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Establishing molecular genetics for *Phanerochaete chrysosporium*

By Ute Raeder, Wendy Thompson and Paul Broda

Department of Biochemistry and Applied Molecular Biology, University of Manchester Institute of Science and Technology, P.O. Box 88, *Manchester M*60 1*QD, U.K.*

Genetics provides an approach to the analysis of the complex function of lignin biodegradation, through the isolation of mutants and the creation of gene libraries for the identification of genes and their products. However, white-rot fungi (for example, *Phanerochaete chrysosporium*) have not so far been analysed from this point of view, and there is the challenge of establishing such genetics. *P. chrysosporium* is convenient experimentally because relatively few genes are switched on at the onset of ligninolytic activity. We describe the isolation of clones carrying genes expressed specifically in the ligninolytic phase, the development of a general strategy for mapping such clones, and the elucidation of the mating system of this organism. Another objective is the development of methods for transforming DNA into *P. chrysosporium*. This would allow the use of site-directed mutagenesis to analyse the functioning of ligninases, and the control of expression of the corresponding genes. The use of genetic crosses for strain improvement and the identification of components of the system are also discussed.

1. Introduction

An improved understanding of biological lignin degradation will have broadly two types of applications: (1) use of individual enzymes for specific industrial purposes, as will be discussed by Dr Farrell in this symposium; and (2) use of ligninolytic organisms *in situ* for delignification of plant material for use as upgraded animal food, fermentable substrates, e.g. for ethanol production by yeast, or for treatment of polluting lignocellulosic wastes. Such applications require simple and robust biological systems that work in non-sterile environments, comparable in complexity to solid-substrate fermentations such as mushroom production.

The best-known lignin degraders are the white-rot fungi, of which the most-studied representative, *Phanerochaete chrysosporium*, has already been discussed by Dr Kirk and Dr Eriksson. The work to be described in this paper also concerns *P. chrysosporium*. However, it should be noted that actinomycetes provide an alternative system about which it is known that (1) extracellular enzymes are also involved, (2) lignin attack results in soluble products as well as CO_2, and (3) it occurs during the primary growth phase, rather than only as a secondary metabolic event, as is the case with *P. chrysosporium* (see below) (McCarthy & Broda 1984).

Understanding of the ligninolytic system is necessary for its effective use and for the possibility of targeted manipulation of particular functions such as increased lignin solubilization or reduced cellulolytic activity (Johnsrud & Eriksson 1985). Classical genetics have a role in strain improvement, but can also provide information about complexity, rate-limiting steps and mechanisms of regulation. At U.M.I.S.T. we are combining classical and molecular genetical methods in the investigation of lignin degradation by *P. chrysosporium*. It is hoped that what

is learned about the methodology employed in such investigations will be applicable to other organisms, because inevitably other organisms will also be of great interest in particular contexts for lignocellulose utilization.

2. Lignin degradation by *P. chrysosporium*: a secondary metabolic event

We chose *P. chrysosporium* as the object of study because it was known that lignin degradation (which is not inducible by the presence of lignin) occurs in the secondary metabolic state in response to nutrient depletion, which is normally the level of nitrogen (Keyser *et al.* 1978). It does not occur under conditions of nutrient sufficiency during the primary metabolic growth phase. The possibility of distinguishing between the primary metabolic (non-ligninolytic) and the secondary metabolic (ligninolytic) phases offers several experimental approaches for investigation of the switch mechanism, the isolation of mutants and the cloning of genes. However, it should be noted that the concept of primary metabolism and secondary metabolism may be an over-simplification of the natural situation on lignocellulosic substrates, where nitrogen levels are constantly low (Merrill & Cowling 1966) but growth and lignin degradation nevertheless occur at the same time.

