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Potential Role of Pathogen Signaling in Multitrophic Plant-Microbe Interactions Involved in Disease Protection

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Multitrophic interactions mediate the ability of fungal pathogens to cause plant disease and the ability of bacterial antagonists to suppress disease. Antibiotic production by antagonists, which contributes to disease suppression, is known to be modulated by abiotic and host plant environmental conditions. Here, we demonstrate that a pathogen metabolite functions as a negative signal for bacterial antibiotic biosynthesis, which can determine the relative importance of biological control mechanisms available to antagonists and which may also influence fungus-bacterium ecological interactions. We found that production of the polyketide antibiotic 2,4-diacetylphloroglucinol (DAPG) was the primary biocontrol mechanism of *Pseudomonas fluorescens* strain Q2-87 against *Fusarium oxysporum* f. sp. *radicis-lycopersici* on the tomato as determined with mutational analysis. In contrast, DAPG was not important for the less-disease-suppressive strain CHA0. This was explained by differential sensitivity of the bacteria to fusaric acid, a pathogen phyto- and mycotoxin that specifically blocked DAPG biosynthesis in strain CHA0 but not in strain Q2-87. In CHA0, hydrogen cyanide, a biocide not repressed by fusaric acid, played a more important role in disease suppression.

Molecular and biochemical analysis has demonstrated that, for many beneficial bacteria applied in agriculture as biological control agents to suppress plant diseases, production of various antimicrobial compounds is the primary mechanism of action (21, 26, 60). The polyketide antibiotic 2,4-diacetylphloroglucinol (DAPG) has received particular attention because it plays a key role in the ability of introduced *Pseudomonas fluorescens* strains to suppress a broad spectrum of crop diseases (29). Indigenous DAPG-producing populations have been identified as the driving force behind development of natural disease suppressiveness in certain soils under long-term monoculture (47). Conservation of *phl* genes for biosynthesis of DAPG among ecologically and geographically diverse antagonistic pseudomonads further supports the global importance of DAPG production in biocontrol (31, 66).

All DAPG producers so far described carry *hcn* genes for biosynthesis of the broad-spectrum biocide hydrogen cyanide (HCN), indicating an evolutionary linkage of the two metabolites (31, 69). Production and pathway-specific regulation of each compound, however, appear to be independent of those of the other (5, 6). Biosynthesis of antimicrobial compounds in *Pseudomonas* biocontrol strains is closely regulated by molecules produced by the organism itself (44, 52) and by external environmental factors, including nutritional components, soil chemical and physical properties (17, 19, 55, 56), host plant genotype (38, 43, 57), and nonpathogenic soil bacteria (45). Because these factors can determine the ability of particular strains to suppress disease, identifying them would facilitate the targeted application of strains into environments that are more favorable for effective and consistent biocontrol activity.

In biocontrol research, the focus has almost exclusively been placed on understanding how bacterial antagonists impact fungal pathogen survival and disease-causing activity. Despite the very close interaction between these bacteria and fungi, surprisingly little attention has been given to the potential impact of pathogens on biocontrol agents and their disease-suppressive activity. The best evidence to date supporting such an impact is (i) that root pathogens influence plant colonization by antagonistic pseudomonads (4, 40) and (ii) that tolerance by pathogens of particular antimicrobial metabolites diminishes the efficacy of biocontrol strains producing these compounds (12, 41). Here we report an example of signaling between a pathogen and a biocontrol agent involving genes important in disease suppression. Using promoterless *lacZ* fusions to the *phlA* and *hcnA* biosynthetic genes, we found that the phyto- and mycotoxin fusaric acid (32), produced by the tomato crown and root rot pathogen, *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *radicis-lycopersici* Jarvis and Shoemaker, blocked biosynthesis of DAPG but not HCN in the biocontrol agent *P. fluorescens* CHA0 but had no major effect on DAPG production in Q2-87, another biocontrol strain of *P. fluorescens*. Mutational analysis confirmed that DAPG production was key to the biocontrol activity of strain Q2-87 but that it was not important for the fusaric acid-sensitive strain CHA0, which relied more on HCN production for biocontrol of this disease.

