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Toward an understanding of microbial communities through analysis of communication networks

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Abstract

Bacteria receive signals from diverse members of their biotic environment. They sense their own species through the process of quorum sensing, which detects the density of bacterial cells and regulates functions such as bioluminescence, virulence, and competence. Bacteria also respond to the presence of other microorganisms and eukaryotic hosts. Most studies of microbial communication focus on signaling between the microbe and one other organism for empirical simplicity and because few experimental systems offer the opportunity to study communication among various types of organisms. But in the real biological world, microorganisms must carry on multiple molecular conversations simultaneously between diverse organisms, thereby constructing communication networks. We propose that biocontrol of plant disease, the process of suppressing disease through application of a microorganism, offers a model for the study of communication among multiple organisms. Successful biocontrol requires the sending and receiving of signals between the biocontrol agent and the pathogen, plant host, and microbial community surrounding the host. We are using *Bacillus cereus*, a biocontrol agent, and the organisms it must interact with, to dissect a communication network. This system offers an excellent starting point for study because its members are defined and well studied. An understanding of signaling in the *B. cereus* biocontrol system may provide a model for network communication among organisms that share a habitat and provide a new angle of analysis for understanding the interconnections that define communities.

Abbreviations: AHL – *N*-acyl-homoserine lactone; DFI – differential fluorescence induction; IVET – *in vitro* expression technology

Introduction

Bacteria engage in intra- and inter-species communication. In complex ecosystems, they must simultaneously exchange signals with members of their own species as well as with members of other species of microorganisms and eukaryotes. A signal, in this context, is a small molecule generated by one organism that is sensed by another, resulting in a cellular response, usually manifested in a change in gene expression.

A research challenge for microbiologists of the 21st century is to develop a portrait of the communication networks that link together organisms in communities. A dynamic environment in which to study such communication is the rhizosphere, which is the region surrounding and affected by a plant root. The rhizosphere is characterized by dense microbial life, high growth rates and metabolic activity, and rapid changes in physical conditions. The microbial community in the rhizosphere is a source of diverse chemistry, and the bacteria in it are likely to be exposed to a cacophony of signals that must be interpreted correctly to ensure their survival. In addition to the microbial chemistry, the microbial community is immersed in the complex chemical matrix that plants pour into the rhizosphere. Plants release into the rhizosphere approximately 20% of the C that is allocated to the root, suggesting a highly evolved relationship between plants and their rhizosphere associates. Therefore, both microbial and plant-derived compounds are likely to act as regulatory signals in

Table 1. Examples of bacterial–bacterial signaling molecules in Gram-negative and Gram-positive bacteria

Organism	Signaling molecule	Regulated process	Reference
Vibrio fischeri	*N*-(3-oxo)-hexanoyl-L-homoserine lactone *N*-(3-oxo)-octanoyl-L-homoserine lactone	Bioluminescence	Kuo et al. (1994)
Erwinia carotovora	*N*-(3-oxo)-hexanoyl-L-homoserine lactone	Carbapenem and exoenzyme production	Bainton et al. (1992)
Agrobacterium tumefaciens	*N*-(3-oxo)-octanoyl-L-homoserine lactone	Ti plasmid conjugation	Fuqua & Winans (1994)
Rhizobium leguminosarum	*N*-(3-hydroxy)-tetradecanoyl-L-homoserine lactone (HtDeHL or Bacteriocin *small*)	*rhi* gene expression	Schripsema et al. (1996); Gray et al. (1996)
Pseudomonas aureofaciens	*N*-hexanoyl-homoserine lactone (HHL)	Phenazine antibiotic production	Wood & Pierson (1996); Chancey et al. (1999)
Pseudomonas aeruginosa	2-heptyl-3-hydroxyl-4-quinolone	LasB elastase	Pesci et al. (1999)
Vibrio harveyi	3-(2H)-furanone	Bioluminescence	Schauder et al. (2001)
Bacillus subtilis	ComX peptide pheromone	competence	Magnuson et al. (1994)
Streptomyces griseus	2-iso-capryloyl-3*R*-hydroxymethyl-γ-butyrolactone (A-factor)	Antibiotic production and differentiation	Hara & Beppu (1982); Khokhlov et al. (1967)

members of the rhizosphere community. Because of its complexity and tendency to change abruptly, the rhizosphere is an intriguing and challenging environment in which to dissect communication networks, and one that has a profound effect on plant health.

