Mutational Analysis of Gliotoxin Production by the Biocontrol Fungus *Gliocladium virens* in Relation to Suppression of Pythium Damping-off

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ABSTRACT

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The fungus *Gliocladium virens* is an important biocontrol agent against plant pathogenic fungi, such as *Pythium ultimum* and *Rhizoctonia solani*, that cause damping-off diseases. *G. virens* strain G20 (synonym GL21) has been commercially formulated into the disease-suppressing product Gliogard (W. R. Grace & Co., CT). One possible mechanism of *G. virens* biocontrol may be the production of the fungistatic metabolite gliotoxin. The presence of this metabolite has been associated previously with disease suppressive activity toward *P. ultimum*. The purpose of this study was to critically test, using mutational analysis, the importance of gliotoxin production in the disease-suppressiveness effected against *P. ultimum*. Seven mutants lacking gliotoxin production (Glx^- phenotype) were isolated by selection-based enrichment and screening procedures following UV-treatment of parental strain G20-4VIB (WT). On average, these Glx^- mutants displayed only 54% of the disease-suppressive activity of the wild-type isolate in vivo and experienced a nearly total loss of antagonistic activity in vitro toward *P. ultimum*. This study represents strong genetic evidence supporting a major role for antibiosis in the suppression of a plant disease by a fungal biocontrol agent.

Additional keywords: fungal metabolite.

The Deuteromycete soil fungus *Gliocladium virens* Miller, Giddens & Foster is one of the most promising and studied fungal biocontrol agents (27,30). Gliogard (W. R. Grace & Co., CT), a formulation of *G. virens* strain G20 (synonym GL21) (20), has been registered with the U.S. Environmental Protection Agency to control damping-off diseases of bedding plants caused primarily by the pathogens *Pythium ultimum* Trow and *Rhizoctonia solani* Kühn (16,19,22). Although strain and formulation selection (18,19) has proven useful as a means of obtaining improved biocontrol efficacy, an understanding of the mechanism(s) of biocontrol is necessary if more directed approaches are to be pursued.

The mechanisms by which antagonistic microorganisms are believed to control plant diseases generally are categorized as competition, parasitism, and antibiosis. The evidence supporting competition and parasitism in suppression of other diseases is convincing (7). Genetic manipulation of bacterial biocontrol agents has provided both positive and negative evidence for antibiosis in the action of these antagonists (29). However, the importance of antibiosis in disease control by fungi has been more difficult to establish. Support has come mainly from the identification of metabolites found in culture that inhibit pathogens in vitro (5,7,21,23). *G. virens* strain G20, as well as other naturally occurring strains, produces gliotoxin, an epidithiodiketopiperazine with antibacterial and fungistatic activity (2,35,38). The in vitro inhibition of *P. ultimum* growth and germination by culture filtrates from *G. virens* was attributed to gliotoxin (32). This metabolite is produced by *G. virens* in soil and soilless media suppressive to *P. ultimum* (21), and its maximal accumulation corresponds to the time of greatest disease-suppressive activity (20).

These studies clearly demonstrate an association between gliotoxin production and in vitro inhibition or disease-suppressive ability. However, determining the relative contribution of gliotoxin to the overall biocontrol process is complicated by the production of lytic enzymes (6) and other fungistatic metabolites (3,11,39) that also may contribute to biocontrol activity. Therefore, experimental or genetic manipulation of gliotoxin production independent of other possible biocontrol mechanisms is necessary to critically evaluate and quantify its role. Mutational analysis provides an effective approach, because the lack of a sexual stage in this fungus (33) precludes genetic analysis of natural variants.

In this study, we evaluate the role of gliotoxin biosynthesis in suppression of disease by comparing the biocontrol efficacy of a gliotoxin-producing parental isolate (G20-4VIB) with that of UV-induced mutants lacking gliotoxin production. A selection-based enrichment and rapid screening assay were devised to facilitate the isolation of multiple mutants.

MATERIALS AND METHODS

Microbial strains and culture maintenance. *G. virens* strain G20-4VIB, the parent isolate used in this study, is a single-spored isolate of the previously described strain, G20 (synonym GL21; 20), chosen for high gliotoxin production. All other *G. virens*

strains used in this study were derived from G20-4VIB. *P. ultimum* strain PuZS1 has been described previously (20). Sporangia were collected from cultures on corn meal agar plates flooded with soil extract. *Bacillus subtilis* strain BR151 originated from the Bacillus Genetics Stock Center, University of Ohio. *G. virens* isolates were maintained on V8-juice agar (200 ml of V8 juice, 20 g of agar, 800 ml of water, and 3 g of $CaCO_3$, pH 5.5). Stock slants containing conidiated mycelia were stored at 4 C. Conidia were transferred from stocks to fresh V8-agar slants and incubated under a fluorescent light source at 20 C. Conidia from cultures between 7 and 21 days old were used as the source of inoculum in all experiments.

