

Identification of Mycelial Polypeptides Associated with Gliotoxin-Producing Strains of the Biocontrol Fungus *Gliocladium virens*

C. J. Ridout, R. D. Lumsden, and W. R. Hruschka

First and second authors: Biocontrol of Plant Diseases Laboratory; third author: Instrumentation and Sensing Laboratory, U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD 20705-2350.

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ABSTRACT

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Soluble mycelial polypeptides from two gliotoxin-producing strains of *Gliocladium virens* (G-20 and G-15) were compared with the soluble mycelial polypeptides from two strains unable to produce gliotoxin (G-3 and G-4) by using two-dimensional gel electrophoresis. In single comparisons, there were between 146 and 170 polypeptides that were common to both producers and nonproducers, and between 12 and 14 polypeptides that were uniquely associated with producers of gliotoxin. However, when the data from all experiments were compared, there were four major

polypeptides that were consistently and uniquely associated with strains that have the ability to produce gliotoxin. Molecular weights (kDa) and isoelectric points (pI) of these four polypeptides were estimated to be 33.8, 7.35; 33.8, 7.10; 27.2, 7.25; and 15.1, 4.6. In addition, there were several polypeptides that were consistently more abundant in gliotoxin-producing strains. The results may help to explain physiological differences that affect gliotoxin production and lead to improved methods for selecting and formulating *G. virens* for biological control.

Additional keywords: metabolite, protein, viridin.

Gliocladium virens J. H. Miller, J. E. Giddens & A. A. Foster is an important potential biocontrol fungus for use against several soilborne plant pathogens (24). The fungus has now been developed into a variety of formulations (15-17), two of which were recently registered by the Environmental Protection Agency (EPA registration 11688-4 for WRC-GL-21 and 11688-3 for WRC-AP-1) for potential commercial development. However, to be successful, any such formulation must be optimized so that its performance is consistent under the range of environmental conditions normally encountered during use.

The performance of *G. virens* may be improved by selecting the best strains and by improving the formulation; these procedures require an understanding of the physiology of the fungus and of the mechanisms by which it controls plant pathogens. *G. virens* and the closely related *Trichoderma* spp. are known to produce extracellular enzymes and secondary metabolites, some of which may be involved in biological control (4,10,24,25,29). The antibiotic gliotoxin has been isolated from soil (18,19,33) and is known to have a particularly potent effect on some plant pathogens (2,27); strains that produce quantities of this antibiotic are particularly effective for biological control (19,34). Another important metabolite is viridin. However, this antibiotic is converted to viridiol, which has phytotoxic properties (13). It is therefore possible that the biocontrol performance of *G. virens* could be improved by selecting those strains and formulations that produce more gliotoxin and less viridin.

Gliotoxin is a member of the epipolythiopiperazine-3,6-dione group of fungal metabolites and contains a disulfide bond that confers activity (3,32). The antibiotic is probably derived from phenylalanine via the intermediate cyclo (phenylalanyl-L-seryl) (14). In spite of numerous studies, however, no key biosynthetic steps or enzymes have been conclusively established. The end

stages, involving N-methylation and the addition of the disulfide bridge to the intermediate, are particularly obscure and could probably be resolved only if cell-free synthesis can be achieved (14). Viridin, a steroidlike antibiotic, is structurally unrelated to gliotoxin and lacks both the sulfur and nitrogen atoms (5,6). In a survey of 20 isolates of *G. virens*, all were found to produce viridin, whereas only 14 isolates were able to produce gliotoxin (9).

Comparing the physiology of gliotoxin-producing strains with nonproducing strains should enable some of the processes uniquely involved in gliotoxin production to be determined. All physiological processes involved in the synthesis of a metabolite such as gliotoxin are controlled by proteins, which perform such functions as biosynthesis, intracellular transport, self-resistance to the antibiotic, and export from the cell (20). If the various proteins that are associated with the ability to produce gliotoxin can be identified, it will provide the first step towards understanding how gliotoxin is produced, how environmental factors affect its production, and why strains vary in their ability to produce this antibiotic. Also, if such proteins can be identified and sequenced, DNA probes can be constructed and used for the identification of genes involved with gliotoxin production (22).