The study of signaling and its effect on rhizosphere bacteria has largely focused on Gram-negative plant-invasive bacteria. Gram-positive bacteria are common soil and plant-associated microbes (Gilbert et al. 1993; Felske et al. 1998), but have received less attention from rhizosphere biologists. To address this knowledge gap, we are interested in signaling between plant-associated Gram-positive bacteria and the plants, microbes, and pathogens they encounter during life in the rhizosphere.

Bacillus cereus is a Gram-positive bacterium that is ubiquitous in soil and on plant roots (Stabb et al. 1994). Many strains of *B. cereus* isolated from field-grown alfalfa and soybean roots have biocontrol activity (Halverson et al. 1993; Stabb et al. 1994; Raffel et al. 1996) on many plant species against diseases caused by oomycete plant pathogens (Handelsman et al. 1990; Handelsman et al. 1991; Phipps 1992; Smith et al. 1993; Osburn et al. 1995). We propose that signals from the plant, pathogen, and the microbial community that they share regulate gene expression in *B. cereus*, and this gene regulation defines the outcome of the relationship among the organisms. We have adapted a technique called differential fluorescence induction (DFI) (Valdivia & Falkow 1996) for use with *B. cereus* to identify changes in gene expression in this organism in response to compounds from other members of the rhizosphere community. In this paper, we review some of the known modes of communication among bacteria and between bacteria and eukaryotes and then we describe the *B. cereus* biocontrol system that we have developed for study of communication in the rhizosphere.

Bacterium-to-bacterium signaling: from squid to plants

Bacterial signals in bacterial communication

Bacteria communicate with themselves. Quorum sensing, also known as autoinduction, is a sensing system in bacteria used for monitoring population density and coordinating gene expression with population growth. Small diffusible compounds, autoinducers, are secreted by bacteria during growth. These autoinducers accumulate in the environment surrounding the bacteria and after reaching sufficient concentration, activate the expression of genes important for bioluminescence, virulence, antibiotic production, competence, and sporulation (Salmond et al. 1995; Kaiser & Losick 1993). The first report of quorum sensing was

