

# Characterization of cDNAs encoding putative laccase-like multicopper oxidases and developmental expression in the tobacco hornworm, *Manduca sexta*, and the malaria mosquito, *Anopheles gambiae*

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## Abstract

Laccase (EC 1.10.3.2) is an enzyme with *p*-diphenol oxidase activity that is a member of a group of proteins collectively known as multicopper, or blue copper, oxidases. Laccase is hypothesized to play an important role in insect cuticle sclerotization by oxidizing catechols in the cuticle to their corresponding quinones, which then catalyze protein cross-linking reactions. To facilitate studies of the structure, function and regulation of insect laccases, we have cloned two cDNAs for laccases from the tobacco hornworm, *Manduca sexta* (*MsLac1* and *2*), and one from the malaria mosquito, *Anopheles gambiae* (*AgLac1*). The *MsLac1* and *2* cDNAs encode proteins of 801 amino acids (aa) and 760 aa, respectively, while the *AgLac1* cDNA encodes a protein of 1009 aa. All three cDNAs contain putative secretion signal sequences, and the 10 histidines and one cysteine that form the copper-binding centers, as well as a methionine in the T1 copper center. Novel to the insect laccases, relative to both fungal and plant laccases, is a longer amino-terminal sequence characterized by a unique domain consisting of several conserved cysteine, aromatic, and charged residues. Northern blot analyses identified single transcripts of approximately 3.6, 3.5, and 4.4 kb for *MsLac1*, *MsLac2*, and *AgLac1*, respectively, and also showed that *AgLac1* was expressed in all life stages of the mosquito. RT-PCR revealed that the *MsLac1* transcript was most abundant in the midgut, Malpighian tubules, and epidermis, whereas the *MsLac2* transcript was most abundant in the epidermis. *MsLac2* showed strong expression in the pharate pupal and reduced expression in the early pupal epidermis, consistent with the laccases' presumed role in cuticle sclerotization.

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## 1. Introduction

The exoskeleton or cuticle of insects has contributed greatly to their evolutionary success. It not only provides protection against the environment but also plays important roles in locomotion, respiration, and communication. The cuticle is secreted by epidermal cells and consists of a thin, waxy, outer layer (epicuticle) and a thicker inner layer (procuticle) comprised primarily of chitin, proteins, and lipids (Neville, 1975; Hepburn, 1985). Stabilization of the cuticle occurs in part when proteins in the procuticle become cross-linked by highly

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reactive quinones, a process known as sclerotization (Andersen, 1985, 1990; Hopkins and Kramer, 1992). The quinones are produced by the oxidation of catechols, mainly the derivatives of dopamine and dihydroxyphenylethanol (Hopkins and Kramer, 1992). Oxidation may be carried out by two different types of phenoloxidases present in the insect cuticle, tyrosinase and laccase. Tyrosinase is able to hydroxylate monophenols to *o*-diphenols (monophenol monooxygenase, EC 1.14.18.1), and oxidize *o*-diphenols to their corresponding quinones (catechol oxidase, *o*-diphenol oxidase, EC 1.10.3.1). Laccase lacks the monooxygenase activity of tyrosinase but is able to oxidize both *o*- and *p*-diphenols (benzenediol:O<sub>2</sub> oxidoreductase, *p*-diphenol oxidase, EC 1.10.3.2). While tyrosinases have been extensively studied for their roles in melanization, wound healing, and insect immunity (Ashida and Yamazaki, 1990; Sugumaran and Kanost, 1993; Marmaras et al., 1996; Ashida and Brey, 1997), relatively little attention has been given to characterizing the importance of laccases in insect physiology. We are interested in identifying the roles played by laccases in insect cuticle stabilization and other physiological processes.

Several lines of evidence suggest that the primary role of laccase is in cuticle sclerotization, while tyrosinases function in the cuticle primarily during wound healing (Andersen, 1985, 1990; Ashida and Yamazaki, 1990; Hopkins and Kramer, 1992). For example, phenylthiourea (PTU), an inhibitor of tyrosinase but not laccase, has been shown to prevent melanization of wounds in the cuticle of the caterpillar of *Calpododes ethlius* (Lai-Fook, 1966), and the larva of the sheep blowfly, *Lucilia cuprina* (Binnington and Barrett, 1988). Dennell (1958a) noted that hydroquinone, a *p*-diphenol and thus a substrate for laccase but not tyrosinase, might be involved in hardening of the puparium of the bluebottle fly, *Calliphora vomitoria*. Furthermore, injection of PTU failed to prevent puparium formation (Dennell, 1958b), indicating that the phenoloxidase involved was not a tyrosinase. Similarly, Sugumaran et al. (1992) demonstrated that the phenoloxidase responsible for the hardening of the puparium of *Drosophila melanogaster* was able to oxidize the laccase-specific substrate syringaldazine, and that this enzymatic activity was greatly reduced by the laccase inhibitors sodium azide and potassium cyanide but not by PTU. Finally, the enzymatic activity of laccases was shown to be more closely correlated with molting than was that of tyrosinases. Tyrosinase activity rose during the intermolt period but then dropped during molting in *L. cuprina* (Hackman and Goldberg, 1967; Barrett, 1987), the American cockroach, *Periplaneta americana* (Mills et al., 1968), and the larva of the Egyptian cotton worm, *Spodoptera littoralis* (Ishaaya and Navon, 1974). Contrary to those observations, laccase activity in the cuticle during larval development of *D. virilis* (Yamazaki, 1969) and *L. cuprina* (Barrett, 1987),

as well as the larva of the silk moth, *Bombyx mori* (Yamazaki, 1972), was very low during the intermolt period, then increased dramatically at the time of molting.

Laccases are members of the blue oxidase family of proteins along with ascorbic oxidases and ceruloplasmins. Members of this family are copper-containing enzymes (multicopper oxidases) that couple the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water (Messerschmidt and Huber, 1990; Messerschmidt, 1993; Solomon et al., 1996; Mayer and Staples, 2002). Roles attributed to these enzymes include pigmentation in fungi and bacteria, lignification and delignification of plant cell walls, as virulence factors, and copper and iron homeostasis. Three types of copper-binding centers for these proteins have been classified according to their spectroscopic properties. Type-1 (T1) centers bind a single copper ion and exhibit an absorption maximum in the oxidized state at 600 nm that gives these proteins their characteristic blue color. Type-2 (T2) centers also bind a single copper but show no absorption pattern in the visible spectrum. Type-3 (T3) centers bind two copper ions that are antiferromagnetically coupled and have a strong absorption maximum at 330 nm. Laccase, the simplest of this group of proteins, binds four copper ions, one in a T1 center and three in a T2/T3 hybrid center. The copper in the T1 center serves as an acceptor of electrons from the substrate. These electrons are then passed on to the T2/T3 center, where molecular oxygen is reduced.

In addition to insects, laccases have been found in plants, fungi, and bacteria (Messerschmidt and Huber, 1990; Messerschmidt, 1993; Thurston, 1994; Solomon et al., 1996; Alexandre and Zhulin, 2000; Sanchez-Amat et al., 2001; Hullo et al., 2001; Mayer and Staples, 2002; Martins et al., 2002), with fungal laccases being the most intensely studied. Laccases have a very broad substrate range, being able to oxidize not only polyphenols but also aminophenols, methoxyphenols, and diamines. Laccase has also been shown to have ferroxidase activity, similar to that of other blue oxidase family members Fet3 and ceruloplasmin (Liu et al., 1999). These enzymes have received considerable interest for possible use in paper pulp bleaching, detoxification of textile dyes, removal of phenolic compounds from wine, creation of biosensors to monitor environmental pollutants, and bioremediation (Bollag et al., 1988; Smith et al., 1997; Cohen et al., 2002; Mayer and Staples, 2002; Claus et al., 2002; Soares et al., 2002). In addition, laccase has been identified as a virulence factor in the human pathogen *Cryptococcus neoformans* (Williamson, 1997).

