Interspecies Chemical Communication in Bacterial Development

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Abstract
Our view of bacteria, from the earliest observations through the heyday of antibiotic discovery, has shifted dramatically. We recognize communities of bacteria as integral and functionally important components of diverse habitats, ranging from soil collectives to the human microbiome. To function as productive communities, bacteria coordinate metabolic functions, often requiring shifts in growth and development. The hallmark of cellular development, which we characterize as physiological change in response to environmental stimuli, is a defining feature of many bacterial interspecies interactions. Bacterial communities rely on chemical exchanges to provide the cues for developmental change. Traditional methods in microbiology focus on isolation and characterization of bacteria in monoculture, separating the organisms from the surroundings in which interspecies chemical communication has relevance. Developing multispecies experimental systems that incorporate knowledge of bacterial physiology and metabolism with insights from biodiversity and metagenomics shows great promise for understanding interspecies chemical communication in the microbial world.
INTRODUCTION

Our perception of bacteria has changed dramatically since the discovery of antibiotics. Shifting from focusing primarily on germ eradication, we have embraced the knowledge that bacterial communities are an integral, functional component of most environments and biological systems. We find astounding bacterial diversity within photosynthetic mats, plant root systems, and in the tissues and guts of animals, to name but a few examples (30, 39, 53, 58, 115). In fact, such systems depend upon their bacterial constituents for normal physiological function and defense against potential pathogens. Recognition of bacterial function in the environment traces back to the early efforts of Winogradsky, Beijerinck, and Becking. Yet until recently, the networks of interactions, both metabolic and physical, which bind members of bacterial communities together, have been largely in the realm of our imagination. Given the richness of species interactions across all biological systems, it would be unlikely that individuals within microbial communities ignored the actions of their neighbors as they proceeded only to consume nutrients and reproduce. Indeed, many examples exist to the contrary, but as a field of study, microbial signaling between species is in its infancy. Our emerging view of the microbial world is one in which individual bacteria exert their influence within communities through producing, sensing and responding to an array of chemical signals.

Inherent to the function of any community is the need for information to be transferred between individuals to coordinate behaviors, whether based on cooperative or competitive interactions (26). Microbes survive in the environment by exchanging chemical information, fuel for cell growth, and waste products. The many chemical signals generated and sensed by microbes constitute the language with which members of a community can define the laws of civil, or at times hostile, engagement. The challenge for microbiologists is how to interpret the actions of diverse chemical signals to understand the principles that govern interactions within microbial communities.

The concept of intercellular signaling has been explored to great depths in the study of the development of multicellular organisms and in the context of human disease. Arising from these systems is a paradigm for signaling between cells that invokes binding of a ligand to a receptor, thus triggering activation or repression of one or more biochemical pathways. These signaling pathways direct such events as tissue and appendage development or cellular responses to disease and infection. Bacterial cell signaling circuits share many similarities with those of multicellular organisms, and the same signaling paradigm holds true (14). However, bacterial sensory systems also harbor some significant differences, owing in part to the direct exposure of bacteria to shifting environmental conditions in the absence of the homeostatic mechanisms generated by tissues and organs.

Despite differences in physiology from multicellular organisms, bacteria and other microbes organize communities capable of executing organ-like functions in a given niche. Biofilms exemplify this concept, wherein cell assembly and production of an extracellular matrix allow for individual cells in a population to carry out distinct behaviors in a manner
coordinated across an entire population. *Myxococcus xanthus* and *Bacillus subtilis* both form structured biofilms that display division of labor among individuals (2, 7, 56, 114). How does differentiation of cell types occur within a bacterial biofilm? Many recent studies demonstrate, for example, that stochastic mechanisms operate in microbial cell fate determination (28). The concept of bistability provides one type of switching mechanism for cell fate, but development of ordered structures such as fruiting bodies requires intercellular coordination and control. For a population to carry out cellular differentiation toward a productive and coordinated end, a means of intercellular communication is required. The evidence for the existence of such signals is clear, especially in the case of single-species developmental systems (5). How these signals operate in an ecological context, in multispecies communities, or even in a relatively simple mixed-species community in the laboratory remains largely unknown.

For more than a century, the application of biochemical and genetic approaches in microbiology has uncovered the fundamental principles of microbial growth and development within pure-culture systems. The clear limitation of single-species experiments is the removal of the organism from any context that would give relevance to interspecies interactions. Yet, bacteria exist predominantly in multispecies environments. A combination of culture-based studies and culture-independent approaches allows us to make an accounting of the remarkable species diversity in bacterial communities (27, 30, 87, 100, 104, 107). Strikingly, as knowledge of microbial diversity expands, it brings into focus the fledgling stage of our understanding of bacterial interspecies signaling processes (21).