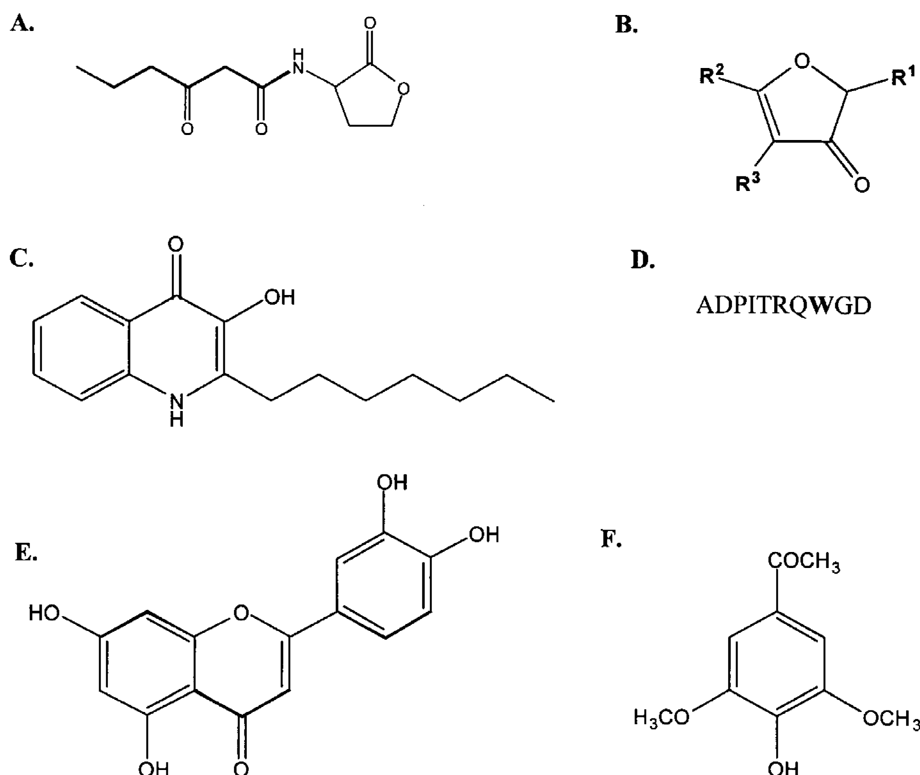

Figure 1. Examples of classes of described signaling molecules. Bacterial-bacterial signals: (A) *N*-(3-oxo)-hexanoyl-L-homoserine lactone; (B) 3-(2H)-furanone; (C) 2-heptyl-3-hydroxyl-4-quinolone; (D) ComX peptide (tryptophan in bold type indicates unknown modification) Plant-bacterial signals: (E) acetosyringone; (F) luteolin.

published in 1970 and described the autoinduction of luminescence in the bacterium *Vibrio fischeri*, which lives in the light organs of marine squid (Nealson et al. 1970). For a thorough discussion of quorum sensing in *Vibrio*, the reader is referred to excellent reviews on the subject (Greenberg 1997; Bassler 1999; Hastings & Greenberg 1999). Quorum sensing appears to be widely used in the bacterial world (Hastings & Greenberg 1999).

The quorum sensing compounds described to date include *N*-acyl homoserine lactones (AHLs), quinolones, and cyclic dipeptides in Gram-negative bacteria (Pesci et al. 1999; Holden et al. 1999; Holden et al. 2000) and peptides and γ-butyrolactones in Gram-positive bacteria (Dunny & Leonard 1997; Kleerebezem et al. 1997; Upton et al. 2001; Khokhlov et al. 1967; Hara & Beppu 1982). Examples of these signaling molecules are shown in Table 1 and Figure 1. A new class of autoinducer, AI-2, predicted to be a furanone (Schauder et al. 2001), is produced by the gene product of *luxS*, whose homologues have been found in both Gram-negative and Gram-positive bacteria (Surette et al. 1999). The widespread nature of *luxS* provides evidence for molecular cross-talk between Gram-negative and Gram-positive organisms. It appears that quorum-sensing is a widely distributed mechanism for communication among bacteria in diverse environments.

In plant-associated bacteria, quorum sensing controls secondary metabolite production, virulence gene expression, and the expression of genes in the rhizosphere of plants. The plant-associated bacteria shown to engage in quorum sensing include *Erwinia carotovora*, *E. stewartii*, *Agrobacterium tumefaciens*, *Xanthomonas campestris*, *Rhizobium leguminosarum*, *Pseudomonas fluorescens*, *P. aureofaciens*, *Ralstonia solanacearum*, and *Serratia liquefaciens* (Pirhonen et al. 1993; Zhang et al. 1993; Ganova-Raeva et al. 1994; Pierson et al. 1994; Beck von Bodman & Farrand 1995; Eberl et al. 1996; Farrand et al. 1996; Schripsema et al. 1996; Flavier et al. 1997; Shaw et al. 1997; Rodelas et al. 1999; Laue et al. 2000; Slater et al. 2000). It is intriguing that one study found that *Pseudomonas* spp. isolated from plants were more likely to produce AHLs than were *Pseudomonas* spp. isolated from the soil (Elasri et al. 2001), suggesting

that quorum sensing, and perhaps molecular communication more generally, is important in communities associated with a plant.

Plants scramble bacterial messages

In addition to the quorum sensing signals derived from bacteria, the rhizosphere contains compounds produced by plants that mimic bacterial AHLs, either inhibiting or stimulating bacterial signaling. The first description of disruption of bacterial signaling by a plant involved the marine red alga *Delisea pulchra* (Givskov et al. 1996; Kjelleberg et al. 1997). Halogenated furanones from *D. pulchra* displaced bacterial AHLs from their receptor proteins, resulting in disruption of quorum sensing (Manefield et al. 1999; Rice et al. 1999). Additionally, exudates from pea seedlings, crown vetch, rice, soybean, tomato, and *Medicago truncatula*, stimulated or inhibited AHL-dependent behavior in several different AHL reporter strains (Teplitski et al. 2000). Preliminary efforts to characterize the pea compounds indicate that they are chemically different from the bacterial AHLs, but the chemical nature of the compound or compounds remains unsolved. Given the diversity of monocotyledonous and dicotyledonous plants that contain AHL mimics, the ability to disrupt quorum sensing may be common among higher plants.

