Cloning of *Trametes versicolor* Genes Induced by Nitrogen Starvation

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Received 3 August 1987/Accepted 10 March 1988

We have screened a genomic library of *Trametes versicolor* for genes whose expression is associated with nitrogen starvation, which has been shown to induce ligninolytic activity. Using two different approaches based on differential expression, we isolated 29 clones. These were shown by restriction mapping and cross-hybridization to code for 11 distinct differentially expressed genes. Northern analysis of the kinetics of expression of these genes revealed that at least four of them have kinetics of induction that parallel kinetics of induction of ligninolytic activity.

Over the past few years, microbial degradation of lignin has been the focus of a number of investigations (4, 16, 25). Still, very little is known about this process, its onset, and its regulation. Lignin degradation has very specific features that distinguish it from ordinary catabolic pathways. Lignin degradation is not induced by its substrate (13). Its appearance is associated with cessation of primary growth (13), and thus ligninolytic activity can be found in cultures in absence of the substrate. Furthermore, ligninolytic activity requires the presence of another carbon source such as glucose or cellulose (15). Finally, it can be suppressed by factors seemingly unrelated to lignin itself such as nitrogen (8). These results suggest a regulation mechanism in which the substrate, lignin, plays a minor role if any and raise the possibility that ligninolytic activity is a secondary if not accidental role for many of the genes involved. Recently a number of prospective enzymes involved in lignin catabolism have been identified (17, 20, 28) in a related fungus, *Phanerochaete chrysosporium*, and one of the corresponding cDNAs was cloned (29). However, the exact role of these enzymes is controversial (11).

We were interested in identifying the structural genes responsible for ligninolytic activity as well as the genes involved in the regulation of this activity. Presumably, the expression of these genes would correlate with the ligninolytic phenotype. Thus we used differential expression as the criterion to identify and clone genes involved in ligninolytic activity. This permitted us to isolate at least four genes whose kinetics of induction were parallel to the kinetics of induction of ligninolytic activity.

MATERIALS AND METHODS

**Library of *Trametes versicolor***. Total cellular DNA of *T. versicolor* was extracted (26) and digested partially with *Sau3A*. The 10-kilobase-pair fraction was isolated from a sucrose gradient and ligated to purified BamHI arms of λ L47.1 (18). After in vivo packaging, we obtained a total of 5 × 10⁶ PFU as determined by titration on *Escherichia coli* 803. Without the addition of the 10-kilobase-pair fraction, the background was 10⁶ PFU. Since λ L47.1 is an Spi+ vector we verified the titer of our library on a P2 lysogen, strain *E. coli* C600(P2), and obtained the same titer. This indicated that most of the phages in the library were recombinants. The library was amplified once before screening.

**Differential growth cultures**. For the standard approach, *T. versicolor* ATCC 42530 was grown without agitation in 300 ml of liquid medium in 2-liter Erlenmeyer flasks at room temperature. The medium composition was 3.7 mM KH₂PO₄, 2 mM MgSO₄, 0.68 μM CaCl₂, 0.36 μM FeSO₄, 5.1 μM MnCl₂, 3.5 μM ZnSO₄, 4 μM CuSO₄, 0.91 mM Dl-phenylalanine, and 0.15 μM thiamine hydrochloride. Dextrose or cellulose was added to a final concentration of 20 g/liter. The differential growth conditions were obtained by adding nitrogen at either high concentration (high-N₂ medium; 33.3 mM L-aspagine or low concentration (low-N₂ medium; 1.25 mM L-aspargin and 1.25 mM NH₄NO₃).

For the differential kinetics of expression approach, *T. versicolor* was grown as described above but in a medium rich in nitrogen (N medium, containing malt extract [Difco Laboratories]). After 7 days the medium was removed, cells were washed with a medium low in nitrogen (I medium, the low-N₂ medium used in the standard approach) and then suspended in 300 ml of either N or I medium. Growth was resumed under the same conditions.