Microorganisms. *F. oxysporum* f. sp. *radicis-lycopersici* strain 22 was isolated from tomato plants with crown and root rot disease and was obtained from C. Alabouvette, Institut National de la Recherche Agronomique, Dijon, France. The pathogen was routinely cultured on 2% malt extract (ME) agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) at

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TABLE 1. *P. fluorescens* strains and plasmids used in this study

Strain or plasmid	Genotype and relevant characteristics[a]	Reference
Strains		
CHA0	Wild type from Morens, Switzerland; DAPG$^+$ HCN$^+$	63
CHA5	*hcnB*::Ω-Hg insertion derivative of CHA0; Hg^r DAPG$^+$ HCN$^-$	64
CHA207	Chromosomal *hcnA'-'lacZ* fusion of CHA0; DAPG$^+$ HCN$^-$	7
CHA630	*phlH*::Tn5 single-chromosomal-insertion derivative of CHA0; Km^r DAPG$^-$ HCN$^+$	52
CHA631	Δ*phlA* in-frame-chromosomal-deletion derivative of CHA0; DAPG$^-$ HCN$^+$	52
Q2-87	Wild type from Washington State; DAPG$^+$ HCN$^-$	62
Q2-87::Tn5-1	*phlD*::Tn5-1 chromosomal-insertion derivative of Q2-87; Km^r DAPG$^-$ HCN$^-$	3
Plasmids		
pME3013	pVK100 with an 8-kb genomic fragment of CHA0 containing functional *hcnABC* genes conferring HCN biosynthesis; Tc^r	64
pME6259	pACYC177-pVS1 carrying a *lacZ* translational fusion to the *phlA* of CHA0; Tc^r	52
pME6261	pACYC177-pVS1 with a 2-kb fragment of CHA0 containing a functional *phlA* of CHA0 conferring DAPG biosynthesis; Tc^r	52
pMON5118	pCP13/B with a 35-kb genomic fragment of Q2-87 containing the DAPG biosynthetic locus conferring DAPG biosynthesis; Tc^r	62

[a] +, positive for production; −, no production; Hg^r, mecury resistant (20 μg ml^{-1}); Km^r, kanamycin resistant (25 μg ml^{-1}); Tc^r, tetracycline resistant (125 μg ml^{-1} for *P. fluorescens*).

24°C and was stored as mycelia and conidia in ME broth with 40% glycerol at −80°C. *P. fluorescens* strains and plasmids used in this study are described in Table 1. *P. fluorescens* was routinely grown on King's B medium (KB) (34) at 27°C and was stored in KB broth with 40% glycerol at −80°C. *Escherichia coli* was grown in Luria-Bertani broth at 37°C (50). Plasmids were mobilized from *E. coli* to *P. fluorescens* by electroporation or by triparental matings as previously described (52).

Effect of purified DAPG on pathogen growth. The susceptibility of *F. oxysporum* f. sp. *radicis-lycopersici* to DAPG was tested on ME agar (pH 7.0) amended at concentrations of 0 to 300 μg ml^{-1}. Agar plugs (4 mm in diameter) taken from 7- to 10-day-old cultures were placed inverted in the center of test plates. Hyphal radial growth was measured after 7 days of incubation at 24°C in darkness. The experiment with each treatment concentration consisted of three replicate plates and was conducted twice.

Hydroponic assay to determine the role of DAPG and HCN in biocontrol. A soilless noncirculating hydroponic assay that simulates tomato production systems widely used worldwide (18) was used to compare the biocontrol efficacies of mutants with those of wild-type parent strains CHA0 and Q2-87. Pregerminated tomato seeds (*Lycopersicum esculentum* Mill.) were placed in divets of rock wool cubes (3.5 cm^2 by 4 cm deep, one seed per cube; Grodania A/S, Hedehusene, Denmark), with 18 cubes per plastic tray (23.5 by 28.5 cm in diameter by 5.5 cm deep). Rock wool was saturated with nutrient solution (15) and kept at a level of approximately 1 to 2 cm deep throughout the course of experiments by addition of filter-sterilized deionized water as needed (18). Bacteria (approximately 10^7 CFU ml^{-1}) and *Fusarium* (10^6 microconidia ml^{-1}) were added to 400 ml of nutrient solution before being dispensed over the rock wool. Plants were incubated in growth chambers with 16 h of light at 22°C and 8 h of darkness at 18°C and 70% relative humidity. After 14 to 21 days, seedlings were scored for crown and root rot disease severity on a scale of 0 to 4 where 0 means no symptoms and 4 means that plants are dead or nearly so (42).