The specific objective of this study was to identify the soluble mycelial proteins that are consistently associated with the ability to produce gliotoxin. This objective was achieved by the development of a rigorous analytical technique that was used to compare the polypeptide subunits of proteins from gliotoxin-producing strains of *G. virens* with the polypeptides from those strains that were unable to produce gliotoxin. Our long term objectives were to obtain a complete molecular and physiological understanding of gliotoxin production so that improved strains and formulations can be developed.

MATERIALS AND METHODS

Strains and growth conditions. All *G. virens* strains used were from the culture collection of the Biocontrol of Plant Diseases

Laboratory. The cultures were maintained on V8 agar (17). Strains (17) G-20 (a subsolate of GL-21) and G-15 were selected for their ability to produce gliotoxin, and strains G-3 and G-4 were selected for their inability to produce gliotoxin. Two additional strains derived from the parent G-20 were also used. Strain G-20-4VIB was obtained after six consecutive transfers of single spores from the parent G-20, and strain G-20A was isolated from an irregular colony of the parent.

To quantify the amount of gliotoxin and viridin produced, we transferred aqueous conidial suspensions (1 ml containing 10^6 spores) to Erlenmeyer flasks (250 ml) that contained modified Weindling's medium (30 ml) (13) with three replicates for each strain. After 4 days of static incubation at 25 C in the dark, the mycelium was harvested by filtering through Nitex nylon mesh (25 μ m pore size) (Tetko Inc., Elmsford, NY). The dry weight of the mycelium was determined after heating at 100 C for 1 h, and the amounts of gliotoxin and viridin in the culture filtrate were assessed by using the procedures described below.

Each electrophoresis experiment compared the polypeptides of one strain with another. G-20 was compared with G-3, G-15 was compared with G-4, and G-20-4VIB was compared with G-20A. For each pair, the growth conditions, protein extraction procedures, and the electrophoresis conditions were identical. All strains were grown as above except that sterile rectangular polyethylene containers (280 \times 170 \times 130 mm) filled with modified Weindling's medium (250 ml) were used to obtain large quantities of protein.

Determination of gliotoxin and viridin. The amounts of gliotoxin and viridin produced were determined with high performance liquid chromatography (HPLC). The culture filtrate (30 ml) was mixed with chloroform (HPLC grade, Sigma, St. Louis, MO) (30 ml) and shaken vigorously. The chloroform was separated, evaporated to dryness by using a rotary evaporator (Flash Evaporator, Buchler Instruments, Fort Lee, NJ), and the residue was resuspended in chloroform (100 μ l). An aliquot of the concentrated chloroform extract (25 μ l) was injected into a Beckman Ultrasphere reverse phase column (4.6 \times 25 mm) (Beckman Instruments, Fullerton, CA) mounted in a Beckman system made up of a 421A controller, a 427 integrator, and a 165 variable wavelength detector set at 540 nm. The eluent was a mixture of double-distilled water (65%), acetonitrile (20%), and methanol (15%) brought to pH 4.0 with acetic acid at a flow rate of 3.0 ml/min. The system was calibrated by using standard gliotoxin (Sigma) and viridin (a gift from ICI, Jealotts Hill, U.K.).

All cultures used in electrophoresis experiments were routinely analyzed with thin-layer chromatography (TLC) for the production of gliotoxin and viridin. An aliquot of the culture filtrate (1.5 ml) was mixed with chloroform (1.5 ml) and shaken vigorously. The chloroform was separated and concentrated by evaporating to dryness, and the residue was resuspended in chloroform (100 μ l). An aliquot (25 μ l) of this concentrate was spotted onto a TLC plate (Whatman LKDF, Hillsborough, OR) and developed with a chloroform/acetone (70:30, v/v) mixture for 40 min. The standards of gliotoxin or viridin were run on the same TLC plate, and the location of the compounds was determined by viewing at 254 nm with a Mineralight UVGL-58 ultraviolet light (UVP Inc., San Gabriel, CA). Quantitative analysis of gliotoxin was also performed for the G-20-4VIB vs. G-20A electrophoresis experiment by using HPLC with an aliquot (25 μ l) of the TLC chloroform concentrate. Yields were expressed as micrograms of gliotoxin per milligram fresh weight of mycelium, and the same mycelium was then used for protein extraction.