**Ligninolytic activity**. Ligninolytic activity was monitored by oxidation of poly B411 (Sigma Chemical Co.) as described by Glenn and Gold (10). Ligninolytic activity is expressed as the differential absorbance ratio (593 nm/483 nm) per hour.

**cDNA probes**. Total RNA was extracted from *T. versicolor* cultures by the technique of Timberlake et al. (30, 31). The RNA was then enriched for mRNA by adsorption on oligo(dT) cellulose (Bethesda Research Laboratories, Inc.) columns (18). To prepare cDNA probes, 1 μg of mRNA was heated at 68°C for 5 min and then immediately put on ice. Labeling was done with 50 μCi of [³²P]dATP (400 Ci/mmol) in a 25-μl reaction mixture containing 100 mM Tris hydrochloride (pH 8.3), 10 mM MgCl₂, 10 mM dithiothreitol, 50 mM KCl, 4 mM Na₃P₂O₇, 1 mM each dGTP, dCTP, and dTTP, 20 μg of oligo(dT) (Sigma) per ml, 180 U of RNAsin (Amersham), and 50 U of reverse transcriptase (Bethesda Research Laboratories, Inc., or Siekagaku). Incubation was for 1 h at 37°C, followed by 3 h in 3 N NaOH–10 mM EDTA at the same temperature. The probe was separated from [³²P]dATP by gel filtration on a Sephadex G-50 column.
Screening of library and purification of clones. E. coli 803 was infected with the library phages of T. versicolor and spread on a single 500-cm² petri dish. Four filters were blotted on the petri dish (1). Each filter was hybridized with \(10^7\) cpm of the cDNA probe. After autoradiography, plaques giving a differential signal were picked and purified to homogeneity by the same procedure on 90-mm petri dishes.

**Mapping of the genes.** Restriction enzymes were used according to the manufacturers’ instructions. For the characterization of the genomic structure and the cross-hybridization analysis, the insert of each clone was isolated and labeled by random primed synthesis (7). The hybridization procedure was that of van der Ploeg et al. (32), with modifications (2).

**Northern analysis.** Northern (RNA) blots were prepared as follows. mRNA (10 µg) was electrophoresed on formaldehyde-agarose gels as described by Rave et al. (22), with modifications (19). After migration, the gel was washed three times for 30 min each in 0.01 M phosphate buffer (pH 6.5), treated with NaOH as described by Maniatis et al. (18), transferred to nitrocellulose, and hybridized with probes made from each clone (2).

**RESULTS**

In order to produce cultures of *T. versicolor* differentially expressing the ligninolytic activity, two approaches were used. The first was the standard approach used for cloning differentially expressed genes (27), which is based on growth of the organism under conditions of expression and nonexpression of the genes of interest. We grew *T. versicolor* in two media differing only in their nitrogen concentrations. Nitrogen starvation has been shown to induce ligninolytic activity (13). Indeed, we found that in media with low nitrogen concentration we could readily detect ligninolytic activity by using the poly B411 colorimetric technique of Glenn and Gold (10). The ligninolytic activity in the *T. versicolor* cultures with high nitrogen concentration was at least 10-fold lower. From each culture we extracted the mRNAs, and these were used to prepare cDNA probes. However, we had to wait 16 to 18 days to have a high level of ligninolytic activity in the low-N\(_2\) cultures, whereas the high-N\(_2\) cultures had to be harvested while still in the primary growth phase, which was 7 days at the most. Thus, besides nitrogen concentration, the two cultures differed also in the time of growth. This, we felt, could result in differential expression of genes induced by other factors than nitrogen starvation, such as depletion of other nutrients.

