JOURNAL OF BACTERIOLOGY, Nov. 1995, p. 6106-6110 Vol. 177. No. 21
0021-9193/95/$04.00+0
1995, American Society for Microbiology

# *Phanerochaete chrysosporium* Glyoxal Oxidase Is Encoded by Two Allelic Variants: Structure, Genomic Organization, and Heterologous Expression of *glxl* and *glx2*

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Received 13 June 1995/Accepted 21 August 1995

**A cDNA clone (glx-2c) encoding glyoxal oxidase (GLOX) was isolated from a *Phanerochaete chrysosporium* λgt11 library, and its nucleotide sequence was shown to be distinct from that of the previously described clone glx-lc (P. J. Kersten and D. Cullen, Proc. Natl. Acad. Sci. USA 907411-7413, 1993). Genomic clones cm-responding to both cDNAs were also isolated and sequenced. Overall nucleotide sequence identity was 98%, and the predicted proteins differed by a single residue: Lys-308↔Thr-308. Analyses of parental dikaryotic strain BKM-F- 1767 and homokaryotic progeny firmly established allelism for these structural variants. Southern blots of pulsed-field gels localized tbe GLOX gene *(glx)* to a dimorphic chromosome separate from the peroxidase and cellobiohydrolase genes of *P. chrysosporium*. Controlled expression of active GLOX was obtained from *Aspergillus nidulans* transformants when glx- lc was fused to the promoter and secretion signal of the *A. niger* glucoamylase gene. The GLOX isozyme corresponding to glx-2c was also ethciently secreted by *A. nidulans* following site-specific mutagenesis of the expression vector at codon 308 of glx-lc.**

The white rot fungus *Phanerochaete chrysosporium* has been extensively studied for its lignin-degrading ability (29). *P. chrysosporium* secretes three known classes of extracellular enzymes under ligninolytic (secondary metabolic) conditions in defined glucose media: glyoxal oxidase (GLOX), lignin peroxidases (LiPs), and manganese peroxidases (MnPs). GLOX is a source of the extracellular $H_2O_2$ that is required for the oxidations catalyzed by the ligninolytic peroxidases. In nutrient-limited *P. chysosporium* cultures, the 68-kDa glycoprotein appears as two isozyme forms of pl 4.7 and 4.9. The purification, physical characteristics, and kinetics of GLOX have been reported (26, 28).

Multiple roles for GLOX in lignocellolose degradation by *Phanerochaete* spp. are suggested by several lines of evidence. In addition to the obvious function of peroxide supply, modulation of GLOX activity in vitro supports a role in extracellular regulation of ligninolytic activity in vivo. Purified GLOX is inactive unless activated by a coupled peroxidase system including both peroxidase and peroxidase substrate. The molecular basis for this extracellular regulation has not been elucidated. Furthermore, GLOX substrate production in vivo is likely to involve multiple metabolic pathways. Substrates for purified GLOX include formaldehyde, acctaldehyde, glycolaldehyde, glyoxal, glyoxylic acid, dihydroxyacetone, glyceraldehyde, and methylglyoxal. Both glyoxal and methylglyoxal have been detected in ligninolytic cultures, but the derivation and the full complement of physiological substrates from carbohydrate metabolism are still under investigation. Interestingly, products produced by lignin peroxidase activity on lignin model compounds are also substrates for GLOX; an efficient sequence of oxidations (glycolaldehyde to glyoxal to glyoxylate to oxalate) is catalyzed (23). This finding suggests that ligninolysis can, in part, be perpetuated by GLOX and the action of lignin peroxidase on lignin itself.

Of the *P.* chrysosporium germs implicated in lignocellulose degradation, those encoding LiPs, MnPs, and cellobiohydrolases (CBHs) have been studied in some detail (reviewed in references 1 and 12). All are encoded by large, complex families of structurally related genes. Eight of the ten known LiP genes are linked, and three of these are clustered within a 30-kb region (18, 19). Three of the six known CBH genes are also tightly linked (9). Chromosomic length dimorphisms have been observed for the allelic homologs of LiP genes (17, 35) and CBH genes (9).

Relatively little is known concerning the molecular genetics of GLOX, but recently a cDNA (glx- 1c) encoding GLOX was characterized (27). In reasonable agreement with native GLOX, the predicted enzyme had several potential N-glycosylation sites, a molecular mass of 57 kDa, and a pI of 5.1. The number and distribution of tyrosine, histidine, and cysteine residues suggested an active site similar to that of galactose oxidase, and potential copper ligands were tentatively identified at Tyr-377 and His-378 (27). GLOX expression is transcriptionally regulated and coordinate with the LiP and MnP genes (27, 35). We show here that GLOX is encoded by a single gene with two alleles, that the gene is located on a dimorphic chromosome unlinked to known LiP, MnP, and CBH genes, and that GLOX is efficiently expressed in the model heterologous expression system, *Aspergillus nidulans.*