Protein extraction. The mycelium was washed in distilled water (250 ml), filtered through nylon mesh (25 μ m), and blotted to remove excess moisture. The mycelium was weighed, transferred to a mortar chilled to -20° C, mixed with acid-washed sand (0.3 g) and liquid nitrogen, and ground to a fine powder. Extraction buffer (urea [Sigma], 5.4 g; Nonidet NP-40 [Sigma], 0.4 ml; Pharmalyte 3-10 [Pharmacia LKB Biotechnology Inc, Piscataway, NJ], 0.2 ml; β -mercaptoethanol [Sigma], 0.2 ml; and distilled water to 10 ml) was added in a volume (μ l) equal to the weight (mg) of the original mycelium; this constant ratio ensured that

the protein concentration (μ g/ μ l) was similar for all samples. The resulting paste was centrifuged at 200,000 *g* at 10 C for 1 h to remove cell debris, and the supernatant containing the polypeptides was collected and used immediately for electrophoresis. Reagents in the extraction buffer interfere with protein determination, so the approximate protein concentration was determined by using the Bradford assay (1) with preliminary samples extracted into 0.1 M Tris-HCl buffer, pH 6.8. A volume of buffer (μ l) equal to the fresh weight (mg) of mycelium was added, and a protein concentration of approximately 3.5 μ g/ μ l of buffer was consistently obtained. The extraction buffer may have resulted in a higher protein concentration because it contains urea, sodium dodecyl sulfate (SDS), and β -mercaptoethanol; these reagents disrupt membranes and assist the release of membrane-bound proteins. However, the absolute quantity of protein applied was not critical, providing that the same quantities were applied for each isolate in each comparison.

Two-dimensional electrophoresis. All two-dimensional electrophoresis procedures were based on those of O'Farrell (21) and were performed with a Protean II apparatus (Bio-Rad, Richmond, CA). The entire procedure consisted of three stages: isoelectric focusing (IEF) in the first dimension, an equilibration stage, and then denaturing electrophoresis in the second dimension.

IEF in the first dimension. Isoelectric focusing over the pH range 3.0–10.0 was performed. Tube gels (175 \times 1.5 mm, i.d.) were prepared by mixing aqueous acrylamide (9.7%, w/v)/bisacrylamide solution (0.3%, w/v) (2.5 ml), Nonidet NP-40 (Sigma) (0.1 ml), urea (Sigma) (2.7 g), Pharmalyte 3-10 (Pharmacia) (0.32 ml), and ammonium persulfate (Bio-Rad) (760 μ g). Eight gels were prepared and prefocused at 500 V for 500 V h by using fresh NaOH (0.1 M) as the catholyte and phosphoric acid (0.06%, v/v) as the anolyte. The gels were first overlaid with an aqueous solution containing Pharmalyte 3-10 (5%, v/v) and Nonidet NP-40 (2%, v/v). Then, 30 μ l (100 μ g) of the freshly prepared protein extract was layered between the gel surface and the overlay solution. Four replicate gels were used for one isolate, and four replicate gels were used for the other isolate in each comparison. The gels were focused at 500 V and 15 C for a further 8,000 V h.

Equilibration of first dimension gels. After focusing, the gels were extruded into microcentrifuge tubes (1.5 ml) filled with an equilibration buffer (1.0 ml) that consisted of 0.12 M Tris-HCl, pH 6.8, containing glycerol (10%, v/v), SDS (5%, w/v), and β -mercaptoethanol (2%, v/v). The cathodic (basic) ends were stained with bromophenol blue, and the gels were stored at -80° C in the equilibration buffer. Immediately before electrophoresis in the second dimension, the gels were transferred to fresh equilibration buffer (0.5 ml) and incubated at 40 C for 10 min.

Electrophoresis in the second dimension. The equilibrated gels were laid on the top of a slab gel (16 \times 16 cm) that contained polyacrylamide (10%, w/w) and SDS (0.1%, w/w) (7) and were electrophoresed with molecular weight standards (Bio-Rad) at 30 mA until the bromophenol blue tracking dye reached the bottom of the gel. Two slab gels were freshly prepared and run at the same time, one for each strain in each comparison. Electrophoresis in the second dimension was repeated three times for each pair of comparisons. The entire experiment (three gels per strain) was performed three times for G-20 vs. G-3, and once each for G-15 vs. G-4 and G-20-4VIB vs. G-20A. One complete set (three pairs) of the G-3 vs. G-20 gels was stained with a silver-staining kit (Bio-Rad); all other gels were stained with Coomassie Brilliant Blue R250 (Bio-Rad) (7).

