Genetics

# Detection of Double-Stranded RNA in *Phytophthora infestans*

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## ABSTRACT

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Total nucleic acids were isolated from mycelial mats of *Phytophthora infestans* grown in V-8-juice broth at 18 C in darkness, and double-stranded RNA (dsRNA) was selectively purified by Whatman CF-11 cellulose chromatography. Polyacrylamide gel electrophoresis (PAGE) indicated that dsRNA was present only in Mexican isolates of *P. infestans*, most of which originated in the Toluca region. Thirty-six percent ($n$ = 40) of the Mexican isolates tested contained dsRNA, and three distinct PAGE profiles were observed. Sizes of dsRNAs of *P. infestans* were estimated at 2,750, 2,500, 1,600, and 1,500 base pairs. No dsRNA was detected in 20 isolates of *P. infestans* from diverse locations within the United States and Europe. Attempts to purify virus particles from isolates that contained dsRNA were unsuccessful. Isolates containing dsRNA showed a wide range in virulence and showed increased growth in V-8-juice broth compared with a group of randomly chosen dsRNA-free Mexican isolates. The presence of dsRNA in *P. infestans* provides a valuable new marker for genetic and epidemiological studies.

*Additional keywords*: mycovirus, potato late blight.

More than 40 species of plant-pathogenic fungi have been reported to contain viruses or viruslike particles (8,22). The majority of fungal viral genomes appear to consist of double-stranded RNA (dsRNA) (5,8–10,22).

In the genus *Phytophthora*, researchers have observed viruslike particles (VLPs) in nuclei of certain strains of *P. drechsleri* (12) and *P. infestans* (17). Attempts were not made by these workers to characterize the genomes of these VLPs, to extract dsRNA from the strains, or to purify viruses or VLPs from the strains.

Nuclear genes have been identified that code for isozymes in *P. infestans* (13), and the potential use of recombinant DNA techniques (3) to generate many additional nuclear markers appears promising. Cytoplasmic genetic markers, however, are lacking in *P. infestans*. Because dsRNA is usually present in the cytoplasm of virus-infected strains (9,10,22), its discovery in *P. infestans* would provide a valuable tool for genetic studies and for comparing strains of the pathogen from different geographic regions.

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New approaches to the control of potato late blight caused by *P. infestans* are also needed to reduce the current heavy dependence on fungicides. Suppression of virulence (hypovirulence) in several species of fungi has been shown to occur by cytoplasmically transmissible factors believed to be dsRNA (9,23). If such factors could be identified in strains of *P. infestans*, they could perhaps be introduced more widely into the fungal population to reduce the fitness and virulence of the pathogen.

Because of the need for cytoplasmic genetic markers in *P. infestans* and the potential use of dsRNA for conditioning fungal hypovirulence, our objectives were to determine whether isolates from different geographic regions contained dsRNA, to attempt to purify viruses from isolates of *P. infestans* containing dsRNA, and to determine whether dsRNA affects the growth or virulence of *P. infestans*.

## MATERIALS AND METHODS

**Isolates of *P. infestans*.** Forty isolates from Mexico and 20 isolates from the United States and Europe were assayed for the presence of dsRNA. The exact sources of the isolates were listed previously (18,19) except for isolate 139, which originated from Maine, isolate 162 (ATCC 16981) from West Virginia, and isolate

581 from Toluca, Mexico. The isolates exhibited wide diversity in isozyme phenotype, virulence, fitness components, and nuclear DNA content (18–20). Isolates from Mexico were of both A1 and A2 mating type, while all non-Mexican strains were of A1 mating type (19). The fungus was grown in 20% V-8-juice broth in 800-ml Roux bottles or in 2,800-ml Fernbach flasks at 18 C in darkness. Mycelial mats were harvested after about 4 wk of growth by vacuum filtration on Whatman No. 1 filter paper and were stored frozen at −80 C.