Media preparation and additions. Weindling's modified minimal medium (13), containing (per liter) 15 g of glucose, 2 g of ammonium sulfate, 1 g of magnesium sulfate, 0.7 g of H_2O, 0.5 g of monobasic potassium phosphate, and 1.1 mg of iron-EDTA was amended as follows. For medium buffered at pH 6.5, 2-[*N*-morpholino]ethanene-sulfonic acid (MES, 25 mM) was included prior to adjusting the pH with sodium hydroxide. Medium buffered at pH 3.5 was prepared by adjusting the pH of the base medium with phosphoric acid.

p-Fluorophenylalanine (FPA) was prepared as a 0.1 M solution in water by adding concentrated HCl dropwise to the FPA suspension and heating to 35 C until the FPA was dissolved (HCl concentration did not exceed 0.25 N). Freshly prepared FPA solutions were sterilized by filtration just before adding to autoclaved water agar (final concentration of 1.5%) cooled to 55 C.

B. subtilis indicator plates were prepared by diluting late log-phase Luria broth medium cultures, grown at 35 C, 1:100 into autoclaved potato-dextrose agar (PDA) medium cooled to 50 C. Ten milliliters of cooled medium containing bacteria was dispensed into 100-mm-diameter plates and cooled to 4 C to prevent growth of the bacteria. Indicator plates worked best if stored overnight but lost viability after 2–3 days.

Time course of metabolite production. Conidia of wild-type or mutant strains were transferred to 250 ml of Weindling's modified medium (pH 3.5) at concentrations of 5×10^4 conidia per milliliter in 1-L Erlenmeyer flasks. Cultures were incubated at 24 ± 2 C in the dark on a rotary shaker at 150 rpm. Ten-milliliter samples were removed at 4–8 h intervals over 4 days and filtered through Whatman GF/A glass fiber filters (Whatman, Maidstone, England). Culture filtrates were stored for up to 3 days at −20 C before extraction. Mycelial mats were lyophilized to dryness, and the dry weights were recorded.

Measurement of gliotoxin resistance. The biosynthesis of an antibiotic is often linked to the expression of resistance in producing microorganisms (24,25), and thus resistance may be lost as a consequence of disrupted biosynthesis. Agar plugs from the edge of a *G. virens* culture were transferred to solid Weindling's modified media amended after autoclaving with gliotoxin (Sigma Chemical Co., St. Louis) dissolved in ethanol at a stock concentration of 2 mg/ml. Colony growth was measured at nine intervals during a 36-h period, and the linear growth was analyzed with linear regression to determine a growth rate.

Metabolite extraction and quantification. Thin-layer chromatography (TLC) was performed to identify and quantify gliotoxin, viridin, and viridiol from culture extracts (22). Chloroform extracts of culture filtrates were concentrated and applied to channeled TLC plates containing a fluorescent indicator (Whatman LK6DF). Plates were developed in chloroform/acetone (7:3) and visualized under ultraviolet light by fluorescence quenching. Metabolites were identified by comparison to standards in adjacent lanes. Gliotoxin standard was obtained from Sigma, whereas viridin and viridiol standards were from purified samples previously described (23). Video-image analysis of the UV-quenching was used to quantify metabolites (23).

The presence of gliotoxin on the TLC plates also was determined by two more-specific tests. First, TLC plates were visualized by spraying with 0.1 M silver nitrate (14). The sensitivity of this detection method, determined by running a series of gliotoxin standards, was approximately 0.25 μg on a single TLC channel. Second, culture filtrates were analyzed by mass spectroscopy. Preparative TLC plates (Analtech 31012, Analtech, Inc., Newark, DE) were loaded with chloroform extract from 100 ml of culture filtrate, developed in 7:3 chloroform/acetone, and visualized under short-wave ultraviolet light. The silica medium was scraped from the region of the plate corresponding to an adjacent gliotoxin standard. The excised silica medium was extracted with a mixture of chloroform/acetone (7:3) for 1 h. Silica was removed by filtration, and the chloroform was concentrated. Mass-spectral data was obtained with a HP5988A quadrapole instrument equipped with a direct insertion probe (Hewlett-Packard Co., Palo Alto, CA). The sample was applied to the probe and heated to 1,000 C at a rate of 20 mA/s.