Computerized analysis of gels. The computerized analysis was based on the method described by Hruschka (11,12). Color Ektachrome ASA 64 (Eastman Kodak Co., Rochester, NY) slides of the gels were taken and viewed with a videocon camera (Sierra Scientific, Mountain View, CA) connected to a Grinnell 270 image processor (Grinnell Co., San Jose, CA); the resulting digitized images were stored in a Hewlett Packard 1000F computer (Hewlett Packard, Rockville, MD). The images were smoothed by horizontal and vertical passes of a three-pixel Gaussian filter, and background was removed with a 5 \times 5 Laplacian filter.

The images of the three replicate gels from one strain were summated to form a single composite image; this was then compared with the composite image from the other strain in each comparison between gels. Before images were summated and compared, they were brought into registration with each other by the following procedure. Sixteen landmark locations were chosen to cover the whole area of one gel image. For each location, the green image from one gel replicate was scrolled over the red image from another gel replicate so that matching protein spots in that region appeared in yellow. The pair was marked, and the locations were stored in memory. A two-dimensional quadratic least squares polynomial was calculated between the corresponding spot locations and was applied to all green pixels to bring them into registration with the red image. The third replicate was incorporated in the same way to produce a single composite image of all gel replicates. The composite image from one strain was compared to the composite image from the other strain by using the same polynomial matching procedure. In the resulting image, common spots appeared yellow, whereas unique spots were red or green, which allowed accurate identification of gel similarities and differences.

RESULTS

Metabolite identification and quantification. The quantities of gliotoxin and viridin produced by all strains are shown in Table 1. HPLC retention times for gliotoxin and viridin were 6.6 and 8.5 min, respectively. In addition, TLC was used routinely to detect metabolites in all Weindling's medium culture filtrates

TABLE 1. Quantification of gliotoxin and viridin produced in Weindling's medium (30.0 ml) by strains of *Gliocladium virens*^a

Strain of <i>G. virens</i>	Gliotoxin ($\mu\text{g}/\text{mg}$)	Viridin ($\mu\text{g}/\text{mg}$)
G-15	1.36 \pm 0.20	1.38 \pm 0.28
G-20	1.53 \pm 0.26	1.28 \pm 0.19
G-3	0.00	3.97 \pm 0.61
G-4	0.00	6.09 \pm 0.85
G-20-4VIB	2.35 \pm 0.62	2.28 \pm 0.97
G-20A	0.69 \pm 0.10	0.21 \pm 0.12

^a Yields ($\mu\text{g}/\text{mg}$ of mycelial dry weight) are expressed as the means of three replicates \pm the standard deviation.

before protein extraction and electrophoresis. Relative TLC migration distances (R_f) for gliotoxin and viridin were 0.54 and 0.59, respectively. Strains G-15 and G-20 always produced gliotoxin and viridin, and strains G-3 and G-4 always produced viridin. On no occasion did strains G-3 and G-4 produce gliotoxin.

Visual appearance of electrophoresis gels. The method resulted in well-separated polypeptides with little gel-to-gel variation. The overall appearance of gels was similar for all strains, indicating their similar physiology and genetic expression. The intensities of spots on single gels varied considerably. Those spots that were on the lower detection limit with Coomassie Brilliant Blue R250 were considered to be less abundant cell constituents (minor polypeptides), and those spots that stained intensely with Coomassie Brilliant Blue R250 were considered to be relatively abundant cell components (major polypeptides). Although the silver stain could detect faint polypeptide spots, gel regions with large quantities of protein were so intensely stained that it was not possible to delimit individual spots; Coomassie Brilliant Blue R250 was more effective at delimiting spots in such regions. However, the different colors of the polypeptides on silver-stained gels were useful for orientation and visual comparison of the spots. Thus, by combining the results from the silver-stained gels with the results from the Coomassie Brilliant Blue R250 stained gels in the G-20 vs. G-3 experiments, an accurate comparison of both major and minor polypeptides could be achieved.

The objective of these experiments, however, was not to identify every polypeptide but rather to compare patterns from different strains so that polypeptides unique to gliotoxin-producing strains could be identified. Therefore, Coomassie Brilliant Blue R250 staining was considered to be sufficient for comparison of G-4 vs. G-15 and G-20-4VIB vs. G-20A. The appearance of the Coomassie Brilliant Blue R250 stained gels from G-3 and G-20 is shown in Figure 1; SDS-polyacrylamide gel electrophoresis (PAGE) and IEF axes are oriented by using the standardized convention of the International Electrophoresis Society.

