All of these bacteria contain a synthase gene named *luxS* that if inactivated results in the loss of AI-2 production. Scientists are just beginning to understand the AI-2 signal. So far, AI-2 has clearly been shown to be involved in signaling in two species, *Salmonella enterica* serovar Typhimurium and *Vibrio harveyi* (see Information Box 20.3) (reviewed in Vendeville *et al*., 2005). A second potential universal bacterial signal, Autoinducer-3 (AI-3), was reported in 2003 (Sperandio

Information Box 20.3 *Vibrio harveyi* Bioluminescence

The bioluminescent bacterium *Vibrio harveyi* has a well-characterized AI-2 regulatory system. Similar to *V. fischeri*, *V. harveyi* can colonize a number of marine organisms and also produces light via a bioluminescence operon (*lux* operon). *V. harveyi* also can exist in high numbers in a free-living state. Fascinatingly, *V. harveyi* has been implicated as the causative agent of milky seas, a phenomenon in which *V. harveyi*-generated bioluminescence can cover areas of the ocean the size of Connecticut. These milky seas have been observed by merchant vessels and are visible from space (see figure on left, from Miller *et al*., 2005). However, *V. harveyi* regulates light production much differently than *V. fischeri* (Information Box 20.1). *V. harveyi* contains a LuxR regulatory protein required for *lux* operon activation, but the LuxR protein does not require an AHL signal. Instead, in *V. harveyi*, there is a reversible phosphorylation cascade involving four proteins, LuxP, LuxQ, LuxU, and LuxO. In the absence of AI-2, there is an induction of a signal cascade mechanism from the periplasmic protein LuxP to the cytoplasmic proteins LuxQ, LuxU, and LuxO. Phosphorylated LuxO (LuxO-PO_4), in conjunction with the sigma factor RpoN (σ^{54}), results in activation of a small noncoding regulatory RNA (sRNA) that results in the degradation of the *luxR* mRNA and therefore no luminescence. Alternatively, when sufficient AI-2 is present, it causes the phosphorylation cascade to go from LuxO to LuxQ, resulting in dephosphorylation of LuxO. In this instance, LuxO is inactive and the luxR mRNA transcript is protected, resulting in production of luminescence.



The milky sea phenomenon photographed from space.



From: http://www.lifesci.ucsb.edu/~biolum/organism/milkysea.html. Steve Miller (2005)·Naval Research Lab. Used with permission.

et al., 2003). AI-3 is chemically distinct from AI-2, although its exact structure is not yet known.

20.4.2 Bacterial Muropeptides

Although the exact structure of bacterial cell walls can vary among different organisms (i.e., Gram-negative versus Gram-positive bacteria; Figure 2.3), all bacterial cell walls contain peptidoglycan as a major component. This peptidoglycan layer is composed of repeating disaccharide subunits of β(1-4)-*N*-acetylglucosamine—β(1-4)-*N*-acetylmuramic acid. While the basic disaccharide subunits are conserved, there can be large variations in the specific structures and chain lengths of these peptidoglycan components. Enzymatic digestion of the peptidoglycan layer results in the generation of fragments referred to as muropeptides (Boudreau *et al.*, 2012). Because the integrity of the cell wall is of paramount importance for survival, bacterial cell walls have characteristics that make them more recalcitrant to degradation. For example, bacterial cell walls contain unique components, such as the presence of D-amino acid stereoisomers (e.g., D-alanine), which are rarely found in other organisms. This is why many antibiotics used to treat bacterial infections target the cell wall, which makes them specific to prokaryotes. Recently, muropeptides have been recognized as signals utilized by bacteria and their hosts. For example, bacteria use the detection of muropeptides to signal that cell wall-degrading antibiotics are present; bacteria that form long-term survival structures such as spores use muropeptides as germinants to trigger vegetative growth; and eukaryotic organisms can use muropeptides to sense that a bacterium is present within host tissues.