In view of this possibility, we used a second approach, which we called differential kinetics of expression, that was aimed at minimizing differences in growth conditions except for nitrogen starvation. *T. versicolor* was grown in a rich nitrogen medium (N medium) for 7 days, at which time it was still in the primary growth phase. Then the medium was removed, and cells were washed; in one set of cultures fresh N medium was added, whereas in another set the medium added was low in nitrogen concentration (I medium). We then monitored ligninolytic activity (Table 1). In the cultures where the medium was replaced by the I medium, the ligninolytic activity was induced from background on day 0 to a level at least fourfold higher after the first day and continued increasing for at least 7 days. In cultures where the N medium was added, the increase in ligninolytic activity was much slower and remained much lower than in the I-medium culture. To screen for induced ligninolytic genes, we prepared three probes made from mRNA extracted on day 0 (N0) and on day 2 from both the low (I2)- and high (N2)-nitrogen cultures. The I2 probe was to permit identification of genes whose expression was induced by nitrogen starvation when compared with that on day 0 (N0), whereas the N2 probe would allow us to eliminate from the induced genes the ones that had been turned on by factors other than nitrogen starvation.

**Screening of *T. versicolor* library.** The genome size of *T. versicolor* is unknown, but the genome size of another white rot fungus, *P. chrysosporium*, has been estimated at about 5 \(\times 10^7\) kilobase pairs (23). This is similar to the genome sizes of other fungi (5, 6). Presuming that the genome of *T. versicolor* is of similar size and having cloned fragments of about 10 kilobase pairs, we estimated that the genome of *T. versicolor* would be represented on average once in every 5 \(\times 10^5\) PFU of our library.

For the standard approach, we screened \(5 \times 10^6\) PFU with cDNA probes made from mRNAs extracted from the low-N\(_2\) culture and the high-N\(_2\) culture. We then compared the hybridization patterns (Fig. 1). Most plaques gave similar signals with both probes. Some plaques gave a stronger signal with the high-N\(_2\) probe than with the low-N\(_2\) probe, and just a few plaques gave a stronger signal with the low-N\(_2\) probe than with the high-N\(_2\) probe (arrows). Eighteen plaques identified as belonging to the last category were isolated and purified to homogeneity.

In the case of the differential kinetics of expression approach, we hybridized 1.25 \(\times 10^5\) clones of the *T. versicolor* library with the N0, N2, and I2 probes described above. We looked for clones that would give a stronger signal with the I2 probe than with both N0 and N2 probes. We found 11 such clones, which we picked and purified to homogeneity. Examples of the differential signals obtained with purified clones are given in Fig. 2. Clones 101 and 113 hybridized much more strongly with the I2 probe than with the N0 and N2 probes. Note that low levels of expression were detected with the N0 and N2 probes. This correlates with low levels of ligninolytic activity (Table 1). Presumably, due to variations in microenvironment, even during the active growth phase, some of the cells were already under nitrogen starvation.

**Mapping of the clones.** In Fig. 3 we have drawn the physical map of the inserts present in the 18 phage clones isolated by the standard approach, as deduced by restriction enzyme analysis. These were subdivided into five groups. The first one contained clones 3, 8, 13, 15, 18, 20, 21, 23, 24, 25, 26, and 27. The inserts in these clones had overlapping restriction maps, such that they could be aligned to give a continuous restriction map (top of Fig. 3). It should be noted that this DNA segment contained at least two distinct differentially expressed genes, since some clones did not share overlapping sequences. Clones 10, 12, and 22 also gave
FIG. 1. Screening of the genomic library of *T. versicolor*. Library phages (5 x 10⁴) were plated on a single 20- by 20-cm petri dish. Replica filters were blotted on this petri dish and hybridized with cDNA probes prepared from a high-nitrogen culture (n) or a low-nitrogen culture (i). The probes had identical specific activities, and 10⁷ cpm of the cDNA probe was used on each filter. Arrows indicate examples of plaques showing induction of expression on the i autoradiogram compared with that on the n autoradiogram.

overlapping restriction maps, whereas clones 11, 31, and 34 each gave a unique restriction map. Thus, we isolated at least six distinct genes.