Comparisons of polypeptide differences. A rigorous analysis of gels could be achieved with the computer image-enhancing system, because composite images from the replicates were used and one image could be scrolled precisely over the other image. Regions of interest could also be magnified for detailed analysis.

The computer-generated composite images of Coomassie Brilliant Blue R250 stained gels of the G-3 vs. G-20 comparison are shown in Figure 2A and B; polypeptide differences from that comparison are shown in Figure 2C. The differences shown in

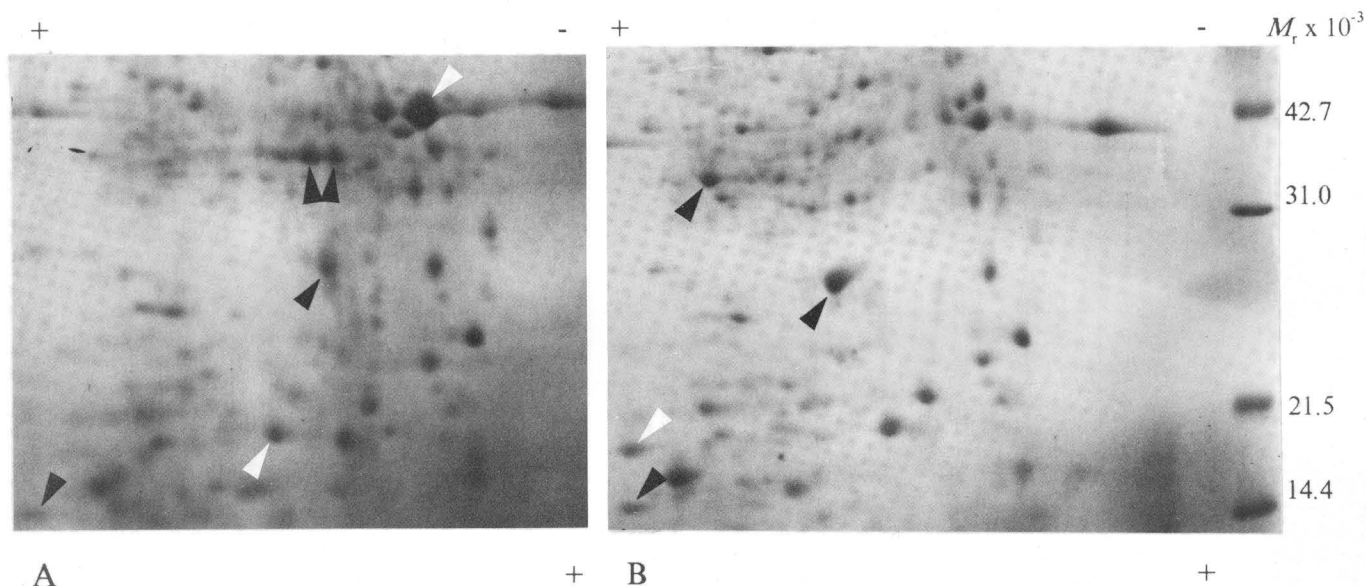


Fig. 1. Sections from gels stained with Coomassie Brilliant Blue R250 that show comparison of mycelial polypeptides from A, gliotoxin-producing *Gliocladium virens* G-20 with B, nonproducing strain G-3. Major polypeptides consistently present in one strain and absent from the other strain are shown by the black arrows. Polypeptides that are consistently more abundant in one strain than in the other are shown by the white arrows. Molecular weights (M_r) of standard proteins are shown in kilodaltons.

Figure 2C were compiled by investigating at maximum sensitivity every spot on the color monitor. Certain regions that were obvious artifacts were ignored in the compilation. For example, there are apparently a number of polypeptides near the left margin in Figure 2B; this was caused by a vertical streak from material that accumulated in the phosphoric acid at the bottom of the IEF gel. Therefore, because Figure 2A and B are actual printouts from the computer, there may be certain differences that are not represented in the composite Figure 2C. Differences from all other comparisons were compiled in the same way and are shown in Figure 2D-F. Gels were scanned for the presence or absence of polypeptide spots, and the observed differences between each comparison are summarized in Table 2.