Previously Thomas et al. (1989) used trypsin digestion to solubilize a phenoloxidase present in the pharate pupal cuticle of the tobacco hornworm, *Manduca sexta*, and demonstrated that it possessed laccase-like activity

through the use of specific substrates and inhibitors. Here, we expand upon that study, and introduce a laccase from the malaria mosquito, *Anopheles gambiae*. We have cloned three laccase cDNAs, two from *M. sexta* and one from *A. gambiae*. Analysis of their deduced amino acid sequences confirmed that these cDNAs contain all of the residues necessary for the formation of the copper-binding centers. Furthermore, the putative insect proteins have a relatively long amino terminus with a unique domain not found in either plant or fungal laccases. Northern blotting and RT-PCR analyses revealed that their genes are expressed in multiple tissues in both a constitutive and regulated manner. Significantly, the transcripts for the *M. sexta* laccases are upregulated in the pharate pupal and pupal epidermis at the time when pupal cuticle sclerotization occurs.

## 2. Materials and methods

### 2.1. Insect cultures

*M. sexta* larvae were reared using an artificial diet at 27 °C as described by Bell and Joachim (1976), with a photoperiod of 16 h of light and 8 h of darkness. *A. gambiae* (strain 4arr) were maintained at 26–29 °C and approximately 70% relative humidity as described previously (Gorman and Paskewitz, 1997).

### 2.2. Cloning of *A. gambiae* and *M. sexta* laccase cDNAs

Degenerate primers were designed based on conserved regions in fungal and plant laccases, as well as a bilirubin oxidase from the insect *Trachyderma tsunoda* (GenBank AB006824). The forward primer 5'-ACGAGYATHCAYTGCCAYGG-3' (where H = A, C, or T, and Y = C or T) was derived from the amino acid sequence TSIHWHG, and the reverse primer 5'-GTGGAAGTCDATRTGRCARTG-3' (where D = A, G, or T, and R = A or G) was derived from the amino acid sequence HCHIDFH. Lambda phage DNA isolated from a *M. sexta* bacterial-challenged, larval fat body cDNA library (F. Schultz and M. Kanost, unpublished data) was used as a template in a polymerase chain reaction (PCR) under the following conditions: initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 90 s. One microliter from this reaction was used directly as a template in a second, nested PCR under the same cycling conditions. The nested forward primer 5'-GGAACAAAYTGGCNGAYGG-3' (where N = A, G, C, or T) was derived from the amino acid sequence GTNWADG, and the nested reverse primer 5'-GAACCACGGRCNGGRTTRTC-3' was derived from the amino acid sequence DNP GPWF. A cDNA of

approximately 1.2 kb was amplified and cloned into the pGEM-T vector (Promega). Sequencing of this product confirmed that it encoded a laccase-like protein. However, screening of the *M. sexta* cDNA library with this product failed to identify any positive clones.

To obtain a full-length cDNA sequence (*MsLac1*), rapid amplification of cDNA ends (RACE)-PCR was performed using the GeneRacer Kit (Invitrogen) according to the manufacturer's instructions. Single-stranded cDNA to be used as template for RACE-PCR was reverse transcribed from total RNA isolated from the epidermis of pharate pupae using the Glassmax RNA Microisolation Spin Cartridge System (Invitrogen). 3'-RACE was performed using the MsLac1-specific forward primer 5'-GCGTTTGACGAAGCAGGGCTGCTGAAAAGA-3', and a reverse primer supplied with the GeneRacer Kit. Touchdown PCR was performed as follows: initial denaturation at 94 °C for 2 min, followed by five cycles of denaturation at 94 °C for 30 s, annealing and extension at 72 °C for 3 min; five cycles of denaturation at 94 °C for 30 s, annealing and extension at 70 °C for 3 min; 25 cycles of denaturation at 94 °C for 30 s, annealing at 68 °C for 30 s, and extension at 72 °C for 3 min. A cDNA of approximately 1.3 kb was amplified, cloned into the vector pCR4-TOPO (Invitrogen), and sequenced to confirm that it overlapped with the original 1.2 kb clone.

The same cycling conditions were used for 5'-RACE with a forward primer supplied with the GeneRacer Kit, and the MsLac1-specific reverse primer 5'-TCCCCTGAAGAGTGGTGGTGGTCCGGTGAAC-3'. One microliter from this reaction was used in a second PCR using a nested forward primer supplied with the GeneRacer Kit, and the MsLac1-specific nested reverse primer 5'-ACAGAGACTCATAAGGCTCTTGGGACTTG-3'; touchdown PCR was performed as described above, except that the annealing and extension temperatures were lowered to 65 and 68 °C, respectively, for the last 25 cycles. A cDNA of approximately 1.1 kb was amplified, cloned into the vector pCR4-TOPO, and sequenced to confirm that it overlapped with the original 1.2 kb clone.

A second *M. sexta* laccase cDNA (*MsLac2*) was identified when a primer used in 3'-RACE-PCR (5'-GGTACCGCTCTTCAGTTAGG-3') for the *M. sexta* chitin synthase cDNA (Zhu et al., 2002) amplified a 1.36 kb fragment that encoded the first 412 amino acids of a laccase-like protein. Again, 3'-RACE-PCR was used to clone the remainder of the cDNA, utilizing the *M. sexta* bacterial-challenged, larval fat body cDNA library as template. Amplification was performed using the MsLac2-specific forward primer 5'-TGACGCTGCTGAGAGATATCC-3', and the nested forward primer 5'-GGTAAAGGACAATTCAGGGA-3', with a reverse primer specific to the T7 RNA polymerase promoter in the library vector. PCR was performed for 45 cycles using the same cycling conditions as described above for

the amplification of the 1.2 kb *MsLac1* fragment. A cDNA of approximately 2 kb was amplified, cloned into the pGEM-T vector, and sequenced to confirm that it overlapped with the 1.36 kb clone.

The approach used to clone *MsLac1* was also taken with *A. gambiae*, using a larval cDNA library (Barillas-Mury et al., 1996) as template for PCR. The second, nested, PCR amplified a cDNA of approximately 1.0 kb, and sequencing confirmed that it also encoded a laccase-like protein. This PCR product was subsequently used to screen and identify clones in the *A. gambiae* cDNA library.

### 2.3. Sequence analysis

All programs, except for PileUp, were available through links with the Swiss Institute of Bioinformatics ExPASy Molecular Biology Server (<http://us.expasy.org/>). The SignalP (v. 2.0) prediction server (Nielsen et al., 1997) was used to predict the putative signal peptides of the deduced amino acid sequences for the laccase cDNA clones. The mature proteins were then aligned using the PileUp program of SeqWeb (v. 2) (a web-based sequence analysis programs of the GCG Wisconsin Package), and the signal peptides then added to the aligned sequences. The program parameters used were the BLOSUM62 scoring matrix with a gap creation penalty of 8 and a gap extension penalty of 2 (all default settings). Potential glycosylation sites were identified using the NetNGlyc 1.0 (Gupta et al., in preparation) and NetOGlyc 2.0 (Hansen et al., 1995, 1997, 1998) prediction servers. The big-PI Predictor (Eisenhaber et al., 1999) was used to identify potential GPI anchor sites.

### 2.4. Phylogenetic analysis

Insect, plant, and fungal laccase protein sequences were aligned using the T-Coffee program (Notredame et al., 2000) available on the ExPASy molecular biology server (<http://usa.expasy.org/>). Preliminary analysis identified several regions with varying degrees of similarity. Areas where the alignment was poor were omitted from further analysis to avoid undue influence by these ambiguous regions. The final alignment was constructed from a region of approximately 280 amino acids in the amino-terminal halves of the proteins. This corresponded to I193-M475 in *MsLac1*, V200-P478 in *MsLac2*, I334-Y497 plus I598-P703 in *AgLac1*, and the equivalent regions in the plant and fungal proteins. The gap in *AgLac1* is due to the presence of a 100 amino acid sequence that does not have a homologous region in the other laccases except the *D. melanogaster* clone CG3759. PAUP\* 4.0b10 (Swofford, 2002) was used for data analysis. Gaps in the alignment were treated as characters. The phylogenetic tree was constructed by the parsimony method using a heuristic search algorithm.

Statistical analysis was performed by the bootstrap method using 10,000 repetitions.

