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Symbiosis and DNA Methylation in the *Cladonia* Lichen Fungus

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Abstract

In eukaryotes, the modification of DNA by addition or removal of specific methyl groups is thought to affect gene activity and differentiation. We began to investigate the relationship between DNA methylation and differentiation in lichens, organisms in which the symbiosis between a fungus and a unicellular alga or cyanobacterium generates remarkable morphological and biochemical complexity. Restriction analysis of DNA from the lichen *Cladonia grayi* indicated that overall DNA methylation is low in the fungus cultured in absence of the alga and high in the natural lichen. Within the lichen, however, fungal DNA methylation is not uniform: it is high in the body of the goblet-shaped thalli (podetia) and in the vegetative propagules (soredia), somatic tissues in which the fungus is associated with the alga; it is low in the fungal ascocarps (apothecia) which develop without algae on the upper rim of the podetia and produce meiotic spores. Methylation remains low in the mycelia derived from spores in axenic culture. These results suggest a correlation between symbiosis and methylation of fungal DNA in *Cladonia*. DNA methylation was also observed in two other lichens tested, a *Parmelia* and an *Usnea*. To relate the overall genomic changes found in *Cladonia* to the behavior of individual genes, we evaluated through Southern blotting the methylation of four fungal genes presumably

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involved in the production of lichen secondary compounds: three appear more methylated in the lichen than in the isolated fungus, while one shows the opposite behavior.

Keywords: DNA methylation, symbiosis, lichens, *Cladonia*, fungi

1. Introduction

Our knowledge of development and differentiation in lichens is primarily morphological (reviews: Büdel and Scheidegger, 1996; Honegger, 1996). Few studies have used biochemistry (Green and Smith, 1974; Bubrick et al., 1982; Culberson et al., 1983; Raineri and Modenesi, 1988; Culberson and Armaleo, 1992; Galun and Kardish, 1995) or molecular biology (Kardish et al., 1990; Armaleo and Clerc, 1991) to approach differentiation in lichen symbiosis.

To the developmental geneticist, lichens are unique for several reasons. In most other multicellular organisms, development involves interactions among clonal cell lineages sharing essentially the same genome and evolutionary history. The differentiation of a lichen, however, requires communication between the unrelated genomes of a fungus and a unicellular alga. After diverging for hundreds of millions of years (Knoll, 1992), those two organisms evolved a set of novel interactions intricate enough to guarantee the stability and specificity of the symbiosis, but also tenuous enough to allow the survival of the isolated symbionts in culture and possibly in nature. This alternation of dependence and independence is the likely basis of another feature of lichen differentiation, plasticity: in the lichens *Sticta* and *Pseudocyphellaria* for instance, a single mycobiont undertakes separate symbiotic pathways, interacting either with a green alga or with a cyanobacterium and producing morphologically different thalli or thallus sections (James and Henssen, 1976; Armaleo and Clerc, 1991); the morphological variability of lichens in general continues to vex and fascinate lichenologists (Jahns and Ott, 1994; Honegger, 1995); in the laboratory, undifferentiated mycobionts or photobionts can arise from practically any differentiated tissue or stage of the lichen thallus (Yoshimura et al., 1993; Stocker-Wörgötter, 1995). It is therefore likely that most or all changes in gene expression supporting differentiation in lichens are reversible.

To experiment with the genetic basis of lichen differentiation, we began by concentrating on the fungus and on the following questions. Is it possible to identify large-scale changes in the mycobiont's genome that can be correlated with the presence or absence of symbiosis? Are such changes reversible? Lichen symbiosis is a complex, multi-step process (Armaleo, 1991; Honegger, 1993), which we reduced here to two states: a non-symbiotic state in which the fungus

has no obvious interaction with the photobiont, and a symbiotic state comprising all levels of fungus-alga association in a mature lichen thallus. We used as model system the lichen *Cladonia grayi* and its isolated symbionts, the mycobiont and the unicellular alga *Trebouxia.* We found that overall DNA methylation in the fungus was very low in the non-symbiotic condition and increased dramatically in the symbiotic condition. Highly methylated DNA was also found in two other lichens tested. Through Southern analysis, we observed that methylation of individual fungal genes often mirrored that of the genome as a whole.

In eukaryotes, DNA methylation is thought to be involved in the control of gene expression during differentiation (Razin and Cedar, 1993; Grässman and Grässman, 1993). Accordingly, methylation levels and distribution vary in different tissues, developmental stages, and genes. Eukaryotic DNA methylation occurs on the 5' carbon of the cytosine ring in CpG sequences and is reversible: it can be lost through DNA replication and requires the activity of specific enzymes to be initiated and maintained (Leonhardt and Bestor, 1993; Adams et al., 1993). Most prominent in prokaryotes, plants, and vertebrates, some DNA methylation is also found in fungi (Antequera et al., 1984; Magill and Magill, 1989; Selker, 1993).

2. Materials and Methods

Biological samples and media

Two types of mycobiont were used: a single spore isolate (30-197#8) of *Cladonia grayi* Merr. *ex* Sandst. (North Carolina: Mitchell Co., Roan Mountain, *Culberson19971,* DUKE), and polyspore cultures obtained from apothecia of freshly collected *C. grayi* using standard methods (Ahmadjian, 1993). Lichen fungus cultures were grown in Lilly and Barnett medium (LB), as described (Culberson and Armaleo, 1992). *C. grayi, Usnea strigosa* and *Parmotrema hypotropa* were collected near Duke University. The *Trebouxia* alga, a gift from Dr. Elfie Stocker, was isolated from *Cladonia furcata* and was grown on MY medium (2% malt extract, 0.2% yeast extract, 1.5% agar).