Effect of fusaric acid on bacterial gene expression. Strains CHA0 and Q2-87 carrying a *lacZ* reporter gene fusion to the *phlA* gene of CHA0 on plasmid pME6259 were grown in 20 ml of liquid PCG medium (15, 61) in 100-ml Erlenmeyer flasks sealed with cotton plugs. Strain CHA207, carrying the chromosomal *hcnA'-'lacZ* fusion, was grown in OSG minimal medium (52). Culture media were inoculated with 40 μl of exponential-growth-phase cultures of the bacteria diluted to an optical density at 600 nm (OD$_{600}$) of 0.05. Just prior to inoculation with bacteria, a fresh preparation of fusaric acid (5-butylpicolinic acid; molecular weight, 179.2; Sigma-Aldrich Corp., St. Louis, Mo.) dissolved in 10% (vol/vol) methanol was added to give 50 and 500 μM. Controls received the same amount of methanol. Cultures were incubated at 30°C with rotational shaking at 180 rpm. β-Galactosidase-specific activities of at least four independent cultures were determined by the method of Miller (50). After kinetics of *phlA'-'lacZ* reporter gene expression were obtained, acidified cultures (pH 2 with 2 N HCl) were extracted with equal volumes of ethyl acetate and the DAPG and fusaric acid concentrations were quantified by high-pressure liquid chromatography methods as previously described (15, 17). DAPG levels obtained by direct isolation were consistent with those indicated by reporter activity (data not shown), as has previously been reported (52).

Pathogen sensitivity to DAPG. The first objective in examining molecular interactions between a tomato root pathogenic fungus and a biocontrol agent was to determine the inhibitory activity of a bacterial antimicrobial metabolite that has been identified to be primarily responsible for suppression of several other plant diseases (29, 60). We found that *F. oxysporum* f. sp. *radicis-lycopersici* was highly sensitive to pure DAPG in vitro, with considerable growth inhibition at concentrations as low as 25 μg ml^{-1} and nearly complete inhibition at 75 μg ml^{-1} (Fig. 1). This is similar to the toxicity levels that have been observed for other fungi, including other *Fusarium* spp (30). It also demonstrates that in situations when this antibiotic is present it is an effective growth inhibitor of this particular pathogen. The exact mechanism of action of DAPG against *Fusarium* is un-
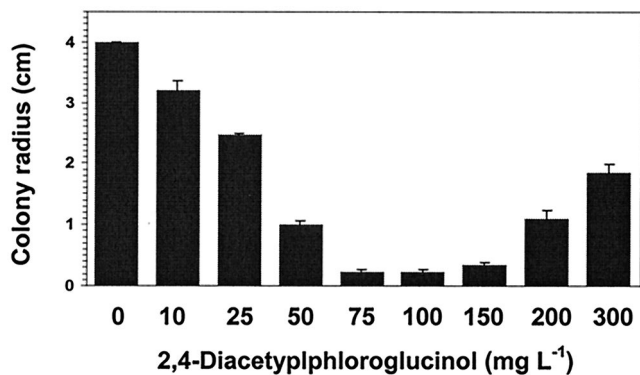
FIG. 1. Inhibition of *F. oxysporum* f. sp. *radicis-lycopersici* by synthetic DAPG. Radial growth of hyphae on 2% ME agar was measured after 7 days of incubation at 27°C. Values (± standard errors [error bars]) represent the means of six replicates in two experiments.

known. A recent report, however, indicates that DAPG at extremely low concentrations (0.4 ng ml^{-1}) lysed zoospores and caused extreme hyphal tip disorganization in phytopathogenic *Pythium ultimum* (12).