Bacteria scramble each others' messages

Plants may not be the only organisms that disrupt bacterial quorum sensing. Recent reports indicate that bacteria could disrupt AHL signaling by degrading AHLs. A *Bacillus* isolate contains *aiiA*, a gene that encodes a 250-amino acid AHL-lactonase protein (Dong et al. 2000; Dong et al. 2001), which hydrolyzes the lactone bond of AHLs. When *aiiA* is expressed transgenically in tobacco and potato plants, it disrupts quorum sensing in the pathogen *Erwinia carotovora* and increases resistance of the plants to disease caused by this bacterium. Degradation of quorum sensing signals may turn out to be a common trait among bacteria. A strain of *Variovorax paradoxus* isolated from soil grew on AHLs as a sole carbon and nitrogen source (Leadbetter & Greenberg 2000). Quorum sensing mimics and degradative functions from plants and bacteria introduce new dimensions of complexity into our portrait of the communication networks among bacteria associated with plants, and suggest new models to be tested in the environment.

While the quorum sensing mimics produced by plants were discovered recently, communication between plants and bacteria with other signaling compounds is a well-studied and exquisitely tuned phenomenon.

Plant-bacterium signaling: from symbionts to pathogens

The canonical systems – Rhizobium *and* Agrobacterium

Much of the research involving the study of interactions between plants and bacteria has focused on invasive relationships resulting in visible changes in the plant, such as the formation of root nodules, galls, or disease. Two of the best-studied plant-bacterial interactions are those of *Rhizobium* spp. with leguminous plants and *Agrobacterium tumefaciens* with dicotyledonous host plants. In each of these systems, bacterial gene expression is induced by plant-derived phenolic compounds. In *Rhizobium*, plant flavonoids, such as luteolin (Figure 1), induce genes in *Rhizobium* spp. that are important in the formation of nitrogen-fixing nodules in the plant (Peters et al. 1986; review: Stacey et al. 1995). In the crown gall system, acetosyringone (Figure 1) and α-hydroxyacetosyringone, compounds exuded from plant wounds, induce expression of virulence genes in *A. tumefaciens* (Stachel et al. 1985: review: Winans 1992). These exciting discoveries led to the search for genes in other plant-associated bacteria whose expression was influenced by plant compounds, focusing largely on pathogenic bacteria. Several of these studies are described below.

Global searches for plant-induced genes

A novel and global approach for identifying pathogenicity genes in a plant disease-causing bacterium was introduced by Osbourn et al. in 1987. Their strategy employed a promoter-probe plasmid to identify genes in *Xanthomonas campestris* that were selectively expressed when the bacterium was growing on the plant. A library of *X. campestris* DNA was constructed containing genomic fragments upstream of a promoterless chloramphenicol-resistance gene on the plasmid. This library was introduced into *X. campestris* and the clones were screened for pathogenicity on turnip seedlings that had been grown in a solution containing chloramphenicol, which is readily taken up by plant tissues. This process selected

for clones containing promoters driving expression of the chloramphenicol-resistance gene, thereby enabling the appropriate clone to grow and induce disease on the treated plant. Promoters induced *in planta* were distinguished from constitutively expressed promoters by discarding clones that grew on laboratory medium containing chloramphenicol. Using this technique, 14 plant-induced genes were identified, although little is known about the nature of the gene products or the inducing compounds from the plant. This work laid the foundation for many studies of plant- and animal-associated microbes that led to identification of host-regulated bacterial genes.

The promoter-probe technique described by Osbourn et al. was further developed by researchers studying the pathogenicity of *Salmonella* in its host, and is referred to as *in vivo* expression technology or IVET (Mahan et al. 1993). IVET was subsequently used to identify plant-induced genes in a non-pathogenic bacterium, *Pseudomonas fluorescens* (Rainey 1999). In IVET, a gene essential for growth on or in the host is used to select for promoters expressed only in the presence of the host. To identify induced promoters in *P. fluorescens*, *panB*, which encodes ketopantoate hydroxymethyltransferase, was used as the selective gene as it had previously been shown to be essential for rhizosphere colonization of sugar beet seedlings by *P. fluorescens*. Rainey (1999) identified 20 rhizosphere-induced genes, 14 of which contained sequence similarity to previously identified genes involved in acquisition of nutrients, response to stresses, or secretion. The remaining genes had no homologues in the database. These genes are currently under study to determine their functions and ecological roles and identify the rhizosphere signals that induce their expression.