The 11 clones isolated with the differential kinetics of expression approach could be ascribed to five individual genomic fragments (Fig. 4). Clones 49, 101, 102, 111, and 114 shared overlapping restriction maps, as did clones 105 and 112 and clones 104 and 107. Clones 109 and 113 each represented distinct segments. Thus, with this approach we isolated at least five distinct differentially expressed genes.

All of the clones from both approaches were hybridized to each other. There was no cross-hybridization between clones of different groups, which indicated that the 11 genes isolated from both approaches were different from one another. Each clone was used as a probe to map the corresponding genomic DNA; these were identical to the maps of the cloned fragments. The polymorphism of the EcoRI sites of clones 104 and 107 (Fig. 4) was also seen in the genomic DNA of *T. versicolor*. Thus, clones 104 and 107 probably represent two alleles present in the *T. versicolor* genome.

To delineate more precisely the DNA sequences coding for the differentially expressed genes, we digested each clone with a series of restriction enzymes and hybridized the resulting fragments with the same cDNA probes used to isolate the clones. Only certain fragments of each digest of each clone hybridized with the probes, and this permitted us
FIG. 3. Restriction maps of the clones isolated by the standard approach. When multiple overlapping clones were isolated, a composite restriction map is presented at the top. Clones 3 and 20 were identical, as were clones 13 and 18. The open boxes correspond to the regions coding for the induced genes. S, SalI; H, HindIII; E, EcoRI; B, BamHI; X, XhoI; Kb, kilobase.

to delineate which segments of the cloned fragments carried the differentially expressed genes. These segments are illustrated in Fig. 3 and 4 as open boxes. Note that there is one box common to clones 13, 15, 18, 21, 23, 24, 25, 26, and 27 and another box common to clones 3, 8, and 20. Notice also that there are two boxes in clone 109, which means either that there are two separate genes or that the mRNA of gene 109 is a spliced transcript. By the position of the boxes, we can also predict that, except maybe for clones 11 and 109, we have full copies of the induced genes.

Analysis of the mRNAs coded by the clones. Northern analysis of all the genes isolated by both approaches confirmed that these were differentially expressed (data not shown). To establish more precisely the relation between these genes and nitrogen starvation, as well as ligninolytic activity, we did the following analysis. T. versicolor was
grown as described above for differential kinetics of expression. We extracted mRNA at the time of medium change and at days 1, 2, 4 and 7 after medium change, both in cultures where the medium was replaced by nitrogen-rich (N) or low-nitrogen medium (I). These mRNA preparations were used to prepare multiple Northern blots that were hybridized with the 11 distinct genes isolated from both approaches. With the six genes isolated by the standard approach, we observed two patterns of expression (Fig. 5). The mRNA corresponding to clone 11 was weakly present at day 0. With the N medium the expression remained at the same low level through the 7 days, whereas with the I medium the expression remained weak until day 4 but increased to a high level on day 7. This clearly indicates that clone 11 contains a gene that is induced by low nitrogen concentration. However the kinetics of mRNA induction did not correspond to the kinetics of induction of ligninolytic activity (Table 1). We conclude that gene 11 is a nitrogen starvation gene but not a ligninolytic gene. Clone 34 showed induction of expression both with the N and I media. This indicates that induction is not directly linked to nitrogen starvation per se but to another, undetermined factor. All the other clones had an expression pattern similar to that of clone 34. Thus out of six differentially expressed genes isolated by the standard approach, five were not linked to nitrogen starvation.

The results obtained with the genes isolated by the differential kinetics of expression approach were quite different (Fig. 6). At day 0 and also with the N medium there was very little mRNA in all of the clones. However, with the I medium mRNA was induced at the time of induction of ligninolytic activity (Table 1). However, in clones 101 and 113 there appeared to be a lag in mRNA induction compared with ligninolytic activity induction (see Discussion). Clone 105 is not presented because the signal we obtained with this clone was very weak. These analyses were repeated with other mRNA preparations and gave similar results.