Early observations of signaling in microbial systems began with a community of a single species and led to an understanding that chemical signaling in its many forms is widespread in the microbial world (83, 111). Quorum sensing (QS) has largely dominated our discussion of microbial signaling, arguably because we can detect QS signal activity operating in traditional pure culture, thus leading to an astounding wealth of information (5). However, questions remain about QS as a general model for density-dependent microbial communication versus alternative models of diffusion sensing or efficiency sensing (47, 57, 95). Nonetheless, our recognition of the remarkable species diversity within many microbial communities highlights the need for creative, new experimental systems and a modified conceptual framework to understand the multifarious chemical exchanges and signaling interactions between microorganisms. Several recent reviews thoughtfully examine the types of chemical signaling interactions likely to occur between bacterial species, including the evolutionary implications for different forms of information transfer (22, 57, 74, 82, 101). Chemical signaling occurs between two species when a metabolite from one species induces a change in the other species that is not due simply to the metabolism of the signal compound. On the basis of a scheme proposed by Keller & Surrnette (57), the consensus derived from these works suggests that chemical signaling be defined using three categories: chemical communication, environmental cues, and chemical manipulation. Adopting these definitions establishes a guidepost for thinking about the types of chemical signals produced by bacteria and their operation in microbial interspecies interactions. In all likelihood, a bacterial cell must integrate multiple signals of varied form and function to execute the full scope of microbial interaction processes, which include primary metabolic outputs, shifting environmental conditions, and random encounters.

In this review we examine the roles of chemical signaling between bacterial species from the perspective of developmental processes and consider systems for experimentation that may illustrate underlying mechanisms of diverse extracellular signaling. microbial development has long been recognized as cellular differentiation in response to changing environmental conditions. Development occurs in bacteria through activation of regulated and dedicated pathways, which begin with signal inputs and conclude with altered cell form or behavior. When the external conditions encountered by...
a population of bacteria have been shaped by the activities of surrounding species of bacteria, the signals to initiate developmental processes may well be of microbial origin. Therefore, to begin to understand the chemical connections between distinct species of bacteria, we look to developmental processes, envisioning regulation of development to be particularly attuned to the metabolic activity of bacterial neighbors. Taking a broad view of chemical forms of signaling, we begin our discussion with an example of multiple chemical exchanges between plants and bacteria in a developmental progression. This two-way exchange utilizes a diversity of chemical signals, examples of which we anticipate are widespread in the microbial world.

**BACTEROIDS: HOST-BACTERIA ASSOCIATION AND DEVELOPMENT**

A series of reciprocal signal exchanges occurs during the establishment of the symbiosis between bacteria of the family *Rhizobiaceae* and legumes. Bacteria that successfully enter into this symbiosis develop into bacteroids—differentiated bacterial cells within root nodules that mediate N₂-fixation (42). Although this symbiosis is not an interspecies communication between plants and bacteria, it illustrates that a diverse set of chemical signals can direct a progression of developmental events. A brief summary of the process helps highlight the structural diversity of signal molecules employed by plants and bacteria during communication.

The formation of root nodules begins with a population of bacteria sensing and responding to metabolites, principally flavonoids, exuded from the roots of the plant (Figure 1) (38). Root exudates activate a chemotactic response in nitrogen-fixing bacteria, enticing cells within signal range to congregate near tiny root hairs on the root surface. Increased bacterial cell density in the vicinity of the root hair sets the stage for a QS-dependent transition to production of nodulation (Nod) factors by the bacteria. Nod factors are lipochitoooligosaccharide metabolites released by the bacteria that instruct the plant to prepare for a controlled invasion. Nod factors induce the production of an infection thread in the plant through binding a specific receptor and triggering a Ca²⁺-spiking signal cascade that leads to tissue invagination (reviewed in Reference 86). The infection thread forms the conduit through which bacteria travel from the soil to the cortex of the plant roots, where nodule formation occurs. This signaling progression underlies the basic model of *Rhizobium*-legume chemical communication that establishes N₂-fixing symbiosis, and makes use of structurally distinct chemical signals: flavonoids, lipochitoooligosaccharides, and acylated homoserine lactones (HSLs).

Until recently, the function of Nod factors was thought to be the universal signal from all *Rhizobiaceae* for initiation of infection threads. However, sequence analyses of *Bradyrhizobium* genomes (strains BTAi1 and ORS278) reveal the absence of the *nodA* and *nodC* genes and the presence of a degenerate form of the *nodB* gene, rendering this organism incapable of producing Nod factors (40). Despite this fact, these bacteria invade via cracks in the plant tissues and initiate nodulation on the stems of *Aeschynomene* plants. The identity of the infection-thread-inducing signal from these strains is yet to be determined.

Although *Bradyrhizobium* may simply provide the exception to the rule, these findings also suggest plasticity in the generation of specific chemical signals during evolution of this interspecies interaction. Nonnodulating soil bacteria harbor well-conserved orthologs of the *nodA-C* genes that are missing in *Bradyrhizobium* (40), underscoring the significance of other specificity determinants in correct selection of bona fide symbionts from diverse soil microflora. Furthermore, the *Bradyrhizobium* strain BTAi1 synthesizes and responds to a novel quorum signal, p-coumaroyl-l-homoserine lactone (pC-HSL), originally discovered in *Rhodopseudomonas palustris* (103). The pC-HSL is produced by chemical modification of bacterially derived acyl-HSL and plant-derived p-coumarate excreted by the root.
A two-way chemical exchange occurs between bacteria in the family *Rhizobiacae* and leguminous plants during the establishment of *N₂*-fixing symbioses. (a) Nodules on the plant roots (*magenta*) arise as a result of a progression of developmental changes in bacteria and plant during a controlled infection. Flavonoid compounds excreted by the roots signal the bacteria to assemble near the root surface. At a sufficiently high cell density, bacterial acylated homoserine lactone (acyl-HSL) quorum signals activate the production of Nod factors. The Nod factors instruct the plant root hairs to prepare for invasion. Bacterial exopolysaccharides [succinoglycan (EPSI) and EPSII] facilitate entry into root hairs and passage into the root cortex. Modified with permission from Reference 42. (b) Chemical structures of a flavonoid (genistein), a quorum signal (acyl-HSL), and a Nod factor (lipochitooligosaccharide).