Cleaning of soredia, podetia, and apothecia from Cladonia

Soredia

Freshly collected podetia were allowed to hydrate and were vortexed extensively in several changes of water to detach soredia. The podetia were set aside for further cleaning (see below). The water washes with the soredia were pooled, and the suspension was centrifuged for 2 minutes at 7,000 x g. The

centrifugation separated two populations of soredia: a bottom population containing water-logged soredia, mycelia, thallus fragments and soil residue, and a population of clean, air-filled soredia floating at the water surface. The two populations were approximately equivalent in mass. The top population was harvested, washed once more, lyopilized, and stored at –20°C until DNA extraction. From 45 mg of air-dry podetia, 5 mg of clean, lyophilized soredia were obtained.

Podetia

Excessive moisture was blotted from the washed podetia. Residual soredia were removed by careful scraping of the podetial outer surfaces with fine tweezers, monitoring the removal through a dissecting microscope. About 30 mg of thoroughly cleaned podetia were obtained from 45 mg of intact podetia. Cleaned podetia were vortexed once in water, the water was discarded, and podetia were lyopilized and stored at –20°C until DNA extraction.

Apothecia

Apothecia were removed from fresh, abundantly fruiting podetia at environmental moisture, with as little carryover of somatic tissue as possible. Individual apothecial fragments were then allowed to hydrate in a water droplet and, under a dissecting microscope, scraped clean of all residual somatic tissue and algae while submerged. As the removed soredia, algae and somatic tissue became dispersed in the water droplet, the apothecial fragment was transferred to a clean droplet and scraping was continued. The process was repeated until all algae and practically all somatic tissue were removed. Cleaned apothecial fragments were collected in microcentrifuge tubes, lyopilized and stored at –20°C. DNA was extracted from 10 mg samples.

DNA extraction materials

With more than 20 mg of material, grinding was performed in an acid-washed ceramic mortar and pestle cooled with liquid nitrogen, as described (Armaleo and Clerc, 1995). Less than 20 mg of material were ground in microcentrifuge tubes, using a Craftsman electric 140 rpm screwdriver (suggested by Ulrik Søchting) to which an acid-washed Kontes pellet pestle was fastened (Fig. 1). The Kontes pestle was sanded to fit perfectly the rounded bottom of the microcentrifuge tubes. During grinding, the sample tubes were held in a microcentrifuge rack cooled with liquid nitrogen. The rack (polypropylene, Fisher cat.# 05-541) was embedded in styrofoam on the bottom and sides (Fig. 1), and all its wells were filled with liquid nitrogen. The following chemicals were used for extraction: pancreatic RNAase A (Sigma), prepared as described (Armaleo and Clerc, 1995); DTAB buffer [5.5% (w/v) DTAB (dodecyltrimethyl

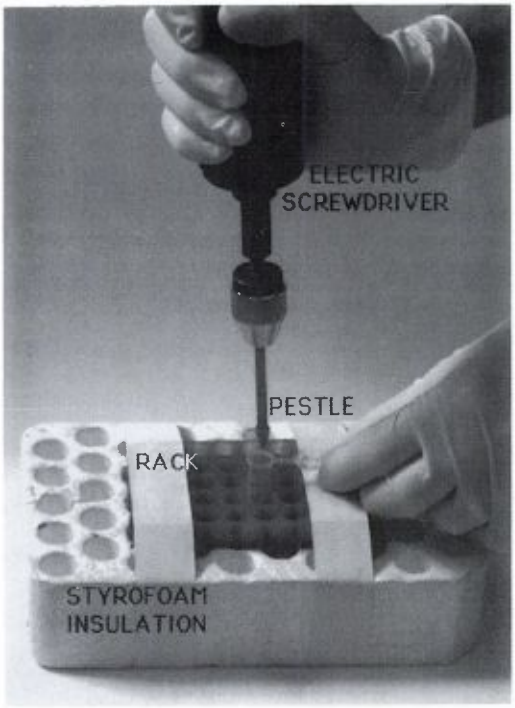

Figure 1. Setup for grinding small amounts of material at liquid nitrogen temperature. The polypropylene rack holding the nitrogen is fastened by tape to styrofoam insulation. The electric screwdriver is shown with pestle attached.

ammonium bromide, Sigma); 700 mM NaCl; 70 mM Tris, pH 8.6; 30 mM EDTA; RNAase A (100 µg/ml, added just before use)]; a 50:50 mix of water-saturated phenol:chloroform; CTAB solution [10% (w/v) CTAB (hexadecyltrimethyl ammonium bromide, Sigma); 700 mM NaCl]; chloroform; isopropanol; 0.5 M Na acetate, pH 5; 100% ethanol; 70% ethanol; TE buffer [1 mM EDTA; 10 mM Tris, pH 7–8]; deionized, autoclaved water.

DNA extraction protocol

An earlier method (Armaleo and Clerc, 1995) was modified to improve the yield of high molecular weight DNA. The modifications were derived from Wilson (1994). Centrifugations were at room temperature.

Very thorough grinding in liquid nitrogen was necessary for good yields. When using a mortar, a ground sample weighing up to 50 mg was transferred to a microfuge tube. Larger samples were transferred to Falcon tubes. DTAB buffer

was added to a final volume equivalent to 10–20 x the dry weight. The sample was incubated at 65°C for 5 minutes, with occasional gentle but thorough mixing using a pipet. Repeated clump disruption while heating improved yield, but vortexing was avoided.

The setup illustrated in Fig. 1 was used with amounts of material too small to be ground in a mortar. The tissue was lyopilized in a microfuge tube and its packed volume was kept below 30–40 µl. The open tube with the sample was placed in one of the rack's wells filled with liquid nitrogen. Throughout the procedure, liquid nitrogen never came in direct contact with the sample. While the sample became frozen (1–2 minutes), the pestle was mounted on the screwdriver and dipped into liquid nitrogen for about 30 seconds. The cooled pestle was immediately inserted into the sample tube, and the screwdriver was operated by applying strong downward pressure on the sample while firmly holding the tube in place by its lid. As the nitrogen evaporated around the tube during grinding, every 15–30 seconds the tube with the pestle inserted was moved from one well to the next filled with nitrogen. The grinding was continued for 2–3 minutes or until the material was pulverized. The tube with the pestle inserted was moved into the well of a heating block at 65°C and 30 µl of DTAB buffer were added. The thawed sample was ground for about 30 seconds in the small amount of buffer, and the pestle was rinsed while adding DTAB buffer to a final volume equivalent to 20 pellet volumes or weights. The sample was kept at 65°C for 5 minutes, with occasional disruption of tissue clumps with a pipet.