The sequences used for phylogenetic analysis were as follows: for insects, *A. gambiae*, *AgLac1* (GenBank AY135184), *agCP15239* (GenBank EAA10244), *agCP15381* (GenBank EAA10258), *agCP15439* (GenBank EAA10119), *agCP5969* (GenBank EAA11475), *D. melanogaster*, CG30437 (GenBank NM165431), CG5959 (GenBank NM143184), CG3759 (GenBank NM135443), CG32557 (GenBank NM133021), *M. sexta*, *MsLac1* (GenBank AY135185), *MsLac2* (GenBank AY135186), *Pimpla hypochondriaca*, *PhLac1* (GenBank AJ427356); for fungi, *Coriolum hirsutum*, *ChLac1* (SwissProt Q02497), *Phlebia radiata*, *PrLac1* (SwissProt Q01679), *Thanatephorus cucumeris*, *TcLac3* (SwissProt Q02079), *Trametes versicolor*, *TveLac4* (SwissProt Q12719), *Trametes villosa*, *TviLac2* (SwissProt Q99046); for plants, *Lolium perenne*, *LpLac2-1* (Genbank AF465469), *Liriodendron tulipifera*, *LtLac2-1* (GenBank AAB17191), *Nicotiana tabacum*, *NtLac1* (GenBank U43542), *Pinus taeda*, *PtLac1* (GenBank AF132119). *Rhus vernicifera*, *RvLac1* (GenBank AB062449).

### 2.5. RT-PCR and northern blot analyses

For *M. sexta*, three insects were dissected for each data point and total RNA was prepared using TRI Reagent (Sigma) (Chomczynski, 1993). To examine the tissue distribution, a cDNA pool for each tissue was created. One microgram of total RNA from each time point was combined (7 µg total) and reverse transcribed with an oligo(dT) primer (5'-GGAGTACTCTAGAAGC(T)<sub>17</sub>-3') and SUPERSCRIPT II RNase H<sup>-</sup> Reverse Transcriptase (Invitrogen) in a total volume of 30 µl, according to the manufacturer's directions. One microliter of each cDNA pool was then utilized for PCR under the following conditions: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 90 s. The primers used for amplification were (forward) 5'-CAAGTCCCAAGAGCCTTATGAGTC-3' and (reverse) 5'-GGGTTATCAGCTTTGAATCTGATC-3' for *MsLac1*; (forward) 5'-GTTGAGAACCACATGGAAGGTATGG-3' and (reverse) 5'-CACGAAGAAACGGAACGGCAAGAA-3' for *MsLac2*.

The developmental expression of *MsLac1* and 2 in various tissues was examined by northern blot analysis. Ten micrograms of total RNA were loaded per lane and fractionated in a 1% agarose/1.2% formaldehyde gel. After electrophoresis, the RNA was transferred to a neutral nylon membrane (GeneScreen, Perkin Elmer) by standard capillary blotting and stained with 0.02% methylene blue in 0.3 M sodium acetate, pH 5.2, for 5 min to verify that an equal amount of RNA was present in each lane. The membranes were first prehybridized in

ULTRAhyb buffer (Ambion), then hybridized overnight at 42 °C with  $\alpha^{32}\text{P}$ -dCTP (Perkin Elmer) labeled probes at a concentration of  $1 \times 10^6$  dpm per milliliter of buffer. The *MsLac1* and 2 cDNAs were used as probes and radiolabeled by the random primer method (RadPrime DNA Labeling System, Invitrogen). Following hybridization, the membranes were washed twice in  $2\times$  SSC/0.1% sodium dodecyl sulfate (SDS) at 42 °C ( $2\times$  SSC is 0.3 M sodium chloride/30 mM sodium citrate/pH 7.0), twice in  $0.1\times$  SSC/0.1% SDS at 60 °C, and twice in  $0.1\times$  SSC/0.1% SDS at 65 °C; each wash was for 15 min. The membranes were then subjected to autoradiography with an intensifying screen at  $-70$  °C.

For *A. gambiae*, total RNA was isolated from embryos (0–14 h), larvae (days 5 and 9), pupae, and adults (days 0 and 4) using Ultraspec reagent (Biotecx). Ten micrograms of total RNA were loaded per lane and fractionated in a 1% agarose/2.5% formaldehyde gel, and transferred to a nylon membrane (Hybond-N, Amersham Pharmacia) by standard capillary blotting. After transfer, the membrane was stained with methylene blue as described above. A 2.3 kb *AgLac1* cDNA fragment was gel purified and radiolabeled with a random primer kit (Boehringer, Mannheim). The membrane was hybridized with the laccase probe in ULTRAhyb buffer at 42 °C, then washed twice at 65 °C with  $2\times$  SSC/0.1% SDS for 5 min each, and twice with  $0.1\times$  SSC/0.1% SDS for 15 min each. Autoradiography was performed with an intensifying screen at  $-70$  °C.

### 3. Results

#### 3.1. cDNAs and N-terminal domains

##### 3.1.1. Cloning and analysis of laccase cDNAs

We have cloned two putative laccase cDNAs from *M. sexta* and one from *A. gambiae* by a combination of library screening and RT-PCR methods. The first *M. sexta* laccase cDNA, which we have designated as *MsLac1*, is 3.5 kb long and contains an open reading frame (ORF) of 2403 bp that encodes a putative 801 amino acid protein with a theoretical molecular weight for the mature protein of 88,561 Da and an isoelectric point (pI) of pH 5.15. Surprisingly, the GTNWADG sequence, from which the nested forward primer was designed, is not conserved between fungi and the insect laccase cDNAs that we isolated. However, the last 10 nucleotides of the nested forward primer matched the codon usage for the sequence RADG, similar to the sequence WADG of the nested forward primer, located at positions 289–292 of *MsLac1* (Fig. 1). The nested reverse primer hybridized to the sequence encoding the amino acids DNPgyWL, residues 697–703, and the resulting PCR amplified product was 1.2 kb. 5'- and 3'-

RACE-PCR were then used to clone the remainder of the cDNA.

The second *M. sexta* laccase cDNA, *MsLac2*, is 3.3 kb long with a 2280 bp ORF that encodes a 760 amino acid protein. The calculated molecular weight and pI for the mature protein are 81,426 Da and pH 6.36, respectively. As described in Section 2.2, it was fortuitously identified during the cloning of the *M. sexta* chitin synthase cDNA. Because *MsLac1* and *MsLac2* were constructed from various PCR products and not isolated as complete clones from a cDNA library, we designed primers that flanked the ORFs of their sequences and performed PCR using cDNA that had been reverse transcribed from total RNA isolated from the epidermis of pharate pupae. cDNA fragments of the expected size were amplified, and sequencing confirmed that they matched the sequences of the *MsLac1* and *MsLac2* clones, although some polymorphism was observed (data not shown). Additionally, the calculated molecular weights of the mature proteins were within the 70–90 kDa range previously estimated for a partially purified laccase-like enzyme from the integument of *M. sexta* (Thomas et al., 1989).

The longest *A. gambiae* cDNA identified by library screening, *AgLac1*, is 4 kb long with a 3027 bp ORF that encodes a 1009 amino acid protein. The calculated molecular weight and pI for the mature protein are 110,162 Da and pH 5.52, respectively. Similar to the initial PCR amplification for *MsLac1*, the last nine nucleotides of the nested forward primer matched the codon usage for the sequence GRR, located at positions 608–610 of *AgLac1* (Fig. 1). The *AgLac1* sequence from G608 through W934 was encoded by the 1 kb product initially amplified by PCR. We mapped the position of *AgLac1* on polytene chromosomes by in situ hybridization to a single band in division 16B (data not shown). The map location was assigned based on the polytene chromosome maps available on the *Anopheles* Genetic Resource Information Project website (<http://klab.agsci.colostate.edu/~mbenedic/>), which is an updated interpretation of an older map (Coluzzi and Sabatini, 1967). This location is consistent with its estimated position as determined by the *A. gambiae* sequencing project.

All three insect laccases contain the 10 histidines and one cysteine important for the binding of copper ions (Fig. 1). Surprisingly, nearly unique to the insect laccases is a methionine in the T1 copper center (M716 in *MsLac1*, M728 in *MsLac2*, and M948 in *AgLac1*), whereas most fungal and plant laccases have either a phenylalanine or a leucine at this position. We utilized several computer software programs available to further analyze the deduced amino acid sequences. All three putative proteins were predicted to have secretion signal peptides (Fig. 1). In addition, several potential N- and O-linked glycosylation sites were identified. Seven N-



*driaca* (PhLac1) (Parkinson et al., 2003). The percentage identity between their deduced amino acid sequences varies from 33% to 80%, with the highest similarity between MsLac2 and the *D. melanogaster* clone CG30437-PA (Table 1). We performed a translated BLAST search (Altschul et al., 1997) of the *Drosophila* genome (Adams et al., 2000), using MsLac2 for comparison, and identified potential alternative exons that encode the last 262 amino acids of the *Drosophila* protein, resulting in the possibility of several splice variants (data not shown). The *D. melanogaster* clone CG30437-PC (GenBank NM 136326) likely represents one such variant as it is 96% identical with CG30437-PA.

