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The Multifactorial Basis for Plant Health Promotion by Plant-Associated Bacteria[▽]

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On plants, microbial populations interact with each other and their host through the actions of secreted metabolites. However, the combined action of diverse organisms and their different metabolites on plant health has yet to be fully appreciated. Here, the multifactorial nature of these interactions, at the organismal and molecular level, leading to the biological control of plant diseases is reviewed. To do so, we describe in detail the ecological significance of three different classes of secondary metabolites and discuss how they might contribute to biological control. Specifically, the roles of auxin, acetoin, and phenazines are considered, because they represent very different but important types of secondary metabolites. We also describe how studies of the global regulation of bacterial secondary metabolism have led to the discovery of new genes and phenotypes related to plant health promotion. In conclusion, we describe three avenues for future research that will help to integrate these complex and diverse observations into a more coherent synthesis of bacterially mediated biocontrol of plant diseases.

Diverse plant-associated bacteria can positively impact plant health and physiology in a variety of ways (19). Three well-studied mechanisms of biological disease control and plant health promotion conferred by plant-associated bacteria have recently been reviewed (38). However, the focus on identifying and characterizing individual mechanisms has obscured the complex, multifactorial nature of biological control. So, while many different biocontrol bacteria have been identified and much has been learned about different types of plant-bacterium interactions for some of these populations, we are just beginning to appreciate the number and complexity of interactions actually taking place *in situ*. Indeed, many different bacteria and many different bacterial metabolites have been identified as important contributors to the biological control of plant diseases. However, we still lack a clear understanding of how the populations and activities of the diverse populations of microorganisms that colonize every plant are connected and integrated in natural and agricultural environments.

Here, we review recent work that indicates the multifactorial nature of biocontrol (Fig. 1). Specifically, we review a number of the studies indicating that biocontrol arises from the combined actions of multiple bacterial populations, each expressing several different classes of bioactive metabolites under the control of multiple genes and regulons. And we highlight recent work in the authors' laboratories that begins to identify the diverse factors contributing to changes in plant growth and health status. We conclude by providing a road map for future research that will lead to a greater understanding of the complex, dynamic, and multifactorial nature of biological control phenotypes expressed by plant-associated bacteria.

THE DIVERSITY AND BIOGEOGRAPHY OF BIOCONTROL BACTERIA

It is well established that there are large and diverse numbers of bacteria found on plants, and a diverse set of bacteria have been identified with biocontrol activities (19). To date, isolates of over two dozen genera of bacteria have been reported to have biocontrol and/or plant growth-promoting activities (61), and new genera and species of biocontrol bacteria are still being discovered (3) (B. B. McSpadden Gardener, unpublished data). Furthermore, among some bacterial genera, multiple species and subspecies of biocontrol agents have been identified and can be found across multiple spatial scales, from the global to the farm level, and even on single plants. Interestingly, individual isolates may display biocontrol activity not only on the crops from which they were obtained but also on other crops (18). This may reflect the generalist nature of some genotypes, especially those with a wide geographic distribution (48). Clearly, if introduced in sufficient numbers and active for a sufficient duration, a single bacterial population can have a significant impact on plant health. However, such active strains do not act alone in a vacuum. Rather, they exist among a diversity of other bacteria which may also contribute to or antagonize biocontrol. For example, one class of biocontrol pseudomonads found to suppress the take-all pathogen (73) were found to coexist in the rhizosphere with other bacteria, including other *Pseudomonas* antagonists and