Following grinding and heating at 65°C, the steps were the same for large or small samples. After shaking by hand for ~2 minutes with 1 volume of phenol / chloroform, the extract was centrifuged for 3 minutes at 5,000 x g. The volume of the upper phase was measured while transferring it to a new tube at 65°C, and 0.11 volumes of CTAB buffer were mixed in (under these conditions, CTAB precipitates polysaccharides, not DNA). The sample was kept at 65°C for 5 minutes and mixed periodically by pipetting. After adding 1 volume of chloroform, the sample was agitated by hand for ~2 minutes and centrifuged for 3 minutes at 5,000 x g. The upper phase was mixed with 0.5 volumes of isopropanol, and kept at room temperature for 5 minutes before centrifugation at 15,000 x g for 5 minutes. After complete removal of the supernatant, 0.5 M Na acetate was added (0.2 x volumes of the aqueous phase before isopropanol addition) without resuspending the pellet. The sample was incubated at 65°C for 5 minutes and, during heating, the pellet was broken up as much as possible by repeated but gentle pipetting. Insoluble material was removed by brief centrifugation, the supernatant was transferred to a new tube, and the nucleic acid was precipitated at room temperature by adding 2.5 volumes of 100% ethanol. The precipitate was collected by centrifugation, rinsed with 70% ethanol, and the excess ethanol was briefly evaporated in ambient air without

drying the pellet completely. Deionized sterile water was added (1–10 µl/mg of original weight) to the pellet, without resuspending it. After heating at 65°C for 5 minutes, the sample was pipetted gently and was stored on ice for at least one hour. The sample was heated again at 65°C for 5 minutes, and gently mixed. Unresuspended polysaccharides, even when invisible, were pelleted with a 10-minute centrifugation. The supernatant was transferred to a new tube and stored at –20°C. Typical lichen yields were 0.5–4 µg DNA/100 mg of dry material. The yield was approximately 10-fold higher with the cultured lichen alga *Trebouxia*.

DNA digestion, electrophoresis and Southern analysis

Restriction enzymes (Promega and Boehringer) were used according to the manufacturer's instructions. For newly prepared samples of genomic DNA, the following was done to ensure completeness of restriction. Test restrictions were set up using increasing dilutions of the genomic DNA sample and, when necessary, increasing amounts of enzyme. The lack of interference from soluble inhibitors was monitored by adding 0.3 µl (100 ng) of intact lambda DNA (Promega) to 10 µl of the reactions containing the genomic sample to be tested. The digests were compared by electrophoresis with identical digests of lambda DNA lacking the genomic sample. Complete digestion was indicated by identical lambda DNA band patterns in the different digests. The tests consistently indicated complete digestion of all genomic DNA preparations used.

To prepare the DNA for electrophoresis after restriction, the reactions were diluted with double-distilled water to a volume of 50–200 µl, and extracted once with chloroform. After adding Na acetate to 0.3 M, the DNA in the aqueous phase was precipitated at –20°C with 2.5 volumes of ethanol. The precipitate was collected by centrifugation at 4°C, rinsed with 70% ethanol, briefly dried and resuspended in appropriate volumes of water. Storage was at –20°C. Agarose gels were run in presence of ethidium bromide following standard protocols. Gels were placed onto a UV transilluminator and images of the DNA fluorescence were captured with a high performance CCD camera (Cohu). The images were stored in a Macintosh computer and processed using the programs "NIH Image" (version 1.55) and "Adobe Photoshop" (version 2.0).

The restricted mycobiont and lichen DNAs to be used for Southern analysis were loaded in duplicate lanes on an agarose gel (500 ng/lane). In preparation for Southern blotting, the gel was kept on the transilluminator for 3 minutes, equilibrated in 0.4 M NaOH for 15 minutes, and washed three x 15 minutes in 20 x SSC (3 M NaCl; 0.3 M Na citrate; pH 7.0). The DNA was then tranferred onto a nylon membrane (Amersham, Hybond N+) by capillarity in 20 x SSC, by

standard procedures. After transfer, the membrane was rinsed briefly with 2 x SSC, dried, and the DNA was crosslinked to it by UV irradiation. As the gel contained two identical replicas of the mycobiont and lichen DNA digests, the membrane was cut in two equivalent halves and kept at room temperature until hybridization.

The probes used for hybridization were obtained by PCR from four polyketide synthetase-like gene fragments cloned from the cultured *C. grayi* mycobiont (Lee and Miao, unpublished; Miao and Davies, 1997) in the plasmid pGEM-5Zf (Promega). Each of the four plasmid clones (pA1, pA2, pA3, pA5) was used as template (0.01 ng) in a standard PCR reaction with M13 forward and reverse primers. PCR reactions were extracted with chloroform and the amplified probes were precipitated with 2.5 volumes of ethanol after adding $MgCl_2$ to 10 mM. Each probe was quantified by electrophoresis and labeled with peroxidase (Amersham, ECL system). Hybridization and washes followed the ECL protocol's high stringency conditions. Overnight hybridization (probe concentration: 10 ng/ml) at 42°C in ECL hybridization buffer + 0.4 M NaCl was followed by two 20-minute washes at 42°C in 0.1 x SSC / 6M urea / 0.4% SDS, and three 5-minute washes at room temperature in 2 x SSC. One half of the blot was hybridized separately with probes A1 and A3, the other with probes A2 and A5. The light signal generated using the ECL kit was recorded on Kodak Biomax-MR film. Between each hybridization, the blots were stored for 48 hrs to allow decay of the first light signal. Peroxidase-labeled lambda DNA was used to highlight the molecular weight standards.

