Viruslike Particles in Tentoxin-Producing Strains of *Alternaria alternata*

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Double-stranded (ds) RNAs associated with viruslike particles (VLPs) have been found in six isolates of *Alternaria alternata* which produce tentoxin. Isolates had from one to three dsRNAs ranging in size from 1.0 to 5.1 kilobase pairs. In two isolates the dsRNAs were associated with 30-nm particles. No dsRNA was detected in any of six other tentoxin-producing isolates or nine isolates which did not produce tentoxin.

Double-stranded (ds) RNA molecules associated with viruslike particles (VLPs) have been found in a large number of fungal species (3). In general, these VLPs are isodiametric and 35 to 45 nm in size. They contain one to several dsRNA segments and one major protein. They are transmitted only vegetatively, have no infectious activity, and are not associated in most cases with a distinct phenotype. Despite their similarities, VLPs found in different species are immunologically distinct. In all these regards, fungal VLPs resemble cryptic dsRNA plant viruses (1).

The fungus *Alternaria alternata* (Fr.) Keissler produces a tetrapetide toxin, tentoxin, which causes chlorosis in seedlings of sensitive plants (8). In studies on the biosynthesis of tentoxin, dsRNAs have been found in six strains which produce tentoxin.

Isolates of *A. alternata* were obtained from surface-sterilized cotton seeds at Southern Regional Research Center. Still cultures of the isolates were grown in 50 ml of Fries medium (19) in a 125-ml flask under continuous light at 28°C for 3 weeks. The resulting fungal mat, weighing between 2 and 3 g, was removed and used for the isolation of RNA and VLPs. The culture medium was assayed for the presence of tentoxin (17). Isolates described as producers of tentoxin contained greater than 80 μg tentoxin in the medium. Nonproducers contained less than the minimum detectable level of 30 μg tentoxin in the medium.

RNA was isolated initially by the method of Chirgwin et al. (6), with 5 M guanidine isothiocyanate as a protein denaturant. VLPs were isolated by grinding a fungal mat in 5 ml of 30 mM sodium phosphate buffer per g, pH 7.6 (P buffer), in a chilled mortar and pestle or with a Tekmar Tissumizer. After cellular debris was pelleted for 20 min at 17,000 × g in an SS-34 rotor (Ivan Sorvall, Inc., Norwalk, Conn.), VLPs were pelleted from the supernatant at 150,000 × g for 6 h in a TH-641 rotor (Sorvall). This VLP pellet was suspended in 100 μl of P buffer, mixed with 11 ml of 40% (wt/wt) CsCl in P buffer, and spun at 128,000 × g for 42 h in a TH-641 rotor (Sorvall). 0.5-ml fractions were collected. Fractions containing dsDNA were identified by adding 500 μl of dH2O and 100 μl of 10% sodium dodecyl sulfate to 100 μl from each fraction, precipitating the nucleic acids by the addition of 1.4 ml of ethanol, and then analyzing the pellet on a 1.4% agarose gel (14). The buoyant density of fractions containing dsRNAs was determined from the refractive index measured with a refractometer (Valentine Instruments, Richmondville, N.Y.). After the addition of 4 volumes of P buffer, VLPs were pelleted from fractions containing dsRNA at 175,000 × g maximum for 18 h in a TH-641 rotor (Sorvall). The final VLP pellet was resuspended in 50 μl of P buffer and stored at −20°C.

One-third of the resuspended VLP pellet was treated with 2% lithium dodecyl sulfate and 1% β-mercaptoethanol for 3 min at 100°C and run on a 9% polyacrylamide gel (12), and the proteins were stained with Coomassie brilliant blue. For analysis of dsRNAs in the VLPs, one-third of the resuspended VLP pellet was solubilized in 2% sodium dodecyl sulfate at room temperature, phenol extracted, and then separated on a 5% polyacrylamide gel. For electron microscopy, samples were negatively stained with 1% phosphotungstic acid (pH 7.0) and examined with a Philips 200 electron microscope.

dsRNAs from fungal mats were isolated by the CF-11 cellulose method of Jordan (11), with 19% ethanol. For electrophoresis, 5 μl of the dsRNA preparation per g of fungal mat was run on an agarose gel.

dsRNAs were first noted as extra bands in total RNA preparations from two isolates of *A. alternata*. The extra bands were resistant to digestion by RNase A in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), DNase I, and RNase but sensitive to RNase A in 10 mM Tris, pH 7.6 (data not shown). RNAs were totally digested by RNase A both with and without 2× SSC. These RNA species were bound by CF-11 cellulose (Whatman, Inc., Clifton, N.J.) in 19% ethanol, which is also characteristic of dsRNA. A screening of isolates of *A. alternata* revealed the presence of dsRNAs in 6 of 12 tentoxin-producing isolates but in none of 9 nonproducing isolates (Table 1). Isolates 8 and 64 were selected for further characterization. The dsRNA of isolate 8 migrated to approximately 5 kilobase pairs, and the dsRNAs of isolate 64 were approximately 1.4, 1.5, and 1.7 kilobase pairs, compared with bacteriophage lambda DNA fragments.

