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

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**ABSTRACT**


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 exhibited against *P. ultimum.* Seven mutants lacking gliotoxin production (Gix phenotype) were isolated by selection-based enrichment and screening procedures following UV-treatment of parental strain G20-4V1B (WT). On average, these Gix 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 antibiotics 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 antibiotics. 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 antibiotics in the action of these antagonists (29). However, the importance of antibiotics 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 epidiiodiketopiperazine 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 (G20-4V1B) 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 G20-4V1B, 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 CaCO3, 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 H2O, 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-morpholinol]ethane-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 suspen-
sion and heating to 35°C until the FPA was dissolved (HCl con-
centration 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 modi-
fied medium (pH 3.5) at concentrations of 5 × 104 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 glutoxin 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 medium amended after autoclaving with glutoxin (Sigma
Chemical Co., St. Louis) dissolved in ethanol at a stock concentra-
tion 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 chromato-
genography (TLC) was performed to identify and quantify glutoxin,
viridin, and viridiol from culture extracts (22). Chloroform ex-
tracts of culture filtrates were concentrated and applied to
channeled TLC plates containing a fluorescent indicator
(Whatman K&GF). 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. Glutoxin standard was obtained from
Sigma, whereas viridin and viridiol standards were from pure samples
previously described (23). Video-image analysis of the
UV-quenching was used to quantify metabolites (23).

The presence of glutoxin on the TLC plates was also 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 glutoxin
standards, was approximately 0.25 µg on a single TLC channel.
Second, culture filtrates were analyzed by mass spectroscopy.

**Preparative TLC plates** (Analtech 30112, 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
scrapped from the region of the plate corresponding to an adjacent
glutoxin 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 quadrupole 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 glutoxin production.**
Mutants deficient in glutoxin production would greatly facilitate
understanding of the role of glutoxin in *G. virens* biocontrol.
However, direct screening for loss of glutoxin 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 glutoxin
production. UV-mutagenized conidia were selected for colonies
that displayed faster growth on FPA-supplemented plates. Glutoxin
nonproducing mutants would be expected to have increased
resistance to FPA due to elevated levels of intracellular phenyl-
alanine, a biosynthetic precursor of glutoxin (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 glutoxin 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
lamp (Sylvania G30T8) positioned 20 cm above the plates. After 20–30 h in total darkness at 24 °C, germinations 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, germination 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 Weidling'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 41 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 spores.

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 clear 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 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 spectrometry 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 GIX phenotype because they produced less than 1% of the gliotoxin produced by the parent strain, designated as GIX+ (Table 1). This FPA-directed approach resulted in the isolation of approximately three GIX mutants per 100 FPA-resistant mutants screened (3.1%), whereas random screening of 100 similarly UV-irradiated germinations without FPA selection resulted in the isolation of none (<1%).

Related phenotypes of GIX+ mutants. Only one GIX+ mutant maintained wild-type levels (23) of gliotoxin resistance (Table 1). Parent G20-4VIB and GIX+ 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, -30, -96, -107, -128, and -149 displayed 92, 94, 77,

<table>
<thead>
<tr>
<th>Table 1. Characteristics of Gliocladium virens strains</th>
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<tr>
<td><strong>Strain</strong></td>
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<tr>
<td>G20-4VIB</td>
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<tr>
<td>G20-M2</td>
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<tr>
<td>G20-M103</td>
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<tr>
<td>G20-M36</td>
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<tr>
<td>G20-M37</td>
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<tr>
<td>G20-M50</td>
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<td>G20-M96</td>
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<tr>
<td>G20-M107</td>
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<tr>
<td>G20-M128</td>
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<td>G20-M149</td>
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*Maximum accumulated gliotoxin over a period of 4 days of incubation in Weidling's modified minimal medium at pH 3.5.
* Determined in vitro by a coculture assay. Phenotypes: + = limitation of Pythium ultimum growth around the G. virens colony, zone of inhibition indicative of antibiotic; – = no limitation of P. ultimum growth around the G. virens colony.
* Determined as the reduction of radial growth rates on Weidling's modified agar (pH 6.5) with gliotoxin at 20 µg/ml compared to unamended medium. Phenotypes: + = growth rate reduced by ≥90%; – = growth rate reduced by ≥50%; – = growth rate reduced by ≤50%.
* Measured as total viridin (viridin + viridical) produced after 4 days of culture in Weidling's modified medium (pH 3.5). Phenotypes: – ≤5 µg/ml; + = 10–40 µg/ml.
* The parent isolate from which all other strains were derived after UV-treatment and p-fluorophenylalanine selection.

