Double-Stranded RNAs Associated with La France Disease of the Commercial Mushroom

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ABSTRACT

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Double-stranded RNA (dsRNA) analysis was used to clarify the purported viral etiology of La France disease of the commercial mushroom, *Agaricus bisporus*. Healthy and diseased sporophores were collected at 65 sites on commercial farms, and dsRNA patterns were compared after phenol extraction, cellulose column chromatography, gel electrophoresis, and ethidium bromide staining. DsRNA was detected in 19 of 22 diseased sporophore isolates and none of 43 healthy sporophore isolates. The presence of a characteristic dsRNA pattern correlated positively with La France disease symptoms. The dsRNA pattern consisted of 2.50, 2.05, 1.90, 1.70, 1.10, 0.89, 0.58, and 0.53×10^6 molecular weight (MW) segments. Variation in the disease-specific dsRNA pattern between sporophore isolates involved deletions of various dsRNA segments and additions of 4.5, 0.34, 0.33, 0.28, and 0.27×10^6 MW dsRNAs. Symptoms associated with the dsRNAs manifested as a reduced yield and, at some sites, as sporophores with elongated stems and small caps. The disclosed association of a characteristic dsRNA pattern with diseased sporophores provides strong circumstantial evidence for the etiologic role of specific virus(es) in La France disease.

Additional key words: mycovirus, X-disease.

La France disease of the cultivated mushroom, *Agaricus bisporus* (Lange) Imbach, was first described after a severe outbreak at a commercial farm located in southeastern Pennsylvania (26). Today the disease occurs in most mushroom-growing countries (32). Numerous symptom syndromes have been ascribed to the disease (4,9,25,26) including a delay in the appearance of sporophores, reduced yield, and development of sporophores that are misshapen, show a premature opening of the veil, and an accelerated postharvest deterioration. The disease is difficult to control because of the unavailability of resistant germ plasm (25) and probably because of factors relating to an incomplete understanding of the disease cycle.

Hollings (9) proposed a viral etiology for La France disease based on the presence of viruslike particles (VLPs) in affected sporophores. Studies from several countries (4,9,12,15,17,20) have associated random mixtures of 25- and 35-nm spherical, 19- × 50-nm bacilliform and, less frequently, 19-, 29-, and 50-nm spherical particles with the disease. Biochemical and biological evidence supporting the viral nature of the VLPs has emerged slowly (2,27–29) because of the difficulty encountered in purifying the particles and lack of a reliable infectivity assay to satisfy Koch's postulates. Consequently, the number of viruses in *A. bisporus* and

which of them may be the causative agent of La France disease remain unclear. Further, the discovery that VLPs are widespread in healthy sporophores (20) has raised questions concerning the role of viruses in the disease (7).

Double-stranded RNA (dsRNA) analysis has proven valuable for the detection and diagnosis of virus infections in plants (16,31) and is ideally suited for fungi (5,11,16,18,22) because mycoviruses typically possess dsRNA genomes. Our objective was to determine the relationship between the occurrence of dsRNA and a pathological condition in *A. bisporus*. We report that the presence of a specific dsRNA pattern in sporophores of *A. bisporus* correlates positively with symptoms previously described for La France disease. The evidence, although circumstantial, corroborates the etiologic role of viruses in the disease.

MATERIALS AND METHODS

Source of tissue. Sporophores of white, off-white, and light cream varieties were collected from 65 crops representing 38 farms located in Chester County, PA. Samples were scored as healthy or diseased based on yield and sporophore morphology. Sporophores were washed, frozen in liquid nitrogen, and stored at −20 C for dsRNA analysis.