Specificity test of probes A1, A2, A3, A5 toward fungal and algal DNA

Probe DNA, DNA from *C. grayi* podetia, and DNA from cultured *Trebouxia* algae were prepared as described above. Individual DNA samples were diluted in 100 µl of 20x SSC (probes: 50 pg each; lichen and alga DNA: 300 ng each). The six samples were denatured by incubation in a boiling water bath for 4 minutes, and rapidly cooled in ice-water. Each was applied separately onto a nylon membrane (Amersham, Hybond N+) using a filtration manifold (Life Technologies, "Hybri-Slot"). The membrane was briefly rinsed in 2 x SSC and air-dried. The DNA was crosslinked to the membrane by heating at 80°C for 2 hours. Before hybridization, the membrane was submerged for 1 minute in distilled water at 100°C to ensure complete denaturation of the DNA. The membrane was air-dried and the DNA was hybridized to the four pooled, peroxidase-labeled probes (each at a concentration of 10 ng/ml in the hybridization buffer). The conditions used for hybridization, washes and signal detection were identical to those of the Southern analysis.

3. Results

A large-scale modification distinguishes Cladonia DNA from that of its cultured mycobiont

DNAs from *Cladonia grayi* and from its mycobiont cultured in liquid medium were digested with EcoR I and BamH I. The double digests were size-fractionated by agarose gel electrophoresis (Fig. 2). As with all complex genomes, this resulted in large populations of fragments yielding continuous distributions. However, the expectation that nearly identical genomes such as these would produce similar distributions was not met. Instead, many of the lichen DNA fragments were larger than those from the cultured mycobiont. As EcoR I and BamH I do not cut at restriction sites with methylated cytosines (review: Nelson and McClelland, 1991), the observed difference was best explained by assuming that the lichen genome was methylated while that of the cultured fungus remained largely unmethylated. The small amount of algal DNA from the *Trebouxia* present in the lichen does not account for the large discrepancy between lanes b and c (see Figs. 5 and 8).

To estimate the degree by which digestion of lichen DNA was reduced, the DNA mass distribution was measured in each lane by densitometry. The two scans are superimposed in Fig. 3 and show that the median of the DNA mass is 1.3 Kb in the cultured mycobiont and 2.8 Kb in the lichen. This approximate doubling of the median fragment length indicates that about half of the total EcoR I and BamH I sites remain uncut in the lichenized fungus, compared to nearly complete cutting in the cultured mycobiont (see below). Estimating the percentage of methylated cytosines that could account for a 50% inhibition of cutting would be premature, because of the unknown distribution of methylation across the genome. However, it is likely that this much inhibition requires significantly more methylation than that observed in most fungi (reviews: Antequera et al., 1984; Magill and Magill, 1989; Selker, 1993).

Southern analysis of four fungal gene fragments

Does the behavior of individual gene fragments confirm that fungal DNA methylation is the cause of the overall difference between the two fragment populations described above? We used four different DNA segments (A1, A2, A3, A5) cloned from the genome of the *C. grayi* mycobiont (Lee and Miao, unpublished; Miao and Davies, 1997) as probes to identify the corresponding fungal DNA fragments in the gel in Fig. 2. The four probes were homologous to fungal genes likely to encode polyketide synthetases (pks), the enzymes that initiate the biosynthesis of many lichen and nonlichen secondary compounds (review: Hutchinson and Fujii, 1995). We chose to focus on the methylation of

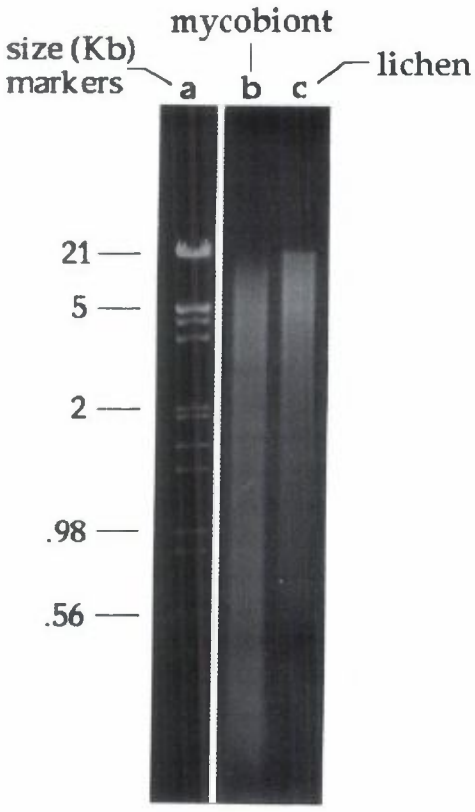

Figure 2. Restriction pattern of genomic DNA from the natural *Cladonia* lichen and from its isolated mycobiont. EcoR I and BamH I double digests of each genomic DNA were fractionated by agarose gel electrophoresis. The DNA fragment distribution was visualized through ethidium bromide fluorescence. Lane a: size standards in Kilobase pairs (lambda DNA cut with HindIII and EcoR I; only some sizes are indicated). Lane b: digest of DNA (500 ng) from a *Cladonia grayi* mycobiont (isolate 30-197#8) grown in liquid LB medium. Lane c: digest of *Cladonia grayi* DNA (500 ng).

these genes because of their relevance to lichen biology and because they are likely to be expressed differently under different developmental conditions (Culberson and Armaleo, 1992; Armaleo, 1995).