20.4.2.1 Gram-Negative Bacterial Muropeptide Sensing

Growth and cell division of Gram-negative bacteria requires the constant synthesis and turnover of peptidoglycan. Peptidoglycan synthesis involves the formation of a cytoplasmic lipid precursor known as Lipid II that is transported through the cytoplasmic membrane into the periplasmic space, where it interacts with a group of proteins collectively known as penicillin-binding proteins (PBPs). Existing peptidoglycan is rapidly turned over during cell growth and division. One product of the breakdown of peptidoglycan is the formation of anhydromuropeptides. These can leave the cell, but are transported across the membrane into the cytoplasm by the enzyme AmpG permease. Inside the cell, the muropeptide is further broken down and recycled for peptidoglycan synthesis. Although peptidoglycan recycling occurs in Gram-negative bacteria, loss of this pathway does not result in reduced cell growth or any obvious phenotypic deficiency, suggesting that this recycling pathway plays another role for the cell. It was observed that reductions in available muropeptide levels, similar to the result of the addition of β-lactam antibiotics that degrade the peptidoglycan layer, results in the induction of resistance pathways to this class of antibiotics. The current hypothesis is that the relative concentrations of muropeptides synthesized *de novo*, versus transported into the cytoplasm by the AmpG permease, control the expression of a regulatory protein known as AmpC. Reductions in *de novo* muropeptide synthesis increase *ampC* transcription that results in the activation of a series of β-lactamases that convey high level resistance to β-lactam antibiotics.

20.4.2.2 Gram-Positive Muropeptide Sensing

Gram-positive bacteria such as *Staphylococcus aureus* have cell walls that lack an outer membrane. Instead, they have a single cytoplasmic membrane surrounded by a thick exposed peptidoglycan layer. Antibiotics effective against Gram-negative bacteria are often ineffective against Gram-positive bacteria, attributed partly to the presence of this thicker peptidoglycan layer. One exception to this is the glycopeptide vancomycin, which is effective against Gram-positive bacteria. Vancomycin binds to the peptidoglycan biosynthetic precursor Lipid II at the —D-Ala-D-Ala- stem. Resistance to vancomycin occurs by two mechanisms. The first, identified in enterococcal Gram-positive lactic acid bacteria, has the —D-Ala-D-Ala- moiety of Lipid II replaced with —D-Ala-D-Lac-, which has a lower affinity for vancomycin. The multidrug-resistant pathogen known as methicillin-resistant *S. aureus* (MRSA) contains a thicker and more cross-linked peptidoglycan believed to confer increased vancomycin resistance as compared to *S. aureus*. MRSA is often acquired in hospitals, where β-lactam antibiotics are commonly used to control Gram-negative bacteria (which usually co-occur with *S. aureus*). However, the combination of β-lactam antibiotics in conjunction with vancomycin can result in MRSA strains with high levels of vancomycin resistance. This is due to the fact that β-lactam antibiotics act by mimicking the —D-Ala-D-Ala- moiety of Lipid II, and by binding irreversibly to the PBPs involved in peptidoglycan biosynthesis. The resulting high-level vancomycin resistance is hypothesized to be the combined result of the replacement of the susceptible PBP by a spontaneous variant that does not bind to β-lactam antibiotics, and the replacement of —D-Ala-D-Ala- with —D-Ala-D-Lac- in Lipid II, which does not bind vancomycin efficiently. These β-lactam-/vancomycin-resistant MRSA derivatives are known as BIVR-MRSA (β-lactam-induced vancomycin-resistant-MRSA). Interestingly, BIVR-MRSA cells release more muropeptides into the surrounding medium than *S. aureus* or MRSA cells. Specifically, analysis of culture filtrates has identified high levels of a specific muropeptide (GlcNAc-MurNAc-L-Ala-D-*i*Gln-L-Lys-(e-Gly_4)-D-Ala-Gly_2). When this purified muropeptide was added to cultures of BIVR-MRSA cells in the absence of β-lactam antibiotics, the cells grew much more rapidly than vancomycin-only treated cells. Currently, the basis for this enhanced growth rate is unclear, but is problematic to the control of MRSA infections.

Gram-positive bacteria were originally thought to lack a mechanism for peptidoglycan recycling. This was thought to be due to the lack of an enclosed periplasmic space, since Gram-positive bacteria lack an outer membrane. Additionally, up to half of the peptidoglycan layer appeared to be released during cell growth (Mauck *et al.*, 1971). However, several homologues of genes for muropeptide-recycling have been identified in *Bacillus subtilis* (Litzinger *et al.*, 2010). Hence, it is possible that Gram-positive bacteria could also alter transcriptional patterns resulting in drug resistance in response to perturbations in peptidoglycan recycling.