**Extraction and purification of dsRNA.** Double-stranded RNA was isolated from mycelial mats using a modification of method 1 of Morris and Dodds (11). Five grams of mycelium (about 1 g dry weight) was ground to a powder in a mortar and pestle containing liquid nitrogen. The following mixture of buffers and solutions was added to each sample: 5 ml of extraction buffer, 125 $\mu$l of mercapto-ethanol, 10 ml of chloroform-pentanol (24:1, v/v), and 10 ml of phenol saturated with 2× STE buffer (1× STE is 0.1 M NaCl, 0.05 M Tris, 0.001 M $Na_2EDTA$, pH 7.0). The extraction buffer consisted of 2× STE buffer, 1.5% SDS, 1% PVP-10, and 0.5% mercapto-ethanol.

Samples were homogenized with a Tissumizer homogenizer (Tekmar Co., Cincinnati, OH) for 1 min, stirred for 30 min, and then centrifuged at 6,780 *g* for 10 min at 4 C. The aqueous phase of the supernatant was recovered, adjusted to 16.5% ethanol, and subjected to CF-11 (Whatman, Clifton, NJ) cellulose column chromatography by adding 2.5 g of cellulose to the samples, stirring for 30 min, and pouring the suspension into 10- × 1.5-cm econo-columns (Bio-Rad, Rockville Centre, NY). The columns were washed with 150–200 ml of STE/16.5% ethanol, and the dsRNA was eluted with 10 ml of STE buffer. Three volumes of cold (−20 C) absolute ethanol and 1 ml of 3 M sodium acetate, pH 6.0, were added to the samples, which were then placed at −20 C overnight to precipitate the dsRNA. The precipitate was collected by low-speed centrifugation (7,710 *g* for 30 min at 4 C) and suspended in 250 $\mu$l of STE buffer, followed by a second cycle of precipitation with ethanol and sodium acetate (−80 C, 1 hr).

**Polyacrylamide gel electrophoresis.** Samples were prepared for electrophoresis by resuspending centrifuged ethanol precipitates in electrophoresis buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.8) containing 10% glycerol, 0.01% bromphenol blue, and 0.015% xylene cyanol. Samples were electrophoresed for 15 hr at 20 mA on vertical 5% polyacrylamide slab gels (16× 18× 0.15 cm) or for 2 hr at 110 V on 5% polyacrylamide minigels (7 × 8 × 0.075 cm). Gels were stained with 1 $\mu$g of ethidium bromide per milliliter for 15 min and then photographed on an ultraviolet trans-illuminator (Ultra-Violet Products Inc., San Gabriel, CA) on Polaroid type 55 or type 57 film using Wratten 9 (yellow) and 23A (red) gelatin filters.

Molecular weight standards included lambda DNA digested with *Hin*dIII (International Biotechnologies Inc., New Haven, CT) and brome mosaic virus (BMV) replicative form dsRNAs isolated from Henry barley. The four different BMV replicative form dsRNAs contain 3,234, 2,864, 2,117, and 875 base pairs, respectively (1,2), which correspond to approximate molecular weights of 2.26, 2.00, 1.48, and $0.61 \times 10^6$. Approximate molecular weights of the *P. infestans* dsRNAs were determined from a standard curve plotting electrophoretic mobility (on 5% poly-acrylamide gels) versus log of the molecular weight (4).

Nucleic acid preparations were tested for sensitivity to ribonuclease (RNase) under high and low salt conditions and for sensitivity to deoxyribonuclease (DNase) as follows. Samples were incubated for 30 min at 37 C with 0.1 $\mu$g of RNase per milliliter (bovine pancreatic ribonuclease A, Sigma Chemical Co., St. Louis, MO) in buffer consisting of 2× SSC (high salt) or 0.1× SSC (low salt) (1× SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0). Samples were incubated for a further 2 hr at room temperature with 4 $\mu$g/ml of proteinase K in 2× SSC and 0.05% SDS (26), and then precipitated in ethanol at −80 C before resuspension for gel analysis. Other samples were resuspended in TNM buffer (0.1 M Tris, 0.1 M NaCl, 0.1 M $MgCl_2$) and treated with 5 $\mu$g/ml of DNase (deoxyribonuclease 1, Sigma Chemical Co., St. Louis, MO) from a DNase stock solution consisting of 5 mg/ml of DNase in TNM buffer, 0.01 M $CaCl_2$, 5 mg/ml of proteinase K, and 50% glycerol (21). Lambda DNA digested with *Hin*dIII was included as a control treatment to verify DNase activity.