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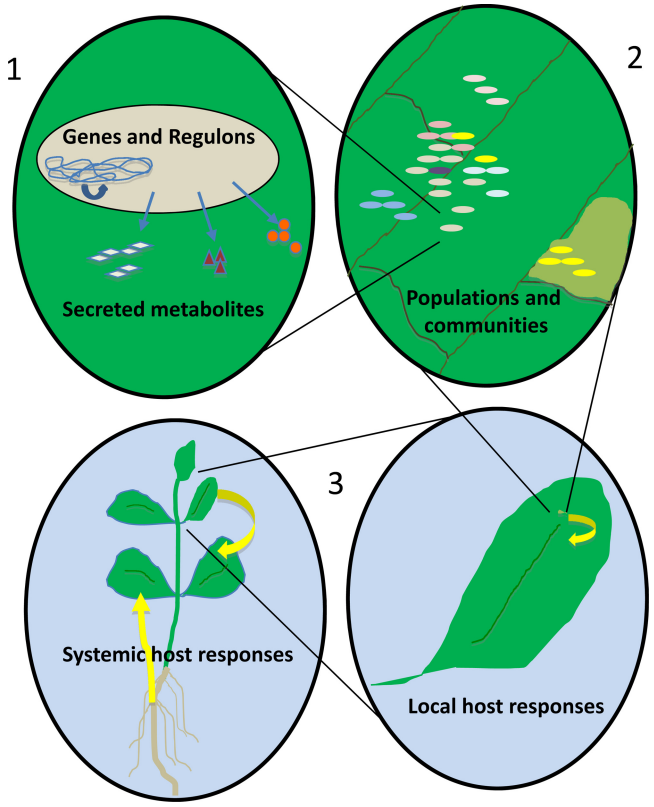

FIG. 1. The multifactorial nature of biological control of plant diseases. (1) Cellular expression of biocontrol-related genes and secretion of bioactive metabolites. Production of bioactive metabolites occurs within the complex network of cellular metabolism, which is influenced by internal regulons as well as external signals. The diversity of these genes, metabolites, and networks has not been fully elucidated. (2) Distribution and dynamics of plant-associated microbial populations with biocontrol capacities. Subsets of these populations may act to alter plant health status across a complex mosaic of aggregates and biofilms found on all host tissues. Such assemblages may contain biocontrol (beige ovals) and/or pathogenic bacteria (yellow ovals), but the distribution, diversity, and dynamics of such populations are not fully characterized. (3) Host responses to microbial activities integrated through the symplast at the cell, organ, and whole-plant levels. The stimulus from multiple contact points between plant hosts and diverse microbial epiphytes and endophytes certainly occurs and can affect the partitioning of nutrients and the development of phenotypes indicating plant health status. However, how such multiple signals are processed and integrated locally and systemically remains a mystery.

Chryseobacterium agonists, whose populations all changed in response to pathogen infection (47). That multiple populations of biocontrol bacteria can contribute additively and sometimes interfere with one another when applied in mixtures (18, 38) further supports the conclusion that multiple bacterial populations can and do contribute to biocontrol *in situ*.

PHYTOHORMONE PRODUCTION AND CATABOLISM BY PLANT-ASSOCIATED BACTERIA

The production of plant hormones is a characteristic of many plant-associated microorganisms. For all five classical phytohormones, i.e., auxin, ethylene (ET), abscisic acid, cytokinin, and gibberellin, synthesis as a secondary metabolite has been demonstrated for at least one bacterial and/or fungal species (16). Some microorganisms can also produce secondary metabolites that affect phytohormone production in plants (6). Plant hormones govern growth of the plant through spatial and temporal control over cell elongation, division, and differentiation. They also play a key role in a plant's response to biotic and abiotic stresses. Given the capacity to contribute to their host's hormone pool, plant-associated microorganisms have the potential to manipulate plant physiology and steer it toward outcomes that favor their own survival.

Probably the best-known hormone-related activity among plant-associated microorganisms is the production of the auxin indole acetic acid (IAA). Plant-associated IAA producers include many species of bacteria, as well as fungi and yeasts (12). Some are plant pathogens and others are beneficials; however, for many no effect on plant health has been demonstrated. The microbial genes underlying IAA production are well characterized, as are the various biosynthetic pathways (66). The amounts of IAA produced *in vitro* by bacteria and other microorganisms can be quite substantial, with concentrations secreted into the medium in the nano- to millimolar range. Only for a few microbial IAA producers has *in vivo* production of IAA been demonstrated to impact bacterial survival and/or plant growth. For example, wild-type *Erwinia herbicola* 299R outcompeted an isogenic *ipdC* mutant, which is unable to produce IAA, on bean leaf surfaces, while it also was shown to stimulate radish root elongation more than the same mutant (5). Similarly, in a canola seedling assay, wild-type *Pseudomonas putida* GR12-2 caused primary roots to be up to 50% longer than an IAA-deficient mutant (53). These results demonstrate that the ability to produce IAA can affect both bacterial survival and plant growth, and future studies will need to reveal whether there is a correlation between the two.