An additional method for identifying plant-induced genes involves the use of a transposon containing a promoterless reporter gene. The transposon is randomly inserted throughout the chromosome of the bacterium of interest and reporter gene activity is assayed in the presence and absence of plant compounds. This approach has been used successfully in *E. chrysanthemi* (Beaulieu & van Gijsegem 1990) and *P. fluorescens* (van Overbeek & van Elsas 1995) to identify plant-induced genes. Among the *E. chrysanthemi* mutants, 10 had promoter activity only when extracts from the plant *Santipaulia ionantha* were present in the agar medium. Among the *P. fluorescens* mutants, four contained reporter gene fusions that were plant-induced. One contained a fusion that responded to proline only, while the other three responded to many different carbon sources including sugars, organic acids, and amino acids.

Plant induction of phosphate solubilization

The studies described above are examples of global searches for genes in plant-associated bacteria whose expression is influenced in the presence of a plant host or compounds from the plant host. One interesting recent study of specific gene induction in plant-associated bacteria involved the effect of compounds from plants growing in a phosphate-limited desert environment on gluconic acid production in a population of bacteria isolated from the plant roots (Goldstein et al. 1999). The authors hypothesized that the bacteria associated with the roots of *Helianthus annus jaegeri* were important for solubilizing mineral phosphate for use by the plant. The ability of the bacteria to solubilize the mineral phosphate lies in gluconic and 2-ketogluconic acid production, which acidifies the extracellular environment. Bacteria isolated from the roots did not solubilize mineral phosphate in culture unless a solution of concentrated root washings was added to the medium. The results of this study suggest that signals from the plant could regulate the ability of the bacteria to acidify the rhizosphere and solubilize phosphate, which is necessary for plant growth.

The application of global techniques such as promoter-probe plasmids, IVET, and reporter transposons coupled with classical mutant hunts to identify genes required for the establishment of plant-microbe relationships promises to yield new insight into the chemical exchange between plants and bacteria. Given the chemical virtuosity and phylogenetic diversity of both plants and soil microorganisms (Heywood 1993; Ueda et al. 1995; Ørreås & Torsvik 1998; Whitman et al. 1998), the opportunity for discovery of new small molecules and genetic mechanisms that regulate plant-bacterial interactions seems boundless.

Oomycete-bacterium signaling

In addition to their contact with plants and bacteria, soil bacteria share their space with eukaryotic microorganisms such as fungi and protists. Recent work demonstrated the potential for signaling between a plant pathogenic oomycete (a protist) and a bacterium that suppresses disease caused by the oomycete. The oomycete *Pythium ultimum* represses genes in the biocontrol bacterium, *P. fluorescens*, resulting in altered ecological fitness of the bacterium (Fedi et

al. 1997). Two of the genes were further characterized and found to be *rrn* operons (Smith et al. 1999). Repression of these operons by *P. ultimum* results in reduced growth rates under conditions of rapid growth, and this repression could be an effective strategy for preventing the establishment of the biocontrol strain in the rhizosphere.

This example of possible chemical communication between *P. ultimum* and *P. fluorescens* is one of many examples of signaling in biocontrol systems. In addition to interacting with the pathogen, the biocontrol agent must interact with the plant host and the microbial community associated with it, providing multiple contexts for chemical communication. The multifaceted communication that contributes to or detracts from successful disease suppression offers a model for understanding communication networks and also will contribute to our ability to manipulate and enhance the efficacy of biocontrol.

Biocontrol systems to study multichannel communication networks

The portrait of biocontrol that has emerged over the last decade indicates that successful disease suppression depends on carefully calibrated signal exchange among the many partners of the system. The principles that have emerged are broad because of the diversity of microorganisms and plant hosts that have been studied. Diverse bacteria, both Gram-negative and Gram-positive, have been used as biocontrol agents (Cook & Baker 1983; Lumsden et al. 1995). Small molecules have been shown to be important in the mechanisms of disease suppression, which include antibiotic production, iron competition, or parasitism of the pathogen (Handelsman & Stabb 1996). Much of the research has focused on the detrimental effects of the biocontrol bacterium on the plant pathogen. Recent studies, such as the oomycete-bacterial signaling described above, have shown that antagonism of the plant pathogen by the biocontrol bacterium is only one side of the story. There appear to be many levels of communication between the organisms involved that contribute to the success or failure of biocontrol, making these systems exciting opportunities to study molecular communication.