(*a*) *Mutants*

Direct screening for mutants defective in lignin degradation is difficult as lignin degradation is not a vital growth function on artificial media, and because it is presently not feasible in a plate test to screen large numbers of colonies for lignin degradation. However, Ander & Eriksson (1976) and Gold *et al.* (1982) had found that mutants for phenol-oxidase activity (another secondary metabolic activity that is easier to screen for) were also non-ligninolytic. Therefore we sought further such mutants (Liwicki *et al.* 1984). In our case, not all phenol-oxidase-defective mutants were also defective in lignin degradation; some of these mutants showed lignin degradation rates similar to or even higher than the wild type. Moreover, some of these mutants showed a difference in the timing of onset of lignin degradation. Further comparison with respect to other secondary metabolic properties displayed by the wild-type, such as increased cyclic adenosine monophosphate (cAMP) levels (MacDonald *et al.* 1984, 1985) and veratryl alcohol production (Shimada *et al.* 1981), showed that (1) several mutants differed from the wild type in a number of respects and (2) the differences in the four secondary metabolic properties tested occurred in various combinations. It therefore appears that phenol-oxidase activity is not a necessary component of the lignin-degrading system. Thus it is likely that the strains defective in phenol-oxidase activity and lignin degradation are not altered in genes coding for these functions, but in other functions not directly related to phenol oxidase, lignin degradation or even secondary metabolism. It could be, for instance, that there are alterations in cell-wall structure with general effects on protein secretion (Lysek & Esser 1970). The frequent occurrence of mutants affected in several secondary metabolic functions, but in different combinations, confirms that lignin degradation depends on a complex network of functions; disturbances in this system can result in increased, as well as decreased, activity of individual components.

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(*b*) *Cloning of secondary-metabolism-specific genes*

Comparison of the primary and the secondary metabolic states of the wildtype showed changes in the populations of total protein (Wallace *et al.* 1984) and messenger RNA (mRNA) (Haylock *et al.* 1985); of these the changes in proteins appeared more prominent. Genomic libraries of *P. chrysosporium* were screened with copy DNA (cDNA) probes derived from RNA of the primary and secondary metabolic states (3 day non-ligninolytic, high-nitrogen cultures and 6 day ligninolytic, low-nitrogen cultures, respectively). In this way we identified a small number of clones containing genes expressed only or mainly in secondary metabolism. Out of 8000 clones with an average insert size of 15 kilobases (kb), equivalent to 2.7 times the haploid genome size (see below), only 38 were of this type. This analysis also showed that only about 55% of the clones containing chromosomal non-ribosomal DNA showed detectable hybridization signals with either or both probes; of this 55%, about a quarter showed stronger signals with primary metabolism-derived cDNA than with secondary metabolism-derived cDNA.

Further analysis of the 38 clones by Southern cross-hybridization and genetic mapping (see §2(*d*)) revealed that they were derived from only 17 distinct loci. However, it is very unlikely that this screen has identified all genes specifically expressed in secondary metabolism. Apart from inherent limitations of the screening procedure (which favours the detection of strongly expressed genes in isolated positions, or co-regulated clusters), it is also possible that some mRNA species specific for secondary metabolism occur only early in the ligninolytic phase (which starts at day 3 in a low-nitrogen medium).

Measurements of mycelial dry mass of low-nitrogen-grown cultures showed a steady increase until day 3 (when lignin degradation starts), followed by a rapid decrease until day 6, when lignin degradation rates reach their maximum, and after which there are only minor changes (Raeder 1986). This suggests that recycling of mycelium is another secondary metabolic function, providing the main source of nutrients and energy for lignin degradation; in our system the rapid decline in fungal biomass ceases at day 6. It may therefore be that lignin degradation on day 6 and later depends on the presence of stable enzymes rather than *de novo* synthesis of their RNAs.

The experiments described above suggest that gene expression plays a role in the switch to secondary metabolism. We also have preliminary evidence that there are metabolic state-dependent size differences between transcripts that are present in both states (Raeder 1986). In view of the low nitrogen levels in the natural habitat of the fungus (which includes woodchips and jute) where growth and lignin degradation occur at the same time, thus allowing penetration of the substrate, one can envisage that the shifts between expression of growth and ligninolytic functions must occur rapidly. Such shifts may be controlled not only at the transcriptional level, but also at the post-transcriptional level, e.g. through differential rates of processing of constantly present pre-mRNA pools, with coding capacity for both growth and ligninolytic functions. We are currently investigating this further.