VLPs of isolate 8 had a buoyant density in CsCl of 1.43 g/cm³, while those of isolate 64 had a density of 1.37 g/cm³. VLPs were disrupted with sodium dodecyl sulfate or lithium dodecyl sulfate and separated on a 5% polyacrylamide gel for dsRNA (Fig. 1) or a 9% polyacrylamide gel for proteins (Fig. 2). The dsRNAs contained in the VLP pellet were the same size as the dsRNAs isolated in total RNA preparations or as those isolated with CF-11 cellulose. Isolate 8 had two major protein components of approximately 90 and 95 kilo-
TABLE 1. Detection of tentoxin and dsRNA from isolates of A. alternata

<table>
<thead>
<tr>
<th>Isolate(s)</th>
<th>Tentoxina</th>
<th>dsRNA sizesb</th>
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<tbody>
<tr>
<td>2</td>
<td>+</td>
<td>2.2</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>5.1</td>
</tr>
<tr>
<td>64</td>
<td>+</td>
<td>1.4, 1.5, 1.7</td>
</tr>
<tr>
<td>242</td>
<td>+</td>
<td>4.5</td>
</tr>
<tr>
<td>309</td>
<td>+</td>
<td>3.0, 4.0</td>
</tr>
<tr>
<td>251</td>
<td>+</td>
<td>1.0, 2.5, 3.0, 4.5</td>
</tr>
<tr>
<td>23, 245, 247, 2013, 2020, 2022</td>
<td>+</td>
<td>—c</td>
</tr>
</tbody>
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a Detected by bioassay (17). Minimum level of detection was 30 μM.
b Detected by agarose gel electrophoresis following CF-11 cellulose procedure (11). Size in kilobase pairs compared with bacteriophage lambda DNA markers.
c —, None detected.

daltons (Fig. 2, lane 2), while isolate 64 had major protein bands at 40 and 45 kilodaltons (Fig. 2, lane 1).

Electron microscopy showed an isodiametric or hexagonal particle 28 to 30 nm in size in VLP preparations from both isolates (Fig. 3).

In an attempt to alter the amount of VLP in the fungus, cultures were grown at 35°C (20). Temperatures higher than 37°C resulted in no growth. Because of the difficulty in obtaining single cells, these experiments utilized whole fungal mats; however, after 3 weeks, dsRNA was barely detectable or absent from the cultures (Fig. 4) and tentoxin was not detectable in the medium. When those cultures were used to inoculate fresh media and were grown at 28°C, both dsRNA and tentoxin were restored to usual levels (Fig. 4). Similar results were found when cultures were grown in the

FIG. 1. dsRNAs found in CsCl gradient fractions containing VLPs; separated on a 5% polyacrylamide gel. Lane 1, Isolate 64; lane 2, isolate 8. Size markers are HindIII-EcoRI fragments of bacteriophage lambda DNA, with sizes given in kilobase pairs.

FIG. 2. Lithium dodecyl sulfate-polyacrylamide gel electrophoresis of VLPs from CsCl gradient fraction, disrupted with 2% lithium dodecyl sulfate at 100°C for 3 min. Lane 1, Isolate 64; lane 2, isolate 8. Markers are protein standards (Sigma Chemical Co., St. Louis, Mo.). The 66-kilodalton marker is visible in all lanes.

FIG. 3. Electron micrographs of VLPs from isolate 8 (A) and isolate 64 (B). Bar = 100 nm.
FIG. 4. Agarose gel (1%) of dsRNAs found in 1 g of cultures of isolate 8 grown at 35°C for 3 weeks and then returned to 28°C. Isolate 8 was grown at 28°C (lane 1) and at 35°C (lane 2) or was inoculated from 3-week-old 35°C culture and grown at 28°C (lane 3). Size markers are HindIII-EcoRI fragments of bacteriophage lambda DNA, with sizes given in kilobase pairs.

presence of 0.4 to 1 mM cycloheximide and when cultures were grown on a rotary shaker at 100 rpm (data not shown). Thus, treatments known to reduce either VLP (16, 20) or tentoxin (13) production were found to greatly reduce both VLP and tentoxin production.

The VLPs in these isolates of *A. alternata* are similar to other fungal VLPs in that the genetic material is dsRNA. Most fungal VLPs have only one major protein, but VLPs with two (15) and three (4) major proteins do occur. Both of the VLPs reported here have two major protein components. The sizes and shapes of these VLPs are similar to those previously reported, although these VLPs may be somewhat smaller, at 28 to 30 nm versus 35 to 40 nm (2, 3). An *A. alternata* VLP 40 nm in size previously reported (10) seems to be different from those reported here. Whether the strain produced tentoxin was not reported.

The presence of VLPs or dsRNAs in fungi has been associated with a distinct phenotype in only a few other cases (5, 7, 21). In yeasts, a well-characterized example of VLPs is the killer system of *Saccharomyces cerevisiae* (18). The relation of the presence of VLPs in *A. alternata* to a phenotype is unclear. No morphological or growth differences are apparent when infected isolates are compared with uninfected isolates. VLPs were not found only in tentoxin-producing isolates and not in nonproducing isolates. There are, however, tentoxin-producing isolates in which no VLP or dsRNA has been detected. A similar relation has been found for *Geotrichum candidum* (15), the causal agent of citrus sour rot.

To determine the effect of the presence of VLPs on *A. alternata*, experimental infection is necessary; however, infection of a fungus with a virus has been only rarely reported (3, 9, 16). Schmidt et al. (16) used infection to show the relationship between the presence of a VLP and lack of production of aflatoxin in *Aspergillus flavus*. If there is a relationship between the presence of VLPs and the production of tentoxin, an explanation is required for the lack of VLPs in approximately half of the tentoxin-producing strains. This lack could be due to infection at levels below the limits of detection, or a different type of stress could lead to the production of tentoxin. Alternatively, tentoxin production might predispose the fungus to VLP infection.

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**LITERATURE CITED**