A search of the *A. gambiae* genome (Holt et al., 2002) identified five potential laccases in the mosquito. Our AgLac1 cDNA corresponds to the hypothetical protein ebiP5549 (GenBank EAA05994), though that sequence is truncated and is missing the regions corresponding to amino acids 1–345, 501–594, 680–719, and 956–1009 of our clone. Of notable interest are the theoretical gene products agCP5969 and agCP5995. Similar to the CG30437-PA clone described above for *Drosophila*, agCP5969 shares 81% identity (85% similarity) to MsLac2 and therefore likely represents an ortholog. Significantly, agCP5995 is located only 4 kb downstream of agCP5969 and is 82% identical (87% similar) to the last 260 amino acids of that sequence. Thus, it appears that agCP5995 represents alternative exons of agCP5969, allowing for the possibility of several splice variants. The three remaining potential laccase proteins in the *A. gambiae* genome are agCP15239, agCP15381, and agCP15439. However, since no corresponding cDNAs have as yet been cloned, the validity of their sequences remains to be confirmed.

### 3.1.2. Unique N-terminal domains

Novel to the insect laccases, relative to both fungal and plant laccases, is a much longer amino-terminal region. Whereas many fungal and plant laccases have approximately 60 residues upstream of the first copper-binding histidine in the mature protein, MsLac1 and MsLac2 have 216 and 221 residues, respectively, while AgLac1 has 340 residues. These amino-terminal exten-

sions show little similarity when the deduced sequences are aligned together. Considerable similarity began at a C–X–R–X–C motif located 90 residues upstream of the first copper-binding histidine (Fig. 1). This motif begins an approximately 45 amino acid-long domain present in all insect laccase sequences available in the databases (Fig. 2A), but not present in plant or fungal laccases. This domain contains six conserved cysteines, as well as several conserved charged, hydrophobic, and aromatic residues.

A second unique amino-terminal domain was discovered in the deduced amino acid sequence of AgLac1. The first 250 amino acids of the putative mature protein showed similarity to hemocytin of *B. mori* (b-Hmc) (Kotani et al., 1995) and hemolectin of *D. melanogaster* (d-Hml) (Goto et al., 2001). This similarity was restricted to the von Willebrand factor (vWF) type B and C domains of these proteins. An alignment of the amino-terminus of AgLac1 with these domains indicated that AgLac1 had two vWF type B and two vWF type C domains in tandem (Fig. 2B). An analysis of the deduced amino acid sequences of the other putative insect laccases indicated that the *D. melanogaster* laccase CG3759 also contained two putative vWF type C domains (Fig. 2B). Neither MsLac1 nor MsLac2 showed similarity to any known domains in its amino-terminus.

### 3.2. Phylogenetic analysis

We investigated the phylogenetic relationship of the insect laccases with representatives from plants and fungi. Only the amino-terminal halves of the insect, plant, and fungal laccases were used for analysis since only these regions produced a meaningful alignment between the three taxa (see Section 2.4.). This alignment, including gaps, was 291 characters long, of which 245 were informative. Analysis by the parsimony method produced only one most-parsimonious tree in 1804 steps. The consistency and retention indices were 72% and 69%, respectively. This analysis revealed that laccases from each taxon clustered in their own group (Fig. 3). This outcome, as well as the relationships within the insects, was the same regardless of which group was

Table 1  
Percentage identity (and similarity) between putative insect laccases

	AgLac1	DmCG3759	DmCG5959	DmCG30437	DmCG32557	MsLac1	MsLac2	PhLac1
AgLac1		52	40	38	44	51	38	40
DmCG3759	(61)		39	38	44	51	36	39
DmCG5959	(50)	(47)		34	42	35	36	33
DmCG30437	(48)	(48)	(45)		34	37	80	50
DmCG32557	(51)	(51)	(47)	(43)		41	36	36
MsLac1	(60)	(60)	(46)	(48)	(51)		36	39
MsLac2	(46)	(46)	(46)	(83)	(45)	(47)		49
PhLac1	(50)	(48)	(44)	(59)	(45)	(48)	(59)	

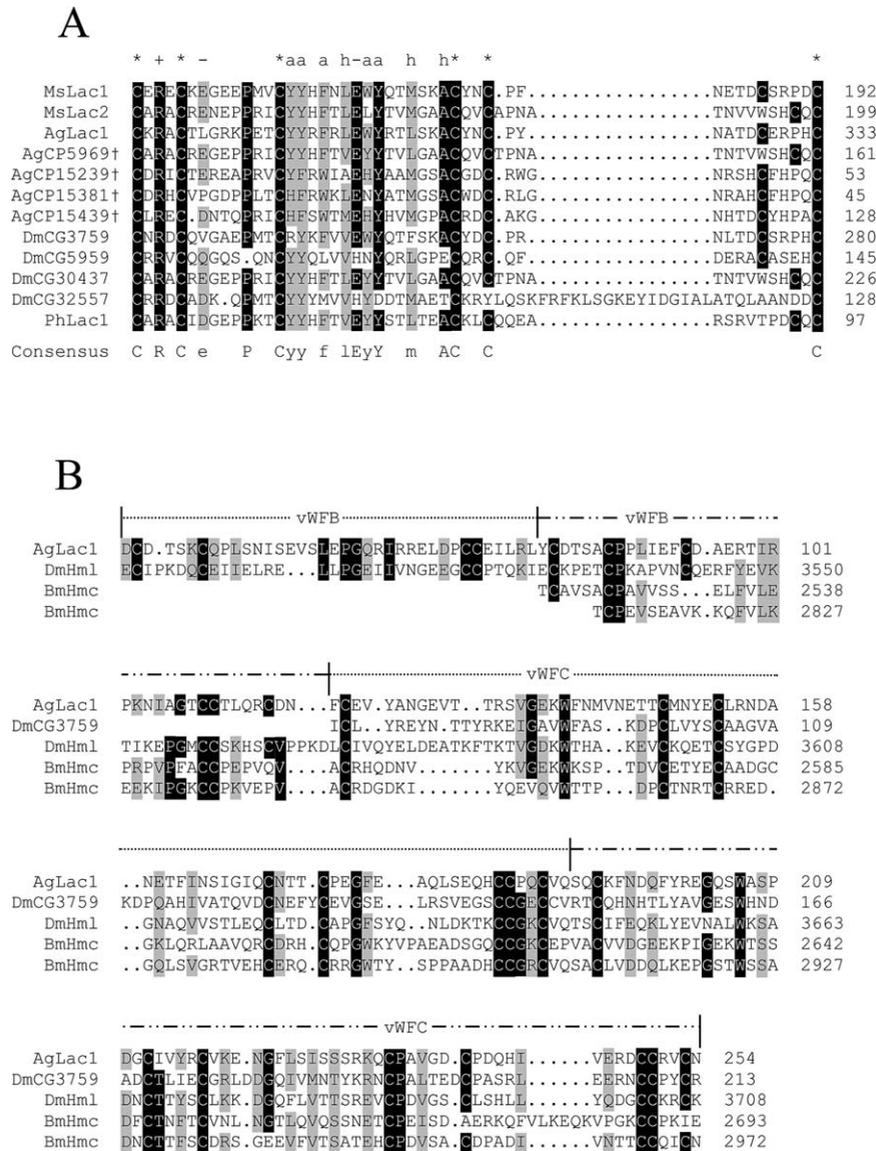


Fig. 2. Unique N-terminal domains. Alignments of the cysteine-rich (A) and von Willebrand factor (B) domains of insect laccases. The position of the last amino acid in a line is indicated to the right of the alignment. Ag, *A. gambiae*; Bm, *B. mori*; Dm, *D. melanogaster*; Hmc, hemocytin; Hml, hemolectin; Ms, *M. sexta*; Ph, *P. hypochondriaca*; vWFB, von Willebrand factor type B domain; vWFC, von Willebrand factor type C domain. Identical residues are boxed in black and written in upper case in the consensus sequence. Conservative residues are boxed in grey and written in lower case in the consensus sequence. A dagger (†) indicates conceptual proteins identified by a search of the *Anopheles* genome database but for which no corresponding cDNAs have yet been cloned. The symbols above the alignment in (A) indicate amino acid characteristics. An asterisk (\*) marks conserved cysteines; (+) or (-), charged residues; a, aromatic residues; h, hydrophobic residues.

designated as the outgroup. Within the insects, MsLac1 and MsLac2 each fell into separate branches with representatives from both *A. gambiae* and *D. melanogaster*. All three genes in the “*Anopheles*-specific” branch lie within a 30 kb region on chromosome 3 (as determined by the *A. gambiae* genome sequence), and potentially represent a duplication event that occurred in mosquitoes. Whether this branch or the “*Drosophila*-specific” branch are species- or family-group specific may only be resolved as more insect laccase genes are discovered and included in the phylogenetic analysis.