The culmination of chemical cross talk up to the point of root colonization is the capture of a bacterial cell in a shepherd’s crook structure at the end of a tiny root hair where the bacteria find the portal into the plant. Further enhancing the pharmacopoeia of compounds involved in this chemical exchange, polysaccharides are required for entry into the root via the infection thread and persistence of bacteria in the nodule. Two exopolysaccharides, succinoglycan (or EPSI) and EPSII, have been described as predominant mediators of bacterial transit through the infection thread (Figure 1) (42). *Sinorhizobium meliloti* succinoglycan functions in part as a protective agent from host immunity and also as a signal to the plant to go forward with infection thread formation (55, 106). In mutants lacking EPSI, Nod factors trigger infection thread formation, but with only ~10% efficiency of wild-type *S. meliloti*, and the bacteria fail to enter the root cortex.

**EPS:**
exopolysaccharide
In addition to exopolysaccharides, lipopolysaccharides (LPSs) play a critical role in bacterial survival within the plant, although the molecular details of LPS function are unclear (38). Similar to EPS, LPS aids the bacteria in surviving the plant defense responses to infection. Variant long-chain forms of LPS produced by differentiated bacteroids are essential for survival in the nodule, possibly through modification of plant defenses within that micro niche. Although it is unclear how LPS acts in promoting symbiosis, the suggestion that a plant uses LPS chemical information to discriminate symbiont from pathogen is strengthened by the function of LPS in other symbioses. In an analogous system, LPS, in conjunction with peptidoglycan, plays a role in establishing symbiosis during *Vibrio fischeri* colonization of the squid light organ (67). Specific forms of EPS and LPS may be particularly adaptable to intimate chemical signals for differentiating potential pathogens from beneficial partners.

Knowledge of chemical signaling between plant and *Rhizobium* becomes much less clear beyond the point of entry into the infection tube. Migration of the bacteria from surface to root cortex must involve communication between the organisms, certainly during the invasion of root cortical tissue, a process much like infection. In part, EPS and LPS can mitigate the host defenses, but unknown signals and modes of communication are likely acting within the plant tissues. Arguably the most profound developmental change exhibited by the bacteria occurs when they transition to the bacteroid form, capable of fixing atmospheric nitrogen. The identities of signaling compounds that instruct the bacteria to begin synthesis of nitrogenase enzymes and to shift from vegetative growth to bacteroid development have yet to be found. One type of bacteroid form, known as indeterminate, is terminally differentiated within the root nodule and incapable of resuming growth outside of the plant (38). Recent evidence suggests that these bacteroids are under the control of plant signals that induce differentiation and endoreduplication of the bacterial genome, readying the cells for N$_2$ fixation (80). The identification of the plant signals and mechanisms controlling bacteroid differentiation will indeed be a remarkable finding.

Implicit in this multistep reciprocal signal exchange between plant and bacteria is the utilization of chemical signals that have undergone evolutionary selection to facilitate a specific symbiotic interaction. Multiple chemical forms, from excreted phenolic compounds to oligomeric sugars, have been functionally co-opted to some degree as signal compounds that direct coordinated development between two species of organism. Parsing out the relative contribution of each signal type has provided great insight into the biochemical mechanisms of generating and processing signals. Yet exceptions to the standing models, such as those provided by *Bradyrhizobium*, as well as the remaining developmental steps that have no known signals, indicate that much is yet to be learned about interspecies communication between plant and bacteria. The formation of root nodules is among the best understood of the chemically mediated interspecies interactions involving bacteria. Many other examples of interspecies interactions involving bacterial development may lack the strict specificity of N$_2$-fixing symbioses but are nonetheless worthy of exploration.

**BIOFILMS IN THE HUMAN ORAL CAVITY**

Observations of the complex microbiota on the tooth surface extend back to van Leeuwenhoek’s initial descriptions of “animalcules” living on his teeth. The many bacteria he observed in dental plaque comprise multispecies biofilm communities, which adhere to the enamel surface of teeth and collect their share of nutrients ingested by the host. In the 1970s, Gibbons & Nygaard (37) observed that individual plaque bacteria could physically interact and aggregate across species lines. Recent studies of bacterial biofilms on tooth enamel reveal the presence of multispecies communities that, following
dental cleanings, repeatedly develop with consistent species composition (65). Assemblage of these communities relies on a chemical interdependence between bacteria that is founded in part on sugar metabolism and thought to involve deterministic patterns of bacterial co-aggregation (8, 66). The communities are established through the binding of specific adhesins and receptors on cell pairs of different species of bacteria. Coaggregation in attached oral communities initiates with primary interactions on the dental surfaces and progresses to secondary interactions between bacteria attached only to other bacteria in the biofilm (reviewed in Reference 66). The combination of bacterial diversity, specific cell-cell contacts, and a metabolic connection through diffusible molecules makes the oral biofilm community a particularly attractive model for the study of chemical communication between species during biofilm development.