## MATERIALS AND METHODS

**Cloning and analysis.** A cDNA library of ligninolytic cultures of *P. chrysosporium* strainBKM-F-l 767(= ATCC 24725) was constructed in λgtl I and probed with polyclonal antibodies as described previously (27). For genomic clone isolation, a pWE15 (36) cosmid library of stain BKM-F-1767 (18) was probed with $^{32}$P-labled cDNA clones under high-stringency conditions (0.125 M $Na_2HPO_4$, 50% formamide, 7% sodium dodecyl sulfate, [SDS], and 1 mM EDTA at 48°C). cDNA and genomic clones were partially mapped and subcloned into M13mp18

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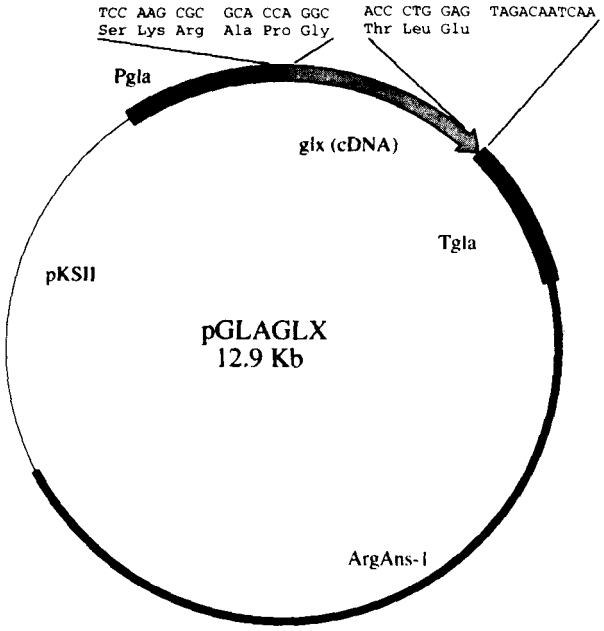


**FIG. 1.** *Aspergillus* expression vector involving translational fusion of the *A. niger* glucoamylase gene (*gla*). At the 5' junction, the *glx-l* cDNA encoding mature GLOX was fused in frame to the glucoamylase propeptide. The precise nucleotide sequences at 5' and 3' junctions are indicated. Abbreviations: Pgla. glucoamylase promoter plus secretion signal plus propeptide; Tgla, glucoamylase terminator; ArgAns-1, *Aspergillus* sequences for high-frequency complementation of *A. nidulans argB (13):* pKSII, Stratagene B1ueScript vector. Vector pGLAGLXD308 (not shown) is identical except that site-specific mutagenesis was used to substitute C for A at nt position 1037 (Fig. 2).

and M13mp19. Single-stranded templates were sequenced by the dideoxy-chain terminating method (32), using Sequenase (United States Biochemical Corporation, Cleveland. Ohio). Both strands were sequenced.

**Allelism.** To test allelism, segregation of the two GLOX variants among homokaryotic progeny (basidiospores) was assessed. Fruiting of BKM-F -1767 was as described previously (21), and germinating single basidiospores were isolated from agar plates with a needle and a dissecting microscope. Putative alleles were then differentiated by a restriction site polymorphism and by hybridization to allele-specific oligonucleotide probes. In the former method, approximately 20 μg of genomic DNA (34) was digested with *Kpn* I, size fractionated in an agarose gel, blotted to Nytran (Schleicher & Schuell, Keene, N.H.), and probed with a nick-translated 1.2-kb *Kpn* I fragment of glx-lc (27), To differentiate putative alleles with oligonucleotide probes, diluted genomic DNA was PCR amplified with primers common to both structural variants: 5'-TCACAC CTTCGCTCTACACG-3' (upstream) and 5'-TATTTACTCCAGGGTCGG CG-3' (downstream). The resulting 680-bp PCR products were blotted to Nytran and probed with 17-mers differing by a single base: 5'-CGAGAC(C/T)CTC-GACCCGC-3'. Hybridization with the probes end labeled with $^{32}$P was in 6× SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as previously described (20) at 58°C. Final washes were at 60°C.

**Pulsed-field gel electrophoresis.** Clamped homogeneous electric field electrophoresis, blotting, and high-stringency hybridization conditions were as previously described (18, 35), Full-length *glxl* cDNA served as a probe.

**cDNA expression in *Aspergillus* spp.** Using overlap extension techniques (24), precise fusions were obtained between the *Aspergillus niger* glucoamylase secretion signal (6, 16) and the cDNA coding region of mature GLOX (27) (Fig. I). Similarly, the *gla* terminator was fused to the last codon of glx- 1c. The entire expression cassette was then combined with *A. nidulans* sequences *ArgB* (5) and ANS1 (13) in Bluescript pKSII (Stratagene Inc., La Jolla, Calif.). A derivative of pGLAGLX, designated pGLAGLXD308, was constructed by site-specific mutagenesis (37) of glx-lc nucleotide (nt) 1037 (C→A). The nucleotide sequences of the mutagenized region and all PCR junctions were confirmed by dideoxy sequencing.