### 3.3. Tissue distribution and developmental expression

We used RT-PCR to examine the expression of *MsLac1* and *MsLac2* genes in various tissues throughout the fifth instar and early pupal stages. The *MsLac1* mRNA was very abundant in the midgut and Malpighian tubules, and slightly less so in the epidermis and fat body (Fig. 4A). The *MsLac2* transcript showed its strongest expression in the epidermis, with reduced expression in the midgut and Malpighian tubules, and very low expression in the fat body (Fig. 4A). Both the *MsLac1*

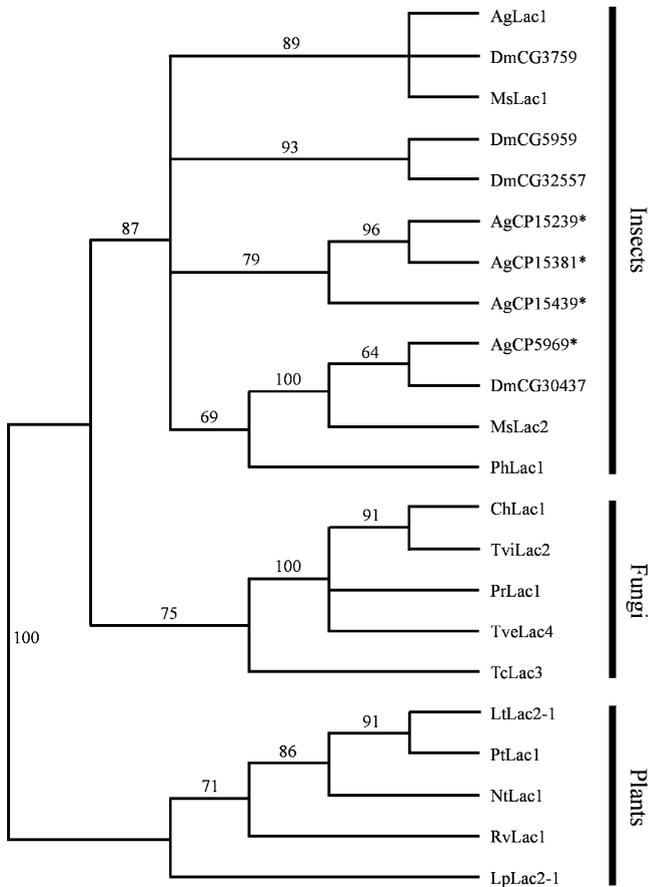


Fig. 3. Phylogenetic relationship of insect, plant, and fungal laccases. The cladogram was constructed by the parsimony method. Full names and database accession numbers of the sequences used are listed in Section 2.3. Bootstrap values, in percentages, are indicated above the branches. An asterisk (\*) indicates conceptual proteins identified by a search of the *Anopheles* genome but for which no corresponding cDNAs have yet been cloned.

and *MsLac2* mRNAs exhibited very low expression in hemocytes.

As we are interested in elucidating the role of laccases in cuticle sclerotization, we looked at the expression of *MsLac1* and *MsLac2* in the epidermis at several time points during the fifth larval instar and pupal stages by northern blot analysis. A single transcript of 3.5 kb was detected for *MsLac2*, having very strong expression in the pharate pupa and low expression during the fifth instar day-0 and pupa day-0 (Fig. 4B); this pattern of expression is similar to that observed for the *M. sexta* cuticle proteins CP20 and CP36 (Suderman et al., 2003). The *MsLac1* mRNA surprisingly showed low basal expression with elevated levels during the fifth instar day-4 and pharate pupal stages (Fig. 4C).

The unexpected high levels of *MsLac1* transcripts detected in the Malpighian tubules and midgut led us to investigate the temporal expression of its gene in these tissues as well. Our results indicate that *MsLac1* is expressed constitutively in these tissues during the feed-

ing stage, then disappears soon after the larva begins wandering behavior (Fig. 4D and E). A single transcript of approximately 3.6 kb was detected for *MsLac1* in all tissues examined.

Northern blot analysis of *AgLac1* detected a single transcript of 4.4 kb in all developmental stages. *AgLac1* mRNA varied in abundance, from barely detectable in eggs to easily detectable in mature adult females (Fig. 4F). Its high expression in adults suggests functions in addition to cuticle sclerotization.

#### 4. Discussion

Cuticle sclerotization is a critical event shared by all insects. Sclerotization occurs when proteins in the cuticle are cross-linked by highly reactive quinones formed from the oxidation of catechols by specific phenoloxidases. Laccase, one such type of phenoloxidase, has been detected in the cuticles of several insect species (see Table 4 in Thomas et al. (1989) and references within; Charalambidis et al., 1994). In the dipterans *D. virilis* (Yamazaki, 1969) and *L. cuprina* (Barrett, 1987), as well as in the lepidopteran *B. mori* (Yamazaki, 1972), this activity has been correlated with periods of molting, suggesting a link to new cuticle formation. The overall aim of our research is to replicate in vitro the events of cuticle sclerotization. As a first step towards this goal, we have cloned three cDNAs for insect laccases from two species.

All three clones contain the conserved histidines and cysteine that were identified in other laccases as being necessary for binding copper. However, nearly unique to the insect laccases is the presence of a methionine in the T1 copper center, whereas most plant and fungal laccases have either a phenylalanine or leucine at this position. The methionine is able to interact weakly with the bound copper ion, while phenylalanine and leucine do not. This difference results in slightly different geometries of their respective copper centers, causing altered redox potentials (Canters and Gilardi, 1993; Xu et al., 1996, 1999). Eggert et al. (1998) have proposed categorizing plant laccases based on the amino acids present in the T1 copper center. Enzymes containing a methionine, which often exhibit the lowest redox potentials, belong to class 1, while enzymes containing a leucine belong to class 2, and those with a phenylalanine, which often have the highest redox potentials, belong to class 3. Under this classification system, all three of these insect laccases would be categorized as class 1 laccases.

In comparison with plant and fungal laccases, insect laccases have an extended amino-terminal region. In addition to the sequences reported here, all putative insect laccases found in the databases (including the *Anopheles* genome sequences) show similarity beginning with the sequence C-X-R-X-C, and all but the *Droso-*