The ordered interactions directing assembly of bacteria at the tooth surface constitute a developmental program of multispecies biofilm formation. Biofilm formation in general is a developmental process undertaken by bacteria (90). The study of the genetic and molecular mechanisms directing adhesion of bacteria to a surface and production of an extracellular matrix, which are two defining features of bacterial biofilms, has largely been undertaken using single-species systems. The oral cavity contains a richly diverse community of resident bacteria composed of several hundred species inhabiting multiple niches, e.g., supragingival and subgingival surfaces (1, 68, 93).

Underlying the complex association patterns that define the biofilms is a chemical exchange involving the consumption of sugars and complex carbohydrates, primarily by the abundant streptococcal bacteria residing in the oral cavity. Lactic acid, which is produced as a by-product of fermentation, is the preferred carbon source for at least two species commonly found within dental biofilms: Veillonella atypica and Aggregatibacter actinomyctematitans (12, 31). In fact, the preference of A. actinomyctematitans for lactic acid is such that cells prevent uptake of other common sugars via phosphoenolpyruvate-dependent phosphotransferase uptake systems (PTS) in the presence of lactate (12). The preference for lactic acid forms the basis of a synergistic chemical exchange between species for multispecies biofilm development in the oral cavity. Sugar fermentation by Streptococcus spp. produces an accumulation of lactic acid, which in turn supports the growth of V. atypica and A. actinomycetematitans. Lactic acid buildup normally causes Streptococcus to repress amylase expression, reducing the availability of fermentable sugars. The metabolic coupling between streptococci and lactic acid-utilizing bacteria minimizes lactic acid accumulation, yielding higher-efficiency nutrient utilization to benefit both partners. A study by Egland and coworkers (31) demonstrated the presence of a diffusible activity of unknown structure produced by V. atypica. This activity induces Streptococcus gordoni to increase production of amylase, enhancing levels of carbohydrate metabolism and lactic acid excretion. In this way, chemical communication boosts nutrient flux through the community, which likely translates into development of robust multispecies biofilms. This microbial metabolic networking is relevant to the health of the host, wherein disruption of balanced sugar metabolism affects tooth decay. In experiments using gnotobiotic rats, single-species biofilms of Streptococcus mutans correlated with a higher level of dental caries than did biofilms of coaggregated Veillonella-Streptococcus (77).

A handful of chemical signals that have a role in biofilm development in the oral cavity have been identified and characterized. Among these signal compounds, autoinducer 2 (AI-2) has been intensely studied. AI-2 was originally described as a second QS signal in Vibrio harveyi (6). Subsequently, AI-2 has blurred the lines between intraspecies QS and interspecies communication, because the signal is generated by many gram-positive and gram-negative bacteria and plays a widespread role in interspecies signaling (45). AI-2 refers to a collection of excreted molecules spontaneously cyclized from...
2-(4,5-dihydroxy-2,3-pentanedione) (DPD), the product generated by the enzyme encoded by the luxS gene. In bacteria with a luxS gene, DPD is produced during detoxification of the S-adenosyl methionine-mediated methyl donor pathway. The association of DPD with detoxification needed in the activated methyl cycle complicates the interpretation of luxS mutant phenotypes, generating ambiguity between signaling and metabolic defects (45). The luxS gene is found in many species of oral bacteria, which suggests that it plays a significant role in communication between species within oral biofilms. Indeed, mutation of luxS disrupts biofilm formation of S. mutans UA159 and S. gordonii (117). Furthermore, development of mixed-species biofilms of S. oralis with A. actinomycetemcomitans, and of S. gordonii with Porphyromonas gingivalis, requires AI-2 (78, 99). Bacteria that have a luxS gene but no identifiable AI-2 receptor (similar to LuxPQ from V. harveyi, or Lsr from S. typhimurium) are abundant, suggesting that the quorum signaling function of AI-2 may be limited to a subset of species (98). The prevalence of the luxS gene throughout genomes of the oral bacteria, many of which lack an AI-2 receptor, implies a general metabolic role for AI-2, something more akin to a membership card in mixed-species communities rather than a business card to facilitate specific interactions. In other words, in some systems AI-2 functions as an intra- and interspecies signal, but the specific action of the AI-2 within natural dental plaque communities remains a question for future study.

**PSEUDOMONAS AERUGINOSA INTERSPECIES INTERACTIONS**

The human body provides a suitable growth environment for many microorganisms, such that bacterial cells associated with the body outnumber human cells by at least one order of magnitude (102). Much of what we know about bacterial interactions with humans arises from studies on disease-causing microorganisms. Opportunistic pathogens are typically harmless bacteria that exploit abnormal conditions within the human host to produce an infection. Such pathogens have gained increased attention owing to the rising numbers of hospital-acquired infections they can cause. One such bacterial pathogen is Pseudomonas aeruginosa (94). P. aeruginosa has risen to prominence in human health as a pathogen in burn wounds and in the lungs of cystic fibrosis (CF) patients. The success of P. aeruginosa is due in part to its ability to manipulate the surrounding environment through development of robust biofilms and excretion of redox-active and toxic metabolites (63). Excreted metabolites provide the bacteria with a competitive advantage against other bacteria and fungi. In this section we highlight developmental interactions between P. aeruginosa and other human-associated microorganisms. Unlike the previous examples of N2-fixing root nodules and the oral cavity, such interactions are incidental to the host, raising the question of the origin of interspecies chemical signaling mechanisms.