Isolation of dsRNA. A modification of the method after Pfannenstiel et al (21) was employed. Twenty grams of frozen sporophore tissue was homogenized for 2 min in a Waring blender at 6 C with 10 ml of 4 M NH_4OH, 10 ml of 0.1 M ethyleneglycol-bis-N, N′ tetraacetate, 30 ml of 10 M LiCl, 25 ml of distilled water, and 100 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline. The emulsion was separated by centrifugation at 9,400 *g* for 20 min (low-speed centrifugation) and the upper aqueous phase was recovered. Nucleic acids were precipitated overnight at −20 C with 2 volumes of cold 95% ethanol, collected by low-speed centrifugation, dried under nitrogen gas, and resuspended in 2 ml of sterile water.

Where indicated, dsRNAs were chromatographed on cellulose using the abbreviated protocol described by Morris and Dodds (16). After low-speed centrifugation, the initial aqueous phase was adjusted to 15% ethanol by the addition of 100% ethanol and to 25 mg of cellulose powder (CF-11, Whatman, Clifton, NJ) per milliliter. The slurry was stirred for 1 hr, and the cellulose was collected by low-speed centrifugation, resuspended in STE buffer (50 mM Tris-Cl, 0.1 M NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.0) containing 15% (v/v) ethanol (STE/15 buffer), and packed into a 0.9 × 15-cm column. The cellulose was washed at the rate of 36 ml/hr with 150–200 ml of STE/15 buffer to elute DNA and single-stranded RNA, and then with 25 ml of STE buffer to elute dsRNA. The dsRNA was precipitated with ethanol as before and resuspended in a minimal volume of sterile water.

Gel electrophoresis. Samples for electrophoresis consisted of a 10–80-μl aliquot of a dsRNA preparation adjusted to 10% sucrose, 0.002% bromophenol blue, 0.5% sodium dodecyl sulfate, and 0.4 mM EDTA (100 μl final volume). Samples were electrophoresed in 9-cm-long, 6-mm-diameter cylindrical 3% polyacrylamide gels (bis:acrylamide, 1:20) at 5 mA per gel for 4 hr. Electrophoresis buffer consisted of 40 mM Tris-Cl, 20 mM sodium acetate, and 0.83 mM EDTA, pH 7.8. Gels were incubated in a 0.002% solution of ethidium bromide containing 1 mM EDTA for 30 min and then overnight in 0.3 M NaCl. Alternatively, nucleic acids were

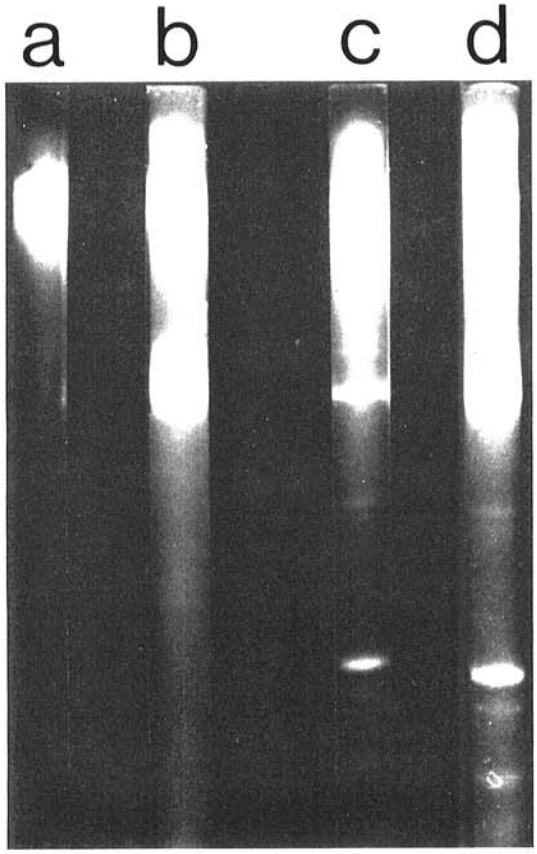

Fig. 1. Polyacrylamide gel electrophoretic analysis of the total nucleic acid fraction from healthy and diseased (isolate 2A17) sporophores. Lanes a and c, nucleic acids from healthy and diseased sporophores, respectively, after treatment for 1.5 hr at 30 C with 0.3 M NaCl and RNase A (25 μg/ml). Lanes b and d, nucleic acids from healthy and diseased sporophores, respectively. Nucleic acids were electrophoresed for 4 hr in 3% polyacrylamide gel and stained with ethidium bromide.