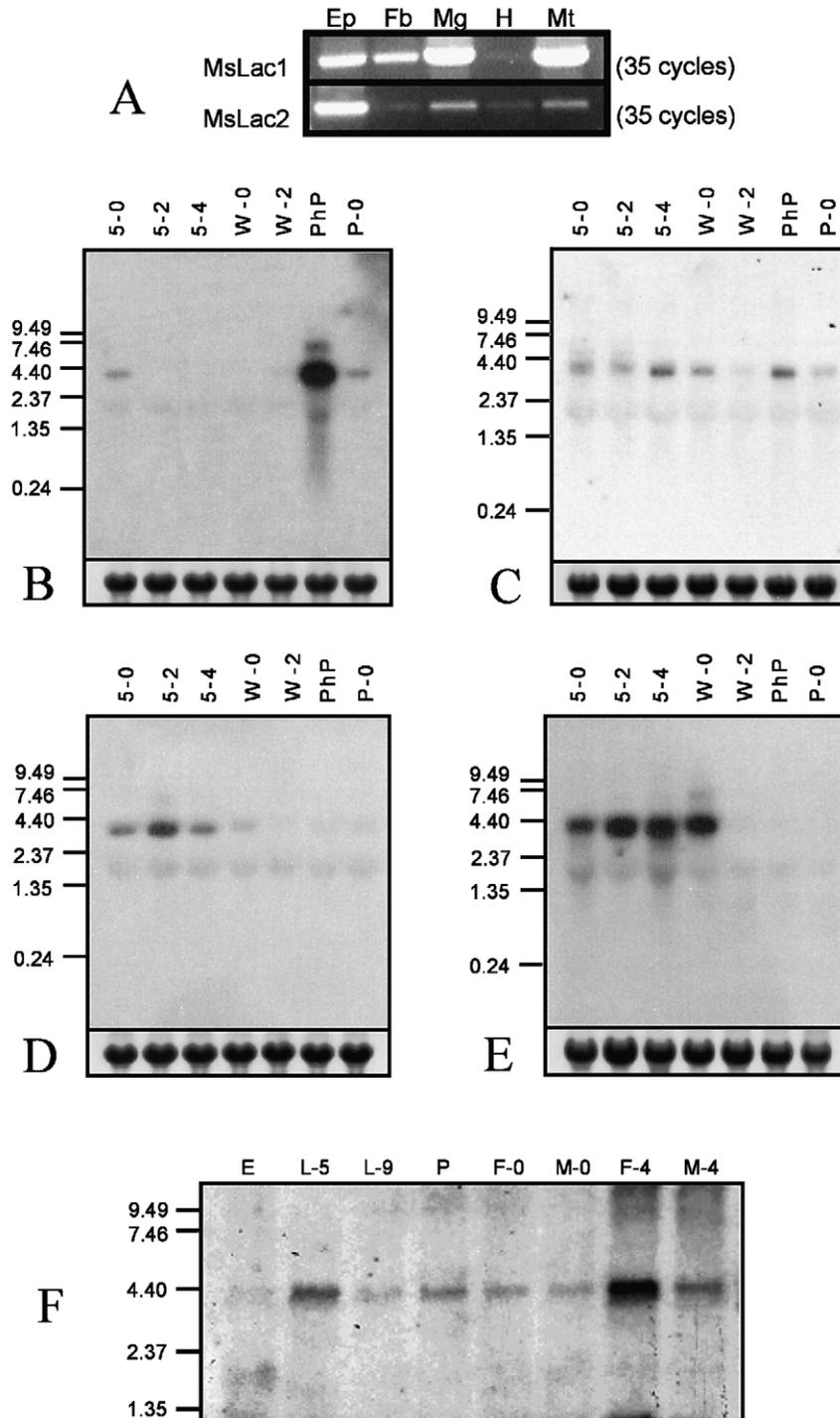


Fig. 4. Developmental expression of laccase genes. (A) Tissue distribution of *MsLac1* and 2 during the fifth instar was determined by RT-PCR; samples were visualized by agarose gel electrophoresis and staining with ethidium bromide. The number of amplification cycles performed is noted to the right of each figure. Ep, epidermis; Fb, fat body; H, hemocyte; Mg, midgut; Mt, Malpighian tubules. (B–E) Northern blot analysis of gene expression in various tissues throughout the fifth instar and early pupal stages in *M. sexta*. Methylene blue stained ribosomal RNA is shown below each autoradiograph to indicate equal loading of RNA in each lane. Abbreviations used are: 5-0, 2, 4, fifth instar feeding larva days 0, 2, and 4; P-0, pupal day 0; PhP, pharate pupal; W-0, 2, fifth instar wandering larva days 0 and 2. *MsLac2* expression in the epidermis is shown in (B). *MsLac1* expression is shown for the epidermis (C), midgut (D), and Malpighian tubules (E). (F) Expression of *AgLac1* during different developmental stages of *A. gambiae* as determined by northern blot analysis. E, embryo; F-0, 4, adult females days 0 and 4; L-5, 9, larval days 5 and 9; M-0, 4, adult males days 0 and 4; P, pupal.

*phila* clone CG32557 have six conserved cysteines in this unique region (CG32557 has five of the six cysteines). This domain also contains several conserved aromatic and charged residues and is similar in characteristics to several classes of carbohydrate-binding proteins (Beintema, 1994; Powell and Varki, 1995; Tomme et al., 1995; Shen and Jacobs-Lorena, 1999; Håkansson and Reid, 2000; Van Dellen et al., 2002). Since chitin is an abundant carbohydrate in the cuticle and peritrophic matrix, it is intriguing to speculate that this cysteine-rich region may serve as a novel chitin-binding domain that may function to anchor laccase to these chitinous structures. Additionally, both MsLac1 and AgLac1 have hydrophobic tails that may serve as GPI anchor sites. If some laccases are integral membrane proteins, this may help to explain the requirement for mild proteolysis to solubilize these enzymes from extracts of integuments (Yamazaki, 1972; Andersen, 1978; Thomas et al., 1989).

The degree of amino acid sequence identity between insect laccases varies widely from less than 40% to over 80%. This amount of variation may well reflect the function of laccases in insect physiology and thus the substrates they oxidize. For example, MsLac1 and MsLac2, which share only 36% identity in their protein sequence, have very different expression patterns in different tissues. The *MsLac2* mRNA is expressed most abundantly in the epidermis in a regulated manner, suggesting that the primary role of MsLac2 is the oxidation of catechols for protein cross-linking during cuticle sclerotization (Kramer et al., 2001). On the other hand, in addition to being expressed in the epidermis, *MsLac1* is also highly expressed in the Malpighian tubules and midgut in a constitutive manner. Since these tissues function in insect detoxification systems (Ahmed et al., 1986; Lindroth, 1991), MsLac1 could be involved in oxidizing toxic compounds acquired from the diet. Alternatively, given the importance of the related multicopper oxidases Fet3 and ceruloplasmin in iron metabolism (Askwith and Kaplan, 1998; Aisen et al., 2001), and the reported ferroxidase activity of a *Cryptococcus neoformans* laccase (Liu et al., 1999), laccase may also play a role in insect iron metabolism (Nichol et al., 2002). Previously Sidjanski et al. (1997) reported the localization of laccase-like phenoloxidases in the midgut of the adult female mosquito *A. stephensi*.

Although we currently do not know the exact function of MsLac1, its expression in tissues other than the epidermis indicates that laccases are involved in physiological processes other than cuticle sclerotization. Using microarray analysis, De Gregorio et al. (2001) identified the *D. melanogaster* putative laccase gene CG3759 as being upregulated in adult flies upon septic injury, suggesting a role for laccase in the melanization pathway during the insect's immune response. Interestingly, both CG3759 and AgLac1 show sequence similarity to b-Hmc and d-Hml in their vWF type C domains. This

domain has been found in mucins, thrombospondins, and fibrillar collagens, and has been proposed to function in oligomerization and complex formation (Bork, 1993). Both *b-hmc* and *d-hml* were found to be upregulated in immune-challenged insects (Kotani et al., 1995; Goto et al., 2001). Significantly, a recombinant C-terminal portion of b-Hmc containing a carbohydrate-recognition domain and the vWF type B and C domains caused hemocyte aggregation in the hemolymph of *B. mori* larvae (Kotani et al., 1995).

The high amino acid sequence similarity between MsLac2 and laccases from two dipteran species suggests that this form of the enzyme has a highly conserved role. The primary catechols present in the insect cuticle are derivatives of dopamine and dihydroxyphenylethanol, which differ only in the presence of either an amino or hydroxyl group attached to the  $\alpha$ -carbon, respectively (Hopkins and Kramer, 1992). It might be expected that fewer amino acid mutations would accumulate in an enzyme whose primary function is to oxidize such structurally similar compounds. It is interesting to note that the putative *Drosophila* and *Anopheles* orthologs to MsLac2 have the potential for alternative splicing over the last 220 amino acids of their respective proteins. These different isoforms may show preferences for the different catechols present in the cuticle. The high sequence similarity between MsLac2 and its dipteran counterparts, along with their variants, allows a unique opportunity to examine the relationship between the primary amino acid sequence and substrate specificity of these enzymes. The continued investigation of gene structures, functions, and regulation of insect laccases will not only further our understanding of the vital process of cuticle sclerotization, but should also contribute to our overall knowledge of this important class of multicopper oxidases and their roles in insect physiology.