Mutant isolation and analysis for gliotoxin production. Mutants deficient in gliotoxin production would greatly facilitate understanding of the role of gliotoxin in *G. virens* biocontrol. However, direct screening for loss of gliotoxin production would be impractical due to the number of isolates to be tested. By applying a selection strategy (34) that enriches for such mutants and employing a rapid biological screen, we used the procedure shown in Figure 1 to isolate *G. virens* mutants lacking gliotoxin production. UV-mutagenized conidia were selected for colonies that displayed faster growth on FPA-supplemented plates. Gliotoxin nonproducing mutants would be expected to have increased resistance to FPA due to elevated levels of intracellular phenylalanine, a biosynthetic precursor of gliotoxin (15), and a competitive inhibitor of FPA toxicity (26). Although other unrelated mutations also could produce FPA-resistance, this selection step serves to enrich for gliotoxin nonproducers. Only those colonies displaying resistance were screened further.

Conidia (15,000) of the parental strain G20-4VIB were plated onto solid Weindling's modified medium (pH 6.5) at densities of 500 conidia per 100-× 15-mm plate. To produce approximately 10% mortality, conidia were exposed for 45 s with a germicidal

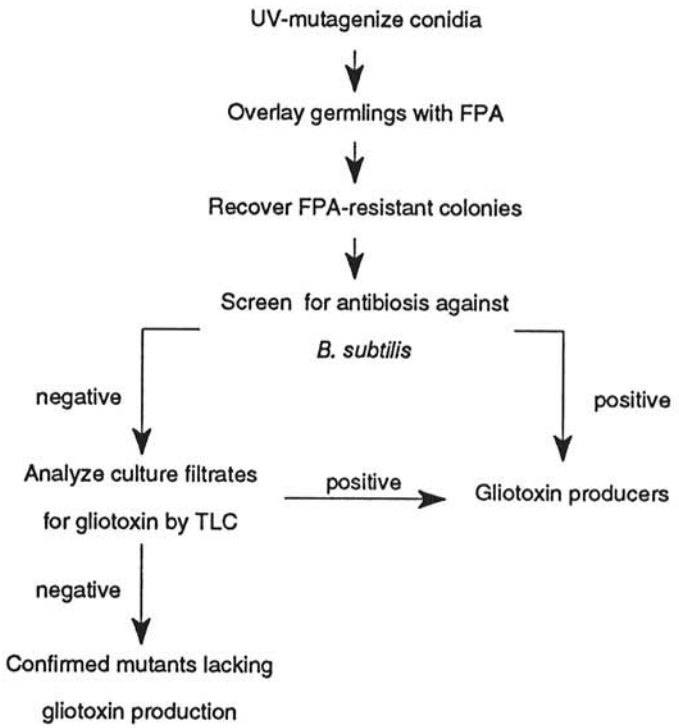

Fig. 1. Selection and screening procedure for isolation of gliotoxin-deficient mutants of *Gliocladium virens*. Mutagenized conidia were germinated on solid media and overlaid with 20 or 50 mM *p*-fluorophenylalanine (FPA) in water agar. FPA-resistant colonies that displayed faster growth were transferred to agar plates containing *Bacillus subtilis*. Positive fungal colonies display a cleared halo of inhibition of bacterial growth due to gliotoxin production. Negative fungal colonies display no halo of clearing in the bacterial lawn. These potential gliotoxin nonproducers were evaluated by thin-layer chromatography (TLC) of liquid-culture filtrates for the presence of gliotoxin.

lamp (Sylvania G30T8) positioned 20 cm above the plates. After 20–30 h in total darkness at 24 C, germlings were overlaid with 20 or 50 mM FPA-agar solution prepared as described above. This overlay was delayed to coincide with the approximate initiation (31) of gliotoxin production. At concentrations of 20 and 50 mM, germling growth was severely restricted but not totally prevented. Plates were incubated for 2 days at 24 C. Actively growing FPA-resistant colonies suspended in the agar matrix were transferred to PDA plates with a dissecting microscope to prevent accidental transfer of the much smaller, sensitive colonies. After 1–2 days of growth at 20 C, mycelial plugs cut from these plates were transferred to *B. subtilis* indicator plates to screen for antibiosis. The age of the intermediate culture was important because plugs from colonies grown for less than one full day on PDA were likely to give a false negative result, having no halo, but producing gliotoxin in liquid culture. Indicator plates, seeded with 8–10 mycelial plugs each, were incubated at 20 C for 8–12 h and then at 35 C for 2–6 h. This switch in incubation temperature was designed to allow preferential growth, first of the fungus and next the bacterium, to ensure that gliotoxin would be produced prior to the growth of the bacteria. Colonies producing no clearing zones in the turbid bacterial lawn were tested further by culturing the isolate in Weindling's modified medium and analyzing the culture filtrate by TLC (as above) to confirm loss of gliotoxin production.