Polypeptides of interest were characterized by using coordinates that corresponded to their relative molecular weight (M_r) and their isoelectric point (pI). In determining M_r , the relative migration distance (R_f) of the polypeptide spot was first determined; R_f was then used to calculate the M_r from a graph of $\log_{10} M_r$ of known standards plotted against their R_f . The pI was determined by measuring the distance that the polypeptide spot had migrated from the cathode. Although this method assumes a linear gradient between pH 3.0 and 10.0 and gives only an estimate of the pI, it was considered to be sufficient for this preliminary characterization of polypeptides.

The technique resolved polypeptides with molecular weights ranging from 100 to 10 kDa; polypeptides outside this range would

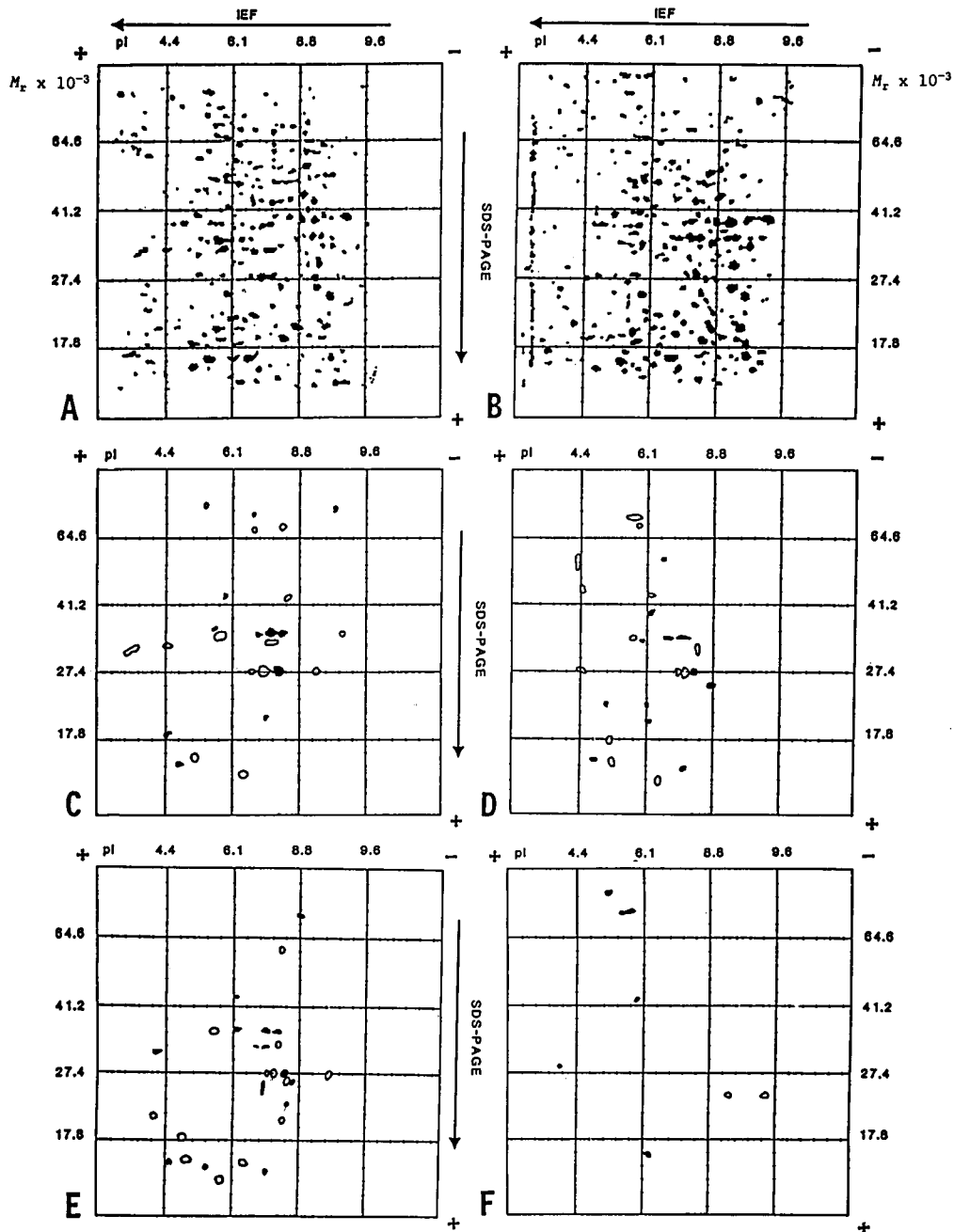


Fig. 2. Comparison of polypeptide differences by using image-enhanced computerized analysis. A, B, computer-generated composite images of Coomassie Brilliant Blue R250 stained gels from A, G-3 and B, G-20. C-F, schematic diagrams from computerized comparisons showing: C, polypeptides detected by Coomassie Brilliant Blue R250 staining that were unique either to G-20 (filled spots) or to G-3 (open spots); D, polypeptides detected by silver staining that were unique either to G-20 (filled spots) or to G-3 (open spots); E, polypeptides detected by Coomassie Brilliant Blue R250 that were unique either to G-15 (filled spots) or to G-4 (open spots); F, polypeptides detected by Coomassie Brilliant Blue R250 that were unique either to G-20-4VIB (filled spots) or G-20A (open spots). Ordinate: electrophoresis in the presence of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with relative molecular weights (M_r) marked in kilodaltons (kDa); abscissa: isoelectric focusing (IEF), which shows isoelectric point (pI) marks.

not be detected. It is also possible that some mycelial polypeptides were not solubilized by the urea buffer. However, because the protein extraction procedures were identical for all strains, these limitations were not considered to be a problem for this comparative study.

Both of the gliotoxin-producing strains, G-15 and G-20, possessed two major 33.8-kDa polypeptides with pIs of 7.1 and 7.35. Although these polypeptides were completely absent from G-4 and G-3, these nonproducing strains possessed instead a major polypeptide with the same molecular weight but with a pI of 5.8 (Fig. 1; Fig. 2C-E). All these polypeptides stained intensely with Coomassie Brilliant Blue R250 and stained a dull red color with silver.

A similar type of difference was also observed with a major 27.2-kDa polypeptide; in the gliotoxin-producing strain the pI was 7.25, and in the nonproducing strain the pI was 6.8. This polypeptide stained a deep blue color with silver. In the Coomassie Brilliant Blue R250 stain and the silver-stain comparisons of G-3 vs. G-20, there were 25 observed differences (Table 2). In the comparison of G-4 vs. G-15, there were 27 observed differences. However, when the differences common to both sets were totaled, there were just eight observed unique differences between the two producers and the two nonproducers (Table 3).