(*c*) *Genomic and genetic properties*

An investigation of basic genomic properties of *P. chrysosporium* (strain ME446) showed that it has a haploid genome size of about 44×10^6 base pairs, which is within the known range for fungi. Of this, about 20–30% is repetitive ribosomal DNA (GC 52%) and mitochondrial DNA (GC 33%). The remainder is chromosomal DNA (GC 59%), of which some but not

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much is also repetitive. These properties also apply to another isolate of *P. chrysosporium*, previously known as *Sporotrichum pulverulentum* Novobranova (ATCC 24725), with which it shows high DNA homology but differs with respect to the positions at which restriction sites occur (Raeder & Broda 1984).

An aspect of this study led to the development of a general mapping strategy for cloned sequences. We observed that the restriction patterns of cloned chromosomal DNA fragments themselves were often simpler than the patterns of the restriction fragments in digests of total DNA, when probed with such cloned DNA (for both strains of *P. chrysosporium*). The high proportion of cloned sequences that showed such relatively complex restriction patterns (in Southern hybridizations to total DNA) made it unlikely that this was due to repetitive DNA sequences. It was more likely that both strains contain two genome equivalents that are basically homologous but differ from each other with respect to the positions where restriction sites occur. That is, restriction fragment-length polymorphisms appeared to be abundant not only between strains but also between haploid genome equivalents within individual strains.

Southern hybridization of cloned sequences to DNAs of single basidiospore-derived cultures provided a test for this hypothesis. Basidiospore-derived DNAs showed either one or other of two simpler hybridization patterns, termed 'A' or 'B', which in combination were equivalent to the parental ('AB') pattern. This showed that two genome equivalents had segregated in the formation of these spores. Moreover, different basidiospores displayed different combinations of A and B alleles when several such cloned DNAs were used as probes. This too is expected of haploid products of meiotic recombination. In contrast, DNAs from 12 single conidiospore-grown cultures all showed the more complex parental hybridization pattern (Raeder & Broda 1986). Consistent with the latter observation, microscopic analysis of large numbers of conidiospores showed that they contained 2 nuclear bodies (Thompson & Broda 1987). It therefore appears that conidiospores of this isolate are heterokaryotic dikaryons, while on this model basidiospores are homokaryotic, although not necessarily monokaryotic.

An analysis of the mating behaviour of *P. chrysosporium* indicated that the one isolate that could be induced to fruit in culture (strain ME446) was not homothallic, as had previously been assumed (see, for example, Eriksson *et al.* 1978; Alic & Gold 1985). In contrast, this isolate possesses a unifactorial homogenic incompatibility (mating) system governing heterokaryon formation, i.e. it exhibits bipolar heterothallism/diaphoromixis (figure 1) (Thompson & Broda 1986). This is the condition whereby dikaryon/heterokaryon formation can only be accomplished following conjugation between homokaryons (i.e. primary mycelia derived from single basidiospores) differing at one mating-type locus, there being numerous alleles of this locus in the natural populations.

(*d*) *Genetic mapping of cloned sequences*

We have described how it is possible, in the DNA of meiotically derived cultures, to distinguish between two polymorphic alleles corresponding to cloned sequences. This allowed us to test for genetic linkage between cloned sequences by determining whether the A and B alleles corresponding to the different cloned sequences had segregated together or independently in a 'test' set of haploid DNAs (Raeder & Broda 1986). If two cloned fragments are allelic, they should give the same patterns of As and Bs. If two cloned fragments derive from different chromosomes, each would give its own independent pattern of As and Bs because the segregation of the chromosomes is independent. Where two cloned sequences, but not allelic