Biological control assays. Studies performed in vitro, which correlate the presence of a metabolite with the biological activity toward a pathogen, may not necessarily predict the in vivo activity of a metabolite (7). To directly determine the role of gliotoxin in vivo, *G. virens* isolates were tested for their ability to suppress damping-off disease caused by *P. ultimum*. Biological control tests, including preparation of antagonist and pathogen inocula, were previously described by Lumsden and Locke (20). *G. virens* conidia from one V8-media slant were inoculated onto 50 g of an autoclaved wheat bran/water mix (1:1). After 3 days of growth at room temperature, 10 g of colonized bran was incorporated into 4 l of Redi-Earth (The Scotts Co., Marysville, OH); approximately 700 g amended with water to a final weight of 1,100 g). A fertilizer solution (500 ml of a 0.64% solution of 20-20-20 [N-P-K]) was added to the soilless mix. After 3 days incubation in a plastic bag at 25 C, the colonized soilless mix was divided into four planting flats (12 × 16 cm). Each flat was planted with 40 zinnia seeds and drenched with 9,600 *P. ultimum* sporangia.

Each treatment was replicated in four flats. The healthy control contained no fungal inoculum, whereas the disease control was treated with *P. ultimum* and sterile bran. Seedling emergence was evaluated after 1 wk at 21 C. These conditions produced maximal biocontrol activity (20). Disease incidence was scored as the percentage of seedlings present after 1 wk compared to the number of seeds planted (percent plant stand). Disease suppression by *G. virens* was indicated when its preinoculation resulted in an increase in plant stand over the diseased control (*P. ultimum* treatment without antagonist). The magnitude of this increase is representative of biocontrol activity. Determination of antagonist population densities and statistical analysis was as described by Lumsden and Locke (20). The entire experiment was repeated; however, the results of one experiment are presented because significantly different plant stands of the diseased controls between experiments prevented averaging them.

RESULTS

Identification and characterization of mutants lacking gliotoxin production. FPA-resistant colonies reached sizes approaching 1–2 mm diameter after 2 days, whereas sensitive colonies (comprising approximately 98% of the population) were much smaller. In a rapid screen for gliotoxin production, FPA-resistant mutants were tested in a plate assay for their inhibition of the gliotoxin-sensitive bacterium *B. subtilis*. The parent G20-4VIB strain produced a clearing zone approximately 6 mm beyond the mycelium on the *B. subtilis* lawn. Seven of 226 FPA-resistant isolates, however, produced no clearing zone beyond the mycelium or below the surface of the colony and, therefore, were likely candidates of disrupted gliotoxin production. These seven putative mutants were analyzed further by TLC analysis of liquid-culture filtrates to confirm loss of gliotoxin production. Little (<10% of the parent strain) or no UV-absorbing band corresponding to the mobility of gliotoxin was detected on the TLC plate, and more specific analysis by reaction with silver nitrate did not produce a brown color (14) characteristic of gliotoxin. Comparison of silver reactivity to gliotoxin standards demonstrated that all seven mutants produced gliotoxin at less than 0.1 μg/ml. The parent isolate produced 13.8 μg/ml when grown under similar conditions (Table 1). Mass spectroscopy of culture extracts confirmed that cultures of the mutants contained gliotoxin at levels below 1% of G20-4VIB, because gliotoxin was readily detected in G20-4VIB extract diluted 100-fold but not in undiluted mutant extract. These mutants were designated as Glx^- phenotype because they produced less than 1% of the gliotoxin produced by the parent strain, designated as Glx^+ (Table 1). This FPA-directed approach resulted in the isolation of approximately three Glx^- mutants per 100 FPA-resistant mutants screened (3.1%), whereas random screening of 100 similarly UV-irradiated germlings without FPA selection resulted in the isolation of none (<1%).

Related phenotypes of Glx^- mutants. Only one Glx^- mutant maintained wild-type levels (23) of gliotoxin resistance (Table 1). Parent G20-4VIB and Glx^- mutant G20-M37 were resistant to gliotoxin at 20 μg/ml (displaying 20 and 14% growth inhibition, respectively), whereas all other mutants were sensitive (mutants G20-M37, -50, -96, -107, -128, and -149 displayed 92, 94, 77,

TABLE 1. Characteristics of *Gliocladium virens* strains

Strain	Gliotoxin[v] (μg/ml)	Phenotype	*P. ultimum* inhibition[w]	Resistance to gliotoxin[x]	Viridin overproduction[y]
G20-4VIB[z]	13.8	Glx^+	+	+	−
G20-M2	14.1	Glx^+	+	+	−
G20-M103	16.1	Glx^+	+	+	−
G20-M36	<0.1	Glx^-	−	−	−
G20-M37	<0.1	Glx^-	−	+	+
G20-M50	<0.1	Glx^-	−	−	+
G20-M96	<0.1	Glx^-	−	(−)	+
G20-M107	<0.1	Glx^-	−	−	+
G20-M128	<0.1	Glx^-	−	−	+
G20-M149	<0.1	Glx^-	−	−	+