One unique aspect of several Gram-positive bacteria is the ability to form long-term survival structures known as spores, in response to adverse environmental conditions, such as limited nutrient availability. The regulation of the development of these resting structures is highly ordered and temporally complex. We know even less about how these metabolically minimized structures sense their surroundings, and the molecular mechanisms involved in initiation of germination. During spore formation, the peptidoglycan undergoes multiple structural alterations that result in enhanced structural integrity and resistance to degradation. Germination of dormant spores involves multiple sensory pathways. One of these sensory pathways involves muropeptides. These muropeptides have been classified as spore germinants, and are believed to bind to cytoplasmic membrane-bound, eukaryotic-like serine/threonine kinases (STKs). Synthetic muropeptides have been identified that also bind to spore STKs. It has been shown that *Bacillus subtilis* spores contain a PrkC STK that responds to one muropeptide, while spores of *S. aureus* contain a similar PrkC STK, but respond to a different muropeptide. Replacement of the *B. subtilis* PrkC with the *S. aureus* PrkC STC resulted in *B. subtilis* spores that only germinated in the presence of the *S. aureus* muropeptide. Recognition of the specific muropeptide occurs via a penicillin-binding-associated and serine/threonine kinase-associated (PASTA) domain in the PrkC STK. Of additional interest is that the PrkC STKs respond preferentially to muropeptides produced by growing rather than non-growing cultures, indicating that the structures of muropeptides change dependent on the growth stage of the cells.

20.4.2.3 Recognition of Bacterial Muropeptides as Part of Eukaryotic Host Immunity

Humans, animals and plants contain evolutionarily conserved innate immunity systems. This innate immunity utilizes pattern-recognition receptors (PRRs) that become activated upon binding to highly conserved bacterial structures known as microbial-associated molecular patterns (MAMPs). These receptors help the host recognize “non-self” molecules. Examples of bacterial structures recognized by PRRs include flagella, lipopolysaccharides (LPS) and muropeptides. These have also been referred to as PAMPs (pathogen-associated molecular patterns) when used in reference to pathogenic bacteria. There are three basic classes of PRR receptors: the extracellular Toll-like receptors; the nucleotide-binding domain/leucine-rich repeat receptors (NLRs); and the retinoic acid-inducible gene receptors (RLRs). In addition to MAMP recognition by plant and animal hosts (termed pathogen-triggered immunity, or PTI), plants recognize strain-specific pathogen protein effectors that are transported into the host cell during the interaction (termed effector-triggered immunity, or ETI). There is considerable cross-talk among PRRs, probably to ensure that an immune response is generated only when the combined inputs of multiple PRRs reach a threshold value. This may increase the ability of the host to distinguish between the presence of the normal beneficial microbiome and the presence of pathogens.

In animal cells, the two NLR receptors (NOD1 and NOD2) recognize different specific muropeptide structures generated from the enzymatic degradation of peptidoglycan. Interestingly, one muropeptide called muramyl dipeptide (MDP) is produced from enzymatic degradation of both Gram-negative and Gram-positive bacterial peptidoglycan, and has been used as an immune adjuvant for over 40 years. Animal cells also contain a second class of muropeptide receptors called peptidoglycan-recognition proteins (PGRPs), which differ from NOD receptors in that they recognize polymeric peptidoglycan.

20.4.3 Eavesdropping on the Party Line

Although many bacteria produce signals, many other bacteria do not produce signals (as far as we know) but still have the ability to “listen in” on the conversations. This eavesdropping may allow an incoming bacterium to detect the metabolic state of the community to determine whether expression of specific traits is appropriate. For example, there is evidence that *Pseudomonas aeruginosa* listens in on the indigenous microbial community during the infection process. *P. aeruginosa* is an opportunistic pathogen that is problematic for immunocompromised patients, and it is a primary cause of morbidity and mortality in patients suffering from cystic fibrosis, a hereditary life-threatening childhood disease. This ubiquitous Gram-negative bacterium primarily colonizes the lungs in cystic fibrosis patients, where it exacerbates mucus formation.

