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Stage-Specific DNA Methylation in a Fungal Plant Pathogen

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Significantly more 5-methylcytosine residues were found in the DNA from the dormant sclerotia of *Phymatotrichum omnivorum* than in the DNA from the metabolically active mycelia of the fungus, as shown by high-pressure liquid chromatography of acid-hydrolyzed DNA digests and by restriction of the DNA with the isoschizomers *Msp*I and *Hpa*II. N^6-Methyladenine was not detected in GATC sequences in the DNA isolated from either stage.

The occurrence of significant amounts of 5-methylcytosine (5-mC) appears to be rare in the DNA of lower eucaryotes. Antequera et al. (1) analyzed DNA isolated from mycelia of 20 species of fungi representing 15 taxonomic families and found only 2 species in which there was detectable methylation of cytosine residues, as demonstrated by comparison of *Msp*I and *Hpa*II restriction patterns. The same authors reported that very little 5-mC was found in mycelial DNA from *Aspergillus nidulans* (24). Proffitt et al. reported that less than 0.01% of the cytosine residues were methylated in the DNA of *Saccharomyces cerevisiae* (19). A high level of 5-mC (21%) was found in *Chlorella* nuclear DNA and in several *Chlorella* viruses, but was not found in other green algae (26). Bull and Wootton demonstrated that amplified transforming DNA from *Neurospora crassa* mycelia was heavily methylated, whereas the nuclear DNA from wild-type mycelia had very low levels of 5-mC based on analysis of *Msp*I and *Hpa*I digests (5).

There is considerable evidence in higher eucaryotic organisms, especially vertebrates, that gene inactivation may be caused by methylation of specific cytosine residues in the DNA. In humans, the developmental switch from fetal to adult forms of hemoglobin (6, 13, 15) and the inactivation of genes on one X chromosome in females (16, 30, 31) are accompanied by the methylation of cytosine residues in 5′-flanking regions of the DNA. However, the lack of significant methylation of DNA from lower eucaryotes such as fungi and from some insects such as *Drosophila melanogaster* (25) indicates that DNA methylation is not a universal mechanism for inactivating genes during development.

The base N^6-methyladenine (6-mA) has been detected in the DNA of a number of lower eucaryotes including the unicellular green alga *Chlamydomonas reinhardi* (12), several dinoflagellates (20), ciliated protozoa (3, 7, 11), and some insects (8). Experiments with *Tetrahymena thermophila* (3) suggest that 6-mA may serve to protect specific DNA sequences from degradation during developmental changes in the life cycle. 6-mA has not been found in fungi and appears to be absent from the DNA of all higher eucaryotes (8).

This work compared the methylation of DNA from differentiated resting structures (sclerotia) and actively growing, proliferative mycelia of the fungus *Phymatotrichum omnivorum* by using restriction by isoschizomers differing in sensitivity to base methylation and by using quantitation by high-pressure liquid chromatography of the 5-mC content in acid-hydrolyzed DNA.

MATERIALS AND METHODS

Strains and culture conditions. The *P. omnivorum* strain which was used was a 1982 field isolate obtained from D. Lyda of Texas A&M University. Mycelial cultures were maintained on solid Vogel minimal medium (27) and were transferred every 3 weeks to maintain viability. *P. omnivorum* mycelium for DNA isolation was grown in large quantities at 28°C on disposable plastic culture plates containing approximately 25 ml of liquid Vogel minimal medium after inoculation with triangular plugs of mycelium. The mycelial mats were allowed to fill the entire culture plates before harvest (generally 21 to 31 days after inoculation). Each mat was harvested through one layer of micracloth in a Büchner funnel, and the agar plug from the inoculum was gently removed.

The sclerotial stage was cultured as described by Dunlap (9). Flasks which each contained Houston Black Clay, sorghum seeds, and mycelial inoculum were incubated at 27°C for 6 to 8 weeks and then stored at 4°C. To harvest sclerotia, the mycelial growth and sorghum seed layer were scraped away from the soil and sclerotial layer. The soil and sclerotial layer were then transferred to a no. 18 sieve and washed thoroughly to remove the soil from the sclerotia. If necessary, the sclerotia were floated free from the shale residue in 2.5 M sucrose.

DNA purification. The freshly harvested mycelial mats were frozen in liquid nitrogen for 15 min and powdered by grinding with a mortar and pestle. Sclerotia were frozen for 30 to 45 min in liquid nitrogen and powdered in a mill before being ground in liquid nitrogen for an additional 20 min with a mortar and pestle. DNA was isolated from powdered sclerotia or mycelia by the method of Zimmer et al. (E. A. Zimmer, C. J. Rivin, and V. Walbot, Plant Mol. Biol. Newsl. 2:93–96, 1981). Digestions were done with 1 μg of DNA and 5 U of restriction enzyme, each in a total volume of 20 μl. Each reaction was terminated by adding 5 μl of a solution containing 0.15% bromophenol blue, 15% Ficoll (type 400) in 0.04 M Tris acetate, and 0.5 M EDTA (pH 7.5).