Two human-associated microorganisms, the fungal species Candida albicans and the bacterial species Staphylococcus aureus, inhabit body cavities and skin as commensal microbes. Provided favorable conditions for infection, both organisms can turn against their host with dramatic results. C. albicans disease appears as oral and vaginal candidiasis, biofilm infections of implanted catheters, and disseminated disease in immunocompromised patients. Likewise, S. aureus exploits damage to host tissues, producing potentially lethal infections, and it is among the most troublesome of the nosocomial infectious agents. In general, some perturbation to normal host function opens the door for these microorganisms to flourish as pathogens. In the cases of catheter implants and dental disease, this bacterial-fungal pair is able to collaborate, forming mixed-species biofilms in patients (19, 116). The prevalence and overlap of C. albicans and S. aureus suggest that favorable conditions for infection are common to both organisms. However, the interactions between bacteria, fungus, and host are reshaped when P. aeruginosa enters the scene.
P. aeruginosa exploits the surface of C. albicans filamentous cells as a suitable surface for biofilm development and a potential food source. When the bacteria encounter filamentous cells of the fungus, they adhere to the fungal surface and kill the cells with a combination of virulence factors (50). C. albicans is prepared for the assault, however, owing to its shape-shifting ability to grow as either yeast-form cells or filamentous cells. The yeast form of C. albicans is impervious to P. aeruginosa attack. By undergoing a developmental shift triggered by a bacterial signal, C. albicans survives under otherwise lethal conditions. The bacterial signal was identified as 3-O-C12 homoserine lactone (30C12HSL), one of two P. aeruginosa quorum signaling compounds (51).

C. albicans is able to eavesdrop on P. aeruginosa planning for biofilm formation and preparing to synthesize virulence factors. Interpreting the chemical signals for bacterial interspecies communication, the fungus escapes the attack. Not only is C. albicans capable of detecting a signal from the bacterium, it also sends an interference signal to P. aeruginosa, altering interspecies communication and development. The signal is farnesol, an isoprenoid molecule excreted by C. albicans that blocks the formation of filamentous cells (49). Cugini et al. (20) demonstrated that farnesol and related isoprenoid metabolites inhibit the production of the Pseudomonas quinolone signaling (PQS) molecule by P. aeruginosa. One effect of inhibiting PQS synthesis is a decrease in production of the virulence factor pyocyanin, whose synthesis is regulated through PQS signaling. Thus, in response to an attack on fungal filaments by P. aeruginosa, C. albicans survives both by shifting to yeast-form cells and by causing diminished virulence of P. aeruginosa through interspecies signal jamming.

A secondary effect of reducing PQS synthesis involves the mechanism by which the molecule is delivered to a target cell by P. aeruginosa. The hydrophobicity of the PQS molecule is considered key for its unique mode of delivery; PQS is embedded within the membranes of microvesicles budded from the outer membrane of the bacterial cell (76). The microvesicles diffuse and fuse with the target cell membrane to deliver not only the PQS in the membrane, but also any payload enveloped in the interior of the vesicles. Among such payload molecules is the redox-active quinoline compound 4-hydroxy-2-heptylquinoline-N-oxide (HQNO), recognized for its ability to inhibit the growth of some gram-positive bacteria, including staphylococci (72). HQNO was recently demonstrated to have activity in an interspecies interaction with S. aureus (48). In addition to the toxic effect of HQNO, the bacteria paradoxically derive a great benefit from the presence of HQNO. The study by Hoffman and coworkers (48) demonstrated that S. aureus cells shift growth morphology to become small-colony variants (SCVs) as a result of prolonged exposure to HQNO, a compound detectable in the sputum of CF patients. SCVs of S. aureus are resistant to aminoglycoside antibiotics commonly used to treat P. aeruginosa infections of the CF lung. The authors show that as a result of HQNO-induced changes, S. aureus survives antibiotic exposure. P. aeruginosa and S. aureus may not be directly communicating via HQNO, but this chemical exchange demonstrates that the developmental outcome of a microbial interaction is modulated by environmental perturbation. The influence of environmental inputs is undoubtedly important for microbial interactions and, as demonstrated by this example of tobramycin-treated infections, may be significant for the health of the host.

SOIL BACTERIA IN DEVELOPMENTAL INTERACTIONS

Soils harbor bacteria that exhibit striking patterns of cellular development. The nature of bacterial growth, development, and community assemblage in situ is not particularly well understood, but on an agar or liquid growth medium, remarkable features of growth and development are observed. Bacteria can alter their cellular morphology, generate new cell types, synthesize pigments, and construct extracellular
Secondary metabolite: a metabolite of limited species distribution that is not essential to basic growth and development of the organism

Small molecule: a biologically active molecule or metabolite that is not polymeric in structure

matrices as a natural result of growth and starvation. Signaling plays a key role in spatial and temporal coordination of cellular developmental processes, and many of the signals involved in development are secondary metabolites. These small molecules are by definition not required for basic growth and reproduction of the organism (thus their designation as secondary metabolites), but they are instrumental in cellular differentiation. Understanding the physiological relevance and ecological significance of secondary metabolism is an exciting challenge for microbiology. In this section, we examine the action of bioactive small molecules that influence development in interspecies interactions.