Enteric bacteria, which include the genera *Escherichia*, *Salmonella*, *Klebsiella*, *Enterobacter* and *Citrobacter*, all contain a single LuxR homologue named SdiA, but lack a corresponding LuxI homologue (Soares and Ahmer, 2011). Genes such as SdiA are considered orphan quorum sensing regulators. Although these bacteria cannot produce AHLs, they respond to a broad range of exogenous AHL signals produced by other bacteria. For example, *Salmonella enterica* serovar Typhimurium, a major cattle and poultry pathogen responsible for salmonellosis, uses a SdiA regulator that recognizes exogenous AHL signals to activate genes in its chromosome (*srgE*), and an operon (*rck*) contained on a virulence plasmid that encodes for resistance to the host immune response and for production of factors such as pili involved in pathogenesis. In some cases, these orphan LuxR genes encode proteins that form dimers with the known LuxR protein making them inaccessible to AHL binding. This may represent a mechanism to prevent premature expression of pathogenicity traits that would induce a host defense response. However, we are only beginning to touch the tip of this fascinating "iceberg."

20.4.4 Quorum Quenching and Quorum Sensing Inhibition

If the accumulation of AHL signals confers a competitive advantage on a microorganism, then other organisms might develop mechanisms to thwart this advantage by breaking down the AHL signal or otherwise interfering with the signaling system. This phenomenon was first termed "quorum quenching" (Dong *et al*., 2001). More than 20 genera of bacteria are known to degrade AHL signals (Uroz *et al*., 2009). The ability to breakdown AHL signals occurs primarily by two enzymatic activities—that of AHL lactonases that open the homoserine lactone (HSL) ring, and AHL acylases that cleave the fatty acid sidechain from the HSL. Although *P. aeruginosa* uses AHL signaling to regulate the expression of genes required for successful pathogenesis, it also has three acylase enzymes that degrade AHL signals (Huang *et al*., 2003). How the concomitant synthesis and turnover of AHL signals influences pathogenicity is currently under study. Some nitrogen-fixing *Sinorhizobium* spp. that colonize and nodulate legume roots (e.g., alfalfa, pea) (Chapter 19) contain at least six AHL-degrading enzymes (Krysciak *et al*., 2011). Some isolates of *Bacillus cereus*, a Gram-positive soil bacterium that does not itself produce AHL signals, encode a lactonase, reducing the activity of AHL signals 1000-fold. The *Bacillus* spp. gene *aiiA*, which encodes the lactonase, was cloned into potato and shown to reduce virulence by the AHL-dependent soft rot pathogen *Erwinia caratovora* (Dong *et al*., 2001). In comparison, a plant pathogenic *Ralstonia* spp. was shown to produce a bacterial AHL acylase (Lin *et al*., 2003). The gene encoding this enzyme, *aiiD*, was cloned and shown in *E. coli* to inactivate several AHL signals by cleaving the homoserine lactone ring from the fatty acid side chain.

Degradation of AHL signals probably is important for more reasons than reducing a competitor's advantage. It is possible that without AHL turnover, the accumulation of AHLs would render them poor signaling molecules (Diggle *et al*., 2006). Additionally, we know that many pathogenic bacteria utilize quorum sensing via AHL signals to control the production of pathogenicity proteins required for successful infection and disease. Premature expression of these genes would trigger strong host responses that would interfere with the ability of the pathogen to invade successfully. Therefore, degradation of AHL signals would prevent this early expression or "giving one's position away," until sufficient bacterial numbers had accumulated to successfully infect the host.

There is quite a range of variation among hosts with respect to the ability to degrade AHL signals. For example, the legume alfalfa degrades AHL signals by hydrolysis (Götz *et al*., 2007), while other plants such as *Arabidopsis* and barley cannot degrade AHLs. AHLs are quite stable on *Arabidopsis* roots, and are taken up by the plant and transported to upper plant parts (von Rad *et al*., 2008). In addition to AHL degradation, some plants also produce compounds that interfere with bacterial quorum sensing. L-Canavanine, an arginine analogue produced by alfalfa, blocks activation of *Sinorhizobium* spp. quorum sensing, which results in loss of production of an exopolysaccharide (EPSII) on the surface of the bacterial cell that is required for colonization of alfalfa roots (Keshavan *et al*., 2005). However, why a plant host would want to block colonization by a potentially beneficial nitrogen fixing root symbiont is unclear.

Some organisms utilize quorum quenching to block colonization by bacteria. A well-studied example is the marine red alga *Delisea pulchra* (Rasmussen *et al*., 2000). This alga produces a halogenated furanone [(5Z)-4-bromo-5-bromomethylene-3-butyl-2(5H)-furanone] (Table 20.1) that interferes with AHL quorum sensing by binding to the AHL receptor protein LuxR which results in its accelerated turnover in the cell. This halogenated furanone has broad effects on quorum sensing as it was also shown to inhibit the AI-2 quorum sensing system in *E. coli* (Ren *et al*., 2001).