A Bacillus cereus *biocontrol model system*

We have studied biocontrol of oomycete diseases by strains of *B. cereus* from many angles, and it seems that whatever the research question, the answer lies in small molecules. In our studies of the mechanism of biocontrol, we discovered that *B. cereus* produces two antibiotics, zwittermicin A and kanosamine, that contribute to disease suppression (Silo-Suh et al. 1994; Milner et al. 1996). Zwittermicin A prevents elongation of germ tubes from cysts of the pathogen, *Pythium torulosum*, either in culture or on plants (Silo-Suh et al. 1994; Shang et al. 1999). The plant enters the chemical equation by affecting both zwittermicin A production and bacterial growth. Production of zwittermicin A in culture is enhanced by addition of exudates from alfalfa roots (Milner et al. 1995), and alfalfa seeds secrete canavanine, an arginine analogue that is highly toxic to *B. cereus* in culture and influences the spatial distribution of the bacterium on and around the seeds (Emmert et al. 1998). Thus, small molecules can govern diverse aspects of the interactions among the biocontrol agent, plant host, and pathogen. While most of the effects we have studied to date are dramatic, effecting gross changes in cell number or morphology, the more subtle effects of small molecules will likely also prove important as indicated by the many recent ecological studies that show quantitative genetic effects to define biological outcomes.

Based on observations in the field (Gilbert et al. 1993), we predict that the interactions between the *B. cereus* and the rhizosphere community will be important for understanding biocontrol. An interesting observation made in our laboratory is that we can isolate certain soil bacteria from apparently pure cultures of *B. cereus* isolated from plant roots. Over the past 10 years in our laboratory, approximately 1–5% of *B. cereus* isolates obtained from alfalfa or soybean roots appear to be pure cultures using standard microbiological techniques. After extended incubation at 4 °C, outgrowth from the *B. cereus* colonies on agar plates indicate the presence of a co-isolated bacterium, undetectable by microscopy in the original culture. The co-isolation phenomenon has only been seen in *B. cereus* isolated from plant roots, not bulk soil, and suggests that this relationship could be important in the ecology of *B. cereus* on plant roots. Because of these interesting interactions between *B. cereus* and other organisms, we have turned our attention to the study of the exchange of signals that govern gene expression among the many partners that influence the outcome of biocontrol (Figure 2).

Our approach for studying the complex communication between *B. cereus*, and the plant, plant pathogen, and rhizosphere microbial community involves the use