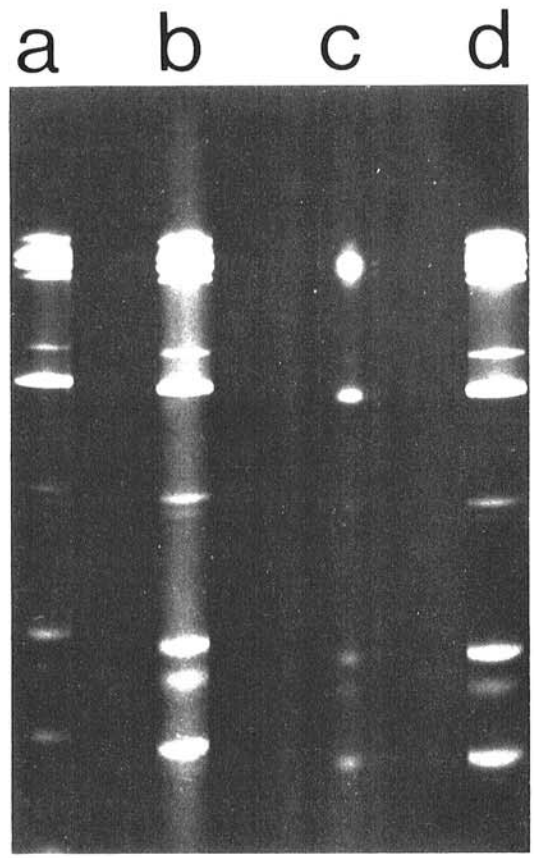

Fig. 2. Ionic strength-dependent sensitivity of the disease-specific nucleic acids in sporophore isolate 2A17 to hydrolysis by ribonuclease. The nucleic acids from diseased sporophores eluting in the dsRNA fraction (STE) upon cellulose chromatography were electrophoresed for 4 hr in 3% polyacrylamide gels and stained with ethidium bromide. Gels were incubated 1.5 hr at 30 C with: 0.3 M NaCl and RNase A (25 μg/ml) (lane a), 0.3 M NaCl (lane b), 0.03 M NaCl and RNase A (25 μg/ml) (lane c), and 0.03 M NaCl (lane d).

electrophoresed in a 1- × 12.5- × 19-cm slab gel of 0.9% agarose at 100 V for 2.5 hr. Electrophoresis buffer was 0.08 M Tris-phosphate and 8 mM EDTA, pH 7.8, containing 50 ng of ethidium bromide per milliliter. Nucleic acids were visualized with ultraviolet light (302 nm) and photographed with Polaroid type 55 film.

Confirmation of dsRNA. After electrophoresis, replicate gels were incubated in either a 0.3 M or a 0.03 M NaCl solution containing 25 μg of ribonuclease A (RNase A, Sigma Chemical Co., St. Louis, MO) per milliliter for 1.5 hr at 30 C. RNAs that hydrolyzed in low but not high salt were regarded as double-stranded.

Molecular weight determination. The molecular weights of the mushroom dsRNAs were estimated from their electrophoretic mobilities relative to the Bst E II restriction endonuclease fragments of lambda DNA assuming molecular weights of 5.49, 4.71, 4.14, 3.70, 3.13, 2.81, 2.39, 1.51, 1.25, 0.89, 0.82, 0.46, and 0.15 × 10^6 (New England Bio Labs, Inc., Beverly, MA). Molecular weights estimated for the replicative forms of tobacco mosaic virus RNA using the DNA standards agreed closely with expected values.