Since lane c in Fig. 2 contained a mixture of fungal and algal DNA, to follow fungal DNA methylation we needed to ensure that the probes would react only with fungal sequences, and not accidentally with algal sequences. We therefore compared the probes' reactivity towards lichen DNA with that towards DNA isolated from an axenic *Trebouxia*, the alga found in *Cladonia* lichens. All four

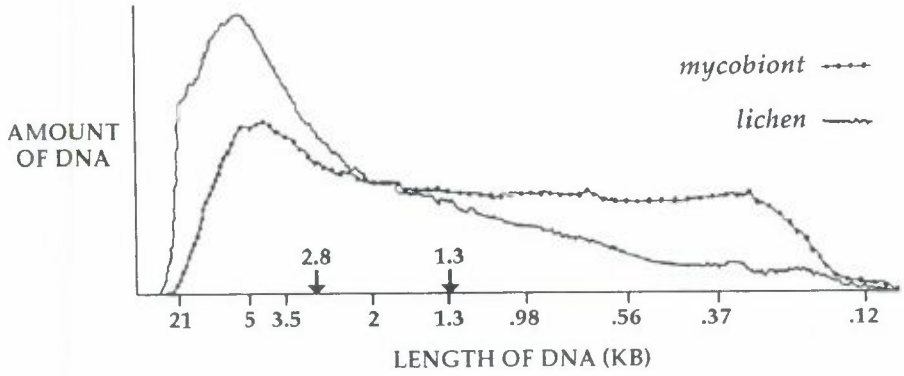

Figure 3. Densitometry profiles of lichen and mycobiont DNA after restriction and gel electrophoresis. The fluorescence intensity in lanes b and c of the gel in Fig. 2 was plotted as a function of distance from the well. The profiles from the individual lanes of mycobiont and lichen DNA are superimposed, and the direction of migration is from left to right. The ordinate measures fluorescence intensity, proportional to DNA mass. The abscissa measures distance from the well: the sizes of representative molecular weight standards are indicated below the abscissa at the positions at which they migrate in other gel lanes. The median fragment sizes for the lichen (2.8 Kb) and the mycobiont (1.3 Kb) are marked by the arrows.

probes recognized lichen DNA and showed negligible reactivity with algal DNA, indicating that they were fungal-specific (Fig. 4).

Once the specificity of the probes was established, the DNA from the gel in Fig. 2 was transferred to a nylon membrane and hybridized, separately and at high stringency, to each of the four probes. Each hybridization is represented in one of the A1, A2, A3, A5 panels in Fig. 5. The dark bands in these panels highlight the positions (sizes) of individual DNA fragments bearing a specific pks-like sequence. Within a given panel, signal intensity is proportional to the amount of DNA in each band. Each probe should highlight a single fragment in unmethylated DNA, as there are no EcoR I and BamH I sites within the probes. Typically, methylation in the region around the probed sequence will reduce the number of cleavable restriction sites. This results in the appearance of larger fragments bearing that sequence, and in the parallel fading or disappearance of smaller fragments seen in less methylated or unmethylated DNA. This "reciprocity" between specific fragments of different sizes in Southern blots is one of the hallmarks of methylation. Specifically, Fig. 5 shows the following.

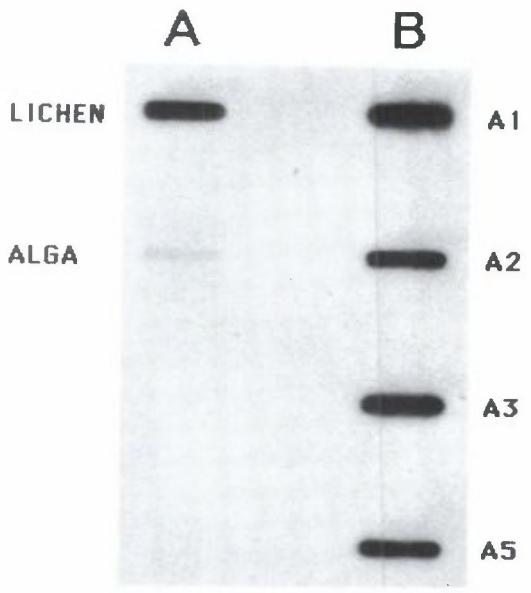

Figure 4. Test of probe specificity for fungal and algal DNA. Using a slot blot device, equal amounts (300 ng) of lichen and algal DNA were bound to a nitrocellulose membrane next to the four fungal DNA segments A1, A2, A3, A5 (50 pg each). The membrane was incubated with all four labeled probes A1, A2, A3, A5. Column A: reactivity of the probes towards lichen and algal DNA. The marginal reactivity with algal DNA demonstrates the specificity of the probes for fungal DNA. Column B: control for probe and assay functionality showing that the probes react with themselves.

First, the DNA fragments highlighted by three of the four probes (A1,A2,A3) were two to three times larger in the lichen digest (L) than in the digest from the cultured mycobiont (M). This matched the behavior of the majority of the fragments in the population and is consistent with the assumption that methylation of individual sequences generally increases in the lichenized mycobiont. However, the M lanes with probes A3 and A5 show that methylation is not completely absent in the cultured mycobiont: some DNA regions can be methylated in a few (A3) or in all (A5) of its nuclei.

Second, reciprocity could be clearly seen among the intensities of different bands, further supporting the occurrence of methylation. Probe A1: compared to the M lane, the lowest 1.3 Kb doublet in the L lane fades dramatically as the two larger and intense bands appear. Probe A2: a decrease in the intensity of the lowest 1.2 Kb band in the L lane is accompanied by the appearance of a single band of 2.2 Kb. Probe A3: the 3 Kb band visible in the M lane is almost