In other cases, hosts may encourage colonization by beneficial bacterial species that degrade AHL signals produced by pathogenic bacteria. An example is the bacterium *Bacillus* isolate QSI-1 isolated from the intestine of Prussian carp (*Carassius auratus gibelio*), the wild form of goldfish (Chu *et al*., 2010). Strain QSI-1 produces an enzyme that degrades AHLs. When strain QSI-1 was fed to fish, it significantly reduced the ability of the

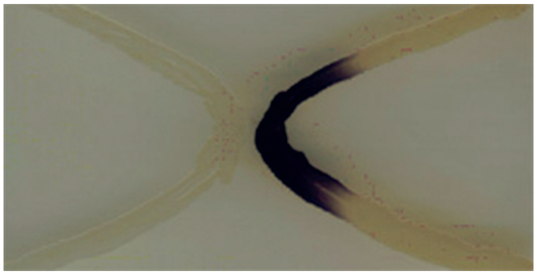


FIGURE 20.9 Production of the purple pigment violacein by an AHL reporter derivative of strain *Chromobacterium violaceum* that lacks its endogenous LuxI AHL synthase. *C. violaceum* strain CV026 (Teplitski *et al.*, 2000) was streaked in a V-pattern on the right side of the plate, and an *E. coli* strain that contains a plasmid with an AHL synthase gene was streaked in a V-pattern on the left. Recognition of sufficient AHL produced by the *E. coli* strain is visible by purple violacein production by CV026. Photo courtesy L.S. Pierson III.

freshwater fish pathogen *Aeromonas hydrophila* to cause infection, consistent with the hypothesis that degradation of the AHL signal required by the pathogen resulted in the protection of the fish. The authors proposed that strain QSI-1 is a probiotic for aquaculture.

Several plants also produce AHL signal mimics that activate or inhibit bacterial quorum sensing (Teplitski *et al.*, 2000). The AHL reporter strain *Chromobacterium violaceum* only produces a purple pigment (violacein) if supplied with exogenous AHL signal (Figure 20.9). Pea seedling root exudates were identified that blocked activation of violacein production by added AHL in *C. violaceum*. In contrast, these pea root exudates activated several other bacterial AHL reporters. The model legume *Medicago truncatula* produces a collection of root exudate compounds that also differ in their effect on quorum sensing depending on the reporter system used (Gao *et al.*, 2003). The unicellular alga *Chlamydomonas reinhardtii*, which is found in soil and fresh water, produces a number of compounds that also affect quorum sensing systems differentially (Teplitski *et al.*, 2004). Multiple biosensor strains are facilitating the identification of quorum sensing-inhibiting compounds, and several of these compounds have promise as treatments for reducing bacterial pathogenicity.

20.4.5 Interkingdom Signaling: Sociobiology and the Concept of Holobionts

Eukaryotic hosts and their associated bacteria have evolved complex mechanisms of both signaling and signal perception in order to monitor each other's status. As we saw earlier in this chapter, bacteria utilize a wide range of small signaling molecules to control the expression of traits important for their optimal growth and survival under different environmental conditions. Known bacterial signals include AHLs, AI-2, AI-3, oligopeptides and muropeptides (cell wall components). The role of quorum quenching in modulating the effects of quorum sensing on the expression of key traits has also been discussed.

The discovery of bacterial signaling has led to a paradigm shift in the way we view the microbial world. It was first believed that quorum sensing allowed a single population of bacteria to coordinate group-specific behaviors such as colonization, light production and pathogenicity by controlling gene expression in response to specific signals (intraspecies communication). This concept alone was revolutionary as it meant that bacteria did not react or behave as single, isolated cells, but instead were capable of organizing their behaviors coordinately, analogous to a multicellular organism. Next it was shown that unrelated bacteria could communicate (cross-talk) with each other via signals, and that some bacteria evolved the ability to eavesdrop on these conversations (interspecies communication). It is now recognized that this signaling communication can occur between bacteria and their eukaryotic hosts, a phenomenon known as interkingdom signaling (Rumbaugh, 2007). Interkingdom signaling is defined as the exploitation of signal transduction pathways by the signaling compounds of one organism to alter the behavior, through changes in gene transcription, of an organism from a different kingdom. Interkingdom communication can result in: (1) the ability of bacteria to eavesdrop on their host by responding to host signals; (2) interference of bacterial signaling via host-produced interfering signals; or (3) recognition of bacterial signals by the host resulting in altered host gene expression, such as that required for an immune response.