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95, 94, and 85% growth inhibition, respectively). On Weindling's modified medium without glotoxin, all mutants grew at rates greater than G20-4V1B (data not shown).

TLC analysis for glotoxin production showed that the production of viridin, another fungistic metabolite of G. virens (23), was increased in most Glx° mutants. Parent strain G20-4V1B 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 viridol (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. Viridol 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 viridol, because little viridol was detected in cultures of G20-4V1B 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 G20-4V1B 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 G20-4V1B 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° G20-4V1B; 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 G20-4V1B (relative biocontrol activity of 1); thus, a 46% reduction in biocontrol activity was associated with the loss of glotoxin production.

To investigate the possibility that reduced plant stands were due to the conversion of viridin to the phytotoxin viridol (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 viridol

![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 Gloecladium 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 G20-4V1B (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.](image)

![Fig. 2. Production of viridin by Gloecladium 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 G20-4V1B (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.](image)

<table>
<thead>
<tr>
<th>Strains°</th>
<th>Plant stand (%)</th>
<th>Relative biocontrol activity</th>
<th>Soil colonization (10^6 cfu/g of soil)</th>
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<tbody>
<tr>
<td>None</td>
<td>87 a</td>
<td>1.0</td>
<td>0.19 (0.047)</td>
</tr>
<tr>
<td>Pu</td>
<td>12 f</td>
<td>1.0</td>
<td>8.7 (0.52)</td>
</tr>
<tr>
<td>Pu + G20-M103 (Glx°)</td>
<td>82 a</td>
<td>1.3</td>
<td>1.0 (0.25)</td>
</tr>
<tr>
<td>Pu + G20-M2 (Glx°)</td>
<td>77 a</td>
<td>1.2</td>
<td>8.5 (0.08)</td>
</tr>
<tr>
<td>Pu + G20-M50 (Glx°)</td>
<td>66 b</td>
<td>1.0</td>
<td>11 (0.79)</td>
</tr>
<tr>
<td>Pu + G20-M96 (Glx°)</td>
<td>49 e</td>
<td>0.69</td>
<td>18 (1.3)</td>
</tr>
<tr>
<td>Pu + G20-M128 (Glx°)</td>
<td>46 c</td>
<td>0.63</td>
<td>12.0 (0.01)</td>
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<tr>
<td>Pu + G20-M107 (Glx°)</td>
<td>38 e</td>
<td>0.63</td>
<td>18 (0.46)</td>
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<tr>
<td>Pu + G20-M36 (Glx°)</td>
<td>35 e</td>
<td>0.63</td>
<td>12 (0.81)</td>
</tr>
<tr>
<td>Pu + G20-M37 (Glx°)</td>
<td>32 e</td>
<td>0.63</td>
<td>12 (0.81)</td>
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</table>

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

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 arc sine transformation.

Relative increase in plant stand from diseased control compared to G20-4V1B, 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 G20-4V1B Glx° treatment − percent plant stand in Pu-inoculated control).

Antagonist 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 glvorin, 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 antibiotic 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 tryptophan (MT), an analog of tryptophan. 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 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 viridol. 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 and sporulation 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-diacylphloroglucinol, 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 antibiotics 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 bifunc-
tional 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 pheno-
types 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 develop-
ment 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 biocon-

LITERATURE CITED

control of Pythium ultimum, a causal agent of damping-off. Phyto-
pathology 82:131-135.
tion, characterization, and synergistic antifungal activity in combi-

29. O'Sullivan, D. J., and O'Gara, F. 1992. Traits of fluorescent Pseudo-

32. Roberts, D. P., and Lumsden, R. D. 1990. Effect of extracellular metabolites from Gliocladium virens on germination of sporon-