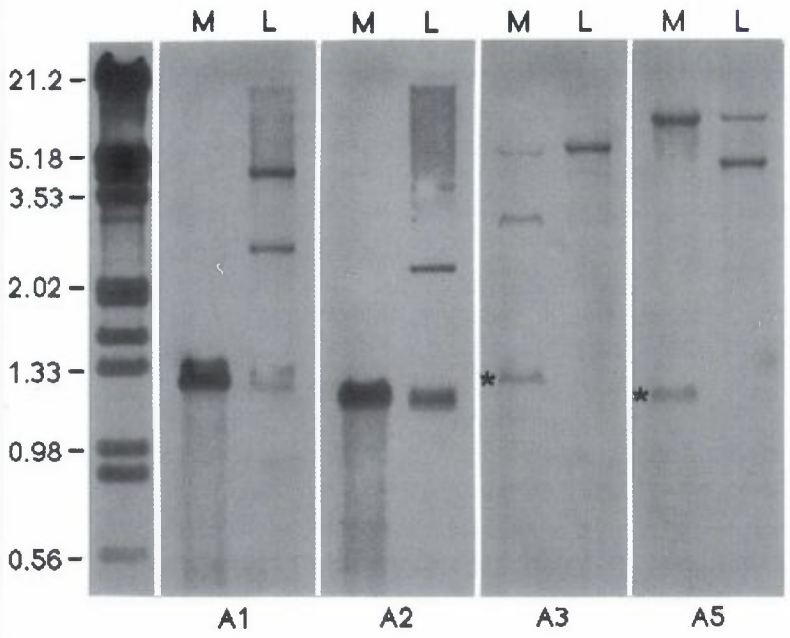

Figure 5. Restriction pattern of specific fungal gene fragments from the cultured or lichenized mycobiont. The DNA from the gel in Fig. 2 was transferred onto a membrane and hybridized separately with each of four labeled probes, A1, A2, A3, A5. Each probe was specific for a different polyketide synthase-like gene from the cultured mycobiont. The hybridization patterns are represented in panels A1–A5. M and L indicate the mycobiont and lichen patterns, respectively. Labeled lambda DNA was used in a separate hybridization to highlight the markers (left lane) whose sizes are indicated in Kb. The two faint bands marked by asterisks in panels A3 and A5 are not A3/A5-specific, but artefactual carryover from the two strongest hybridization signals in panels A1 and A2.

completely lost in the L lane where the 6 Kb band becomes stronger instead. Probe A5: the 8 Kb band fades as the 5 Kb band appears in the L lane.

Third, the frequent presence of more than one band in the gel lane with methylated fragments indicated that the methylation pattern was not necessarily identical across all copies of a given sequence in different fungal nuclei or hyphae.

DNA methylation is high in the lichenized, low in the nonlichenized parts of the Cladonia thallus

The dramatic difference observed between mycobiont cultured in liquid

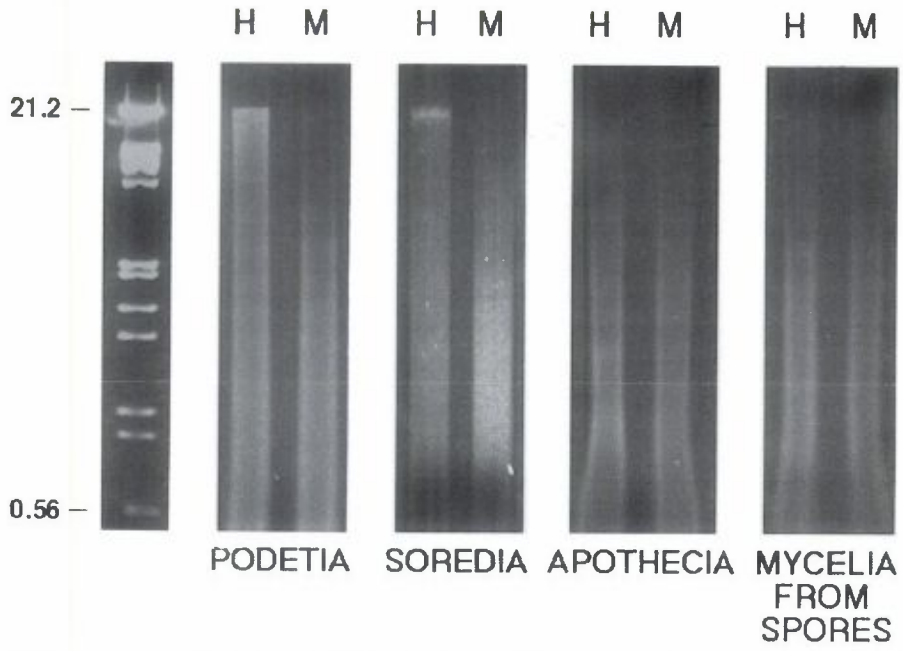

Figure 6. DNA methylation in different phases of the fungus-alga association in *Cladonia*. DNA was extracted from de-sorediated podetia, soredia, dissected apothecia, and mycelia germinating from mycobiont spores. Two equal aliquots (50–70 ng) from each DNA sample were digested: one with Hpa II (H), the other with Msp I (M). The parallel digests were run next to each other on an agarose gel and visualized through ethidium bromide fluorescence. The source of the DNA is indicated for each panel of parallel digests. The two enzymes produce different digestion patterns if the DNA is significantly methylated (podetia, soredia), the same pattern if methylation is low or absent (apothecia, mycelia). Left panel: size markers (Kb).

medium and lichen thallus suggested that DNA methylation may correlate with the symbiotic state of the fungus. To investigate this possibility and confirm that methylation was the discriminating factor, we specifically tested DNA methylation in separate phases of the fungus-alga association in *Cladonia*: we used DNA from soredia and from thoroughly cleaned podetia to represent the lichenized fungus, and DNA from dissected apothecia and from spore germlings to represent the nonlichenized fungus. A common method to assay for DNA methylation uses pairs of restriction enzymes that recognize the same sequence (isoschizomers) but differ in their response to methylation (review: Nelson and McClelland, 1991). We chose the enzymes Hpa II and Msp I. Both cleave at unmethylated CCGG sites, but only Msp I cleaves the methylated C ^{5m}CGG sites.