This section discusses signaling between prokaryotic bacteria and their eukaryotic hosts, i.e., interkingdom signaling. As we will see, signaling occurring between hosts and their associated microorganisms influences the outcomes of the interactions, whether beneficial or detrimental, to both members. Evidence is accumulating that bacterial signals, such as AHLs, can serve as bacterial hormones that modify patterns of gene expression in the eukaryotic host, and serve to alter the host's fitness in its current environment.

20.4.5.1 Signaling Between Bacteria and Fungi

In nature, the majority of bacteria live in mixed communities with other bacteria and fungi, and many bacteria are known to actively colonize fungal hyphae. Not surprisingly, communication among bacteria and fungi can affect their behaviors. For example, an AHL produced by the opportunistic human pathogen *P. aeruginosa* induces morphological changes in the human-associated yeast *Candida albicans* (Hogan *et al.*, 2004). Specifically, McAlester *et al.* (2008) showed that *C. albicans* responded to the bacterial AHL 3-oxo-C_{12}HSL by growing only in the yeast form that is resistant to killing by *P. aeruginosa*. In return, the fungal-produced metabolite farnesol affected *P. aeruginosa* by causing reduced levels of the *Pseudomonas* quinolone signal (PQS) and pyocyanin (Cugini *et al*., 2007).

20.4.5.2 Signaling Between Bacteria and Plants

Since microorganisms were the first life form on Earth, plants evolved in their presence, and it is logical that these groups of organisms evolved mechanisms of communication that are only now being recognized. Research has shown that plants change patterns of gene expression, and alter developmental pathways in response to bacterial AHL signals.

Communication between plants and bacteria was first studied extensively in the beneficial *Rhizobium* spp.—legume symbiotic association (reviewed in Brencic and Winans, 2005). Studies have documented the exquisite signal communication that occurs by both the bacteria and plant, resulting in colonization, infection, nodule development and nitrogen fixation (Information Boxes 16.2 and 16.3; Section 19.3.2.2). Communication between the plant pathogen *Agrobacterium tumefaciens* and its host has also been studied extensively, and was discussed in Section 20.2.3. Both of these plant-associated bacteria interact with their plant hosts by invading plant tissues, the former beneficially while the latter as a pathogen. However, the majority of plant-associated bacteria exist by colonizing the plant surface. Recent work suggests that interkingdom communication between hosts and microbes is both widespread and occurs bi-directionally.

In other examples, AHL signals can alter the expression of plant defense genes, a phenomenon known as induced systemic resistance (ISR) in plants. For example, AHL produced during root colonization by *Serratia liquefaciens* MG1 induced increased resistance against the fungal leaf pathogen *Alternaria alternata* in tomato plants (Schuhegger *et al*., 2006). The AHL signal oxo-C_{14}-HSL, when added to *Arabidopsis thaliana*, increased its ability to resist infection by *Pseudomonas syringae* pv. tomato DC3000 (Schikora *et al*., 2011). Pretreatment of plants with this AHL also blocked infection by the biotrophic fungal pathogens *Golovinomyces orontii* (on *Arabidopsis*) and *Blumeria graminis* (on barley). AHLs produced by *Serratia plymuthica* protected cucumber seedlings from the fungal damping-off disease caused by *Pythium aphanidermatum*, and tomato and bean from *Botrytis cinerea*.

However, not all AHL impacts on plants are beneficial. For example, treatment of *Nicotiana attenuata* by C_6-HSL resulted in reduced production of a protease inhibitor in leaves, resulting in increased herbivory of the leaves by the tobacco hornworm *Manduca sexta* (Heidel *et al*., 2010). Recently, it was shown that the length and modification of the AHL side chain impacts how the plant recognizes the AHL and its effect on host defense responses. Because unrestricted expression of plant defense pathways have detrimental effects on plant growth and development, plants only activate defense pathways upon pathogen recognition. For example, in *Arabidopsis thaliana*, recognition of bacterial pathogens requires the response of two mitogen-activated defense protein kinases (AtMPK3, AtMPK6) to conserved bacterial elicitors called MAMPs (microbial-associated molecular patterns) such as flagella. Treatment of plants with a purified subflagellar peptide induces a strong but transient activation of MPK3 and MPK6. However, pretreatment with AHL resulted in a prolonged activation of MPK3 and MPK6, and a stronger plant defense response against bacterial MAMPs (Schikora *et al*., 2011).