Plants can harbor high numbers of IAA-producing microbes on their above- and below-ground parts (70, 71). For example, up to 60% of all bacteria that were cultured from leaves of field-grown lettuce were capable of IAA production *in vitro* (J. Leveau, unpublished data). Identification by 16S rRNA gene sequencing of a subset of these lettuce isolates revealed that they belong to genera such as *Erwinia*, *Pantoea*, and *Pseudomonas*, which were among the most abundant on these leaves (Leveau, unpublished). This high proportion of bacterial IAA producers suggests that IAA synthesis might be a property that is selected for, in the sense that it contributes to survival in the plant environment. Some data exist in support of this claim, e.g., IAA mutants of *Erwinia herbicola* (5, 41) and *Pseudomonas savastanoi* (66) showed reduced population sizes on plant leaf surfaces. The mechanism by which IAA production aids in bacterial survival remains unclear. It may be that some bacteria can trick the plant into redirecting the flow of nutrients (including photosynthates such as sucrose, fructose, and glucose) toward the bacterial site of colonization. Another proposed mechanism by which IAA production aids bacterial survival is the IAA-induced release of saccharides from the plant cell wall (37).

It was suggested recently that microbially produced IAA not only serves to manipulate host physiology but also acts as a bacterial signal (66). Most remarkable in this context is the stimulation by IAA of its own synthesis in plant growth-promoting *Azospirillum* species, analogous to a quorum sensing

(QS) or autoactivation mechanism. This hypothesis puts the spotlight on another set of plant-associated bacteria, namely, those that can actively destroy IAA. Such bacteria also can be quite common on plant surfaces (35). Some, like *Pseudomonas putida* strain 1290, can use IAA as a sole source of carbon, nitrogen, and energy. Others partially degrade IAA but would be equally able to disrupt IAA-based signaling. Interestingly, some degradation products of IAA are in turn signal molecules (16). For example, salicylate is a plant hormone involved in the plant response to pathogens, and isatin has a demonstrated signaling function in bacterial biofilm formation; yet, both can be derived from the degradation of IAA.

Further research into the role of microbial IAA as a manipulative and/or signaling molecule will likely benefit from tools that allow us to demonstrate and quantify the availability of IAA *in vivo* and at scales that are most relevant to microbes. For this, bioreporter technology (34) holds considerable promise. Because of the known responsiveness of certain gene promoters to chemical or other stimuli, the specificities of such promoters allow bacteria (or other microorganisms) to report on the exposure to a specific stimulus through the synthesis of an easily detectable reporter protein, such as green fluorescent protein (GFP). An exciting source for promoters that respond to IAA is those bacteria that carry the newly identified IAA catabolism (*iac*) genes (33). In *Pseudomonas putida* strain 1290, these genes code for the destruction of IAA and are induced specifically in response to IAA, making them excellent candidates for bioreporter purposes. It is expected that bioreporters such as these, in parallel with plant-based reporter constructs for IAA, will be important assets in the gathering of information on the spatial and temporal variation of IAA availability in plant environments, on the ecology of plant-associated microorganisms that produce and degrade IAA, and on the direct and indirect impact of these microbial interactions on plant health.