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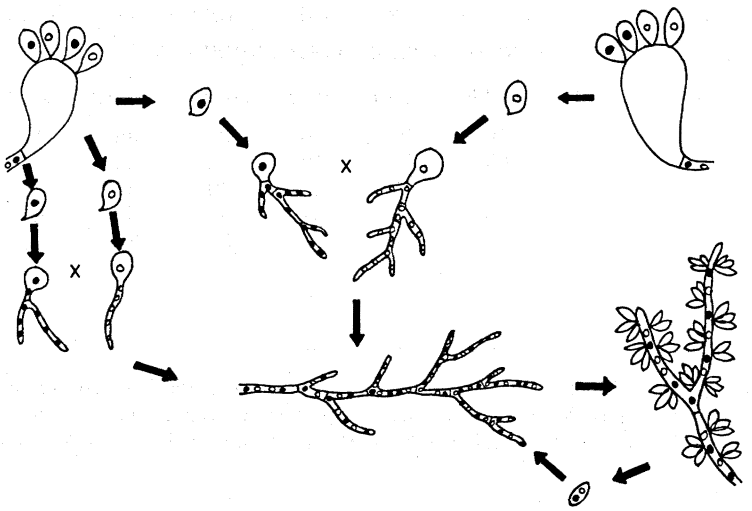

FIGURE 1. Life cycle of *Phanerochaete chrysosporium* ME 446. Sib-related matings have been demonstrated in culture; non-sib-related matings have not yet been demonstrated but are expected on the basis of known out-crossing behaviour of other *Phanerochaete* spp. investigated (A. M. Ainsworth & A. Rayner, personal communication).

sequences, are linked the patterns of A and B will be congruent except where there has been a crossover event between the two.

We therefore isolated DNA of 53 basidiospore-derived cultures by using the rapid DNA preparation method of Raeder & Broda (1985) and prepared Southern blots with *Sal*I-restricted DNAs. The 38 clones containing genes expressed specifically in secondary metabolism (see §2(*b*) above) were used as hybridization probes. Of these, 36 revealed interallelic restriction-site polymorphisms with *Sal*I-restricted DNA. Comparison of their segregation patterns showed, in general, 100% linkage (100% congruence of allelic segregation patterns) for cloned sequences that contained strongly homologous sequences. This confirmed that they were derived from the same locus. Among the non-allelic cloned sequences (17 in all), two groups of three and three groups of two showed close genetic linkage (figure 2, table 1). With one of these clusters of three markers, ordering has been possible by using three-point cross analysis (Raeder & Broda 1986). However, a surprising finding was that two of the cloned sequences that had been shown to have strongly homologous coding sequences (and to hybridize to the same-sized secondary-metabolism-specific and abundant RNA transcript) are, in fact,

no. 6	BAABAABAAB BBAAAABAAA BBAABABBBB
no.14	BBBBBBABBA ABBBAABBAA AAABBBABBB
no.36	BBBBBBABBA ABBBBABBAA AAABBBABBB
no.20	BBBBBAABBB ABBBABBBAA AAABABABBB
no. 7	BBBAAAABAA BAABBABBAA ABBBBBBABA
no.18	BBBABAABAA BBBBBABBBA ABBBBABAAB
no.19	BBBABAABAA BAABBABAAA ABBBABBABA
no. 8	BBBBBBBAAB BABABBAABB BBBABBAABA
no.33	BBBBA?AABB ABBABAAABB BBBABBAABA

FIGURE 2. Meiotic segregation patterns of alleles of cloned loci containing genes specifically expressed in secondary metabolism.

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TABLE 1. PERCENTAGES OF CONGRUENCE BETWEEN SOME OF THE OBSERVED ALLELIC SEGREGATION PATTERNS OF NINE CLONED SEQUENCES

clone	no. 6	no. 14	no. 36	no. 20	no. 7	no. 18	no. 19	no. 8	no. 33
no. 6	—	42	38	40	36	34	38	47	52
no. 14	—	—	**98**	**87**	53	58	55	57	60
no. 36	—	—	—	**85**	56	60	56	58	61
no. 20	—	—	—	—	51	57	53	55	56
no. 7	—	—	—	—	—	**83**	**87**	51	52
no. 18	—	—	—	—	—	—	**81**	53	50
no. 19	—	—	—	—	—	—	—	60	54
no. 8	—	—	—	—	—	—	—	—	**85**
no. 33	—	—	—	—	—	—	—	—	—

As the parental arrangement of alleles is not known (homokaryons were not detected among the conidiospores) the assignment of the two allele types 'A' and 'B' to restriction patterns was arbitrary. Therefore, linkage is revealed by either very high or very low values in the table (e.g. greater than 80% or less than 20% congruence).

genetically unlinked. These two clones therefore contain either two similar genes that are highly expressed only in secondary metabolism, or a gene and a silent pseudogene.