**Extraction and purification of VLPs.** Frozen mycelium (5–10 g) of *P. infestans*, isolate 519, was used for extraction and purification of VLPs using a variety of protocols (7,15,25). After two cycles of differential centrifugation, each partially purified preparation was layered on a 10–40% sucrose gradient buffered to the same molarity as the buffer used to resuspend the final high-speed pellet. The gradients were centrifuged in a Beckman SW40 rotor at 40,000 rpm (201,800 *g*) for 2 hr at 4 C and then scanned with an ISCO UA-5 absorbance monitor at 254 nm.

**Virulence and in vitro growth of dsRNA-infected isolates.** Cultures of *P. infestans* were grown on Rye A agar (6) in 9-cm diameter petri dishes for 10–14 days at 18 C in darkness. Sporangial suspensions were prepared using a cotton swab and by washing cultures with 10 ml of distilled water. Sporangial suspensions were concentrated by centrifugation at 1,000 *g* in a tabletop centrifuge and were adjusted to 50,000 sporangia per milliliter with a hemacytometer.

In vitro plantlets of 11 differential potato genotypes were obtained from L. J. Turkensteen, Research Institute for Plant Protection, Wageningen, The Netherlands. The genotypes and their major genes for late blight resistance (R-genes) were as follows: cultivars Norchip and Bintje (no known R-genes), CEBECO-43154-5 (R1), CEBECO-44158-4 (R2), CEBECO-4642-1 (R3), CEBECO-4431-5 (R4), Black 3053-18 (R5), Black XD2-21 (R6), Black 2182 ef(7) (R7), Black 2424 a(5) (R8), Black 2573 (2) (R9), Black 3618 ad(1) (R10), and Black 5008 ab(6) (R11).

The plantlets were propagated via cuttings, which were trans-planted into pots of Jiffy-Mix Plus (Jiffy Products of America, West Chicago, IL) and grown in a growth chamber at 18–20 C. The chamber was illuminated with 40W cool-white fluorescent tubes and incandescent bulbs on a 16 hr-light/8 hr-dark cycle (about 75 $\mu E\ m^{-2}\ sec^{-1}$).

Leaf disks were cut from leaves of 4–8-wk-old plants, using a no. 8 cork borer, and were placed lower surface upward on moist polyurethane foam in small, 16-compartment plastic boxes (Autogem Ltd., Howley Park, Morley, Leeds, England). Three leaf disks of each differential genotype were placed into each compartment within a box, so that each box contained three disks of each differential. A 40-$\mu$l drop of sporangial suspension of each isolate was placed onto each leaf disk. The boxes were covered with sliding plastic covers, placed at 12 C in darkness overnight, and then placed at 18 C under cool-white fluorescent tubes on a 16 hr-light/8 hr-dark cycle (about 25 $\mu E\ m^{-2}\ sec^{-1}$). All virulence experiments were repeated at least three times.

Five days after inoculation, the leaf disks were examined for sporulation of *P. infestans*. If sporulation was observed, the interaction was rated compatible; if no sporulation was observed, the interaction was rated incompatible. The number of compatible interactions for each isolate was recorded.

For experiments to assess the effects of dsRNA on in vitro growth of *P. infestans*, isolates were grown on Rye A agar plates for 7 days. Cork-borer plugs (no. 3) were cut from the colony margins and placed into 20 ml of 20% V-8-juice broth in 9-cm diameter Petri dishes. Other Rye A plugs were transferred to plates of Rye B agar (6). Five replicate plates of V-8 broth and five Rye B plates were inoculated per isolate per experiment, and five experiments were performed with each isolate. After inoculation, all plates were incubated at 18 C in darkness.

Radial growth on Rye B agar was assessed 7 days after inocula-tion of the plates by measuring colony diameters. Mycelial dry weight of isolates growing in V-8-juice broth was assessed by harvesting mycelial mats 10 days after inoculation. Mats were harvested onto Whatman No. 1 filter paper in a Büchner funnel, and liquid was removed by vacuum filtration. The mats were placed into small paper envelopes in a drying oven at 80 C for 48 hr and then weighed.

Data were analyzed by analysis of variance, and isolate means were separated using Bayes least significant differences (14). Contrasts (16, page 177) were used to compare means of dsRNA-

containing and dsRNA-free groups of isolates.