PRODUCTION AND EFFECTS OF BACTERIAL VOLATILES

Analogous to the role of volatiles in insect resistance (23), volatile organic compounds (VOCs) play a significant role in the induction of plant host resistance. Ryu et al. (63, 64) observed that both *Bacillus subtilis* strain GB03 and *Bacillus amyloliquefaciens* strain IN937a produced volatiles that significantly reduced the severity of disease on *Arabidopsis thaliana* caused by *Pectobacterium carotovorum* subsp. *carotovorum* (formerly known as *Erwinia carotovora* subsp. *carotovora*). Further study of these VOCs identified 2,3-butanediol and 3-hydroxy-2-butanone (also referred to as acetoin) as elicitors of induced systemic resistance (ISR) in *A. thaliana* against *P. carotovorum* subsp. *carotovorum*. Bacterial VOCs were shown to elicit ISR in several *Arabidopsis* mutants, including a jasmonic acid-insensitive line (*coi1* mutant), an ethylene-insensitive line (*ein2* mutant), a salicylic acid (SA)-degrading transgenic line (*nahG* mutant), and a nonexpressor of PR proteins (*npr1* mutant). VOCs from strain IN937a elicited ISR on all of these lines, whereas VOCs from strain GB03 failed to elicit ISR on *ein2* mutant plants. The importance of the ethylene-dependent signaling pathway for elicitation of ISR by VOCs from strain GB03 was confirmed in tests using GUS fusions to the PDF1.2 gene, which is an indicator for the ethylene response. Recently, the relationship between ethylene signaling and bacterial VOC-elicited ISR was further substantiated by proteomic analysis that demonstrated significant upregulation of three major ethylene biosynthesis proteins (i.e., aspartate aminotransferase, *S*-adenosylmethionine synthetase 2, and methionine adenosyltransferase 3) following bacterial VOC treatment (31).

At low doses, the biological activity of 2,3-butanediol in *Arabidopsis thaliana* is to activate ISR. The priming activity of 2,3-butanediol to reduce a plant's susceptibility to disease was confirmed using *Bacillus* sp. strains that were altered to no longer produce this VOC (63). In a separate study, Han and colleagues reported that the application of 2,3-butanediol did not increase ISR against *Pseudomonas syringae* pv. *tabaci* but did induce ISR against *P. carotovorum* subsp. *carotovorum* (21). These results were consistent with previous work showing induction of an ethylene (ET)-responsive PDF1.2 gene by bacterial VOC exposure. An ET-dependent plant defense signaling pathway could be more effective against a necrotrophic pathogen like *P. carotovorum* subsp. *carotovorum* than *P. syringae*, which requires a salicylic acid (SA)-dependent resistance response (58). In addition, 2,3-butanediol and acetoin produced by *Bacillus subtilis* strain FB17 can induce ISR against *P. syringae* pv. *tomato* DC3000 (62). Further analysis of *Arabidopsis* mutants and transcriptional profiles suggests that acetoin-elicited ISR is dependent on SA and ET signaling pathways. Collectively, bacterial production of two major volatiles, i.e., 2,3-butanediol and acetoin, orchestrated SA and ET signaling to protect plants against two different types of pathogens, i.e., necrotrophs and biotrophs.

Importantly, VOCs, such as those shown above to contribute to biological control, are actually produced by diverse soil bacteria. In fact, most species of *Proteobacteria* and *Firmicutes* groups produce 2,3-butanediol and acetoin under low oxygen concentrations to provide an alternative electron sink for the regeneration of NAD^+ when aerobic respiration is limited (75). The comprehensive chemical profile of bacterial volatiles indicate that a mixture of more than 30 different compounds was emitted from culture of *Bacillu*s spp., based on headspace solid phase microextraction coupled with software extraction of overlapping GC-separated components (15). Further investigation will be needed to see what other, and potentially more effective, ISR triggering molecules, besides 2,3-butanediol and acetoin, can be found in this mixture.

Recent studies have shown that different plant growth-promoting rhizobacteria (PGPR) can elicit so-called "induced systemic tolerance" to abiotic stresses, such as salt and drought (76) and acid soil stress (59). What role might volatiles play in these phenomena related to plant health? Two recent studies demonstrated that bacterial volatiles conferred resistance to salt and drought (11, 77). Despite their volatile nature, such molecules may be applied practically, because many VOCs, such as 2,3-butanediol, are water soluble, inexpensive (<$1/kg), work at extremely low concentrations (ng/ml), and appear to be safe to animals and humans. Under growth chamber conditions, direct application of acetoin on roots showed a significant reduction of pathogen growth at 96 h after challenge (62). Furthermore, drench application of bacterial volatiles to pepper roots increased ISR under field conditions

(C.-M. Ryu, unpublished data). Taken together, these data suggest that bacterial VOCs are good candidates for improving disease control through enhanced management of induced disease resistance.