AHL signals can also play important roles in modifying plant development. Addition of AHLs can alter approximately 33% of plant protein patterns (Mathesius *et al*., 2003). The structure of the AHL is critical. For example, short chain AHLs (C_4 and C_6) promoted root growth (von Rad *et al*., 2008), long chain AHLs (C_{10}) caused roots to shorten and thicken, while C_{12} AHL caused root hair induction (Ortíz-Castro *et al*., 2008). These data indicate that colonization of plants by different bacteria that produce different AHL signals can clearly alter plant root architecture, and hence plant growth and development.

The bacterial rice pathogen *Xanthomonas campestris* pv. oryzae (Xoo) contains a LuxR homologue (OryR), but lacks a LuxI-type AHL synthase and produces no AHL signals. Ferluga *et al*. (2007) showed that OryR binds and activates 1,4-β-cellobiosidase, an enzyme required for increased virulence in response to macerated rice, but not to any known AHL signal. Thus, Xoo appears to control virulence by a LuxR receptor protein by interacting directly with a rice plant component.

20.4.5.3 Signaling Between Bacteria and Human Cells

Humans are very interested in the coexistence of bacteria and their hosts since there is a need to understand our human signaling with our human microbial flora. An average human is estimated to be composed of 10^{13} mammalian cells and 10^{14} bacterial cells (Hughes *et al*., 2009). This means that humans are outnumbered 10 to 1 by their bacterial partners. Unfortunately, to date the best understood interkingdom signaling between bacteria and human cells is based on studies with pathogenic bacteria.

As mentioned earlier, the opportunistic pathogen *P. aeruginosa* can colonize the lung tissues of immunocompromised patients such as those with cystic fibrosis or severe burns, where it is a significant cause of mortality (Antunes *et al*., 2010). The bacterium contains two quorum sensing systems (LasR/LasI and RhlR/RhlI). The gene *lasI* encodes for the synthesis of 3-oxo-C_{12}-HSL while *rhlI* encodes for C_4-HSL. The RhlR/RhlI system is hierarchically under the control of the LasR/LasI system. There is an additional orphan LuxR receptor QscR which recognizes 3-oxo-C_{12}-HSL. These genes control the expression of multiple genes required for *P. aeruginosa* to be a successful pathogen, including the production of elastase, alkaline protease, pyocyanin, rhamnolipids and exotoxin A. Besides the effects of these AHLs on bacterial gene expression, 3-oxo-C_{12}-HSL has been shown to influence host defensive responses. For example, 3-oxo-C_{12}-HSL induces host interleukin-8 (IL-8) and cyclooxygenase COX-2 secretion from human bronchial cells, while causing the simultaneous down-regulation of lymphocyte proliferation and production of tumor necrosis factor TNF-α, IL-2 and reduced apoptosis of macrophages and neutrophils.

In other cases, signaling between bacteria and human cells involves the ability to recognize signals from other bacteria and the host. Bacterial fatty acid-based AHL signals and mammalian lipid-based hormones have significant similarities both structurally and in their mechanisms of action. The human pathogen EHEC (enterohemorrhagic *E. coli*) O157:H7 colonizes intestinal epithelial cells in which it must interact with the resident gastrointestinal microflora. Mammalian cells, including intestinal epithelial cells, produce the catecholamine stress hormones epinephrine and norepinephrine which participate in coordinating host adaptive responses to stress (Table 20.1). Strain O157:H7 senses the quorum sensing signal AI-3 produced by the resident gastrointestinal microflora, and the presence of epinephrine and norepinephrine through its histidine kinase QseC, and activates the expression of multiple pathogenicity genes in response to this signal cocktail. Expression of these pathogenicity genes results in hemorrhagic colitis and hemolytic uremic syndrome. Thus, in this bacterium, signals from both the host and bacterium are interlinked. Although not directed involved in AI-3 production, a *luxS* mutant of strain O157:H7 unable to synthesize the universal signal AI-2 (discussed earlier) produces little AI-3 signal. Interestingly, this *luxS* mutant can be restored to pathogenicity by the addition of epinephrine and norepinephrine (Sperandio *et al*., 2003; Hughes *et al*., 2009). Thus, this pathogenic bacterium utilizes host-derived stress hormones to cue when to express its pathogenicity mechanisms.