Samples of digested DNA were added to slots in agarose gels (1.5 or 2.3%), and fragments were separated by electrophoresis. The DNA was visualized by immersing the gels in ethidium bromide (0.5 μg/ml of water) for 20 to 40 min at 20°C and then observing them under a UV transilluminator.

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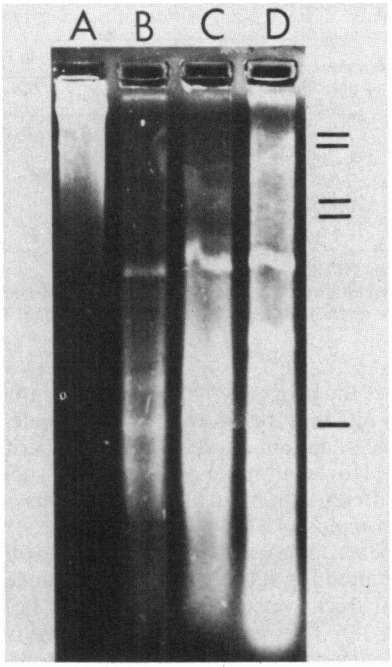

FIG. 1. Restriction analyses of *P. omnivorum* genomic DNA from mycelia with *Hpa*II, *Msp*I, and *Hha*I. The DNA fragments were separated on a 2.3% agarose gel. Uncut mycelial DNA (lane A) and mycelial DNA digested with *Hpa*II (lane B), *Msp*I (lane C), or *Hha*I (lane D). The lines on the right indicate fragment sizes and from the top represent 6.5, 4.4, 2.3, 2.0, and 0.5 kilobase pairs.

Negatives were scanned with a Joyce-Loebl microdensitometer to quantitate the fraction of DNA digested.

Analysis of base composition of digested DNA by HPLC. Purified DNA was made 0.3 M in KOH and incubated at 37°C for 18 h to remove any residual RNA contamination (29). The DNA was then precipitated overnight in ammonium acetate and ethanol, pelleted in a Microfuge for 10 min at 4°C, and dried under vacuum for 6 to 8 h. The dried DNA pellet was digested to individual bases by incubation in 100 μl of 70% perchloric acid at 65°C for 18 h (29). After being neutralized slowly with 3 volumes of 1 M KOH, the sample was centrifuged (5 min in a Microfuge), and the upper aqueous layer containing the individual bases was drawn off. The pH was adjusted to 4.0 with 1 M KOH, and the centrifugation was repeated to remove any residual salts. All samples were filtered (0.4-μm filter) before HPLC was done.

A reverse-phase Spherisorb ODS (5 μm) column (Alltech Associates, Inc.) was used to elute bases from digested DNA samples isocratically with 20 mM ammonium phosphate (pH 2.3). Adenine, thymine, guanine, cytosine, and 5-mC were eluted in 15 min (Table 1). Standard curves to quantitate bases were prepared by using solutions containing all five bases at final concentrations between 10 and 100 μg/ml. Samples (100 μl) were injected onto the HPLC column, and peak heights were measured at characteristic retention times. Mean peak heights derived from five injections of each of two separate solutions of base standards were plotted against the concentration of the base in the standard. Molar concentrations of the bases were extrapolated by using linear regression analysis. The mean moles percent and standard deviation for each base were obtained by pooling all of the samples from each stage of the life cycle. A *t* test was used to analyze differences in base composition between stages.

RESULTS

The results of restriction digests of DNA isolated from mycelia and sclerotia of *P. omnivorum* are shown in Fig.1 and 2, respectively. The isoschizomers *Msp*I and *Hpa*II reveal large stage-specific differences in the methylation of cytosine residues in the 5′-CpG-3′ dinucleotides of the mycelial and sclerotial DNA. The mycelial DNA (Fig. 1) was almost entirely fragmented by both *Hpa*II (lane B) and *Msp*I (lane C). On the other hand, the DNA from *P. omnivorum* sclerotia (Fig. 2) was relatively resistant to *Hpa*II digestion (lane B) but was cut by *Msp*I (lane C), indicating that the sclerotial DNA contained many more methylated CpG doublets in CCGG sequences than did the mycelial DNA. Likewise *Hha*I, which cleaves at the sequence GCGC when neither C is methylated, was much more effective in digesting mycelial DNA than sclerotial DNA (Fig. 1 and 2, lanes D).