RESULTS

Analysis of nucleic acids in healthy and diseased sporophores. Polyacrylamide gel electrophoresis and ethidium bromide staining of total nucleic acid fractions revealed the presence of numerous sharply defined nucleic acid species in diseased sporophore isolate 2A17 that were absent in healthy sporophores (Fig. 1). The disease-specific nucleic acid species were partially obscured by polydispersed nucleic acids considered to be of cellular origin because they also were isolated from healthy sporophores. Subjecting the nucleic acids in the gel to RNase A under high ionic strength conditions had no effect on the disease-specific nucleic acids but hydrolyzed some of the more rapidly migrating polydispersed nucleic acids, which, therefore, were presumed to be ribosomal RNA (rRNA). A slowly migrating polydispersed nucleic acid was thought to be DNA because it resisted digestion under these conditions.

The disease-specific nucleic acids eluted as dsRNA in the STE buffer fraction upon cellulose chromatography (Fig. 2). Conversely, gel electrophoretic analysis revealed that the polydispersed nucleic acids eluted in the STE/15 buffer fraction, a behavior characteristic of cellular DNA and rRNA. Nucleic acid was not detected in the dsRNA fraction from the healthy sporophore isolate used in this study.

Twelve dsRNA segments were detected in diseased sporophore isolate 2A17 in polyacrylamide gels. All segments resisted hydrolysis by RNase A under high, but not low ionic strength conditions, thereby substantiating their duplex nature (Fig. 2). Additional supportive evidence for the dsRNA nature of the disease-specific nucleic acids in sporophore isolate 2A17 was a positive precipitin reaction with antibodies to synthetic dsRNA (Poly I:Poly C) and a hyperchromic effect (T_m = 91.5 C) on thermal denaturation in 0.05 M phosphate buffer, pH 7.0 (Wach, *unpublished data*).

The principal symptom associated with the presence of dsRNA in sporophores was a reduced yield. Conspicuous barren areas existed on the surface of producing beds rather than a carpet of mature sporophores and primordia. At some sampling sites, reduced yield was not as evident. Thus, samples were scored as diseased on the basis of a subnormal yield. Sometimes reduced yields and deformed sporophores occurred concurrently. Most often, sporophores looked like a "drumstick," i.e., a small deformed pileus on an elongated and curved stipe (Fig. 3). Such sporophores were poorly anchored in the casing substrate, usually

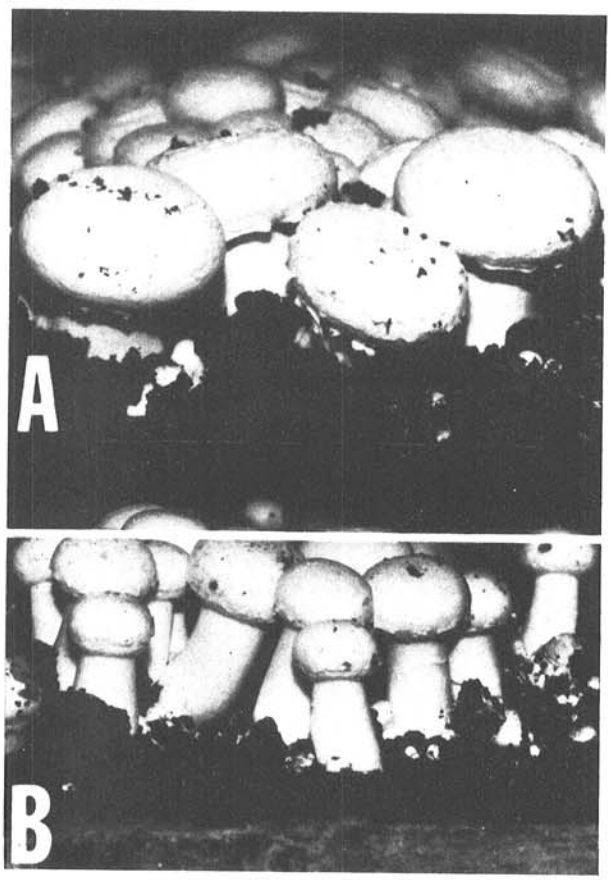

Fig. 3. Typical "drumstick" symptoms associated with the presence of dsRNA in the sporophores. **A,** Healthy sporophores. **B,** Diseased sporophores.