With mirror modification, *A. nidulans (argB; methH2; biA1)* protoplasts were transformed (13), and the *argB*$^+$ transform ants were cultured as previously described (11). For assessing expression of GLOX, cultures were supplemented with methionine, biotin, $KH_2PO_4$ (6.8 g/liter), and either xylose or maltose (50 g/liter). Typically, 50 ml of medium was inoculated in 250-ml Erlenmeyer flasks and incubated at 37°C (150 rpm). As negative controls, *A. nidulans* was also transformed with a vector containing *ArgB* and ANS1 (13) but lacking a GLOX expression cassette.

Culture filtrates were subjected to enzyme activity assays and Western blot (immunoblot) analysis. GLOX activity was determined as described previously, using methylglyoxal as the oxidase substrate and with phenol red or 2,2'-azino-bis(3-ethylbenz-thiazolinc-6-sulfonic acid) as the substrate for horseradish peroxidase in the coupled-enzyme assay (26, 28). A Pharmacia PhastSystem (Pharmacia LKB Biotechnology. Piscataway, N.J.) was used for 10 to 15% SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotting onto nitrocellulose. Polyclonal antibodies against GLOX were produced In rabbits and then parlially purified with Econo-Pac serum immunoglbulin G purification columns (Bio-Rad, Richmond. Calif.). Standard immunodetection techniques were used with an alkaline phosphatse conjugant as the secondary antibody.

**Nucleotide sequence accession numbers.** The nucleotide sequences reported in this paper have been submitted to GenBank/EMBL data banks and assigned accession numbers L47286 *(glxl)* and L47287 *(g;x2).*

## RESULTS AND DISCUSSION

**Structure of the GLOX gene.** Analyses of cDNA and genomic libraries revealed two distinct GLOX-encoding sequences. Among the λgt11 clones reacting with GLOX antibodies, their sequences were identical to those of either glx-1c (27) or a variant type represented by a clone designated glx-2c. Comparison of the two cDNA nucleotide sequences showed 99% identity, with most mismatches occurring as T↔C transitions in the third position of codons. The predicted proteins differed by a single residue, Lys-308↔Thr-308.

Genomic clones corresponding to both cDNAs were sequenced and shown to he 98% identical (Fig. 2). Both sequences featurc four introns ranging in size from 53 to 61 bp. the intron/exort splice sites and the internally conserved sequences are similar to those of other filamentous fungi (3, 22). A putative TATA box is located 53 bp upstream of the ATG site. The sequence surrounding the translational initiation site has a purine (A) at −3 (TTACGATGTT), which is highly conserved in filamentous fungi (22). The T at position +4 is more typical of yeast genes. whereas the consensus for filamentous fungi and higher eukaryotes is G (8, 15). As previously noted for glx-1c (27), the canonical eukaryotic polyadenylation signal AATAAA is not present. The 3' untranslated sequence 5'-GCATATGAAATATCTGT-3' (Fig. 2; nt 2016 to 2023) is conserved in both GLOX variants, and it is highly homologous to the 3' untranslated regions of lignin peroxidase genes ***lipA*** (GCTTATGAAATATCGGT) (33) and ***lipD*** (ACG AAATA) (14). Along with the GLOX gene, ***lipA*** and ***lipD*** are expressed under nutrient starvation, although ***lipD*** is preferentially expressed under carbon limitation as opposed to nitrogen limitation (2, 35).

The structural variants of the GLOX gene were conclusively shown to be alleles. Experimental strategies relied on the homokaryotic condition of basidiospores, which are the products of meiosis and fully viable. A *Kpn* I site polymorphism (Fig. 3A) and allele-specific oligonucleotide probes (Fig. 3B) allowed segregation patterns to be monitored in basidiospore cultures. The GLOX variants consistently segregated among homokary - ons, thus proving allelism. In subsequent studies of genetic linkage (19), allelic segregation was examined in 40 basidiospore cultures, and simple 1:1 Mendelian segregation was observed.

**Genomic organization of *glx*.** Southern blots of pulsed-field gels localized *glx* to a dimorphic chromosome containing no previously mapped genes. Comparison with *A. nidulans* chromosomes indicated that hybridizing bands were approximately 3.7 and 3.9 Mb in the dikaryotic parent strain (Fig. 4B, lane 1). Single bands were observed in chromosomes of homokaryotic cultures (Fig. 4B, lanes 2 and 3), indicating a length dimorphism for the *glx* chromosome in the parental strain. The

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  tg                                a  gc                                                                T
ctctaatatattgacctataagtatgagcagcgctggcgcaccacggcggctgagcgacagacttacgATGTTGTCGCTGCTAGCCGTAGTCTCACTCG    31
                                                                                M  L  S  L  L  A  V  V  S  L     10