Lambda DNA mixed with sclerotial DNA was digested into the expected fragments by *Hpa*II, *Msp*I, and *Hha*I (data not shown) indicating that no extrinsic inhibitor of the restriction enzymes was present in these extracts.

Calculations based on microdensitometer scans of photographic negatives of the gels show that 70% of the DNA remained in the high-molecular-weight (undigested) fraction after incubation with excess *Hha*I or *Hpa*II. In each case where substantial digestion occurred, several distinct bands were visible after electrophoresis, as well as a background smear of indistinguishable fragments centered at approximately 500 base pairs.

The presence of 6-mA in the GATC sequences was investigated by using restriction enzyme digests. The enzyme *Dpn*I which requires 6-mA in the sequence GATC for restriction to occur apparently did not digest either sclerotial or mycelial DNA (Fig. 3), whereas DNA from both stages

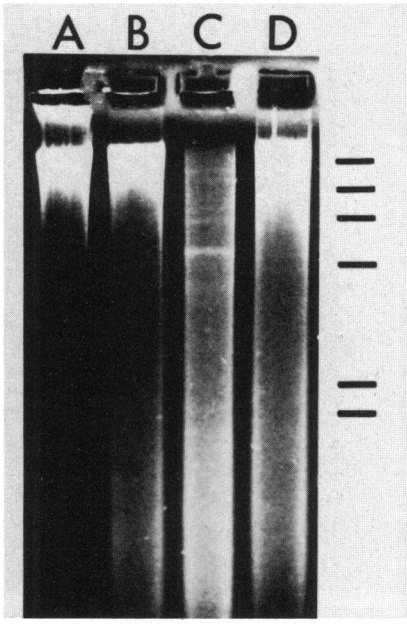

FIG. 2. Restriction analyses of *P. omnivorum* genomic DNA from sclerotia with *Hpa*II, *Msp*I, and *Hha*I. The DNA fragments were separated on a 1.5% agarose gel. Uncut sclerotial DNA (lane A) and sclerotial DNA digested with *Hpa*II (lane B), *Msp*I (lane C), or *Hha*I (lane D). The lines on the right indicate fragment sizes and from the top represent 23.1, 9.4, 6.5, 4.4, 2.3, and 2.0 kilobase pairs.

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was totally digested by *Sau*3AI, which cleaves whether or not 6-mA is present, and by *Mbo*I for which 6-mA in the GATC sequence is inhibitory. The larger average fragment size in *Sau*3AI digests as compared with that in *Mbo*I digests may be due to 5-mC being present in some GATC sequences.

The base composition of the DNA from the two stages was determined by HPLC (Table 1) and was internally consistent in that the moles percent of each base remained constant for the two stages, and the A/T and G/C ratios are equal to one when the cytosine and 5-mC fractions are combined. These data show the significant increase in cytosine methylation in sclerotial DNA (2.27%) as compared with that in mycelial DNA (0.78%). 6-mA could not be separated from adenine by these methods, and therefore 6-mA moles percent were not determined in this HPLC analysis.

TABLE 1. Base composition of purified DNA[a]

Base	Retention time (min) of base standards[b]	Mol% in DNA:	
		Mycelial	Sclerotial
Cytosine	3.23 ± 0.05	21.65 ± 0.48	20.39 ± 0.19
5-mC	6.30 ± 0.22	0.78 ± 0.07	2.27 ± 0.08
Guanine	7.58 ± 0.08	22.38 ± 0.60	22.60 ± 0.30
Adenine	10.84 ± 0.19	27.75 ± 0.62	27.35 ± 0.28
Thymine	12.03 ± 0.05	27.43 ± 0.58	27.36 ± 0.25

[a] Determined by HPLC.
[b] All base standards were dissolved in 0.01 M HCl (pH adjusted to 4.0). Each value is the mean of 10 determinations ± the standard deviation.

DISCUSSION

Although a direct cause and effect relationship is hard to prove, a general rule which has emerged for higher eucaryotes is that DNA methylation is a property of inactive genes. More specifically, in a cell where a gene is active, the promoter region is undermethylated when compared with that in a cell where the gene is not transcribed. Differentiation involves inactivation of specific genes, and it was shown recently that methylation of certain CG clusters is correlated with differentiation in mouse cells (2). In lower eucaryotes the role of 5-mC is not clear. In *Chlamydomonas* sp. matings (21, 22) the chloroplast DNA from the male parent is not methylated and is not expressed, whereas that from the female parent is heavily methylated and gives rise to the chloroplasts of the progeny. Thus, it appears that in *Chlamydomonas*, 5-mC may be protecting the female chloroplast DNA from degradation, thereby playing a critical role in development. However, more recent studies suggest that the control of differentiation in *Chlamydomonas* sp. may be much more complex (4, 10).