MULTIPLE ROLES FOR BACTERIAL PHENAZINES

Phenazines, a diverse class of heterocyclic secondary metabolites, have been studied for many years due to their antibiotic properties and role in virulence (55). However, the complexity of the roles phenazines play in the lifestyle and behavior of the producing organism is only now beginning to be recognized. Phenazines are produced by a wide diversity of *Eubacteria* and some *Archaea* (44). Both pathogenic and beneficial bacteria produce phenazines. For example, *Pseudomonas aeruginosa*, a soil inhabitant and opportunistic pathogen, produces several phenazines, including pyocyanin. Pyocyanin production is correlated with mortality in immunocompromised patients. In contrast, *Pseudomonas fluorescens* and *Pseudomonas chlororaphis* are examples of beneficial phenazine producers, and production of phenazines by these root-colonizing bacteria is responsible for fungal disease suppression on plants (56, 68).

Phenazine biosynthesis is highly conserved among microorganisms. Bioinformatic comparison of phenazine biosynthetic genes among a subset of known producers demonstrated a high degree of conservation in five clustered genes that are considered the core phenazine biosynthetic genes. The conservation across taxa suggests that many bacteria acquired these genes via horizontal gene transfer (44). Although some bacteria produce one phenazine derivative, the majority produce more than one. The differences among natural phenazines are due to one or more accessory genes adjacent to the core genes that encode different terminal-modifying enzymes that alter the substituent added to the basic phenazine structure (43). From an evolutionary perspective, it is interesting to speculate that these single terminal-modifying genes, responsible for the production of different phenazine structures, may play significant roles in determining the success of the phenazine-producing bacterium in diverse ecological niches.

P. chlororaphis strain 30-84, a beneficial root colonizer effective against take-all disease of wheat, produces two major phenazines in different amounts, a yellow phenazine-1-carboxylic acid (PCA) (~90%) and an orange 2-hydroxy-phenazine-1-carboxylic acid (2OHPCA) (ca. 10%) (56). The gene *phzO*, which encodes a monooxygenase, is responsible for the conversion of PCA into 2OHPCA (13). Phenazine production is required for both the persistence of strain 30-84 on plant roots in natural soil that contains other microorganisms and suppression of pathogen growth (46, 56). Phenazine biosynthesis in strain 30-84 is regulated at multiple levels, including PhzR/PhzI QS (57, 74) and GacS/GacA two-component regulation (8). QS mutants of strain 30-84 were defective in biofilm formation, and as expected, it was intriguing to note that a phenazine-specific structural mutant (*phzB* mutant) was equally defective in biofilm formation (40). Complementation of the phenazine mutant restored biofilm formation, demonstrating a role beyond antibiosis for these compounds.

The roles of PCA and 2OHPCA on pathogen inhibition and biofilm development by strain 30-84 were examined by changing the ratio of PCA/2OHPCA. Derivatives of strain 30-84 were constructed in which *phzO* was inactivated or in which additional copies of *phzO* were introduced in *trans* (39). These mutants produced altered ratios of PCA versus 2OHPCA while producing similar total amounts of phenazine. The PCA-only producer was less effective in inhibiting mycelial growth of the pathogen than the wild type or the 2OHPCA overproducer, demonstrating the importance of 2OHPCA production for pathogen inhibition. Compared to the wild type or the PCA-only producer, the 2OHPCA overproducer adhered more quickly to glass surfaces (44% total coverage compared to 1% after 45 min), formed thicker biofilms than the wild type, and had a low dispersal rate. In contrast, the PCA-only producer had a thicker biofilm with a 4-fold-higher biovolume of cells than either the wild type or the 2OHPCA overproducer. These results suggest that 2OHPCA facilitates cellular adhesion, whereas PCA facilitates biofilm growth. These results are consistent with the hypothesis that bacteria produce different phenazine structural derivatives in specific concentrations due to the different roles they serve for the population.