Curiously though, at concentrations above 150 μg ml^{-1} we observed that the pathogen was again able to grow. It is unclear if fungal regrowth was from cells that escaped death from DAPG exposure or if the antibiotic had a fungistatic effect on the fungus. "Reversed toxicity" has been observed for certain fungicides when these are tested at concentrations exceeding those necessary for initial inhibition of fungal growth. A possible explanation for this is that *Fusarium* metabolizes DAPG, as it has been reported to metabolize related phloroglucinol

compounds (65), and that the required genes are up-regulated only after exposure to high toxin concentrations. If this indeed is the case, it would be important to determine if the up-regulated genes specifically affect fungal responses to DAPG or if the efficacy of other key biocontrol compounds (e.g., pyoluteorin, phenazines, pyrrolnitrin) is also compromised. Another explanation that has recently been identified is active efflux with ABC transporter pumps to protect fungi from phenazine antibiotics produced by biocontrol bacteria (53). The ability of the pathogen to grow after exposure to hypertoxic DAPG concentrations has potentially important implications for the efficacy of biocontrol strains genetically modified to either constitutively produce or to overproduce this antibiotic (11, 51). Whether the ability to withstand high concentrations may result in selection of different fungal resistance mechanisms that confer tolerance to lower but inhibitory antibiotic doses, whether tolerance to DAPG confers cross-resistance to other antifungal bacterial metabolites, and whether these could lead to a breakdown in biocontrol should be investigated.

Role of bacterial metabolites in biocontrol of tomato crown and root rot. We applied classical molecular analysis for determining the role of two bacterial metabolites in biocontrol of *Fusarium* on the tomato. Two independent sets of repeated experiments were performed. A DAPG-deficient strain Q2-87 mutant proved significantly less effective at protecting tomatoes from *Fusarium* attack (Fig. 2). Restoration of DAPG production to this mutant with plasmid-mobilized functional biosynthetic genes almost completely restored biocontrol activity relative to that of the wild-type strain (Fig. 2).

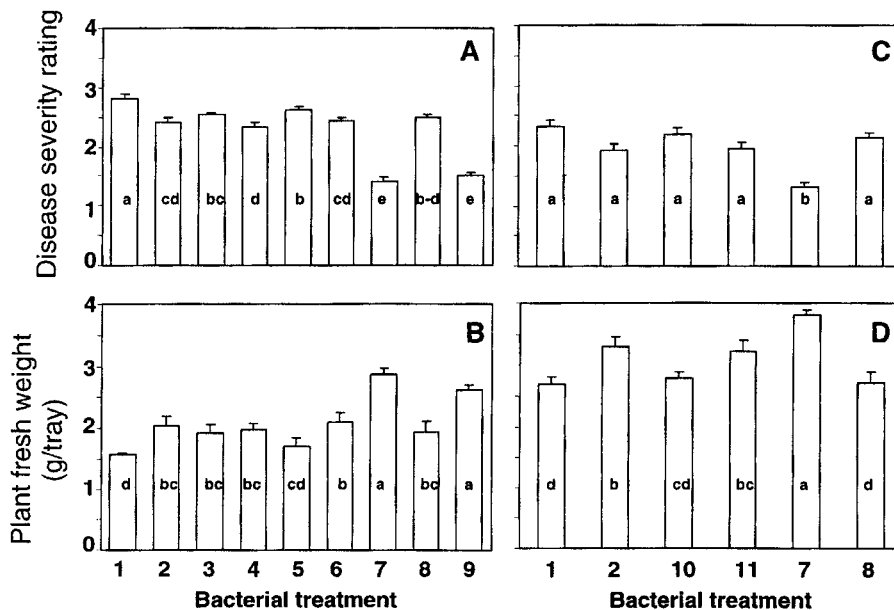Intriguingly, CHA0 mutants deficient for DAPG production