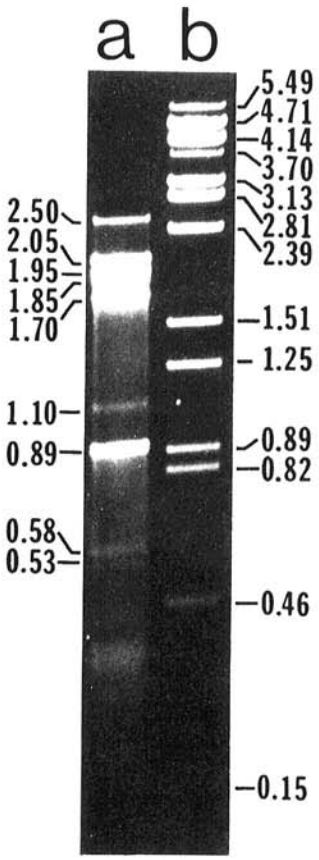

Fig. 4. Relative electrophoretic mobilities of the disease-specific dsRNAs in sporophore isolate 2A17 (lane a) and Bst E II restriction endonuclease fragments of lambda DNA (lane b). Electrophoresis was at 100 V for 2.5 hr through 0.9% agarose gel in 0.08 M Tris-phosphate and 8 mM ethylene diaminetetraacetic acid, pH 7.8. Molecular weights (× 10^6) are indicated.

peat moss, as measured by the ease with which they could be harvested. The disease appeared to be distributed throughout the growing room or confined to one or more discrete foci on the beds. Lipstick mold (*Geotrichium* spp.), a competitor fungus, often flourished in the casing and compost of affected crops.

Molecular weight determination. The molecular weights of the disease-specific dsRNAs in sporophore isolate 2A17 were estimated from their electrophoretic mobilities through a 0.9% agarose gel relative to the Bst E II restriction enzyme cleavage products of lambda DNA. Nine major dsRNA segments with estimated molecular weights (MW) of 2.50, 2.05, 1.95, 1.85, 1.70, 1.10, 0.89, 0.58, and 0.53×10^6 were resolved (Fig. 4). In a 3% polyacrylamide gel, the 1.95 and 1.85×10^6 MW segments appeared as a single band ($MW = 1.90 \times 10^6$) after electrophoresis for 4 hr but were resolved after electrophoresis for 12 hr (Wach, *unpublished data*).

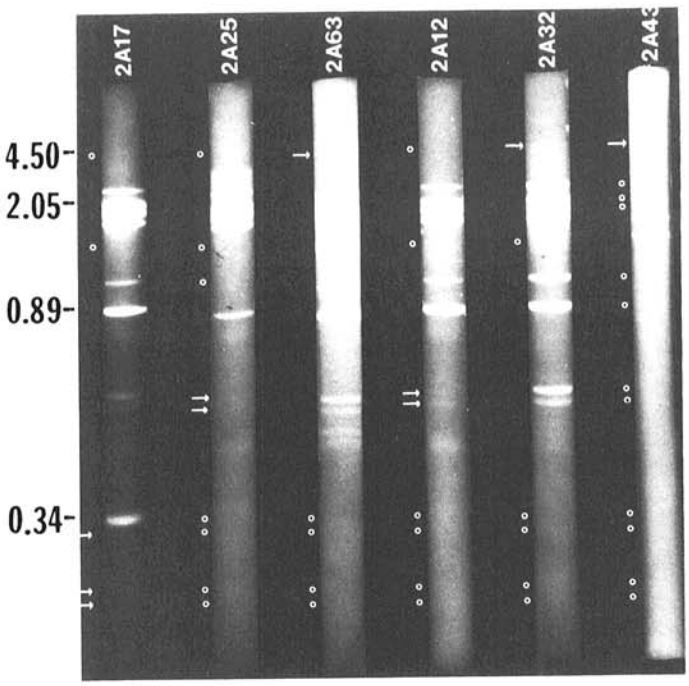

Fig. 5. Variation in the dsRNA pattern among diseased sporophore isolates 2A17, 2A25, 2A63, 2A12, 2A32, and 2A43. dsRNAs were electrophoresed for 4 hr in 3% polyacrylamide gel and stained with ethidium bromide. Arrows (→) are used to indicate faint bands that do not appear in the photograph and circles (o) indicate the absence of corresponding faint bands. Molecular weights ($\times 10^6$) are indicated.