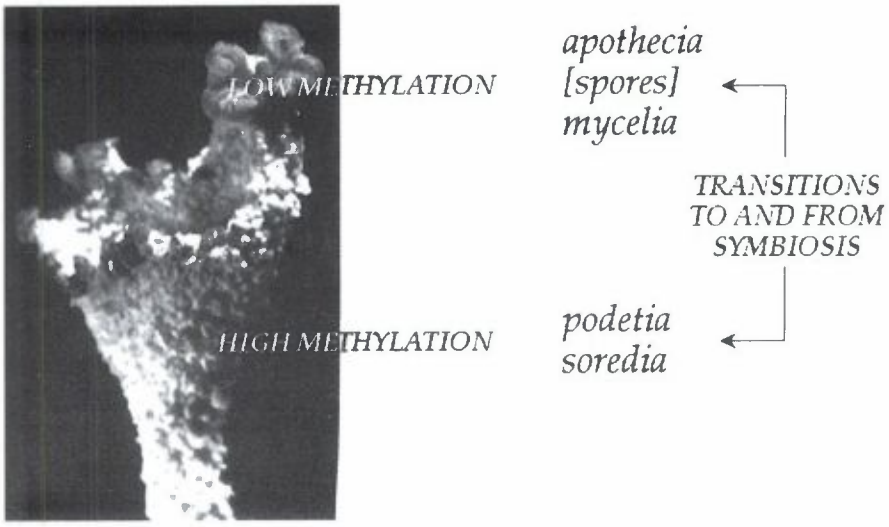

Figure 7. Outline of the relationship between DNA methylation and mycobiont differentiation in *Cladonia*. The figure relates the structure of a *Cladonia* cup-like thallus (left panel) to the DNA methylation levels observed in its various tissues and in the germinating spores obtained from apothecia. Methylation was high where the fungus was associated with the alga: in the cleaned somatic tissue of the cup itself (podetium) and in the vegetative propagules that it produces (soredia, the "warts" covering the cup). Methylation was low where the fungus was not associated with the alga: in the spore-producing ascocarps (apothecia) on the upper rim of the cup and in the mycelia generated from the spores. The brackets indicate that DNA methylation levels have not been measured directly in isolated, ungerminated spores.

Equal amounts of test DNA were treated separately with each enzyme and the two resulting digests were fractionated in adjacent lanes on a gel. Qualitatively, a clear difference between the two electrophoretic patterns indicates that the DNA is methylated, whereas little or no difference means little or no methylation. Fig. 6 compares the results of such a test on the DNA from cleaned podetia, cleaned soredia, cleaned apothecia, and mycelia germinating from mycobiont spores. A large difference was seen between the Hpa II (H) and Msp I (M) digests of podetial DNA, and this large H/M difference persisted in soredial DNA. From gel scans (not shown), Hpa II resistant DNA in soredia was estimated to represent 5–10% of the total genome. On the other hand, no difference was apparent between the H and M lanes of either apothecial or sporeling DNA. These results showed that the DNA of the *Cladonia* fungus was significantly methylated in the lichenized parts of

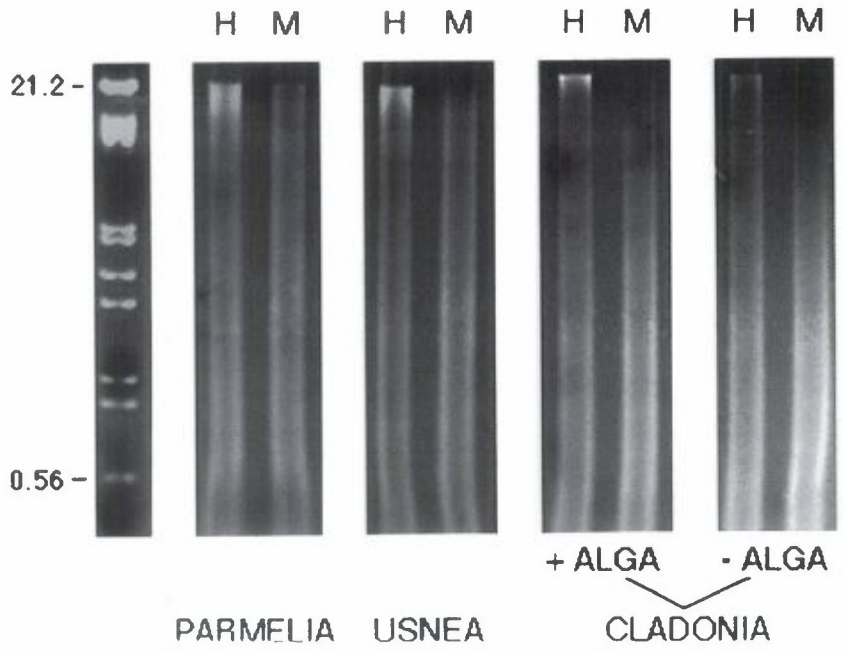

Figure 8. Application of the Hpa II/Msp I methylation test to *Parmelia* and *Usnea*. Effect of the alga on the test in *Cladonia*. The test was conducted as described in Fig. 5 and in the text. The symbols are as in Fig. 5. The source of the DNA is indicated for each panel of parallel digests. In the *Cladonia* panels, "+ Alga" refers to intact podetia covered with soredia, "–Alga" to podetia scraped free of soredia. Left panel: size markers (Kb).

the thallus, i.e podetia and soredia, while methylation was reduced to levels undetectable by this assay in the nonlichenized apothecia and germinating spores (Fig. 7).

The Msp I/Hpa II test was also performed on DNA from intact *Usnea* and *Parmotrema* thalli (Fig. 8), and the resulting patterns indicated that genomic methylation occured also in these lichens. The parallel digests of *Cladonia* DNA in Fig. 8, one of intact podetia and the other of podetia whose algae had been removed, showed that removal of algal DNA enhanced the differential between Hpa II and Msp I digests, confirming that mycobiont DNA was methylated. Whether the DNA from lichenized or cultured photobionts is also methylated or otherwise modified remains to be determined.