Some polypeptides were consistently more intense in either gliotoxin producers or in nonproducers. However, because the dye-binding ability of such polypeptides was unknown, no attempt was made to accurately quantify the relative intensities of the spots. Major polypeptide spots with M_r (pI) coordinates of 38.6 (8.1) and 18.7 (6.75) were consistently more intense in gliotoxin-producing strains; a major polypeptide with the coordinates 18.3 (5.0) was more intense in nonproducing strains (Fig. 1).

The seven observed differences between G-20-4VIB and G-20A were all minor polypeptides (Fig. 2F; Table 2).

DISCUSSION

The crude mycelial extracts used in this study contained mixtures of soluble polypeptides that originated from the cell wall, cell membrane, and the intracellular cytoplasm of *G. virens*. A large number of soluble polypeptides was common to both gliotoxin-producing strains and nonproducing strains, indicating that the general physiology of the two types was similar. There were,

however, some consistent differences. The most striking difference occurred with certain polypeptides that stained intensely with Coomassie Brilliant Blue R250 and so are likely to be major constituents of the cell. These major polypeptides may be the specific biosynthetic enzymes involved with gliotoxin production. Alternatively, they may be involved with more general cell physiology of gliotoxin-producing strains such as membrane structure and function.

Although we have identified polypeptides associated with gliotoxin-producing strains, our results do not prove that the polypeptides are directly involved in gliotoxin production. Instead, the polypeptides may be involved in some other aspect of physiology unique to gliotoxin-producing strains. Further proof of a consistent association could be established if the polypeptides were detected in a much larger number of gliotoxin-producing strains, and we are currently investigating this in our laboratory. However, the most definitive proof of a direct association could be achieved if specific genes were mutated so that the polypeptides were not expressed, and gliotoxin production was prevented. Such site-specific mutation has already been achieved in the fungus *Cochliobolus carbonum* (28). Our preliminary characterization of the polypeptides will now enable such studies to be undertaken. For example, the N-terminal amino acid sequences of the polypeptides could be obtained and used to construct DNA probes for the identification of their respective genes from a *G. virens* genomic library. If defective versions of the gene can be introduced into *G. virens* by using transformation procedures developed for this fungus (23,30), the specific involvement of the polypeptides in gliotoxin production can be determined.