The cloned sequences were also used to test the DNAs of some of the mutants referred to above (§2(*a*)) (U. Raeder, R. Liwicki & P. Broda, unpublished results). Hybridizations of these probes to Southern blots of restricted DNAs of the mutants (which had been selected from γ-irradiated conidiospores) revealed the following. Several mutants showed haploid hybridization patterns with some but not all probe sequences, suggesting partial haploidization (large deletions or chromosome losses). Others showed haploid patterns with all probe sequences that were tested. Still others showed diploid patterns with all probe sequences, as was observed in the wild-type conidiospores.

3. IMPLICATIONS OF GENETIC HETEROGENEITY FOR BREEDING, GENETIC MAPPING AND CLONING

The considerable genomic heterogeneity within individual strains of *P. chrysosporium* and between different strains classified as belonging to this species (Raeder & Broda 1984) gives rise to the expectation that there is also variation in the efficiency of lignin degradation. The observation by McCarthy *et al.* (1984) that *S. pulverulentum* showed higher rates than the closely related strain *P. chrysosporium* (ME446) is consistent with this. With the availability of haploid recombinant basidiospores, a mating system and a mapping system for cloned DNA, it is possible to test strains of differing genomic combinations for lignin degradation and at the same time relate differences in ligninolytic activity to their genomic properties. In the following, we want to outline current experimental strategies.

1. It is possible that the higher lignin-degradation rates observed in some of the phenol-oxidase-defective mutants referred to earlier (§2(*a*) and (*d*)) are caused by derepression resulting from homozygosity rather than to fortuitous up-mutations in limiting ligninolytic functions. Screening of the 53 haploid test strains for their efficiency of lignin degradation will reveal whether significant variation exists and if so whether it can be used in a breeding programme to increase this efficiency (figure 3).

2. Mutants obtained from haploid monokaryotic spores would be superior to those isolated so far, because in these the altered phenotypes should be solely due to mutations, because the

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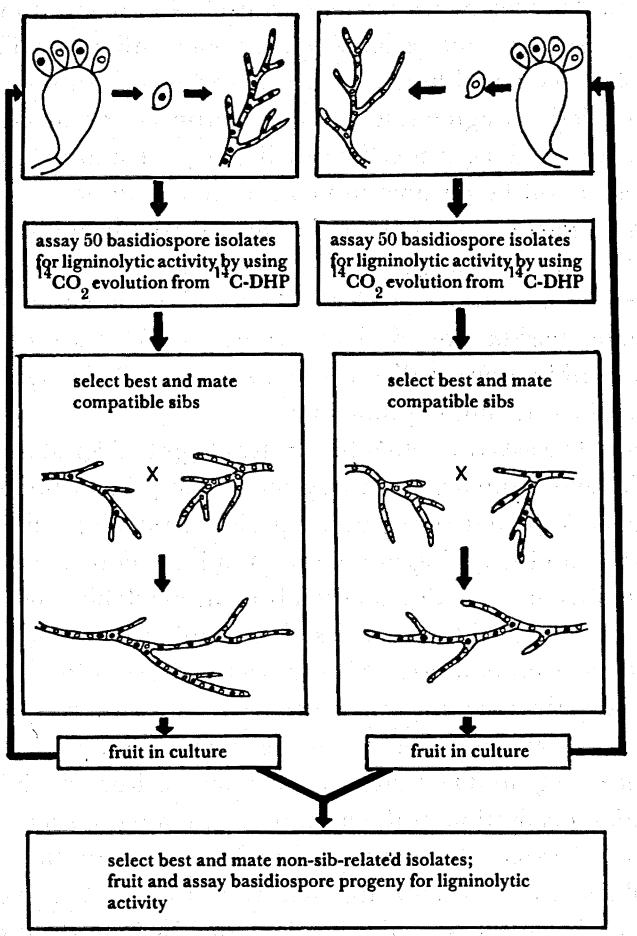

FIGURE 3. Breeding programme envisaged for obtaining isolates of *P. chrysosporium* with enhanced ligninolytic activity.

effects of diploidy and possible haploidization referred to above are excluded. If homozygous 'up-mutations' could be found they would be interesting for the analysis of regulation.