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## References

- Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., et al. 2000. The genome sequence of *Drosophila melanogaster*. *Science* 287, 2185–2195.
- Ahmed, S., Brattsten, L.B., Mullin, C.A., Yu, S.J., 1986. Enzymes involved in the metabolism of plant allelochemicals. In: Brattsten, L.B., Ahmad, S. (Eds.), *Molecular Aspects of Insect-Plant Associations*. Plenum Press, New York, pp. 73–151.
- Aisen, P., Enns, C., Wessling-Resnick, M., 2001. Chemistry and biology of eukaryotic iron metabolism. *The International Journal of Biochemistry and Cell Biology* 33, 940–959.
- Alexandre, G., Zhulin, I.B., 2000. Laccases are widespread in bacteria. *Trends in Biotechnology* 18, 41–42.
- Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.
- Andersen, S.O., 1978. Characterization of a trypsin-solubilized phenoloxidase from locust cuticle. *Insect Biochemistry* 8, 143–148.
- Andersen, S.O., 1985. Sclerotization and tanning of the cuticle. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 3. Pergamon Press, Oxford, pp. 59–74.
- Andersen, S.O., 1990. Sclerotization of insect cuticle. In: Ohnishi, E., Ishizaki, H. (Eds.), *Molting and Metamorphosis*. Japan Scientific Societies Press/Springer-Verlag, Tokyo/Berlin, pp. 133–155.
- Ashida, M., Brey, P.T., 1997. Recent advances in research on the insect prophenoloxidase cascade. In: Brey, P.T., Hultmark, D. (Eds.), *Molecular Mechanisms of Immune Responses in Insects*. Chapman & Hall, London, pp. 135–172.
- Ashida, M., Yamazaki, H.I., 1990. Biochemistry of the phenoloxidase system in insects: with special reference to its activation. In: Ohnishi, E., Ishizaki, H. (Eds.), *Molting and Metamorphosis*. Japan Scientific Societies Press/Springer-Verlag, Tokyo/Berlin, pp. 239–265.
- Askwith, C., Kaplan, J., 1998. Iron and copper transport in yeast and its relevance to human disease. *Trends in Biochemical Sciences* 23, 135–138.
- Barillas-Mury, C., Charlesworth, A., Gross, I., Richman, A., Hoffmann, J.A., Kafatos, C.F., 1996. Immune factor Gambif1, a new rel family member from the human malaria vector, *Anopheles gambiae*. *The EMBO Journal* 15, 4691–4701.
- Barrett, F.M., 1987. Phenoloxidases from larval cuticle of the sheep blowfly, *Lucilia cuprina*: characterization, developmental changes, and inhibition by antiphenoloxidase antibodies. *Archives of Insect Biochemistry and Physiology* 5, 99–118.
- Beintema, J.J., 1994. Structural features of plant chitinases and chitin-binding proteins. *FEBS Letters* 350, 159–163.
- Bell, R.A., Joachim, F.G., 1976. Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Annals of the Entomological Society of America* 69, 365–373.
- Binnington, K.C., Barrett, F.M., 1988. Ultrastructural localization of phenoloxidases in cuticle and haemopoietic tissue of the blowfly *Lucilia cuprina*. *Tissue and Cell* 20, 405–419.
- Bollag, J.-M., Shuttleworth, K.L., Anderson, D.H., 1988. Laccase-mediated detoxification of phenolic compounds. *Applied and Environmental Microbiology* 54, 3086–3091.
- Bork, P., 1993. The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Letters* 327, 125–130.
- Canters, G.W., Gilardi, G., 1993. Engineering type 1 copper sites in proteins. *FEBS Letters* 325, 39–48.
- Charalambidis, N.D., Bournazos, S.N., Zervas, C.G., Katsoris, P.G., Marmaras, V.J., 1994. Glycosylation and adhesiveness differentiate larval *Ceratitis capitata* tyrosinases. *Archives of Insect Biochemistry and Physiology* 27, 235–248.
- Chomczynski, P., 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* 15, 532–537.
- Claus, H., Faber, G., König, H., 2002. Redox-mediated decolorization of synthetic dyes by fungal laccases. *Applied Microbiology and Biotechnology* 59, 672–678.
- Cohen, R., Persky, L., Hadar, Y., 2002. Biotechnological applications and potential of wood-degrading mushrooms of the genus *Pleurotus*. *Applied Microbiology and Biotechnology* 58, 582–594.
- Coluzzi, M., Sabatini, A., 1967. Cytogenetic observations on species A and B of the *Anopheles gambiae* complex. *Parasitologia* 9, 73–88.
- De Gregorio, E., Spellman, P.T., Rubin, G.M., Lemaitre, B., 2001. Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. *Proceedings of the National Academy of Sciences, USA* 98, 12590–12595.
- Dennell, R., 1958a. The amino acid metabolism of a developing insect cuticle: the larval cuticle and puparium of *Calliphora vomitoria*. III. The formation of the puparium. *Proceedings of the Royal Society of London. Series B, Biological Sciences* 149, 176–183.
- Dennell, R., 1958b. The hardening of insect cuticles. *Biological Reviews* 33, 178–196.
- Eggert, C., LaFayette, P.R., Temp, U., Eriksson, K.-E.L., Dean, J.F.D., 1998. Molecular analysis of a laccase gene from the white rot fungus *Pycnoporus cinnabarinus*. *Applied and Environmental Microbiology* 64, 1766–1772.
- Eisenhaber, B., Bork, P., Eisenhaber, F., 1999. Prediction of potential GPI-modification sites in proprotein sequences. *Journal of Molecular Biology* 292, 741–758.
- Gorman, M.J., Paskewitz, S.M., 1997. A genetic study of a melanization response to Sephadex beads in *Plasmodium*-refractory and -susceptible strains of *Anopheles gambiae*. *American Journal of Tropical Medicine and Hygiene* 56, 446–451.
- Goto, A., Kumagai, T., Kumagai, C., Hirose, J., Narita, H., Mori, H., Kadowaki, T., Beck, K., Kitagawa, Y., 2001. A *Drosophila* haemocyte-specific protein, hemolectin, similar to human von Willebrand factor. *Biochemistry Journal* 359, 99–108.
- Gupta, R., Jung, E., Brunak, S., in preparation. Prediction of N-glycosylation sites in human proteins.
- Hackman, R.H., Goldberg, M., 1967. The *o*-diphenoloxidases of fly larvae. *Journal of Insect Physiology* 13, 531–544.
- Håkansson, K., Reid, K.B.M., 2000. Collectin structure: a review. *Protein Science* 9, 1607–1617.
- Hansen, J.E., Lund, O., Engelbrecht, J., Bohr, H., Nielsen, J.O., Hansen, J.-E.S., Brunak, S., 1995. Prediction of O-glycosylation of mammalian proteins: specificity patterns of UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase. *Biochemical Journal* 308, 801–813.
- Hansen, J.E., Lund, O., Rapacki, K., Brunak, S., 1997. O-glycbase version 2.0—a revised database of O-glycosylated proteins. *Nucleic Acids Research* 25, 278–282.
- Hansen, J.E., Lund, O., Tolstrup, N., Gooley, A.A., Williams, K.L., Brunak, S., 1998. NetOglyc: prediction of mucin type O-glycosylation sites based on sequence context and surface accessibility. *Glycoconjugate Journal* 15, 115–130.
- Hepburn, H.R., 1985. Structure of the integument. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 3. Pergamon Press, Oxford, pp. 1–58.
- Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., Nusskern, D.R., Wincker, P., Clark, A.G., Ribeiro, J.M.C., Wides, R., et al. 2002. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science* 298, 129–148.
- Hopkins, T.L., Kramer, K.J., 1992. Insect cuticle sclerotization. *Annual Review of Entomology* 37, 273–302.
- Hullo, M.-F., Moszer, I., Danchin, A., Martin-Verstraete, I., 2001.