## RESULTS

**Detection of dsRNA in *P. infestans*.** Double-stranded RNA was detected in 14 of the 40 Mexican isolates tested, but not in any of the 20 isolates from the United States or Europe (Table 1). Three distinct electrophoretic banding patterns were observed for *P. infestans* dsRNA (Fig. 1). Patterns B and C each consisted of two bands that migrated close to one another on the gel. Pattern A appeared to comprise both sets of bands observed for patterns B and C (Fig. 1). The sizes of the dsRNAs observed for pattern B were estimated at 2,750 and 2,500 base pairs (about 1.9 and $1.8 \times 10^6$ daltons, respectively), while those of pattern C were estimated at 1,600 and 1,500 base pairs (about 1.1 and $1.0 \times 10^6$ daltons, respectively), using BMV replicative form dsRNAs as size markers.

Identification of bands observed on 5% polyacrylamide gels as dsRNA was confirmed by RNase treatment under high- and low-salt conditions and by DNase treatment. The dsRNA purified from BMV-infected barley and from isolates 547 (pattern A), 519 (pattern B), and 560 (pattern C) of *P. infestans* was degraded by RNase when incubated in 0.015 M NaCl and 0.0015 M trisodium citrate, but was unaffected by RNase incubation in 0.3 M NaCl and 0.03 M trisodium citrate (data not shown). These same dsRNAs were not digested by DNase, whereas the lambda DNA used as a control was completely digested by DNase treatment (data not shown).

**Attempts to purify VLPs.** None of the various methods we attempted allowed successful purification of VLPs from tissue known to contain dsRNA. No absorbance peak near 260 nm was observed when pellets from the final high-speed centrifugation were scanned from 220 to 320 nm on a Beckman DU-7 spectrophotometer, and no absorbing zones were observed on sucrose density gradients.

**Virulence and in vitro growth of dsRNA-containing isolates.** Virulence and growth on liquid and solid media were assessed for the 14 Mexican isolates found to contain dsRNA and for eight randomly chosen, dsRNA-free Mexican isolates (Table 2). Isolates with dsRNA showed high overall levels of virulence, with two of them able to attack all 11 differential potato genotypes (Table 2). However, one dsRNA-containing isolate (560) was avirulent and failed to infect even the susceptible control cultivars (Table 2). Two of the isolates with dsRNA (515 and 519) and one dsRNA-free isolate (520) consistently failed to produce enough sporangia for virulence assays to be performed.

Isolates differed significantly in their mycelial dry weights in V-8-juice broth and in their radial growth rates on Rye B agar (Table 2). A wide range in growth rates was observed for isolates within both the dsRNA-containing and dsRNA-free groups (Table 2). To compare growth of the two groups, contrasts were calculated between the means of the isolates in each group (Table 3). Isolates containing dsRNA were found to have significantly higher mycelial dry weights than dsRNA-free isolates, but no differences were observed between the two groups for radial growth rates on Rye B agar (Table 3).

TABLE 1. Isolates of *Phytophthora infestans* containing double-stranded RNA (dsRNA)[a]

| Isolate no. | Mexican source[b] | Mating type | dsRNA pattern[c] |
|---|---|---|---|
| 511 | Toluca | A2 | A |
| 536 | El Tecolote | A2 | A |
| 543 | La Puerta | A1 | A |
| 547 | Raices | A1 | A |
| 509 | Toluca | A1 | B |
| 515 | Toluca | A1 | B |
| 519 | Toluca | A2 | B |
| 526 | Toluca | A2 | B |
| 533 | El Tecolote | A1 | B |
| 534 | El Tecolote | A1 | B |
| 544 | La Puerta | A1 | B |
| 545 | La Puerta | A1 | B |
| 512 | Toluca | A1 | C |
| 560 | Chapingo | A1 | C |

[a] Additional Mexican isolates tested and found not to contain dsRNA were as follows: 501, 503, 510, 513, 517, 518, 520–522, 525, 527, 529, 535, 539, 541, 542, 546, 550, 561, 562, 564–566, 568, 570, and 581. Isolates from the United States and Europe found not to contain dsRNA included isolates 102, 106, 107, 111, 112, 115, 118, 123, 127, 128, 135, 136, 139–141, 144, 146, and 161–163.