REGULATION OF SECONDARY METABOLISM AND BIOCONTROL

The GacS/GacA two-component system in biological control pseudomonads has a central role in regulating the expression of biocontrol factors in several pseudomonads. Unknown signals trigger activation of the sensor kinase GacS, and then the response regulator GacA is activated by phosphorelay. The activated GacA positively controls the transcription of regulatory small RNA when cells reach high population densities. These small RNAs bind to discrete proteins, RsmA and RsmE, relieving translational repression from specified genes (32). Microarray analysis with *P. aeruginosa* reveals 241 genes to be regulated by the GacS/GacA system, with near-complete overlap with genes controlled by the regulatory small RNA (7). Recent studies in *P. aeruginosa*, *P. syringae*, and *P. fluorescens* CHA0 indicate that upstream of the GacS/GacA hybrid sensors, RetS inhibits and LadS activates the Gac/Rsm pathway by direct interaction with the GacS sensor kinase (25, 60, 72). Even though many intermediates in the signaling pathways of the GacS/GacA system are identified, the processes are not fully elucidated, especially with respect to variability between isolates.

A nonpathogenic aggressive root colonizer, *Pseudomonas chlororaphis* strain O6, also illustrates the regulatory power of the GacS/GacA signaling pathway. Strain O6 produces several secondary metabolites, including phenazines, pyrrolnitrin, hydrogen cyanide, and siderophore, as beneficial traits. Root colonization by *P. chlororaphis* strain O6 induces systemic resistance against various plant pathogens (67) and abiotic stresses of drought and salinity (11). Recent proteome and mutational analyses of the GacS/GacA system in strain O6 demonstrated that the GacS/GacA system plays key roles in beneficial trait regulation as well as in general metabolism and control of secretion (Y. C. Kim, unpublished data). The GacS/GacA system positively regulated production of the intra- and intercellular signaling compounds, acyl homoserine lactones (AHSL) (67); production of antimicrobial compounds, including phenazines, hydrogen cyanide, and pyrrolnitrin (28); induction of induced systemic resistance (67), through positive reg-

ulation of the volatile component 2,3-butanediol (21); and RpoS expression and, thus, other genes dependent on this alternative sigma factor (29). Additional functional analysis showed that GacS in *P. chlororaphis* strain O6 is involved in production of biofilms, although this isolate possesses plasticity to form biofilms in a GacS-AHSL-independent manner (2, 24). Interestingly, synthesis of another inducer of systemic resistance in plants, 4-carbamoyl acetic acid, is not under GacS control (52), and production of indole acetic acid is negatively regulated by GacS (27).

Proteomic analyses of the function of regulator mutants have been used to discover which pathways are coregulated. Recently, analyses of GacS mutants identified novel proteins with as yet largely undetermined roles in biological control, including a serine protease (PspB) connected with phase variation and aggregation, two proteins involved in DNA repair (a putative single-strand binding protein, Ssb, and a recombination-associated protein, RdgC), an isoprenoid biosynthesis protein, GATase1_ES1, and glucose-1-phosphate thymidylyltransferase, RmlA, possibly involved in polyketide biosynthesis, cell surface structures, pilus assembly protein (CpaC and peptidoglycan-binding LysM), and outer membrane protein (OprF) (51) (Kim, unpublished). These results point to the complexity of regulons related to the expression of biological control phenotypes within a single agent. And in pseudomonads as well as other bacteria, other global regulators are likely to be present and relevant. For example, a *clp* gene homolog has been shown to be an important regulator of multiple biocontrol phenotypes in *Lysobacter enzymogenes* (30).

INTERSECTION OF SECONDARY METABOLISM, GLOBAL REGULATION, AND THE ECOLOGY OF BIOCONTROL BACTERIA

In Gram-negative bacteria, secondary metabolite production usually is controlled by two-component regulatory systems such as GacS/GacA (34). It was observed that secondary metabolite production is unstable during *in vitro* growth in rich media, with secondary metabolite mutants becoming the majority population, and such spontaneous mutants can also appear on plant roots (9). Previous work demonstrated that these phenotypic variants had mutations in either the *gacS* or *gacA* gene, encoding a two-component sensor kinase or response regulator, respectively, that controls the production of multiple secondary metabolites, including phenazines, proteases, and hydrogen cyanide (8). Gac mutants may be considered "ecological cheaters" because they displace the wild type in nutrient-rich medium. However, in the rhizosphere in natural soil, the outcome of the interaction is very different, with the wild type benefiting from the presence of the Gac mutant. For example, when starting from 50:50 mixtures with Gac mutants, the final rhizosphere populations of wild-type strain 30-84 were significantly greater than populations of the wild type starting from a 100:0 treatment despite being introduced as only half the inoculum in mixture (9).