                                                                                                  T
CCGCCGCCACACTCGCTGCTCCAGCTGCCAGCGATGCACCAGGCTGGCGGTTCGACCTGAAGCCCAACCTTTCCGGGATCGTGGCTCTCGAGGCGATCGT   131
A  A  A  T  L  A  A  P  A  A  S  D  A  P  G  W  R  F  D  L  K  P  N  L  S  G  I  V  A  L  E  A  I  V    44

                                                                                                      C
CGTGAACAGCTCACTGGTGGTCATATTCGACCGCGCCACCGGCGATCAGCCTCTCAAGATCAACGGCGAATCGACCTGGGGTGCGCTCTGGGATCTCGAT   231
  V  N  S  S  L  V  V  I  F  D  R  A  T  G  D  Q  P  L  K  I  N  G  E  S  T  W  G  A  L  W  D  L  D    77

ACGAGCACTGTCCGCCCGCTTTCGGTCCTGACGGACTCCTTCTGCGCCAGCGGTGCGCTGCTCAGCAATGGCACTATGgtaggctaatagcctttggcat   331
 T  S  T  V  R  P  L  S  V  L  T  D  S  F  C  A  S  G  A  L  L  S  N  G  T  M                          103

                    g                                                                 A
ttggtgagagagttctgacgcacgctcttcgatgcgcagGTCAGCATGGGAGGAACCCCTGGCGGTACTGGAGGGGATGTTGCTGCTCCTCCTGGCAACC   431
                                         V  S  M  G  G  T  P  G  G  T  G  G  D  V  A  A  P  P  G  N    123

AGGCGATCCGGATCTTCGAGCCCTGCGCCTCGCCTTCGGGAGATGGCTGCACGCTCTTCGAAGACCCTGCGACTGTTCATCTCCTTGAGGAGCGGTGGTA   531
Q  A  I  R  I  F  E  P  C  A  S  P  S  G  D  G  C  T  L  F  E  D  P  A  T  V  H  L  L  E  E  R  W  Y   157

CCCATCGTCCGTGCGCATTTTCGATGGCAGTCTCATGATCATTGGTGGTTCGCATGTCCTCACACCGTTCTACAACGTCGATCCAGCCAACTCCTTCGAG   631
  P  S  S  V  R  I  F  D  G  S  L  M  I  I  G  G  S  H  V  L  T  P  F  Y  N  V  D  P  A  N  S  F  E    190

                                        T
TTCTTCCCAAGCAAGGAGCAGACGCCCAGGCCTTCAGCCTTCTTGGAACGCTCGCTGCCAGCGAATCTTTTCCCACGgcaagtctttagactttttgact   731
 F  F  P  S  K  E  Q  T  P  R  P  S  A  F  L  E  R  S  L  P  A  N  L  F  P  R                          216

         t                                                  C                    T
acaaacctgctgaccaaagtcattgctcagGGCTTTCGCCTTGCCAGACGGAACCGTCTTTATCGTCGCTAATAACCAGTCTATCATCTACGATATCGAG   831
                                 A  F  A  L  P  D  G  T  V  F  I  V  A  N  N  Q  S  I  I  Y  D  I  E    239

                   T
AAGAATACTGAGACCATCCTCCCGGATATCCCCAACGGTGTTCGTGTCACGAACCCCATCGATGGAAGCGCCATCCTTCTCCCGTTGTCGCCCCCTGACT   931
 K  N  T  E  T  I  L  P  D  I  P  N  G  V  R  V  T  N  P  I  D  G  S  A  I  L  L  P  L  S  P  P  D    272

TCATTCCCGAGGTTCTCGTCTGTGGAGGCTCGACTGCGGACACCTCGCTCCCGTCCACCAGCTTGTCCTCCCAACATCCCGCTACCAGCCAATGCAGCCG  1031
F  I  P  E  V  L  V  C  G  G  S  T  A  D  T  S  L  P  S  T  S  L  S  S  Q  H  P  A  T  S  Q  C  S  R   306

C    C                                                C  A
GATCAAGCTCACCCCCGAAGGTATCAAGGCCGGCTGGCAGGTCGAACATATGCTTGAGGCGCGCATGATGCCTGAGCTCGTGCACGTCCCGAACGGCCAG  1131
  I  K  L  T  P  E  G  I  K  A  G  W  Q  V  E  H  M  L  E  A  R  M  M  P  E  L  V  H  V  P  N  G  Q    339
     T

                         C
ATCCTCATCACCAACGGCGCTGGTACAGGCTTTGCCGCACTGTCTGCCGTCGCGGACCCCGTCGGCAACTCGAACGCGGACCACCCCGTGCTCACACCTT  1231
 I  L  I  T  N  G  A  G  T  G  F  A  A  L  S  A  V  A  D  P  V  C  N  S  N  A  D  H  P  V  L  T  P    372

CGCTCTACACGCCCGACGCACCTCTTGGAAAGCGCATCAGCAACGCGGGCATGCCGACGACGACGATTCCGCGCATGTACCACTCGACCGTGACGCTCAC  1331
S  L  Y  T  P  D  A  P  L  G  K  R  I  S  N  A  G  M  P  T  T  T  I  P  R  M  Y  H  S  T  V  T  L  T   406