FIG. 2. Biocontrol activity of wild-type *P. fluorescens* mutants defective for DAPG or HCN and mutants restored for DAPG or HCN production. Bacterial treatments were as follows: 1, no bacteria, 2, CHA0; 3, CHA630; 4, CHA630/pMON5118; 5, CHA5; 6, CHA5/pME3013; 7, Q2-87; 8, Q2-87::Tn5-1; 9, Q2-87::Tn5-1/pMON5118; 10, CHA631; 11, CHA631/pME6261. Biocontrol assays were conducted in a noncirculated hydroponic system (15, 18). Two sets of repeated experiments were conducted (A and B and C and D). Values (± standard errors [error bars]) represent the means of 6 (A and B) and 11 (C and D) replicates. Bars within a graph with the same letters are not significantly different according to Fisher's protected least-significant-difference test at a *P* of ≤0.0001.
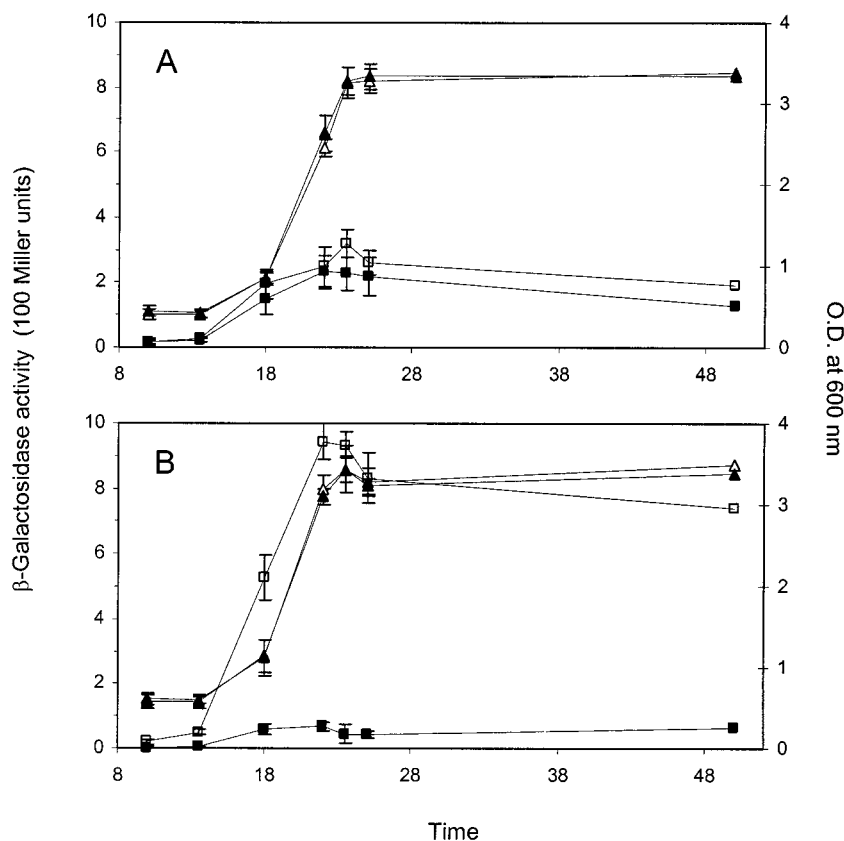
FIG. 3. Kinetics of *phlA'-'lacZ* expression in *P. fluorescens* strains Q2-87 (A) and CHA0 (B) without (□) or with (■) fusaric acid (500 μM) added to PCG liquid culture media at 27°C. OD_{600} values for cultures grown in the absence (△) and presence (▲) of fusaric acid are shown. Values are means ± standard deviations from three experiments. Some of the error bars are too small to be shown.

were not significantly reduced in biocontrol efficacy. This is not to say that the CHA0 mutants were effective biocontrol agents, rather that the already relatively low level of protection provided by wild-type CHA0 was not significantly further reduced by loss of DAPG production (Fig. 2). The lack of a role for DAPG in tomato crown and root rot suppression by CHA0 was confirmed by using two different mutational events (producing CHA630 and CHA631) to abolish production of this compound. However, when HCN production was abolished in the insertion mutant CHA5, biocontrol activity was significantly impaired relative to that of the wild-type parental strain, CHA0 (Fig. 2). Restoration of HCN production with plasmid-mobilized functional *hcnABC* genes in CHA5/pME3013 also restored biocontrol activity (Fig. 2). This demonstrates a role for this biocidal metabolite in tomato crown and root rot biocontrol and is the first example of HCN contributing to biocontrol of *Fusarium* or of a plant disease in hydroponic culture. Biosynthesis of HCN is regulated by the FNR-like transcriptional regulator ANR, which up-regulates the expression of the *hcnABC* genes under oxygen-limited conditions (6, 27), such as those in our noncirculating hydroponic system. However, whereas these conditions may have favored HCN biosynthesis and a greater role of HCN in biocontrol, they did not preclude DAPG production. Under identical conditions, we have isolated DAPG from the rhizosphere of tomato plants inoculated with CHA0 (15).