Relationship between dsRNA and pathological status. From a total of 65 crops, 22 diseased and 43 healthy sporophore isolates were collected. Nineteen of 22 sporophore isolates showing disease symptoms contained dsRNA. In two of these cases, dsRNA was detected in apparently healthy sporophores collected early during the harvest cycle. However, the growers reported that the disease developed later in the cycle. Conversely, dsRNA was not detected in the 43 healthy sporophore samples and three diseased sporophore samples from crops showing inconspicuous yield losses.

Variation in dsRNA between sporophore isolates. A characteristic dsRNA pattern composed of 2.50, 2.05, 1.90, 1.70, 1.10, 0.89, 0.58, and 0.53×10^6 MW segments was detected in a majority of the diseased sporophore isolates (Table 1 and Fig. 5). The frequency at which any one particular dsRNA segment occurred among the isolates ranged from 63 to 95%. Because polyacrylamide gel electrophoretic analysis was used in this study, it remains unclear what proportion of the isolates contained the 1.90×10^6 MW dsRNA as a doublet. More recently, however, approximately 25 diseased sporophore isolates were analyzed and found to contain the 1.95 and 1.85×10^6 MW dsRNAs (Romaine, *unpublished data*). All of these isolates also possessed the 0.58 and 0.53×10^6 MW dsRNA segments, which in some instances could only be observed by overloading the gel.

Variation in the characteristic dsRNA pattern between diseased sporophore isolates involved the addition of 4.5, 0.34, 0.33, 0.28, and 0.27×10^6 MW segments. Isolate 2A63 contained an additional major 1.40×10^6 MW segment, which was resolved as a doublet after electrophoresis for 12 hr through a 3% polyacrylamide gel (Wach, *unpublished data*). Two isolates, 2A25 and GL, showed deletions of the 1.10 and 0.89×10^6 MW dsRNAs, respectively.

Three isolates, 2A37, 2A43, and p681, possessed dsRNA patterns that were clearly distinct from the characteristic pattern observed in the other 16 isolates. Because these distinctive dsRNA patterns occurred at a low frequency, it was not possible to determine their relationship to the disease.

DISCUSSION

The etiologic role of viruses in La France disease of *A. bisporus* is supported principally by electron microscopic studies that have revealed the presence of random mixtures of several types of VLPs in affected sporophores (4,9,12,15,17,20). A viral etiology for the disease was advanced after induction of characteristic symptoms in healthy sporophores through hyphal anastomosis with mycelium and spores from diseased cultures (9,25) and by mechanical inoculation with cell-free VLP preparations (4,9). However, the unauthenticated viral nature of the VLPs, widespread reports of

TABLE 1. Summary of the dsRNA segments detected in diseased sporophore isolates of *Agaricus bisporus*[a]

DsRNA segments	Sporophore isolate																			Frequency
($MW \times 10^6$)	Lap	HGWh	2A12	2A16	2A17	2A25	2A26	2A31	2A32	2A37	2A43	2A63	2A66	p681	Raf	GL	4082	4282	4882	(%)[c]
4.50	–[b]	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	21
2.50	+	+	+	+	+	+	+	+	+	–	–	+	+	–	+	+	+	+	+	84
2.05	+	+	+	+	+	+	+	+	+	–	–	+	+	–	+	+	+	+	+	84
1.90	+	+	+	+	++	+	+	+	+	–	–	+	+	+	+	+	+	+	+	89
1.70	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	95
1.40	–	–	–	–	–	–	–	–	–	–	+	++	–	–	–	–	–	–	–	11
1.10	+	+	+	+	+	–	+	+	+	–	–	+	+	+	+	+	+	+	+	84
0.89	+	+	+	+	+	+	+	+	+	–	–	+	+	–	+	–	+	+	+	79
0.58	+	–	+	–	+	+	+	+	+	–	–	+	+	–	–	–	+	+	+	63
0.53	+	–	+	–	+	+	+	+	+	–	–	+	+	–	–	–	+	+	+	63
0.34	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	11
0.33	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	11
0.28	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5
0.27	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5