**DISCUSSION**

The purpose of this work was to isolate genes related to ligninolytic activity. The number of genes involved and the exact nature of their products are still unknown (14). Keyser et al. (13) have shown that nitrogen starvation induces ligninolytic activity. Thus we have decided to clone genes whose expression would be induced by nitrogen starvation. To do so we used a technique of differential hybridization that has been used successfully to clone differentially expressed genes (3, 9, 12, 21, 27, 33). However, instead of preparing a subtractive cDNA probe (33) or picking individual plaques (27), we made total cDNA probes of each mRNA preparation and hybridized these directly to replica filters of plaque spreads. This very simple approach had two important advantages: it permitted us to screen numerous plaques (>10³) in one assay and to simultaneously compare hybridization of the same plaques to a number of different cDNA probes (>4). This last point permitted the isolation not only of genes differentially expressed in cultures with or without
ligninolytic activity but also of genes that had the expected kinetics of expression in relation to the kinetics of ligninolysis.

In our first approach, T. versicolor was grown in high- or low-nitrogen medium, and cDNA probes were made from the mRNA extracted from both cultures. However, the two cultures had to be grown for different lengths of time. We felt that this increased the probability that other genes not related to nitrogen starvation would also be differentially expressed in these cultures. Indeed, from the six differentially expressed genes that were isolated by this approach, five were not linked to nitrogen starvation per se; the sixth, although clearly linked to nitrogen starvation, did not have kinetics of induction compatible with the kinetics of induction of ligninolytic activity.

In our second approach, we grew T. versicolor in a rich medium. After 7 days, while cells were still in primary growth, we replaced the medium with either the same medium or a medium with low nitrogen concentration. The mRNA was extracted at various days after the medium change, and gene expression was compared among various cultures. This permitted us to compare gene expression in cultures that had been grown separately for only 2 days and yet showed marked differences in levels of ligninolytic activity. We isolated five distinct T. versicolor genes that showed differential expression. Kinetics of induction of expression could be determined for four of these five genes and were shown to be parallel to induction of ligninolytic activity. Kinetics of expression of clone 104 and 109 could be superimposed on the kinetics of ligninolytic activity, whereas the kinetics of expression of clones 101 and 113 were slightly delayed compared with ligninolytic activity. Such a discrepancy between induction of expression and activity has also been observed for the ligninase gene of P. chrysosporium (29) and could represent mixed mRNA species. The fifth differentially expressed gene that we cloned (clones 105 and 112) coded for low levels of mRNA as judged by Northern analysis. Some genes, especially the ones involved in regulation, would be expected to express low levels of mRNA. To further characterize these five genes and to try to determine their relation to ligninolytic activity, we will clone them in an expression vector and have them expressed at high levels in another fungus system, Aspergillus species (work in progress).

How many genes are involved in ligninolytic activity? This is still unclear (14). The situation might be complicated by the fact that ligninolytic genes are not specific to their substrate and thus probably are part of other metabolic pathways that involve other genes not directly related to lignin metabolism. Furthermore, we believe that there is not a single set of genes that is responsible for ligninolytic activity but that various combinations of genes can produce this phenotype. It should be noted, however, that in our approach, active or inactive genes were isolated repeatedly from distinct clones; by using a differential kinetics of expression approach, we can identify a limited number of genes whose expression parallels ligninolytic activity. While this work was in progress, another group reported the isolation of genes of P. chrysosporium induced by nitrogen starvation, by using an approach similar to our first approach (24).

ACKNOWLEDGMENTS

We acknowledge the expert technical assistance of Johanne Proulx, Diane Pankert, and Diane Rivard. We thank Nadine Gusew for her help in producing the genomic library.
P.C. is the recipient of a scientist award from the Medical Research Council of Canada. D.C. is the recipient of a studentship from the National Science and Engineering Research Council of Canada, and P.T. is the recipient of a studentship from F.C.A.C. of Quebec. This work was supported by grants from the National Science and Engineering Research Council of Canada and le Ministére de l’Énergie et des Ressources du Québec.

LITERATURE CITED