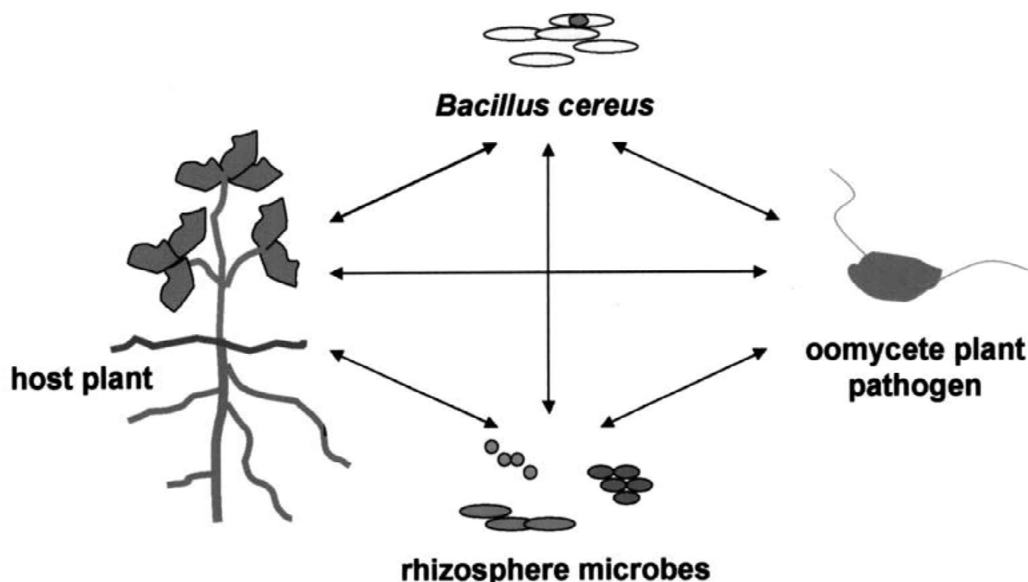

Figure 2. Molecular communication networks between organisms in the *Bacillus cereus* biocontrol system. An example is the production of the antibiotic, zwittermicin A, by *B. cereus*. Zwittermicin A production in culture can be increased by the addition of unknown compounds from alfalfa roots (Milner et al. 1995). This antibiotic has been shown both in culture and on plant roots to reversibly inhibit germ tube elongation of the oomycete pathogen *Pythium torulosum* (Silo-Suh et al. 1994; Shang et al. 1999).

of differential fluorescence induction (DFI). This gene fusion technique was developed by Valdivia & Falkow (1996) as a method to rapidly isolate promoters induced or repressed under a condition of interest. The approach relies on the use of a promoter-trap (or promoter-probe) plasmid with a promoterless reporter gene, which in this case is a red-shifted mutant version of the green fluorescence protein gene, *gfpmut*3a (Cormack et al. 1996). Chromosomal DNA fragments of the study bacterium are cloned upstream of the reporter gene effectively 'trapping' promoters for study. The library of promoters is introduced into the bacterium from which the chromosomal DNA was obtained and the library is screened using multiple rounds of flow cytometry with cell-sorting to rapidly enrich for clones containing promoter fusions showing regulation under the condition of interest. This technique has several advantages for studying regulation of gene expression. DFI facilitates the rapid identification of promoters whose expression is either induced or repressed under the condition of interest and the use of an expression plasmid allows the study of essential genes that would be lost in a transposon study.

Using the Valdivia and Falkow method as a model, we have developed a promoter-trap vector for use in *Bacillus* spp. and have used this vector to generate a promoter-trap library of *B. cereus* chromosomal DNA (Dunn & Handelsman 1999). We have used this approach to identify *B. cereus* genes that are regulated by compounds originating from the plant host and other members of the microbial community in the rhizosphere, focusing on bacterial species that co-isolate with *B. cereus* such as *Pseudomonas aureofaciens*. Using tomato seed exudate as a source of host-plant compounds, we identified two genes whose expression is up-regulated when exudate is present. Sequence similarity indicates these genes potentially encode extra-cytoplasmic proteins that could be involved in stress-response or proper secretion of proteins (Dunn & Handelsman, unpublished data). Using cell-free culture supernatant from *P. aureofaciens* 30–84 (Kluyver 1956; Pierson & Pierson 1996) in our screening process, we identified 12 clones that showed a decrease in GFP-expression when supernatant was present. Sequence analysis of these clones identified similarity to genes encoding products involved in processes such as nutrient acquisition and cell division, as well as several sequences with no homologues in the database (Dunn & Handelsman, unpublished data). We are currently characterizing the plant-regulated and *Pseudomonas*-regulated genes, and will continue our search for similar genes as well as those regulated by the presence of the plant-pathogenic protist, *Pythium torulosum*. The DFI approach will be a useful method for beginning to understand the influence

of chemical signaling on the biocontrol ability of *Bacillus cereus*.

Conclusions

The emerging image of microbial communication networks is one of numerous bacterial signals, with interference, scrambling, and amplification of those signals by other members of the community. At this early stage in our understanding it is difficult to imagine how bacteria make sense of their chemical environment as they are bombarded with small molecules from all sides. But the study of carefully chosen systems will reveal the networks that provide order to the communication and yield new insight into the functioning of microbial communities. We predict that biocontrol systems will provide good models for dissecting communication among diverse organisms, which is vital to our understanding of microbial community function.

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Note added in proof

"Since the preparation of this manuscript, the structure of the inducer, AI2, was published (Chen, X., Schauder, S., Poteir, N., Van Dorsselaer, A., Pelczer, I., Bassler, BL., and Hughson, FM. 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415: 488–489.

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