A physical relationship appears to exist between some of those major polypeptides that are unique to gliotoxin producers and those that are unique to nonproducers. For example, gliotoxin producers possess two 33.8-kDa polypeptides that stain red with silver and have pIs of 7.35 and 7.1. These polypeptides are completely absent from nonproducers, which possess instead a red-staining 33.8-kDa polypeptide with a pI of 5.8. A similar type of difference occurs at 27.2 kDa when the isoelectric point of a polypeptide that stains blue with silver is changed from pI 7.25 to 6.8 in the nonproducing strains. Thus, although the pIs may differ, these major polypeptides may be essentially the same in both gliotoxin producers and in nonproducers. However, the altered pIs may indicate that certain amino acid sequences have been modified, thus affecting the structure and function of such polypeptides.

Synthesis of a secondary metabolite is a polygenic characteristic (20). For example, over 200 genes are involved in tetracycline production; coding for biosynthetic enzymes and other proteins involved in synthesis of precursors, coenzymes, and cofactors; energy metabolism; cell permeability; architecture; and internal resistance to the antibiotic (20). In spite of this, there were only between 25 and 28 observed polypeptide differences between gliotoxin-producing strains and nonproducing strains (Table 2). However, biosynthetic enzymes associated with secondary metabolism are often transient and are produced in low amounts (31). Thus, a higher resolution technique may enable more gliotoxin-associated polypeptides to be identified. A more effective technique for identifying low abundance polypeptides would be to extract mRNAs uniquely expressed by gliotoxin-producing strains; these could then be translated to polypeptides in vitro or used directly to prepare a cDNA library of genes uniquely expressed by gliotoxin-producing strains.

Although strains G-20A and G-20-4VIB were derived from a single parent strain G-20, there was a 3.4-fold difference in their respective yields of gliotoxin. This may have practical significance, because improved strains could possibly be isolated by using single-sporing techniques. Although the gel profiles were similar, some polypeptide differences could be detected, and these may influence the yield of gliotoxin. However, a higher resolution technique such as mRNA isolation may have revealed more differences. The comparison of G-20A with G-20-4VIB is significant, because these strains must have a similar genetic makeup. However, the difference in gliotoxin yield was probably not suffi-

TABLE 2. Summary of comparisons of polypeptides extracted from gliotoxin-producing strains of *Gliocladium virens* (G20, G-15, G-20-4VIB) with polypeptides extracted from strains that produce reduced amounts of gliotoxin (G-20A) or no gliotoxin (G-3, G-4)

Stain ^a	Comparison (strain A/strain B)	Number of polypeptides		
		In common	Unique to strain A	Unique to strain B
CB	G-20/G-3	170	11	14
S	G-20/G-3	154	12	13
CB	G-15/G-4	146	15	13
CB	G-20-4VIB/G-20A	153	5	2

^a CB = Coomassie Brilliant Blue R250; S = silver stain.

TABLE 3. Molecular weight (M_r) and isoelectric point (pI) estimates for polypeptides consistently and uniquely associated with gliotoxin-producing strains of *Gliocladium virens* and with strains that do not produce gliotoxin

Gliotoxin-producing strains ^a		Nongliotoxin-producing strains ^b	
M_r	pI	M_r	pI
33.8	7.35	33.8	5.8
33.8	7.1	27.2	6.8
27.2	7.25	27.2	6.5
15.1	4.6	15.8	5.0

^a G-20, G-15, G-20-4VIB, G-20A.

^b G-3, G-4.

cient to detect any major polypeptide differences. An ideal method of identifying gliotoxin-associated polypeptides or mRNA would be to grow a single strain under two nutrient conditions that either enhance or repress completely the production of gliotoxin.

Our results show that certain soluble polypeptides are associated with the mycelium of gliotoxin-producing strains of *G. virens*. Recently, we have prepared antibodies against two of these polypeptides by excising the relevant spots directly from two-dimensional gels (8) and by using them directly as antigens (26). Such antibodies could be used in an ELISA procedure to confirm the association between the polypeptides and gliotoxin production in a screen that involves more strains. Labeled antibodies could also be used to identify where within the cell these unique polypeptides are located; this information might help in determining the function of such polypeptides. The influence of pH and substrate and environmental factors on the expression of such polypeptides could also be investigated with antibodies, and a sensitive method for monitoring gliotoxin-producing strains in soil could even be developed.

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