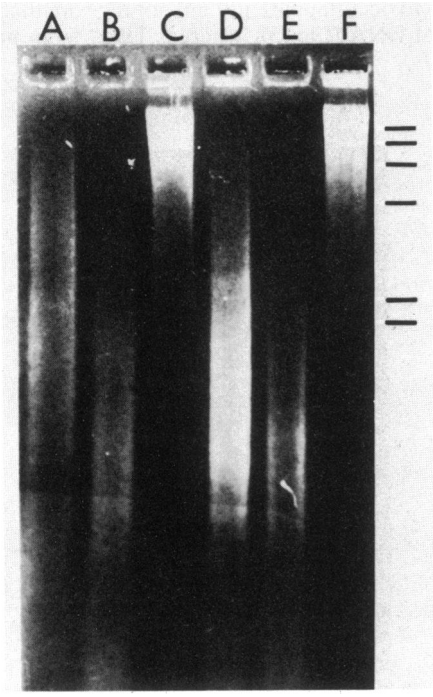

FIG. 3. Restriction analyses of *P. omnivorum* genomic DNA from mycelia and sclerotia with *Sau*3AI, *Mbo*I, and *Dpn*I. The DNA fragments were separated on a 1.5% agarose gel. Mycelial DNA digested with *Sau*3AI (lane A), *Mbo*I (lane B), and *Dpn*I (lane C) and sclerotial DNA digested with *Sau*3AI (lane D), *Mbo*I (lane E), and *Dpn*I (lane F). The lines on the right represent fragment sizes as indicated in the legend to in Fig. 2.

Sclerotia of *P. omnivorum* are specialized differentiated structures adapted for survival under adverse conditions by elaboration of thick walls and deposition of large stores of glycogen (14, 23). They are dormant, and survival for more than a decade deep in the soil has been recorded. As such, sclerotia would seem to be potential sources of inactive genes and therefore methylated bases. Our results show that DNA isolated from sclerotia of this plant-pathogenic fungus is indeed highly methylated in comparison with the mycelial DNA. The inability of methylation-sensitive restriction endonucleases to digest sclerotial DNA and the threefold increase of 5-mC in sclerotial DNA clearly suggest a role in gene regulation for DNA methylation during development of *P. omnivorum*.

The rare base 6-mA appears to play a role in the developmental changes which occur during the life cycle of the lower eucaryote *T. thermophila* (3, 18). The HPLC methods used for base analysis in this study did not separate 6-mA from adenine sufficiently to allow us to conclude that no 6-mA was present. However, the results with this set of restriction endonucleases do show that very little, if any, 6-mA is present within the GATC sequences of either the mycelial or sclerotial DNA of *P. omnivorum*.

Whether *P. omnivorum* is exceptional among fungi, or whether the relative absence of DNA methylation reported for other fungi results from measurements being made only on DNA from actively growing mycelia, awaits clarification. It is interesting in this regard that the slime mold *Physarum polycephalum*, which is often used in developmental studies, is reported to contain 5-mC, with about 20% of the DNA resistant to *Hpa*II restriction (28). In *P. polycephalum* most of the methylation appears to be in highly repeated sequences since specific bands are produced on subsequent digestion with *Msp*I (17). The bands which are visible after *Hpa*II, *Hha*I, and *Msp*I digestion of *P. omnivorum* DNA are most logically explained by the presence of restriction sites within repeated sequences, especially since the visible bands are found at multiples of a unit size. It seems unlikely that methylation is limited to repeated sequences since the few visible specific bands produced by digestion of sclerotial DNA cannot account for the 70% of DNA resistant to *Hpa*II.

Whereas transforming DNA in *N. crassa* was methylated at almost all testable cytosines (5), the methylation of cytosine in *P. omnivorum* DNA appears to be limited to those which are adjacent to guanines, based on digestibility by *Msp*I and *Sau*3AI but not by *Hpa*II or *Hha*I, and is thus

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comparable to that in mammals. If, as in higher eucaryotes, the CG dinucleotide is greatly underrepresented in the DNA (8), the 2.7% of methylated cytosine residues in sclerotia may represent a substantial proportion of the CG nucleotides present. In any case, the alternation of different levels of DNA methylation between the resting and actively growing stages of *P. omnivorum* provides an excellent opportunity to investigate the mechanisms of differential methylation and demethylation.

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