GCAGCAGGGCAACTTCTTCATCGGGGGCAACAACCCGAACATGAACTTCACGCCGCCTGGCACACCCGGGATCAAGTTCCCCAGCGAGCTGCGCATCGAG  1431
  Q  Q  G  N  F  F  I  G  G  N  N  P  N  M  N  F  T  P  P  G  T  P  G  I  K  F  P  S  E  L  R  I  E    439

  T
ACCCTCGACCCGCCGTTCATGTTCAGGAGCCGCCCCGCGCTTCTCACGATGCCTGAGAAGCTCAAGTTCGGCCAGAAGGTGACCGTGCCTATCACGATCC  1531
 T  L  D  P  P  F  M  F  R  S  R  P  A  L  L  T  M  P  E  K  L  K  F  G  Q  K  V  T  V  P  I  T  I    472

                                                c
CGAGCGACCTGAAGGCGAGCAAGGTCCAAGgtgcgtgcaccccgctctcgcgcagggcgcgttcgctgaatggcgtggcatgatctagTCGCCCTGATGG  1631
P  S  D  L  K  A  S  K  V  Q                                                                V  A  L  M    486

ACCTCGGCTTTTCGAGCCACGCCTTCCACTCCAGTGCGCGCCTCGTCTTCATGGAGTCGTCCATCTCGGCGGACCGCAAGTCGCTCACCTTCACGGCTCC  1731
D  L  G  F  S  S  H  A  F  H  S  S  A  R  L  V  F  M  E  S  S  I  S  A  D  R  K  S  L  T  F  T  A  P   520

GCCCAACGGACGCGTCTTCCCACCGGGCCCGGCTGTCGTTTTCCTCACGgtacgcacagtggtttatctctggtgcgacgaacgcggctgacgtgcaatt  1831
  P  N  G  R  V  F  P  P  G  P  A  V  V  F  L  T                                                        536
                                                                                                      c
tcagATTGACGACGTGACAAGTCCGGGAGAGAGAGTCATGATGGGCAGTGGCAACCCTCCGCCGACCCTGGAGTAAatatggagtgttcgcgggtacctc  1931
     I  D  D  V  T  S  P  G  E  R  V  M  M  G  S  G  N  P  P  P  T  L  E  .                            559

   t       aa caa a ag            aa                    c                ga                          a
ggcgcagacc--t---tgt--cttgactgttgtggtgcggtatgtacgttgtttttgttgtgagctcgaaagttgcatatgaaatatctgtttgggcga  2031

tatcatgggcgcagattgctaggcgagaagctgtcgtac                                                                2064
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**FIG. 2.** Nucleotide and deduced amino acid sequences of GLOX-encoding genomic clones. Within coding regions, the nucleotide sequences of *gxl-1* is identical to that of the previously reported cDNA(26), The sequence of the allelic homolog *glx-2* is identical to that of *glx-* I except for the bases shown (upper line). Untranslated regions are indicated by lowercase letters. The mature peptidc begins at amino acid residue 23, A single amino acid difference was observed at position 308. A restriction site polymorphism was used to experimentally establish the allelic relationship between *glx-1* and *glx-2;* there are two *Kpn* I sites *glx-1* (nt positions 530 and 1926) but only one site in *glx-2* (nt position 530). Cosmid mapping indicated another *Kpn* I site located approximately 5.0 kb 3' to the translational strip of *glx-2,*

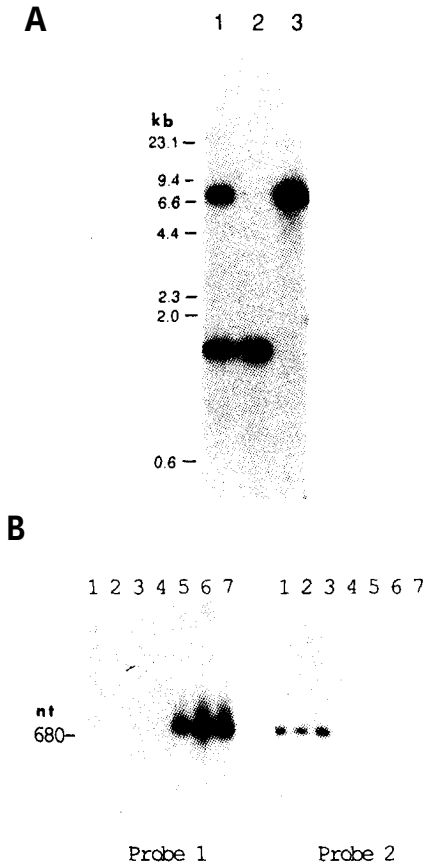


**FIG. 3.** Southern blots showing allelism of *qlx-1* and *glx-2*. (A) *Kpn* I digests of parental BKM-F-1767 (lane 1) and two single-lbasidiospore cultures (lanes 2 and 3) probed with a $^{32}$P-labeled 1.4-kb *Kpn* I fragment of *glx-1*. As expected of allellic pairs, the dikaryotic parent contains both alleles and hybridizes at 1.4 and 6.6 kb. In contrast, the homokaryotic cultures contain a single allele. (B) Single-basidiospore genomic DNA was PCR amplified and probed with allele-specific oligonucleotide probes 1 and 2. Lanes 1 to 3 and 5 to 7 contain amplified product from single-basidiospore cultures. Lane 4 contained a size standard, Again, the homokaryotic cultures contained a single allele.