Recent work in the L. S. Pierson lab has compared the relative fitness of wild-type strain 30-84 and mutants defective in *gacA* or *phzB* (a phenazine-specific mutation) in mixed biofilms using a replacement series containing specific proportions of wild-type strain 30-84 and either the *gacA* mutant or the *phzB* mutant (percentages of 100:0, 50:50, and 0:100, respectively, keeping the total inoculation density constant). As predicted, in the absence of phenazine production, neither mutant alone formed biofilms. However, in the 50:50 mixture of wild-type and 30-84 GacA, the biovolume of 30-84 GacA was as high or higher than the biovolume of the wild-type GacA in pure culture. The combined biovolume of both strains in the mixed population was 3-fold higher than the biovolume of the wild type alone, indicating that both strains benefited by the presence of the mixed community. In contrast, in the 50:50 mixture of the wild type and the *phzB* mutant, the *phzB* mutant competitively displaced 30-84 (W. W. Driscoll and E. Pierson, unpublished data). These data support previous findings that better survival of wild-type populations in mixtures with Gac mutants may benefit the survival of the wild type in the rhizosphere, and thus the Gac mutant phenotype may be selectively maintained, whereas phenazine-specific mutants do not benefit the population and therefore should not be maintained as phenotypic variants. Previously, van den Broek et al. (69) proposed that spontaneous *gac* mutations, including reversible switching between functional and nonfunctional Gac phenotypes, may be a conserved strategy among rhizosphere pseudomonads, which improves their success in the "heterogeneous and challenging environment of the rhizosphere." Thus, it seems likely that substantial variation can occur, even among populations of active biocontrol strains.

EXPERIMENTAL PATHS TOWARD A GREATER UNDERSTANDING OF HOW SECONDARY METABOLISM AFFECTS BIOLOGICAL CONTROL

The studies described above show that individual secondary metabolites can play multiple roles in the ecology of plant-associated bacteria. And because the production of these metabolites is under complex and hierarchical regulatory control, the production and effects of such metabolites need to be considered in light of their pleiotropic activities. Given the complexity of bacterial genomes, their secretomes, and the communities of organisms to which they belong, it is a daunting task to make sense of the phenomenon known as the biological control of plant pathogens. Nonetheless, newer tools and analytical approaches are providing fruitful resources for tackling the fundamental questions of biocontrol (i.e., which populations matter? When do they matter? What do they do? And to what extent can we control their activities?). To better answer those questions and fuel the success of a growing biopesticide industry, future research in biocontrol will need to progress along three main avenues (Fig. 1). These will focus on the biocontrol agents themselves and examine more carefully the complex biotic and abiotic environments within which biological control occurs.

The first avenue of research will entail the identification and characterization of secondary metabolites and genes that contribute to biological control, along with the regulatory networks that determine their expression. It has long been known that more than one mechanism can contribute to biocontrol activity expressed by a single organism, but the full repertoire of biocontrol-related genes expressed (and metabolites se-

creted) by a single strain has yet to be fully investigated. This, however, is about to change. Since the first reported sequencing of biocontrol bacterial genomes just a few years ago (10, 54), the technology to rapidly sequence, annotate, and mutate bacterial and fungal genomes has become widespread. Can a combination of "omic" and mutant analyses lead to the discovery of the full suite of biocontrol genes and products presented by a single agent? Can distinct or integrated regulons of these genes be defined? Current research in a number of laboratories is focusing on characterizing and substantiating the importance of various secondary metabolites (e.g., phytohormones, bacterial volatiles, lytic enzymes, polyketides, and nonribosomal lipopeptides) as well as their associated transporters and regulators. But many other genes are also likely to be important. For example, Mavrodi et al. (45) found that three genes related to pathogenicity in some pseudomonads were important for the superior root colonization of a biocontrol strain. To be sure, much work needs to be done in this area, because there are hundreds of genes per genome that are good candidates for mutational studies based on what we already know about biocontrol. But there are also hundreds more unannotated genes, many of which can be found in operon-like structures. These, too, will need to be investigated for their primary and potential pleiotropic effects on metabolism of bacteria and their plant hosts. And though much of this work is being done in biocontrol pseudomonads, where genetic tools are well developed (20), more work in other plant-associated bacterial genera (e.g., *Bacillus*, *Burkholderia*, *Lysobacter*, *Mitsuaria*, and *Streptomyces*) is warranted because of the potential to discover completely new genes and chemistries with relevance to biological control.