[v] Maximum accumulated gliotoxin over a period of 4 days of incubation in Weindling's modified minimal medium at pH 3.5.
[w] Determined in vitro by a coculture assay. Phenotypes: + = limitation of *Pythium ultimum* growth around the *G. virens* colony, zone of inhibition indicative of antibiosis; − = no limitation of *P. ultimum* growth around the *G. virens* colony.
[x] Determined as the reduction of radial growth rates on Weindling's modified agar (pH 6.5) with gliotoxin at 20 μg/ml compared to unamended medium. Phenotypes: + = growth rate reduced by ≤30%; − = growth rate reduced by ≥85%; (−) = growth rate reduced by 77%.
[y] Measured as total viridin (viridin + viridiol) produced after 4 days of culture in Weindling's modified medium (pH 3.5). Phenotypes: − = ≤3 μg/ml; + = 10–40 μg/ml.
[z] The parent isolate from which all other strains were derived after UV-treatment and *p*-fluorophenylalanine-selection.

95, 94, and 85% growth inhibition, respectively). On Weindling's modified medium without gliotoxin, all mutants grew at rates greater than G20-4VIB (data not shown).

TLC analysis for gliotoxin production showed that the production of viridin, another fungistatic metabolite of *G. virens* (23), was increased in most Glx$^-$ mutants. Parent strain G20-4VIB accumulated detectable levels of viridin only after 4–5 days of culture. In contrast, all Glx$^-$ mutants except G20-M36 initiated significant viridin accumulation after only 2–3 days of culture (Fig. 2). The enzymatic reduction of viridin to viridiol (13) resulted in a decline in the concentration of viridin after its initial accumulation (data not shown). Thus, a peak in viridin concentration occurred approximately 12–36 h after viridin production was initiated, depending on the experiment and isolate. Viridiol accumulation was significant for all mutants showing the elevated phenotype and exceeded the amount of viridin by as much as threefold at the peak of viridin accumulation. Elevated viridin production was not the result of a reduced conversion to viridiol, because little viridiol was detected in cultures of G20-4VIB and G20-M36 compared to the overproducing mutants.

In vitro antagonism against *P. ultimum*. The in vitro antagonistic ability of the Glx$^-$ mutants was tested by coculturing of *P. ultimum* and *G. virens* on PDA medium (Fig. 3). All seven Glx$^-$ mutants were clearly reduced in their antagonistic activity. *P. ultimum* mycelial growth was restricted around the colony of the parent G20-4VIB strain but mostly was unrestricted by the Glx$^-$ mutants and overgrew portions of these colonies. All mutants possessed at least some ability to restrict *P. ultimum* growth through the *G. virens* colony, but whether this was due to physical or other interactions was unclear.

Suppression of *P. ultimum* damping-off. Preinoculation of the soil with the parent Glx$^+$ strain G20-4VIB resulted in significant suppression of disease, increasing plant stand from 12% in the *P. ultimum* disease control to 66% (Table 2). The seven Glx$^-$ mutants, however, possessed considerably lower biocontrol activity. All Glx$^-$ treatments resulted in plant stands significantly lower ($P = 0.05$) than that observed with Glx$^+$ G20-4VIB; this result was consistent for two separate experiments. The relative biocontrol activity of the Glx$^-$ mutants was on average 0.54 relative to that of G20-4VIB (relative biocontrol activity of 1); thus, a 46% reduction in biocontrol activity was associated with the loss of gliotoxin production.

To investigate the possibility that reduced plant stands were due to the conversion of viridin to the phytotoxin viridiol (12) in the soilless mix, a viridin overproducing mutant (G20-M37) was tested in a biocontrol assay without *P. ultimum* (data not shown). This treatment showed no signs of phytotoxicity, as its plant stand (91% emergence) was not significantly ($P = 0.05$) reduced when compared to the healthy (uninoculated) control (97% emergence). The extent of conversion of viridin to viridiol