observed pattern of hybridization is unlike those for all known LiP (18, 35), MnP (30), and CBH (9) genes. Thus, *glx* is not physically linked to any genes implicated in lignocellulose degradation, a result entirely consistent with recent genetic linkage data (19).

These results, together with earlier studies, show no clear relationship between genomic organization and expression of the genes involved in lignocellulose degradation. Structurally related members of the same gene family are often unlinked. Some (e.g., *lipD)* reside on dimorphic chromosomes (35), while others ***(cbhl-6, mnp2,*** and ***mnp1)*** hybridize to single bands (9, 30). Even among clustered members within these families, transcription is often not coordinate. For example, *cbhl-1* and *cbhl-2* are constitutively expressed at low levels, whereas *cbhl-3,* lying only ***13*** kb downstream, is induced by cellulose and highly expressed (10). Further, among the closely linked LIP genes, *lipC* and *lipJ* are derepressed by nitrogen limitation, *lipE* is derepressed under carbon limitation, and *lipA, lipB,* and *lipI* are derepressed under carbon or nitrogen limitation (25, 31, 35). Transcript levels of *glx* also accumulate during carbon or nitrogen limitation (27, 35), although it remains to be shown if *glx* expression is coordinate with these genes in complex woody substrates.

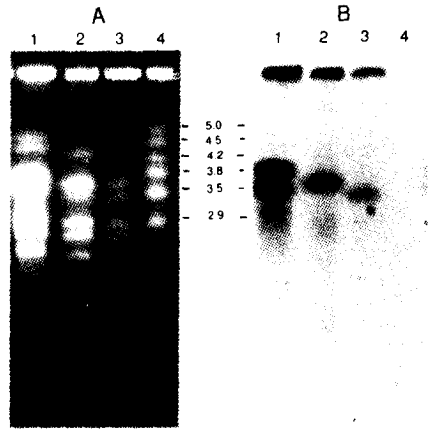


**FIG. 4.** Chromosomal location of *glx.* An ethidium bromide-stained clamped homogeneous electric fieldblot (A) was blotted to Nytran and probed with the *glx-1* cDNA (B). Lanes contain BKM-F- 1767 (lane 1). single-basidiospore derivatives (lanes 2 and 3), and *A.. nidulans* DNA (lane 4) as a size standard. Approximate sizes are shown In megabases. Sample preparation, electrophoretic conditions, andSouthern hybridization were as previously described (7, I8). Meiotic recombination between chromosome homologs typically yields bands of various lengths amoung the basidiospore progeny. The parental doublet and homokaryotic singlets indicate a length dimorphism in the *glx* chromosome.

**Heterologous expression.** When, *glx* was fused to the *A.* niger glucoamylase promoter and terminator, efficient *glx* expression was obtained in *A.* ***nidulans*** (Fig. 5). Two expression vectors were tested: pGLAGLX, encoding the *glx1-c* sequence; and pGLAGLXD308, encoding the *glx1-c* sequence except that a single base was mutated to encode Thr at residue 308. Thus, the protein encoded by pGLAGLXD308 corresponds to the allelic variant *.glx-2.* Secretion of active protein was observed with transformants harboring either plasmid when grown on maltose. Several pGLAGLX transformants were grown in submerged culture, and yields as high as 10 to 20 mg/liter were obtained. This activity is more than 50-fold greater than that of optimized *P. chrysosporium* cultures (26, 28).

The allelic variation in *glx* predicts mature peptides slightly differing in sequence (Lys-308↔Thr-308), isoelectric points (pI 5.1 and 5.0), and potentially O glycosylation. Although post-translational modifications could easily explain the isozymic forms (pI 4.7 and 4.9) of GLOX observed in submerged cultures of *P. chrysosporium,* the possibility that these proteins could be separate allelic products was considered. Recombinant GLOX preparations from the culture supernatants of *Aspergillus* transformants pGLAGLX and pGLAGXD308 were