Four species of spore-forming bacteria are the subject of intensive laboratory studies on development and are thus excellent model systems: Myxococcus xanthus, Bacillus subtilis, Streptomyces coelicolor, and Streptomyces griseus. All four bacteria share the ability to form spores on aerial structures, which the organisms develop through the collective effort of differentiated cell types. Recent reviews have summarized the current state of our knowledge on spore development in each of these systems (16, 35, 52, 56, 69, 118). For M. xanthus and B. subtilis, spore formation occurs within the context of fruiting bodies and aerial projections on biofilm surfaces, respectively. The Streptomyces are filamentous bacteria that form spores on aerial hyphae arising from a substrate mycelium, much like filamentous fungi. In all cases, development is tied to regulatory pathways modulated in part through the action of diffusible signals. The structures of the signals take many molecular forms, sometimes confounding the distinction between specific chemical signals and chemical morphogens, which in turn may influence our interpretation of interspecies signaling interactions that impinge upon bacterial development.

Starvation is a key environmental indicator for bacteria to divert growth toward spore formation. M. xanthus derives nutrients both from the breakdown of organic matter in soils and from predation on other bacteria (96). The very nature of the predatory lifestyle ties interspecies interactions to nutrient sensing and bacterial development. Yet, how M. xanthus senses and responds to changes in prey abundance is unknown. During starvation, the diffusible A-signal—a combination of six amino acids—sends a QS signal for fruiting body formation (29). One could speculate that amino acids excreted by potential prey would disrupt the concentration or balance of amino acids, delaying the sporulation response to the A-signal. In a recent study, Berleman & Kirby (7) reveal that patterns of fruiting body formation by M. xanthus correlate with changes in bacterial prey abundance (Figure 2a). Their experiments show that a shift in prey density supersedes soluble hydrolyzed protein (a source of amino acids) in the medium, suggesting the presence of prey-specific signals. The asgA gene encodes a histidine kinase required for A-signaling during fruiting body formation (73). A mutation in the asgA gene normally blocks fruiting body formation in monoculture experiments. However, Berleman & Kirby demonstrate that a shift in prey abundance induces fruiting body formation, even in the absence of AsgA. Discrete signals may function to monitor prey abundance rather than saprophytic nutrient abundance during development by M. xanthus. Discovering the physical form of signals from prey organisms may reveal novel signaling mechanisms for development in M. xanthus that are tied to interspecies interactions.

Secondary metabolites are predicted to play an important role in M. xanthus predatory behavior. The M. xanthus genome contains multiple gene clusters encoding polyketide synthases and nonribosomal peptide synthases that direct the synthesis of small-molecule bioactive metabolites (97). Such metabolites are proposed to function in competitive interactions and as a means for subduing prey organisms, or alternatively, in development such as the recently described family of pigments, the DKxanthenes (Figure 2d) (79). Taking a mass spectrometry approach, Krug et al. (70) assessed chemical diversity from isolates of M. xanthus taken at varying geographical distances and revealed unique sets of secondary
Free-living soil bacteria such as the myxococci, bacilli, and streptomycetes undergo dramatic developmental changes in response to environmental stimuli and are the advanced organic chemists of the microbial world. Secondary metabolism and bacterial development collide when these soil bacteria encounter each other in dual-species interactions. (a) Swarms of Myxococcus xanthus converge from four directions on a colony of prey bacteria, leaving a cross pattern of fruiting bodies where their paths meet, and the prey is exhausted. (Image courtesy of J. Kirby). (b) Sporulation by a lawn of Streptomyces coelicolor, visible as white aerial hyphae, is disrupted by Bacillus subtilis (colony in center of plate) through the action of surfactin. (c) Two streptomycetes, S. svieus (vertical) and S. griseoflavus (horizontal), are plated as spots in a crosswise pattern on agar medium. A diffusible substance from S. svieus induces aerial development by S. griseoflavus (white on colony surface) (image by J. Stewart). (d) The structure of a yellow polyene pigment from M. xanthus representing the DKxanthene family required for sporulation. (e) The structure of surfactin from B. subtilis, the agent active in blocking aerial growth by S. coelicolor (see panel b) and signaling development via K⁺ efflux (see text). (f) The A-factor from S. griseus, γ-butyrolactone. (g) Chemical structure of pamamycin-607, a macrodiolide antibiotic from Streptomyces alboniger, which acts to induce development by multiple species of Streptomyces.

Metabolites among isolates of a single species. M. xanthus represents a much more diverse group of soil myxobacteria that, by extension, are likely to encode the production of many bioactive metabolites (36, 119). Although little information exists for M. xanthus on the roles of secondary metabolites in interspecies signaling, recent studies suggest a functional role for these small molecules. Fiegna & Velicer (34) examined pair-wise interactions by soil isolates of M. xanthus and found patterns of both cooperation and antagonism between isolates with differing secondary metabolite profiles during fruiting body formation. A plausible hypothesis to explain the differences in interaction outcome includes signaling interactions mediated by multiple small molecules that in part determine compatible or incompatible associations across myxobacteria.