[b] Toluca isolates originated from potatoes growing at the international late blight screening trials sponsored by the Mexican National Potato Program. Isolates from El Tecolote, La Puerta, and Raices originated from small commercial potato fields on the slopes of the Toluca volcano several miles from the Toluca late blight trials. Isolate 560 originated from *Solanum cardiophyllum*, a wild species growing at Chapingo, and was kindly provided by J. Galindo of Chapingo University (19).

[c] Pattern B consisted of two bands about 2,750 and 2,500 base pairs in size. Pattern C consisted of two bands ca. 1,600 and 1,500 base pairs in size. Pattern A consisted of both sets of bands observed for patterns B and C (Fig. 1).

## DISCUSSION

Double-stranded RNA was detected in 14 of 40 Mexican isolates of *P. infestans*, but not in 20 non-Mexican isolates. This is, to our knowledge, the first report of dsRNA occurring in any member of the class Oomycetes. The sizes of the dsRNA components of *P. infestans* fall within the size range observed for dsRNAs from other fungi (5,8).

The occurrence of dsRNA in Mexican isolates provides an additional means to distinguish Mexican populations from those in other regions of the world. It was found previously that Mexican isolates of *P. infestans* contained more variability at isozyme loci (18), greater virulence (19), and lower nuclear DNA content (20) than non-Mexican isolates. Double-stranded RNA may thus serve as another useful character for monitoring the migration of Mexican strains to other parts of the world.

Genetic studies with *P. infestans* have recently advanced due to improvements in methodology and availability of isolates of both mating types from Mexico (13,18). The discovery of dsRNA in this

**Fig. 1.** Double-stranded RNA (dsRNA) banding patterns observed for *Phytophthora infestans* (lanes 1, 2, and 4) and brome mosaic virus (BMV) (lane 3) on a 5% polyacrylamide gel following DNase treatment. Lane 1, *P. infestans*, isolate 533 (pattern B); lane 2, isolate 560 (pattern C); lane 4, isolate 511 (pattern A). The four BMV (replicative forms) dsRNA bands (from top to bottom in lane 3) correspond to sizes of 3,234, 2,874, 2,117, and 875 base pairs, respectively (1,2).

fungus offers an additional new tool for genetic studies of the fungus. Since dsRNA is usually associated with the cytoplasm of the fungal host (9,10,22), the dsRNA of *P. infestans* may be of particular use in genetic studies involving cytoplasmic inheritance. For example, if dsRNA of *P. infestans* were found to exist solely in the cytoplasm, traits that cosegregated with dsRNA in appropriate genetic crosses could be presumed to be under cytoplasmic genetic control.

We were not able, using a variety of methods, to purify virus particles from an isolate of *P. infestans* that contained dsRNA (isolate 519, pattern B). It is possible that virions were present at undetectably low concentrations or were unstable in the reagents or conditions we employed for purification. Electron microscope studies are desirable to determine whether virus particles can be observed in dsRNA-infected isolates.

Interestingly, we were not able to isolate dsRNA from *P. infestans*, isolate 162 (ATCC 16981), nor from strain 6503 of *P. drechsleri*, in both of which other workers had observed intranuclear VLPs (12,17). Apparently, the VLPs observed by these workers either do not contain dsRNA genomes, were present in concentrations below the detection limits of our methods, or were not present in the cultures we received.

Isolates containing dsRNA were, in general, highly virulent, and two isolates (one with pattern A, one with pattern C) were able to infect all 11 of the differential potato genotypes. However, another isolate with dsRNA banding pattern C was avirulent and unable to attack even the susceptible controls. Thus, a strong association does not appear to exist between the presence of dsRNA and virulence in *P. infestans*, or between particular banding patterns and virulence phenotype. The results show clearly, however, that isolates containing dsRNA do not as a group show reduced virulence as in the case of *Endothia* (*Cryphonectria*) *parasitica* (5,8,23).

Isolates containing dsRNA showed higher mycelial dry weights following growth in V-8-juice broth than did dsRNA-free isolates. It is possible that gene products coded for by the dsRNA may enhance fungal growth. However, the occurrence of different genetic backgrounds in each of the dsRNA-containing isolates may have interfered with our ability to assess the phenotypic effect of a specific dsRNA by simply looking at associations between isolate banding patterns and growth. Nuclear genes could be affecting the expression of dsRNA phenotypes, as for *Saccharomyces cerevisiae* (24). Therefore, isogenic fungal strains with and without dsRNA would be desirable in future studies designed to characterize the phenotypic effects of dsRNA infection in *P. infestans*.