[a] A dsRNA fraction was prepared from sporophore tissue by phenol extraction and cellulose chromatography. DsRNAs were resolved by electrophoresis in 3% polyacrylamide gel and visualized by staining with ethidium bromide. Data are based on one extraction of a 25-g sporophore sample and either two or three gel electrophoretic analyses for each isolate.

[b] + Denotes the presence and – the absence of the dsRNA segment. ++ Indicates a doublet.

[c] The number of sporophore isolates containing the dsRNA segment divided by the total number of isolates and multiplied by 100.

VLPs in healthy mycelial cultures and sporophores (19,20,30), the limitations of the pathogenicity trials (7), and an understanding that mycoviruses typically are innocuous in their hosts (3) have rendered the proposed viral etiology of the disease somewhat speculative.

Our study establishes a correlation between the presence of specific dsRNAs in sporophores and symptoms characteristic of La France disease. The disease-related dsRNAs described herein are identical to those we previously described (33) and probably correspond to many of those isolated from two diseased sporophore samples by Lemke (13) and Marino and co-workers (14). Further, the observation that a characteristic dsRNA pattern is associated with the disease implies a specific virus or a complex of viruses as the causal agent of the disease. Although dsRNA was not detected in the healthy sporophore isolates examined, we cannot exclude the possibility of their presence at levels below the resolution of our assay. In fact, several lines of evidence support a subliminal virus infection of healthy mushroom strains (8,9,20,30). Nevertheless, our findings confirm and extend the proposed viral etiology of La France disease predicated on dsRNA analysis rather than VLP observations. We acknowledge the evidence is correlative and that conclusive proof for a viral etiology requires purification of the putative virus(es) and pathogenicity tests.

Sporophores containing the dsRNAs did not always manifest the classical symptoms of La France disease (4,9,25,26). One easily recognized diagnostic symptom, barren areas delimited by malformed sporophores and the presence of *Geotrichium* spp., was observed; however, reduced yield was the only consistent indicator of infection. Consequently, we suspect the biological and economic significance of viruses in mushroom cultivation may be underestimated as virus-related crop losses may go unnoticed or may be easily confused with cultural malpractice. The determinants of symptom severity are largely unknown; however, virus genotypes, host nutrition, developmental stage at the time of infection, and the extent of infection are probably important. There is suggestive evidence that opportunistic secondary organisms, such as saprophagous nematodes, may exacerbate symptoms associated with the presence of disease-specific dsRNAs (24).

The relationship between the dsRNAs reported here and the various VLPs previously associated with the disease needs to be addressed. An obvious possibility is that the dsRNAs represent replicative forms of the 1.4×10^6 MW single-stranded RNA of the bacilliform virus (28,29) or the genomes of the spherical viruses (2,27). Presumably, variation in the dsRNA pattern reflects differences in the residing viruses. Some dsRNAs, in particular the $0.34–0.27 \times 10^6$ MW segments, could be truncated forms of the predominant larger segments (1) or possibly defective interfering or satellite RNAs (6,10). In support of the latter idea, there is correlative evidence for the role of small dsRNAs in moderating symptom severity (8). Results of a recent investigation suggest that the predominant disease-specific dsRNAs copurify with a 25-nm spherical particle (23). Studies are under way to establish the definitive relationship between disease-related dsRNAs and VLPs and to provide a succinct answer to the questions concerning pathogenicity.

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