Indeed, for soil bacteria from many genera, the production of multiple bioactive metabolites by a single species appears to be the norm,
not the exception (15, 17, 70). Understanding the outcome of an interspecies interaction involving multiple bioactive metabolites presents an experimental challenge. Traditionally, experiments have focused on the activity of a single, purified compound. Recently, genetic analysis of mixed-species cultures has been used to study developmental interactions across species lines. The activity of two *B. subtilis* metabolites, bacillaene and surfactin, was recently uncovered by genetic screens of developmental phenotypes in mixed-species cultures with *S. coelicolor* (13, 108, 109). Surfactin is a nonribosomally synthesized lipopeptide produced by *B. subtilis* and is required for surface motility and biofilm formation (Figure 2e) (9). Surfactants synthesized by both organisms promote production of aerial structures. *S. coelicolor* synthesizes SapB, a lantibiotic-like peptide morphogen that promotes aerial growth of filaments (64). The model of aerial development by streptomycetes invokes the action of surfactants to diminish surface tension at liquid-air interfaces, removing the principal barrier to growth away from a surface. A fungal surfactant, SC3 from *Schizophyllum commune*, can substitute for SapB in aerial growth, leading to a corresponding prediction for surfactin (110). However, this prediction was not borne out; using a mixed-species culture approach, Straight et al. (109) found that surfactin antagonizes—rather than stimulates—aerial development by *S. coelicolor* (Figure 2b). Aerial development in *S. coelicolor* depends on the *ramCSAB* and *cspa*-*h* genes, which encode production of the SapB peptide and the chaplin family of hydrophobic peptides, respectively (18, 32, 64, 84). Although the precise mode of action remains to be determined, surfactin disrupts development by interfering with the production of SapB and the chaplins. Surfactin is a molecule with multiple apparent functions for the producer *B. subtilis*, including surface motility, antibiotic activity, and developmental signaling. Metabolites such as surfactin, for which multiple activities have been demonstrated in vitro, may play even more roles in community formation and environmental information transfer in soil ecosystems.

The activity of secondary metabolites in modulating development across species lines has also been studied using purified compounds in single-species experiments. In a recent study by López and colleagues (75), purified bacterial metabolites that function as potassium-selective channels induced development of biofilms by *B. subtilis*. Among the compounds tested were nystatin, known as an antifungal polyene produced by *Streptomyces noursei*, and surfactin. The observed effect on biofilm development led to a novel function for surfactin through a signaling mechanism involving K⁺ ion efflux that somehow activates the signaling histidine kinase, KinC (71). Activated KinC in turn phosphorylates the Spo0A master regulator to a level that induces biofilm formation by *B. subtilis*. The authors propose a mechanism whereby K⁺ leakage caused by surfactin or nystatin triggers KinC phosphorylation of Spo0A to initiate biofilm formation. This hypothesis was further substantiated by an experiment in which the key signaling components KinC, Spo0A, and the Spo0A-dependent reporter P₉₅-cfp were produced in *Listeria monocytogenes*. The signal transduction pathway was activated by treatment with surfactin and abrogated with high concentrations of K⁺. In terms of interspecies signaling, this study demonstrates that structurally diverse small molecules from different species can share a common mechanism in modulating a developmental pathway. The molecules that activate KinC in *B. subtilis* share the property of forming membrane ion channels with some K⁺ selectivity even though their structures are diverse. It is possible that synthesis of secondary metabolites that interfere with potassium homeostasis is widely used as a signaling mechanism in soil and other environments.

Perhaps the most prolific secondary metabolite producers in soils are the actinomycetes, and prime among these are streptomycetes. Linkages between development and secondary metabolite production are well established for streptomycetes. The transition from substrate to aerial growth (idiophase) is an important juncture for
regulation of secondary metabolism, during which the synthesis of small molecules (id-lytes) is activated (25). The transition for many species involves hormone-like signaling using butyrolactones [γ-butyrolactone (A-factor) in S. griseus, SCB1 and derivatives in S. coelicolor, and the virginiae butanolides in S. virginiae, for example] to mediate the shift from substrate to aerial growth (Figure 2f) (52, 60).

The A-factor molecule from S. griseus has been investigated in great detail. A-factor binds with nanomolar affinity to its ArpA receptor protein, a DNA-binding transcriptional repressor, suggesting a highly specific signal-receptor interaction (81, 88). Nanomolar dissociation constants of the butyrolactones with cognate receptor proteins indicate that the molecules are species-specific signals, which holds true in studies in which species such as S. griseus and S. virginiae are compared. Cross-species signaling by γ-butyrolactone from S. griseus was investigated by Hara and Beppu (44). The authors found that γ-butyrolactone could stimulate aerial growth and antibiotic production in a wide range of Streptomyces species, prompting the question of whether diverse species of Streptomyces in soil communities might coordinate shifts in secondary metabolism through common signaling mechanisms.

Addressing the question of signal species specificity, Nishida and coworkers used a phylogenetic approach to examine the evolution of butyrolactone synthases (AfsA homologues) and receptors (ArpA homologues), and found that AfsA and ArpA phylogenetic trees differ in topology (85). The authors observed that strict pairing of ligand and receptor genes is not a paramount feature of the phylogenetic tree, indicating that AfsA and ArpA homologues have differing evolutionary histories. According to their analysis, the earliest common ancestor gene for an ArpA DNA-binding protein existed before the appearance of a butyrolactone synthase, suggesting that butyrolactone signaling was adopted as a means of communication for controlling expression of genes that are regulated by ArpA-like proteins. Furthermore, the butyrolactone receptor proteins are more variable in ligand-binding domains than in DNA-binding domains, suggesting flexibility in ligand specificity. Signaling interactions could involve strict signal-receptor binding as in the case of γ-butyrolactone for ArpA, whereas other less specific interactions could influence patterns of development and metabolism in mixed-species communities through modulation of regulatory activity across species lines.