TABLE 2. Mycelial dry weight, radial growth rate, and specific virulence (race) of isolates of *Phytophthora infestans* with and without double-stranded RNA (dsRNA)

| Isolate no. | dsRNA pattern[a] | Dry weight[b] (mg) | Radial growth[c] (mm/day) | Pathogenic race |
|---|---|---|---|---|
| 560 | C | 157.2 | 10.3 | avirulent[d] |
| 544 | B | 156.8 | 8.8 | 0,1,2,3,6,7,10,11 |
| 509 | B | 155.5 | 10.5 | 0,1,2,3,6,7,10,11 |
| 581 | none | 150.0 | 8.1 | 0,2 |
| 545 | B | 149.4 | 9.2 | 0,1,2,3,4,6,7,10,11 |
| 515 | B | 144.7 | 9.9 | ...[e] |
| 534 | B | 144.5 | 9.2 | 0,1,2,3,7,10,11 |
| 529 | none | 142.2 | 7.9 | 0,1,2,3,4,6,7,10,11 |
| 526 | B | 140.5 | 8.0 | 0,2,3,5,7, 11 |
| 547 | A | 140.2 | 8.9 | 0,1,2,3,4,5,6,7,8,9,10,11 |
| 536 | A | 140.0 | 5.6 | 0,1,2,3,4,6,7,9,10,11 |
| 533 | B | 139.1 | 8.7 | 0,1,2,3,7,10,11 |
| 506 | none | 139.0 | 11.5 | 0,1,2,4 |
| 519 | B | 137.9 | 7.7 | ... |
| 511 | A | 135.0 | 4.8 | 0,1,2,3,4,5,6,7,10,11 |
| 543 | A | 133.3 | 5.4 | 0,1,2,3,7,10,11 |
| 566 | none | 131.4 | 9.1 | 0,2 |
| 512 | C | 131.3 | 6.4 | 0,1,2,3,4,5,6,7,8,9,10,11 |
| 520 | none | 130.6 | 6.3 | ... |
| 541 | none | 129.9 | 8.0 | 0,3,4,7,10,11 |
| 564 | none | 129.3 | 7.4 | 0,2,7 |
| 550 | none | 99.1 | 6.9 | 0,1,2,3,4,5,6,7,10,11 |
| BLSD[f] | | 7.2 | 2.6 | |

[a] Pattern B consisted of two bands about 2,750 and 2,500 base pairs in size. Pattern C consisted of two bands about 1,600 and 1,500 base pairs in size. Pattern A consisted of both sets of bands observed for patterns B and C (Fig. 1).

[b] Dry weight (mg) of mycelium after growth for 10 days in 20% V-8-juice broth at 18 C in darkness.

[c] Rate of growth of isolates after 7 days on Rye B agar medium, expressed as colony diameter (mm) divided by the number of days grown.

[d] This isolate was unable to infect any of the differentials, including the control cultivars Norchip and Bintje.

[e] Isolates not tested due to insufficient production of sporangia.

[f] Bayes least significant difference, $k = 100$ (14) for comparing isolate means.

TABLE 3. Contrasts between means of isolates of *Phytophthora infestans* with and without double-stranded RNA (dsRNA)

| Growth measure[a] | With vs. without dsRNA: Contrast estimate[b] | $t$[c] | $P > t$[d] |
|---|---|---|---|
| Mycelial dry weight | 11.713 | 9.29 | 0.0001 |
| Radial growth rate | −0.369 | −0.78 | 0.4370 |

[a] Mycelial dry weight (mg) was assessed following growth for 10 days in 20% V-8-juice broth at 18 C in darkness. Radial growth rate was assessed following growth for 7 days on Rye B agar as colony diameter (mm) divided by the number of days grown.

[b] The contrast estimate represents the difference between the means of groups of dsRNA-containing and dsRNA-free isolates.

[c] The $t$ value is calculated by dividing the contrast estimate by its standard error (16).

[d] Probability of obtaining a larger absolute value of the $t$ value.

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