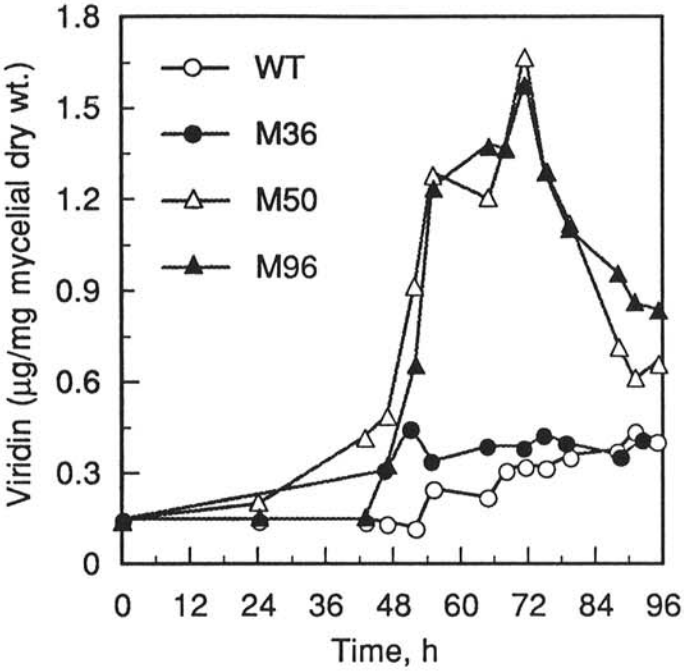

Fig. 2. Production of viridin by *Gliocladium virens* isolates during growth in liquid culture. Viridin was quantified by thin-layer chromatography analysis of filtrates of liquid cultures and normalized to the dry weight of mycelium in the collected sample. The results for *G. virens* isolates G20-4VIB (WT) and Glx$^-$ mutants are shown. The Glx$^-$ mutants not included in the figure produced similar or more viridin than G20-M50 or G20-M96.

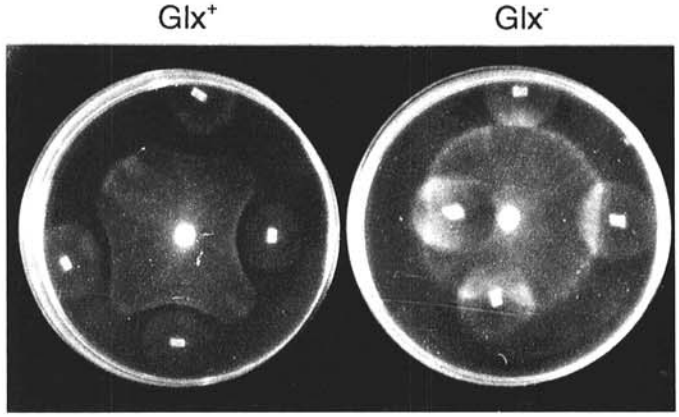

Fig. 3. In vitro antagonism assay. A mycelial plug of *Pythium ultimum* was placed in the center of each potato-dextrose agar plate and surrounded by mycelial plugs of one *Gliocladium virens* isolate at various distances from the center. The multiple placements allow visualization of the temporal dynamics in the fungal interaction. Antagonism is scored as the limitation of *P. ultimum* mycelial growth around the *G. virens* colony, displayed by the parent isolate G20-4VIB (Glx$^+$). The Glx$^-$ mutant shows little inhibition of *P. ultimum* growth, allowing its overgrowth through the *G. virens* colonies with only slight distortion of the mycelial front. A similar result was observed with all seven Glx$^-$ mutants.

TABLE 2. Disease suppression by strains of *Gliocladium virens* differing in gliotoxin production

Strains[w]	Plant stand[x] (%)	Relative biocontrol activity[y]	Soil colonization[z] (10^6 cfu/g of soil)
None	87 a	—	—
Pu	12 f	—	—
Pu + G20-M103 (Glx$^+$)	82 a	1.3	0.19 (0.047)
Pu + G20-M2 (Glx$^+$)	77 a	1.2	12 (0.67)
Pu + G20-4VIB (Glx$^+$)	66 b	1.0	8.7 (0.52)
Pu + G20-M50 (Glx$^-$)	49 c	0.69	13 (0.25)
Pu + G20-M96 (Glx$^-$)	47 cd	0.65	8.5 (0.08)
Pu + G20-M128 (Glx$^-$)	46 cd	0.63	8.3 (0.30)
Pu + G20-M107 (Glx$^-$)	42 cd	0.56	11 (0.79)
Pu + G20-M149 (Glx$^-$)	38 cd	0.48	12 (0.81)
Pu + G20-M36 (Glx$^-$)	35 de	0.43	18 (1.3)
Pu + G20-M37 (Glx$^-$)	32 e	0.37	6 (0.46)

[w] Sporangia of *Pythium ultimum* (Pu) added at time of planting to flats containing a soilless mix colonized by *G. virens* strains.

[x] Determined 1 wk after planting. Values are presented as backtransformed means calculated from four replicates of 40 seeds each. Different letters are significantly different ($P = 0.05$) in Duncan's multiple range test after arcsine transformation.