4. Discussion

Eukaryotic DNA methylation

After the DNA has been replicated, methyl groups may be added by special methyltransferases (Leonhardt and Bestor, 1993; Adams et al., 1993) to the 5' carbon of the cytosine ring in selected target sequences. The methyl groups are exposed on the surface of the major grove of the double helix and do not interfere with basepairing. Methylation can thus change the interactions between DNA and the proteins that recognize specific features of its surface. Once a methylation pattern is established, methyltransferases can maintain it after DNA replication, ensuring "memory" of the pattern across cell generations (Riggs, 1990). However, if not actively preserved, methylation is lost through DNA replication. On this basis, DNA methylation is generally viewed as an epigenetic modifier of gene expression and phenotype that can act both permanently or transiently (Holliday, 1990).

DNA methylation in Cladonia

We find that fungal DNA in the lichen *Cladonia grayi* is highly methylated, whereas the DNA of the nonlichenized mycobiont is mostly unmethylated. The percent of methylated cytosines in the *Cladonia* genome is sufficiently high to inactivate about half of the available BamH I/EcoR I sites (Fig. 3), and the methylated regions comprise 5-10% of the DNA in soredia (estimate from Fig. 6). The actual amount of methylated cytosines and their distribution awaits determination. It is clear, however, that methylation in the *Cladonia* lichen fungus is significantly higher than in nonlichen fungi (reviews: Antequera et al., 1984; Magill and Magill, 1989; Selker, 1993).

Whether algal DNA undergoes any parallel modifications is an important question to address, but is outside the scope of this paper. However, from the behavior of the fungal specific probes (Figs. 4 and 5) and the restriction patterns of the cleaned podetia (Figs. 6 and 8) with no soredia and only a few algae embedded in the fungal tissue, it is clear that the unknown methylation condition of the small amount of algal DNA present in some of our samples does not significantly affect our results.

Is DNA methylation correlated with symbiosis in lichen fungi?

The mycobiont genome is methylated in somatic podetial tissue and in soredia, and unmethylated in apothecia, in freshly germinated spores on solid medium, and in long-term liquid culture of the mycobiont (Figs. 2 and 6). The DNA of two other lichens tested, *Usnea* and *Parmotrema*, is also methylated

(Fig. 8), suggesting that high levels of DNA methylation may be a general feature of fungi in the lichenized state. Free-living fungi show low levels of DNA methylation, and the nonlichenized *Cladonia* mycobiont follows that rule. Its methylation is undetectable by the Hpa II/Msp I test, although the more sensitive Southern technique shows that it is not completely absent (Fig. 5, probes A3 and A5).

In *Cladonia*, dramatic transitions in morphology and function occur within the thallus (Hammer, 1993; Jahns et al., 1995) as well as in germinating spores or vegetative propagules. Overall, our results suggest that these transitions are accompanied by large increases in methylation when the direction is toward symbiosis, and corresponding decreases when symbiosis is abandoned (Fig. 7). It might even be possible to identify cytologically the sites of a sharp epigenetic transition from high to low methylation: they may be where the ascogenous hyphae spring from the lichenized tissue of the podetium and initiate the differentiation of ascocarps. From the smallest primordium, the ascogenous hyphae can be clearly distinguished morphologically and histologically from those of the surrounding tissue (Jahns et al., 1995).

Methylation and gene regulation

The percent and distribution of 5-methylcytosine across the *Cladonia* genome remain to be determined. However, the degree to which BamH I/EcoR I restriction is inhibited (Fig. 3) and the resistance of 5–10% of the soredial DNA to Hpa II digestion (Fig. 6), suggest that large numbers of genes are methylated, unless methylation in *Cladonia* is limited to non-coding regions of the genome. The latter possibility is unlikely since each of the four genes tested individually was found to be methylated (Fig. 5). Large-scale shifts in methylation might therefore reflect large-scale reprogramming of genes to switch between "free-living" and "lichenized" modes.

The data in Fig. 5 represent these two modes for individual genes likely to encode the polyketide synthetases of *Cladonia grayi*. These genes are generally more methylated in the lichen, which is active in secondary product synthesis, than in the mycobiont grown in liquid culture, which is not (Culberson and Armaleo, 1992; Armaleo, 1995). The correlation between methylation and symbiosis becomes one between methylation and gene activity. It still remains a rough correlation, however. We do not know which, if any, of the four probes highlights a pks gene involved in secondary product biosynthesis. We do not know whether some of these putative pks genes are clustered and coordinately regulated with other genes involved in polyketide biosynthesis, as is the case in *Aspergillus* (Brown et al., 1996). The precise correlation for these and other lichen genes must await the individual

identification of each gene and the analysis of its transcriptional activity in relation to methylation. In eukaryotes, higher methylation is often, but not always, associated with lower gene activity (Grässman and Grässman, 1993). Does this mean that a large part of the activity of the fungal genome is turned down during symbiosis? Even just the data in Fig. 5, in which three DNA segments (A1, A2, A3) show higher and one (A5) lower methylation in the lichen, suggest that the answer will be complex. Furthermore, the presence of multiple bands in one lane suggests that a given gene can be found in different methylation (regulatory?) states, even within the limits of a single "lichenized' or "nonlichenized" category.

The complexity of even a partial view of four individual genes reflects the functional and morphological complexity of the fungal mycelium, whether it threads through different tissues in a podetium or radiates through an artificial medium. Mycelial versatility has also been defined as "instability" (Rayner et al., 1995), and DNA methylation may be one of the components involved. Variations in the low methylation levels of some free-living fungi have been correlated with developmental transitions (Antequera et al., 1985; Russell et al., 1987a,b). In *Cladonia*, however, the nature of the phenomenon suggests that lichen mycobionts might have expanded the scope of fungal DNA methylation to cover an uncharted territory: symbiosis.

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