3. The mapping of further cloned sequences by using the 53 meiotically derived test strains will be performed with randomly chosen clones until it is possible to infer linkage groups and an overall genetic map. The number of randomly chosen marker clones required for this purpose depends on the genetic map length of this fungus (determined by the number of chromosomes and crossovers per chromosome), which is presently unknown but should become apparent from the experiment. About 100 randomly chosen marker clones are needed to map 1000 centimorgan at the 30 cM level. With the high frequencies of polymorphisms that we observe with *Sal*I- and also *Pst*I-restricted DNA, we expect to establish a map with 100–300 hybridizations. (Experimentally, 20 such hybridizations are done in parallel against reusable filters from gels with haircombs as slot formers.)

4. One result of such mapping will be to establish whether close genetic linkage found among some of the clones carrying secondary metabolism-specifically expressed genes is from close physical clustering, as in the *SpoC*1 cluster in *Aspergillus nidulans* (Orr & Timberlake 1982; Gwynne *et al.* 1984) or from *P. chrysosporium* having a short genetic map. Subsequent screening

of the cloned DNAs in dot blot hybridizations with cDNAs made from mRNA of different metabolic states (e.g. primary and secondary metabolic mRNA) will reveal whether the apparent clustering of co-regulated functions is a more general feature of this organism.

5. As the map of restriction fragment-length polymorphism (*i.e.* arbitrary) markers can not only be used to map new cloned sequences but can also be related to any phenotypic segregation among the test strains, it will be possible to identify cloned sequences close to or containing the gene(s) specifying these phenotypes. Thus, in the study of the mating system that was done with 12 of the 53 test strains, it emerged that one of the cloned sequences already mapped is probably closely linked to the mating-type locus. If there is variation in the lignin-degradation rates among the 53 monobasidiospore-derived strains, the DNA segregation date might also reveal that particular combinations of alleles occur systematically in high lignin degraders, thus identifying loci coding for rate-limiting functions.

6. In addition, it could be asked whether high rates of lignin degradation are connected with specific changes in the extracellular, secondary metabolic protein population. These can be quantitated, e.g. by enzyme activity or by using specific antibodies. This approach, too, might lead to the identification and mapping of the rate-limiting components.

7. If regulatory mutants in lignin degradation became available it could be possible to map and eventually clone the responsible sequences. This would require one to screen the haploid progeny from a wild-type × mutant cross with respect to segregation of phenotypes and then to repeat the mapping process with their isolated DNAs. However, the number of probe sequences needed for such an operation would be smaller because only genetically equally spaced cloned sequences (e.g. in 30 cM intervals, indentified from the initial mapping) would be needed for the initial localisation of the mutations. Cloning of such sequences would then be done by an informed 'walking' along the chromosome (where the required distance and direction of such walking would become apparent from the DNA segregation patterns). The most closely linked cloned sequences would then be tested by hybridizations against DNA and RNA of wild type and mutant to identify the mutated locus.

With such information on the components of the ligninolytic system of *P. chrysosporium*, it will be realistic to identify regulatory sequences or enzyme-coding genes that are appropriate targets for site-directed mutagenesis. For this, an efficient transformation system is required to reintroduce DNA into *P. chrysosporium*. In our laboratory, efficient protoplasting and regeneration have been achieved and a selectable gene, tryphophan C (*trpC*), has now been isolated by Mr A. Schrank. It is functionally homologous to the *trpC* gene of *E.coli* and shows homology to the *trpC* gene of *Aspergillus nidulans*.

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