[y] Relative increase in plant stand from diseased control compared to G20-4VIB, which increased plant stand 54 percentage points over the disease (Pu) control (from 12 to 66%). Relative biocontrol activity = (percent plant stand in treatment − percent plant stand in Pu-inoculated control) / (percent plant stand in G20-4VIB Glx$^+$ treatment − percent plant stand in Pu-inoculated control).

[z] Antagoinst populations in soilless mix determined at time of planting and inoculation with *P. ultimum*, averaged from three replicates. Values in parentheses are standard errors of the means.

was not measured in soil but may have been much less than that observed in culture. Likewise, the decreased biocontrol activity in the Glx$^-$ mutants was apparently not due to an inability to propagate in soil, because spore populations of the Glx$^-$ mutants were not significantly ($P = 0.05$) decreased compared to G20-4VIB (Table 2).

FPA-resistant isolates G20-M2 and G20-M103, which retained their ability to produce gliotoxin (Table 1), were included in the biocontrol assay. This was to verify that the UV-treatment used to isolate mutants or FPA-resistance itself did not reduce biocontrol activities. These Glx$^+$ isolates displayed significantly ($P = 0.05$) higher biocontrol activity than did G20-4VIB (Table 2). Thus, FPA-selection alone does not appear to compromise biocontrol ability. The increase in activity by these isolates may represent an enhancement of certain characters by growth on FPA, such as those observed with other fungi in which haploidization of aneuploids occurs (36).

DISCUSSION

In this study we describe the isolation of multiple mutants of *G. virens* lacking the ability to produce gliotoxin (Glx$^-$ phenotype). The demonstration that these Glx$^-$ mutants concurrently lost approximately half their disease-suppressive ability against *P. ultimum* indicates that gliotoxin is likely a major factor in this biocontrol activity. To our knowledge, the role of secondary metabolites in disease suppression by biocontrol fungi has been supported only once previously through genetic evidence. Howell and Stipanovic (11) demonstrated loss of disease-suppressive ability in one mutant of *G. virens* unable to produce gliovirin, another antibiotic active against *P. ultimum* that is produced only by strains that do not produce gliotoxin (10,23). The ability to draw strong conclusions from mutagenesis is limited by the potential for multiple characters to be affected in a single UV-induced mutant. Thus, a decrease in biocontrol ability may result from coincident mutagenesis in an independent locus and not directly from loss of antibiotic production. Although the present study on gliotoxin also is subject to similar concerns, our demonstration that all seven independent Glx$^-$ mutants displayed decreased biocontrol activity reduces the likelihood that a mutation in an unrelated locus caused this decrease. Therefore, this is the most conclusive genetic evidence to date supporting antibiosis as an important factor in biological control effected by a fungal antagonist.

Because random screening for gliotoxin production would be time-consuming for large numbers of colonies and not likely to yield multiple Glx$^-$ mutants, we pursued a method to enrich for such mutants prior to screening. One strategy for enrichment of mutants in the biosynthesis of a metabolite is to select for resistance to toxic analogs of a biosynthetic precursor. One class of these resistant mutants would arise through disruption in the metabolite's biosynthetic pathway, because this would increase the amount of precursor and relieve the effect of the toxic analog through competition. Because phenylalanine is a precursor of gliotoxin biosynthesis (15), *G. virens* Glx$^-$ mutants would be one class of mutants resistant to the phenylalanine analog FPA. Further, because gliotoxin biosynthesis consumes a significant portion of cellular phenylalanine (gliotoxin can represent as much as 2% of the mycelial dry weight in culture) and the addition of FPA to liquid cultures did not decrease gliotoxin production at concentrations that impede cell growth (data not shown), the suitability of this enrichment is supported experimentally. This selection would not be expected to work if gliotoxin was produced at low levels or if its synthesis was sacrificed by the fungus to sustain growth during FPA treatment. The usefulness of this strategy is indicated by enhanced isolation of Glx$^-$ mutants, based on our limited comparison to a random screen. A similar strategy was used by Smidt and Kosuge (34) to obtain mutants of *Pseudomonas savastanoi* lacking indole-3-acetic acid (IAA) biosynthesis by selection for resistance to α-methyl trypthophan (MT), an analog of trypthophan. These IAA-deficient mutants were used to demonstrate the role of IAA synthesis by this bacteria in gall formation on oleander (34).

To further facilitate isolation of Glx$^-$ mutants, we developed a rapid screening procedure that differentiates gliotoxin-producing colonies from nonproducing colonies based on the gliotoxin sensitivity of *B. subtilis* (1). This bioassay was rapid and accurate; more than 150 FPA-resistant mutants were screened easily in a single day, and no false positives were detected. Furthermore, the assay was not affected by viridin because *B. subtilis* is not sensitive to this metabolite (3).