20.4.5.4 The Holobiont and Hologenome Hypotheses

Chemical signaling between cells is a basic tenet of multicellularity or multicellular organisms. The ability of bacteria to communicate with, and respond to, their hosts via production and recognition of signals suggests that they be included in the concept of host multicellularity. Thus, if we consider the intracellular microflora (endosymbionts) and the extracellular microflora (exosymbionts) to be essential components of a healthy host, then they become part of its innate multicellularity. The term "holobiont" was coined to describe the collection of all cellular components (microbial and host) that comprise a complete organism such as a plant, animal and human (Zilber-Rosenberg and Rosenberg, 2008). Equally fascinating is the concept of the "hologenome," referring to the hypothesis that the hologenome is the sum of the genetic information contained in the host plus all of its microbiota. The recent completion of the sequencing of several human genomes indicates that humans contain approximately 20,500 genes. Work on the human microbiota (microbiome) estimates that there are approximately 2000 species of bacteria inhabiting the human body. If the average size of a bacterial genome is conservatively estimated to be 2500 genes, this indicates that bacteria potentially contribute 5 million additional genes. Of this total amount, if only 250,000 bacterial genes are considered unique, then the final numbers are 250,000 bacterial genes to 20,500 human genes, indicating that our microflora could be contributing 12-fold more genetic information than we contain. Hence, it could be argued that we humans are technically more "microbial" than we are "human." Continued investigation into human–microbial (and similar) interactions will undoubtedly continue to reveal fascinating details about the diverse and complex communication mechanisms that microorganisms have evolved, and the resulting interconnectedness of life on Earth.

20.5 SUMMARY AND CORE CONCEPTS

Originally, bacteria were thought to be single-celled organisms that sensed and responded to environmental inputs individually. However, bacteria are now believed to be able to communicate among themselves both within a single population and between unrelated populations to essentially behave analogously to multicellular organisms. This communication is dependent on a combination of characterized and uncharacterized signals. Communication between microbes and their hosts is known to directly affect the expression of bacterial genes that encode functions critical to all aspects of bacterial survival and bacterial–bacterial and bacterial–host interactions. These include: (1) coordination of gene expression within a single population; (2) coordination of gene expression among unrelated populations; (3) avoidance of host defense responses; (4) coordination of virulence gene expression; (5) inhibition of a competitor's gene expression; and (6) inhibition or stimulation of host colonization.

Besides allowing communication between bacterial populations, quorum sensing signals have been shown to facilitate communication between bacteria and eukaryotic hosts such as fungi, algae, plants and animals. This signal-dependent communication appears to be a two-way street in that the eukaryotic host can produce quorum sensing signal mimics that directly influence the expression of bacterial genes involved in host−microbe interactions, and bacterial quorum sensing signals can also influence eukaryotic host gene expression patterns. In most higher organisms, many critical processes are dependent on the endogenous microbial flora (microbiome) associated with the host. Thus, these communication networks represent key ecological control points that directly determine the outcome of host−microbe interactions. Understanding these communication networks may facilitate large-scale improvements in bacterial−host interactions, pathogen suppression, host health, bioremediation and treatment of water and wastewater.

QUESTIONS AND PROBLEMS

1. Compare the benefits and limitations of AHL-mediated quorum sensing to peptide-mediated quorum sensing.
2. Design an experiment to determine the effect of bypassing quorum sensing control (i.e., make target gene expression constitutive) on the ability of a human pathogenic bacterium to infect its host.
3. Since higher organisms evolved in the presence of bacteria, it makes inherent sense that these diverse organisms communicate with each other. What processes can you think of that might require bacterial−host cooperation?
4. We have seen examples of both positive cross-talk and negative cross-talk (signal interference) between bacteria and between bacteria and hosts. Are these forms of cross-talk community-wide, or could selected subpopulations be differentially affected? Can you devise an experiment to test this idea?
5. It has become clear that we as humans depend on many of the microorganisms that reside within us to carry out many important activities. Can you identify some of these processes? How would you verify that microbial associations are involved?

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