- CotA of *Bacillus subtilis* is a copper-dependent laccase. *Journal of Bacteriology* 183, 5426–5430.
- Ishaaya, I., Navon, A., 1974. Phenoloxidase activity at various stages of development in the Egyptian cotton worm *Spodoptera littoralis*. *Insect Biochemistry* 4, 67–73.
- Kotani, E., Yamakawa, M., Iwamoto, S.-I., Tashiro, M., Mori, H., Sumida, M., Matsubara, F., Tani, K., Kadono-Okuda, K., Kato, Y., Mori, H., 1995. Cloning and expression of the gene of hemocytin, an insect humoral lectin which is homologous with the mammalian von Willebrand factor. *Biochimica et Biophysica Acta* 1260, 245–258.
- Kramer, K.J., Kanost, M.R., Hopkins, T.L., Jiang, H., Zhu, Y.C., Xu, R., Kerwin, J.L., Turecek, F., 2001. Oxidative conjugation of catechols with proteins in insect skeletal systems. *Tetrahedron* 57, 385–392.
- Lai-Fook, J., 1966. The repair of wounds in the integument of insects. *Journal of Insect Physiology* 12, 195–226.
- Lindroth, R.L., 1991. Differential toxicity of plant allelochemicals to insects: roles of enzymatic detoxication systems. In: Bernays, E. (Ed.), *Insect-Plant Interactions*, vol. III. CRC Press, Boca Raton, pp. 1–33.
- Liu, L., Tewari, R.P., Williamson, P.R., 1999. Laccase protects *Cryptococcus neoformans* from antifungal activity of alveolar macrophages. *Infection and Immunity* 67, 6034–6039.
- Marmaras, V.J., Charalambidis, N.D., Zervas, C.G., 1996. Immune response in insects: the role of phenoloxidase in defense reactions in relation to melanization and sclerotization. *Archives of Insect Biochemistry and Physiology* 31, 119–133.
- Martins, L.O., Soares, C.M., Pereira, M.M., Teixeira, M., Costa, T., Jones, G.H., Henriques, A.O., 2002. Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. *The Journal of Biological Chemistry* 277, 18849–18859.
- Mayer, A.M., Staples, R.C., 2002. Laccase: new functions for an old enzyme. *Phytochemistry* 60, 551–565.
- Messerschmidt, A., 1993. Blue copper oxidases. *Advances in Inorganic Chemistry* 40, 121–185.
- Messerschmidt, A., Huber, R., 1990. The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin. Modelling and structural relationships. *European Journal of Biochemistry* 187, 341–352.
- Mills, R.R., Androuny, S., Fox, F.R., 1968. Correlation of phenoloxidase activity with ecdysis and tanning hormone release in the American cockroach. *Journal of Insect Physiology* 14, 603–611.
- Neville, A.C., 1975. *Biology of the Arthropod Cuticle*. Springer-Verlag, Berlin.
- Nichol, H., Law, J.H., Winzerling, J.J., 2002. Iron metabolism in insects. *Annual Review of Entomology* 47, 535–559.
- Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering* 10, 1–6.
- Notredame, C., Higgins, D.G., Heringa, J., 2000. T-Coffee: a novel method for fast and accurate multiple sequence alignment. *Journal of Molecular Biology* 302, 205–217.
- Parkinson, N.M., Conyers, C.M., Keen, J.N., MacNicol, A.D., Smith, I., Weaver, R.J., 2003. cDNAs encoding large venom proteins from the parasitoid wasp *Pimpla hypochondriaca* identified by random sequence analysis. *Comparative Biochemistry and Physiology Part C* 134, 513–520.
- Powell, L.D., Varki, A., 1995. I-type lectins. *Journal of Biological Chemistry* 270, 14243–14246.
- Sanchez-Amat, A., Lucas-Elio, P., Fernandez, E., Garcia-Borron, J.C., Solano, F., 2001. Molecular cloning and functional characterization of a unique multipotent polyphenol oxidase from *Marinomonas mediterranea*. *Biochimica et Biophysica Acta* 1547, 104–116.
- Shen, Z., Jacobs-Lorena, M., 1999. Evolution of chitin-binding proteins in invertebrates. *Journal of Molecular Evolution* 48, 341–347.
- Sidjanski, S., Mathews, G.V., Vanderberg, J.P., 1997. Electrophoretic separation and identification of phenoloxidases in hemolymph and midgut of adult *Anopheles stephensi* mosquitoes. *Journal of Parasitology* 83, 686–691.
- Smith, M., Thurston, C.F., Wood, D.A., 1997. Fungal laccases: role in delignification and possible industrial applications. In: Messerschmidt, A. (Ed.), *Multi-copper Oxidases*. World Scientific Publishing, Singapore, pp. 201–224.
- Soares, G.M.B., Amorim, M.T.P., Hrdina, R., Costa-Ferreira, M., 2002. Studies on the biotransformation of novel disazo dyes by laccase. *Process Biochemistry* 37, 581–587.
- Solomon, E.I., Sundaram, U.M., Machonkin, T.E., 1996. Multicopper oxidases and oxygenases. *Chemical Reviews* 96, 2563–2605.
- Suderman, R.J., Andersen, S.O., Hopkins, T.L., Kanost, M.R., Kramer, K.J., 2003. Characterization and cDNA cloning of three major proteins from pharate pupal cuticle of *Manduca sexta*. *Insect Biochemistry and Molecular Biology* 33, 331–343.
- Sugumaran, M., Kanost, M.R., 1993. Regulation of insect hemolymph phenoloxidases. In: Beckage, N.E., Thompson, S.N., Federici, B.A. (Eds.), *Parasites and Pathogens of Insects*, vol. 1. Academic Press, San Diego, pp. 317–342.
- Sugumaran, M., Giglio, L., Kundzicz, H., Saul, S., Semensi, V., 1992. Studies on the enzymes involved in puparial cuticle sclerotization in *Drosophila melanogaster*. *Archives of Insect Biochemistry and Physiology* 19, 271–283.
- Swofford, D.L., 2002. PAUP\*. Phylogenetic analysis using parsimony (\*and other methods). Version 4. Sinauer Associates, Sunderland, MA.
- Thomas, B.R., Yonekura, M., Morgan, T.D., Czapl, T.H., Hopkins, T.L., Kramer, K.J., 1989. A trypsin-solubilized laccase from pharate pupal integument of the tobacco hornworm, *Manduca sexta*. *Insect Biochemistry* 19, 611–622.
- Thurston, C.F., 1994. The structure and function of fungal laccases. *Microbiology* 140, 19–26.
- Tomme, P., Warren, R.A., Miller, R.C. Jr., Kilburn, D.G., Gilkes, N.R., 1995. Cellulose-binding domains: classification and properties. In: Sadler, J.N., Penner, M. (Eds.), *Enzymatic Degradation of Insoluble Polysaccharides*. American Chemical Society, Washington, pp. 142–163.
- Van Dellen, K., Ghosh, S.K., Robbins, P.W., Loftus, B., Samuelson, J., 2002. *Entamoeba histolytica* lectins contain unique 6-cys or 8-cys chitin-binding domains. *Infection and Immunity* 70, 3259–3263.
- Williamson, P.R., 1997. Laccase and melanin in the pathogenesis of *Cryptococcus neoformans*. *Frontiers in Bioscience* 2, e99–107.
- Xu, F., Shin, W., Brown, S.H., Wahleithner, J.A., Sundaram, U.M., Solomon, E.I., 1996. A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. *Biochimica et Biophysica Acta* 1292, 303–311.
- Xu, F., Palmer, A.E., Yaver, D.S., Berka, R.M., Gambetta, G.A., Brown, S.H., Solomon, E.I., 1999. Targeted mutations in a *Trametes villosa* laccase: axial perturbations of the T1 copper. *Journal of Biological Chemistry* 274, 12372–12375.
- Yamazaki, H.I., 1969. The cuticular phenoloxidase in *Drosophila virilis*. *Journal of Insect Physiology* 15, 2203–2211.
- Yamazaki, H.I., 1972. Cuticular phenoloxidase from the silkworm, *Bombyx mori*: properties, solubilization, and purification. *Insect Biochemistry* 2, 431–444.
- Zhu, Y.-C., Specht, C.A., Dittmer, N.T., Muthukrishnan, S., Kanost, M.R., Kramer, K.J., 2002. Sequence of a cDNA and expression of the gene encoding a putative epidermal chitin synthase of *Manduca sexta*. *Insect Biochemistry and Molecular Biology* 32, 1497–1506.