The second avenue for progress will be to more fully identify and characterize the diverse microorganisms that affect plant health, both positively and negatively. Historically, this has been done through random phenotypic screens of culture collections (18) and by the profiling of enrichment cultures and/or culture collections for classes of genes already known to be involved in biocontrol, as has been done for some *Pseudomonas* spp. and *Bacillus subtilis* (26, 49). However, more rapid progress has been made recently using culture-independent profiling of soil- and plant-associated microbial communities (4). For instance, community profiling of terminal restriction fragment polymorphisms of 16S rRNA genes has led to the identification and marker-assisted recovery of completely novel bacterial genera and species not previously associated with biological control (3). Recent work has shown that a broad array of bacterial populations may contribute to soilborne disease suppression in take-all decline soils (65), a natural form of suppression once thought to be largely due to the specific suppression of just a few taxa (72). And a tremendous amount of uncultured diversity of *phlD*$^+$ pseudomonads (i.e., a well-studied group of biocontrol bacteria that produce 2,4-diacetylphloroglucinol [72]) have been noted to occur in suppressive and conducive soils and may contribute to disease suppression on a single plant (17). Such diversity needs to be more thoroughly cultured, catalogued, and characterized to determine the extent to which each might contribute to plant health in various contexts. There is much interest currently in metagenomics (36); however, the high degree of sample-to-sample variation, our limited knowledge of bacterial diversity, and relatively high per-sample cost make the approach less attractive than the ribosomal gene-based approaches for now. But there is no reason a metagenomics-based comparison could not be used today to generate candidate markers useful for directing the isolation of novel bacteria as has been done before (3). Can such approaches greatly expand the number of useful biocontrol agents? Will they lead to the discovery of novel strains with new secondary metabolites? Because the breadth of possible pathosystems is so diverse, it seems likely that such work will continue for as long as there is a demand for novel and more effective microbial (and biochemical) biopesticides.

Built on this foundation, and central to progress in understanding the true nature of biocontrol, the third avenue of research will be aimed at understanding the integration of metabolism of biocontrol-related genes in field-relevant multitrophic systems (i.e., with host, pathogen, and biocontrol agents). Early studies of this sort have revealed that in a single species, some biocontrol genes matter more than others for control of a particular pathogen (14), that different strains of biocontrol bacteria can affect the expression of each other's biocontrol genes (42), and that changes in abiotic soil properties alter biocontrol performance (50). Can evidence of the importance of one gene or regulon in one context be extrapolated to other contexts? Will more such studies reduce or expand the number of genes and regulons considered to be important for biocontrol in a particular agent? We think that future studies will not only have to examine "snapshots" of metabolism at particular time points, but they will also need to begin to characterize the shifts of metabolism that occur during the dynamic processes of colonization, infection, and host ontogeny. And such studies will need to be considered in the context of daily and seasonal cycles and variation in key abiotic variables as well. This may be achieved by more intensive sampling and throughput (again justifying the low-cost-per-sample profiling methods, such as terminal restriction fragment length polymorphism analyses) or by the development of *in situ* monitoring based on reporter gene technology (34).

While this review has focused exclusively on plant-bacterium interactions, it is clear that the approaches described above apply equally as well to studies of plant-associated fungi that also promote plant health. In parallel to the work on beneficial bacteria, similar work is likely to proceed in the field of fungal biocontrol, where tremendous inroads have already been made in the study of *Trichoderma* spp. (1, 22). But the genetic tractability and relative simplicity of bacterial genomes will more rapidly provide leads essential to the development of a new synthesis and understanding of plant-microorganism interactions. These types of studies will further bridge microbial genetics and ecology and, by doing so, extend our ability to manage plant health.

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