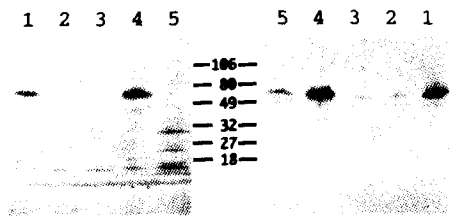


**FIG. 5.** GLOX produced by *Aspergillus* transformants analyzed by SDS-PAGE. Gels were Coomassie stained (left) and Western blotted (right). Three transformants are shown: a pGLAGLX transformant grown in medium with xylose (lane 5) or maltose (lane 4) as the sole carbon source; a pGLAGLXD308 transformant grown in xylose (lane 2) or maltose (lane 1) medium; and a pAR GANS1 transformant grown in maltose medium (lane 3). pARGANS1 contains no *glx* expression cassette. Samples loaded onto the Western blotted gel were diluted 1:100. Molecular size standards are indicated in kilodaltons.

chromatographed by isoelectric focusing, and protein was detected by activity staining of gels. Both transformants gave multiple banding patterns (data not shown) that obfuscated interpretation; therefore, this issue remains unresolved. In contrast to GLOX, no translational differences are indicated for the mature peptides corresponding to the *lip* and **cbhl** alleles of *P. chrysosporium,* although a single amino acid change is predicted in the secretion signal of *lipA (2).*

The *A. nidulans* expression system is suitable for various biochemical investigations, including, as demonstrated here, site-specific mutagenesis. Efficient expression was obtained, although significantly greater yields could be achieved particularly by using commercial strains of *A. niger* var. awamori (4) as hosts.

## ACKNOWLEDGMENTS

This work was supported in part by Department of Energy grant DE-FG02-87ER13712 to D. Cullcn and T. K, Kirk and by USDA Competitive Research Grant 88-33521-4089 to D. Cullen and P. J. Kersten.