Influence of pathogen toxin on antagonist gene expression. Fusaric acid completely abolished DAPG biosynthesis in strain CHA0 but had no effect in strain Q2-87, as determined by using a *lacZ* reporter fusion to the biosynthetic gene *phlA* (Fig. 3). Production by CHA0 of monoacetylphloroglucinol, a putative precursor compound (3), was also completely repressed by 50 and 500 μM fusaric acid (data not shown). Extraction of bacterial cultures and high-pressure liquid chromatography analysis found that fusaric acid was not degraded by either strain (data not shown). Fusaric acid also had no effect on HCN biosynthesis in strain CHA0, as determined by using a chromosomal *hcnA'-'lacZ* reporter fusion (Fig. 4). Fusaric acid can be isolated from our hydroponic tomato assays at a concentration of 0.1 μg ml^{-1}, and even this level is sufficient to completely block DAPG production (15). These results support the conclusions drawn from our bioassays and explain why DAPG contributed to biocontrol in Q2-87 but not CHA0 and why HCN contributed to biocontrol in CHA0. Biosynthesis pathways for both metabolites are regulated via GacS and GacA (8). The fact that HCN and, in a previous study, protease (15) were not affected by fusaric acid indicates a target somewhere downstream. Recent evidence points to the DAPG pathway-specific repressor PhlF as a putative target because mutation in the *phlF* gene relieved sensitivity to fusaric acid in CHA0 (52). Both CHA0 and Q2-87 have *phlF* genes, but, as with another DAPG biosynthetic gene, *phlD*, there is diversity
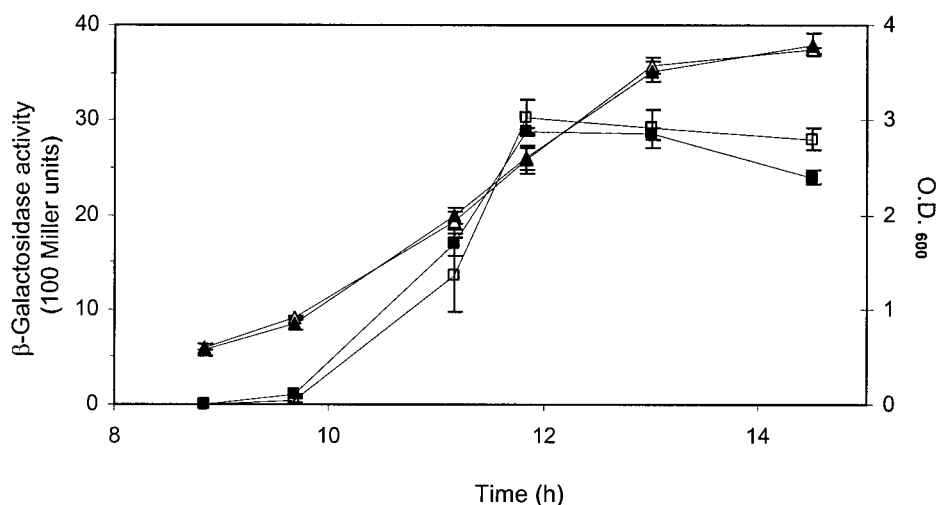
FIG. 4. Effect of fusaric acid on the expression of a chromosomal *hcnA'-'lacZ* fusion in the *P. fluorescens* CHA0 derivative CHA207. Specific β-galactosidase activities were determined for bacteria grown in OSG liquid culture medium at 30°C without (□) or with (■) fusaric acid (500 μM). OD$_{600}$ values for cultures grown in the absence (△) and presence (▲) of fusaric acid are shown. Values are means ± standard deviations from three experiments. Some of the error bars are too small to be shown.

that could explain the differential responses to fusaric acid (48). Interestingly, CHA0 produces metabolites (i.e., pyoluteorin and salicylate) that also regulate DAPG biosynthesis via PhlF, indicating that the pathogen has hijacked a bacterial regulatory cascade for its self-defense purposes (14). The repressive activity of PhlF is thought to be its binding of the inverted repeated sequence phO, located downstream of the *phlA* transcriptional start site (1). Salicylate stabilizes the PhlF-phO complex, and this may also be the case with fusaric acid.