Glx$^-$ mutants clearly lack most of the in vitro antagonism against *P. ultimum* characteristic of parent Glx$^+$ G20-4VIB. *G. virens* strain G20 produces several metabolites that, when tested in purified form, inhibit *P. ultimum* (23). The majority of inhibitory activity was attributed to gliotoxin because of the relatively high level of its synthesis in culture (23). The isolation of Glx$^-$ mutants allowed the direct testing of this relationship. Thus, gliotoxin seems to be the primary inhibitory factor under these culture conditions. When this effect was further tested in disease suppression, all Glx$^-$ mutants had a significantly reduced ability to control *P. ultimum*-induced damping-off of zinnia. Controls eliminated the possibility that the diminished biocontrol ability of the Glx$^-$ mutants was due to impaired colonization ability in the soilless mix, nonspecific changes from the mutagenesis- and FPA-selection procedures, or the overproduction of potentially phytotoxic viridiol. Altogether, these controls and the consistent loss of biocontrol activity in all seven Glx$^-$ mutants suggest that mutations at other loci do not cause the loss of biocontrol activity. Our results agree with studies that have associated the presence of gliotoxin with the biocontrol of *P. ultimum* (21,23,32,40) and support a major role for gliotoxin.

Loss of gliotoxin production under the conditions of this study corresponded to an average decrease of 46% in overall biocontrol ability. This is similar to the relative contribution of oomycin A, 2,4-diacetylphloroglucinol, and phenazine-1-carboxylate to disease suppression by various strains of *Pseudomonas fluorescens*, studied using specific mutations in this bacterial biocontrol agent (29). This partial contribution of gliotoxin suggests other biocontrol mechanisms, such as competition for nutrients, production of lytic enzymes, or antibiosis resulting from production of other antibiotics, are involved in the *G. virens-P. ultimum* interaction as well. This is consistent with the emerging belief that biocontrol results from complex interactions that involve more than a single mechanism (8) or synergism between separate mechanisms (6). The relative contribution of each mechanism may be altered under different environmental and experimental conditions and between *G. virens* strains. Our experiments were inconclusive with regard to the effect of viridin. Little or no inhibition of *P. ultimum* was observed in vitro by the mutants overproducing viridin. By contrast, the one mutant not showing viridin derepression ranked in the lowest statistical grouping of disease-suppressive ability, suggestive of viridin action. This difference could be due to the measurement of *Pythium* mycelial growth alone in the in vitro bioassay, whereas inhibition of sporangial germination also would be a factor in the biocontrol assay. The relative contribution of gliotoxin could be underrepresented, therefore, in this study if viridin overproduction inhibited sporangial germination in vivo, as described in vitro (24), and partially compensate for loss of gliotoxin production. However, the variation in biocontrol activity between the Glx$^-$ mutants could be due to cryptic mutations at other loci independent of viridin production.

The Glx$^-$ mutants demonstrated few other changes in phenotype compared to the G20-4VIB parent strain. Linear growth rate, colony appearance, and sporulation in culture (including the soilless mix) were all unchanged. These observations indicate that multiple mutations have not seriously altered the metabolism of these mutants. However, mutants were altered with respect to viridin production and resistance to gliotoxin. Some Glx$^-$ mutants experienced derepression of viridin production, loss of resistance to gliotoxin, or both. These Glx$^-$-associated phenotypes are probably not the result of independent mutations in the same isolate, because more than one of the Glx$^-$ mutants displayed the

same combination of phenotypes. In other (bacterial) systems, the linkage of such traits has been attributed to 1) an autoregulator that coordinately induces both antibiotic biosynthesis and resistance (24,25); 2) global control of production of multiple antibiotics by a single genetic locus (17); 3) the action of a bifunctional enzyme that participates in biosynthesis and resistance (37); and 4) the action of an antibiotic itself, or a related intermediate, that feeds back to induce resistance (4). Therefore, multiple phenotypes of the *G. virens* mutants could be the result of disruption of proteins with either regulatory or biosynthetic function.

Strain G20 (GL21) of *G. virens* is under commercial development as a biocontrol agent against soilborne pathogens (27) and is an especially interesting model system for studying relevant biocontrol mechanisms possessed by biocontrol fungi. Because *G. virens* lacks a sexual stage (33) and has shown incomplete parasexual recombination (9), the segregation characters among progeny cannot be used to determine which characters contribute to its biocontrol ability. Mutational analysis has provided an alternative strategy to critically test the role of gliotoxin in the biocontrol process. These mutants also will allow more critical testing of this result through complementation by DNA-mediated transformation (28) and subsequent specific gene disruption of cloned genes. The positive evidence presented here substantiates such studies and the effort to improve biocontrol ability through enhancing gliotoxin production.

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