## REFERENCES


1. **Alic, M., and M. Gold.** 1991. Genetics and molecular biology of the lignin-degrading Basidiomycete *Phanerochaete chrysosporium*, p. 319–341. *In* J. Bennett and L. Lasure (ed.), More gene manipulations in fungi. Academic Press, New York.
2. **Andrawis, A., E. Pease, I. Kuan, E. Holzbaur, and E. Tien.** 1989. Characterization of two lignin peroxidase clones from *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **162:**673–680.
3. **Ballance, D. J.** 1986. Sequences important to gene expression in filamentous fungi. Yeast **2:**229–236.
4. **Berka, R., F. T. Bayliss, P. Bloebaum, D. Cullen, N. Dunn-Coleman, K. Kodama, K. Hayenga, R. A. Hitzeman, M. Lamsa, M. Przetak, M. Rey, L. Wilson, and M. Ward.** 1991. *Aspergillus niger* var. *awamori* as a host for the expression of heterologous genes, p. 273–292. *In* T. O. Baldwin and J. W. Kelly (ed.), Applications of enzyme biotechnology. Plenum Press, New York.
5. **Berse, B., A. Dmochowska, M. Skrzypek, P. Weglenski, M. A. Bates, and R. L. Weiss.** 1983. Cloning and characterization of the ornithine carbamoyltransferase gene from *Aspergillus nidulans*. Gene **25:**109–117.
6. **Boel, E., M. T. Hansen, I. Hjort, I. Hoegh, and N. P. Fiil.** 1984. Two different types of intervening sequences in the glucoamylase gene from *Aspergillus niger*. EMBO J. **3:**1581–1585.
7. **Brody, H., and J. Carbon.** 1989. Electrophoretic karyotype of *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA **86:**6260–6263.
8. **Cavener, D. R., and S. C. Ray.** 1991. Eukaryotic start and stop translation sites. Nucleic Acids Res. **19:**3185–3192.
9. **Covert, S., J. Bolduc, and D. Cullen.** 1992. Genomic organization of a cellulase gene family in *Phanerochaete chrysosporium*. Curr. Genet. **22:**407–413.
10. **Covert, S., A. Vanden Wymelenberg, and D. Cullen.** 1992. Structure, organization and transcription of a cellobiohydrolase gene cluster from *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58:**2168–2175.
11. **Cullen, D., G. Gray, K. Hayenga, M. Lamsa, S. Norton, M. Rey, L. Wilson, and R. Berka.** 1987. Controlled expression and secretion of bovine chymosin by *Aspergillus nidulans*. Bio/Technology **5:**369–376.
12. **Cullen, D., and P. Kersten.** 1992. Fungal enzymes for lignocellulose degradation, p. 100–131. *In* J. R. Kinghorn and G. Turner (ed.), Applied molecular genetics of filamentous fungi. Chapman & Hall, London.
13. **Cullen, D., L. Wilson, D. Henner, G. Turner, and J. Ballance.** 1987. High frequency transformation of *Aspergillus nidulans* conferred by novel sequence ANS-1. Nucleic Acid Res. **15:**9163–9175.
14. **de Boer, H. A., Y. Z. Zhang, C. Collins, and C. A. Reddy.** 1987. Analysis of nucleotide sequences of two ligninase cDNAs from a white-rot filamentous fungus, *Phanerochaete chrysosporium*. Gene **60:**93–102.
15. **Edelmann, S. E., and C. Staben.** 1994. A statistical analysis of sequence features within genes from *Neurospora crassa*. Exp. Mycol. **18:**70–81.
16. **Fowler, T., R. Berka, and M. Ward.** 1990. Regulation of the *glaA* gene of *Aspergillus niger*. Curr. Genet. **18:**537–545.
17. **Gaskell, J., and D. Cullen.** 1993. Recent advances in the organization and regulation of lignin peroxidase genes of *Phanerochaete chrysosporium*. J. Biotechnol. **30:**109–114.
18. **Gaskell, J., E. Dieperink, and D. Cullen.** 1991. Genomic organization of lignin peroxidase genes of *Phanerochaete chrysosporium*. Nucleic Acids Res. **19:**599–603.
19. **Gaskell, J., P. Stewart, P. J. Kersten, S. Covert, J. Reiser, and D. Cullen.** 1994. Establishment of genetic linkage by allele-specific polymerase chain reaction: application to the lignin peroxidase gene family of *Phanerochaete chrysosporium*. Bio/Technology **12:**1372–1375.
20. **Gaskell, J., A. Vanden Wymelenberg, P. Stewart, and D. Cullen.** 1992. Method for identifying specific alleles of a *Phanerochaete chrysosporium* encoding a lignin peroxidase. Appl. Environ. Microbiol. **58:**1379–1381.
21. **Gold, M. H., and T. M. Cheng.** 1979. Conditions for fruit body formation in the white rot Basidiomycete *Phanerochaete chrysosporium*. Arch. Microbiol. **121:**37–41.
22. **Gurr, S. J., S. E. Unkles, and J. R. Kinghorn.** 1987. The structure and organization of nuclear genes of filamentous fungi, p. 93–139. *In* J. R. Kinghorn (ed.), Gene structure in eukaryotic microbes. IRL Press, Oxford.
23. **Hammel, K. E., K. A. Jensen, and P. J. Kersten.** 1994. $H_2O_2$ recycling during oxidation of the arylglycerol β-aryl ether lignin structure by lignin peroxidase and glyoxal oxidase. Biochemistry **33:**13349–13354.
24. **Ho, S., H. Hunt, R. Horton, J. Pullen, and L. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
25. **Holzbaur, E., and M. Tien.** 1988. Structure and regulation of a lignin peroxidase gene from *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **155:**626–633.
26. **Kersten, P. J.** 1990. Glyoxal oxidase of *Phanerochaete chrysosporium*; its characterization and activation by lignin peroxidase. Proc. Natl. Acad. Sci. USA **87:**2936–2940.
27. **Kersten, P. J., and D. Cullen.** 1993. Cloning and characterization of a cDNA encoding glyoxal oxidase, a peroxide-producing enzyme from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Proc. Natl. Acad. Sci. USA **90:**7411–7413.
28. **Kersten, P. J., and T. K. Kirk.** 1987. Involvement of a new enzyme, glyoxal oxidase, in extracellular $H_2O_2$ production by *Phanerochaete chrysosporium*. J. Bacteriol. **169:**2195–2201.
29. **Kirk, T. K., and R. L. Farrell.** 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. **41:**465–505.
30. **Orth, A., M. Rzhetskaya, D. Cullen, and M. Tien.** 1995. Characterization of a cDNA encoding a manganese peroxidase from *Phanerochaete chrysosporium*: genomic organization of lignin and manganese peroxidase genes. Gene **148:**161–165.
31. **Reiser, J., I. Walther, C. Fraefel, and A. Fiechter.** 1993. Methods to investigate the expression of lignin peroxidase genes by the white-rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **59:**2897–2903.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
33. **Smith, T. L., H. Schalch, J. Gaskell, S. Covert, and D. Cullen.** 1988. Nucleotide sequence of a ligninase gene from *Phanerochaete chrysosporium*. Nucleic Acids Res. **16:**1219.
34. **Specht, C. A., C. C. DiRusso, C. P. Novotny, and R. C. Ullrich.** 1982. A method for extracting high-molecular-weight deoxyribonucleic acid from fungi. Anal. Biochem. **119:**158–163.
35. **Stewart, P., P. Kersten, A. Vanden Wymelenberg, J. Gaskell, and D. Cullen.** 1992. The lignin peroxidase gene family of *Phanerochaete chrysosporium*: complex regulation by carbon and nitrogen limitation, and the identification of a second dimorphic chromosome. J. Bacteriol. **174:**5036–5042.
36. **Wahl, G. M., K. A. Lewis, J. C. Ruiz, B. Rothenberg, J. Zhao, and G. A. Evans.** 1987. Cosmid vectors for rapid genomic walking, restriction mapping, and gene transfer. Proc. Natl. Acad. Sci. USA **84:**2160–2164.
37. **Weiner, M. P., K. A. Felts, T. G. Simcox, and J. C. Braman.** 1993. A method for the site-directed mono- and multi-mutagenesis of double stranded DNA. Gene **126:**35–41.


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