Our study provides evidence for direct signaling between a pathogen and an antagonist. Previous work identifying *Pythium* signals (58) found that these were likely nonspecific signals from lysed hyphae that affected bacterial trehalose genes (25). Genes in a *P. fluorescens* biocontrol strain which were affected by *Pythium* hyphal extracts appeared to contribute to long-term rhizosphere colonizing ability but were not directly involved in pathogen suppression (22). Trehalose is commonly found as a storage compound in most fungi, as well as in many bacteria and plants (23, 25, 35), and it is known to influence diverse interactions between bacteria and fungi that do not result in microbial competition (24, 37).

Implications of pathogen self-defense in biocontrol and microbial ecological interactions. In addition to this being the first report of roles for DAPG or HCN in *Fusarium* suppression of tomato disease, our results have wider implications for the interpretation of studies designed to elucidate biocontrol mechanisms of action. While our study is the first example where a particular mechanism of action is biocontrol strain specific, it may not be the only case. Often, results obtained with one combination of a pathogen and biocontrol agent are generalized to conclude a role or lack thereof for a particular compound in biocontrol of a particular disease. Our results demonstrate the need to examine several strains that may have different regulatory responses to the same compounds. In situations where DAPG has been found to have no role in biocontrol (49) or in ecological fitness (9), other DAPG-producing strains may perform differently, and likewise, in the many

cases where a role has been demonstrated, it cannot be assumed to be true for all DAPG-producing strains.

Our results offer a new explanation for the variation in efficacy of biocontrol agents (10, 60). Selection of strains based on possession of key biocontrol genes (46) is insufficient because strains must not only have the genes but also be able to express these genes in target environments. Previous studies identified nutritional factors, soil properties, and host plant effects on gene expression (17, 43). Here we further confirm that pathogen signals or pathogen self-defense compounds modulate gene expression in biocontrol agents (14). Moreover, in our case both CHA0 and Q2-87 possess DAPG biosynthetic genes but only Q2-87 is able to express these in the presence of a fusaric acid-producing pathogen. Preliminary data testing a collection of 42 *P. fluorescens* strains (16) and the findings presented here suggest that sensitivity to fusaric acid partly explains the variation in biocontrol efficacy observed with DAPG-producing pseudomonads (54). In terms of strain selection, our results provide the framework for screening DAPG-producing pseudomonads for targeted application to control *Fusarium*, with fusaric acid-insensitive strains likely to provide more reliable protection. A new approach may also be to develop strain mixtures by combining sensitive strains with insensitive strains and/or strains that degrade pathogen toxins (28, 59, 61). *Trichoderma viridae* can down-regulate genes for *Fusarium* mycotoxin biosynthesis (68), and mixtures of bacterial and fungal agents have already been found to improve biocontrol (20). Furthermore, selection of antagonists to control mycotoxigenic fungi (33) must consider the potential gene-regulating activity of these compounds.

Targeted inhibition of antagonist genes by a pathogen metabolite adds a new dimension to microbial ecological interactions. This is further evidence of the ecological importance of interspecies and interphylum signaling that is only now being uncovered. Mycotoxins produced by *Fusarium* spp., including fusaric acid, typically have broad-spectrum antibiotic activity affecting bacteria, fungi, nematodes, insects, and mammals (39,

67), and their activity can be synergistic (13). An unexpectedly diverse range of *Fusarium* species have been found to produce fusaric acid and other mycotoxins in environments that require aggressive saprophytic competition with other organisms (i.e., grain [2] and crop residues [36]). The potential ecological importance of mycotoxins with respect to the saprophytic life cycles of producing fungi is largely unknown, although inhibition of bacteria and other microbial competitors or animal consumers of crop residues may enhance the saprophytic survival of plant-infectious mycotoxigenic *Fusarium* in crops. Understanding the genetic basis for differential responses of two closely related *Pseudomonas* strains carrying similar genes for biosynthesis of DAPG may help us understand the evolution of bacterial genotypes and may reveal a novel type of gene-for-gene interaction among microorganisms.

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