The downstream products of regulation by ArpA and related Streptomyces antibiotic regulatory proteins are themselves bioactive molecules, many of which are clinically useful natural products. The activity of these molecules across species is not limited to antibiotic interactions, as has been described in experiments using subinhibitory concentrations of classical antibiotic compounds (41, 112). In these studies, antibiotics trigger a measurable transcriptional response in target bacteria without inhibition due to antibiotic activity. Dual-activity based on concentration accords with the property of hormesis, whereby antibiotics provide concentration-dependent chemical signal information (22). The differential activity of bioactive molecules when sensed at low concentration raises the intriguing possibility that secondary metabolites convey chemical information between species.

A broader role for antibiotics is also consistent with the findings of D’Costa and colleagues (23), who identified the widespread presence of antibiotic resistance genes in an extensive sampling of soil bacteria. Widespread production and excretion of antibiotics would drive dissemination of resistance genes throughout soil populations that exist predominantly as mixed-species communities. These observations elucidated roles for antibiotics in soil ecology that extend beyond the scope provided by a chemical warfare paradigm. However, small molecules described as antibiotics represent only a fraction of the total potential for structurally diverse compounds synthesized by bacteria (4). A view of secondary metabolism limited to antibacterial activity is insufficient to explain the
relevance of these molecules in bacterial physiology.

Interspecies interactions involving bacterial development lead to a much broader range of assayable activities from small molecules. For example, aerial development and antibiotic synthesis by *Streptomyces* spp. are susceptible to influence by secondary metabolites from neighboring species, as described by Ueda and coworkers (113) (Figure 2c). Compounds that have developmental modulatory activity can be found among many previously described functional categories. For example, Hashimoto et al. (46) demonstrated that the macrolide antibiotic pamamycin-607 stimulates aerial development in 67% of *Streptomyces* strains tested (30/45 strains) (Figure 2g). The siderophore desferrioxamine E stimulated growth, development, and antibiotic activity in several *Streptomyces* strains (120). The microcin B17-like peptide goadsporin stimulates *Streptomyces* development and antibiotic biosynthesis (54, 89). Such examples serve to illustrate what we may expect by investigating bacterial secondary metabolite activity in developmental interactions between species. In our opinion, great insights into bacterial interspecies communication await investigations of developmental interactions between bacteria in soil communities and other microbial communities of diverse bacterial composition.

**FUTURE EXPLORATIONS OF BACTERIAL CHEMICAL CONVERSATION**

The abundance and structural diversity of bacterial secondary metabolites pose a daunting challenge for any attempt to define chemical communication between species of bacteria. We have presented several examples of bacterial development that can be influenced by chemical exchanges between species, ranging from a series of chemical signals in plant nodulation to the wide world of interactions that might occur in the soil among free-living bacteria. This small set of examples highlights the variability in biological signals and chemical cues that bacteria may encounter in the environment. Considering the species diversity of bacterial communities in most environments, a single bacterial species can likely process many signal inputs simultaneously and can respond to them with numerous possible outputs.

Continued study of bacterial development and secondary metabolism coupled with the application of new technologies, genomic and bioinformatic analyses, and mixed-species systems will provide a pathway to understanding at least some of the underlying principles of interspecies chemical communication. A handful of technologies in particular hold great promise for the study of bacterial interactions. Microfluidic devices have proven effective for studying changes in bacterial gene expression patterns with limited numbers of bacteria sequestered in subpopulations, a circumstance more akin to predictions of community structure on soil particles (3, 59, 62). Powerful new technologies blending mass spectrometry and imaging allow the direct visualization of small-molecule production, using simple or complex communities of bacteria (24, 33). Formulation of growth media to better match the natural environment minimizes the disparity between in vitro and in vivo interactions (11, 92). Metagenomic analyses of defined bacterial communities such as the gypsy moth larval gut offer an opportunity to understand networks of interactions independent of isolation and culture (10, 43). Finally, emerging biological systems involving associations between bacteria, fungi, and multicellular organisms provide systems ripe for the study of chemical communication between organisms. Examples include phototrophic consortia of bacteria and insect-fungal-bacterial multipartite symbioses, which blend aspects of chemical exchange and microbial development (91, 105). Interspecies chemical communication in bacterial development is a field about to blossom.
SUMMARY POINTS
1. Bacterial communities are integral components of most environments and biological systems and are often richly diverse in species composition.
2. Bacterial community assembly and function require information exchange in the form of chemical signaling between bacteria, fungi, plants, and other organisms.
3. Cellular differentiation occurs in bacterial communities in many forms such as biofilm formation, sporulation, and virulence factor production.
4. Bacterial communities rely on chemical exchanges to provide the cues for developmental change.
5. Bacteria produce many diverse, bioactive secondary metabolites that act to alter development in neighboring species.

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