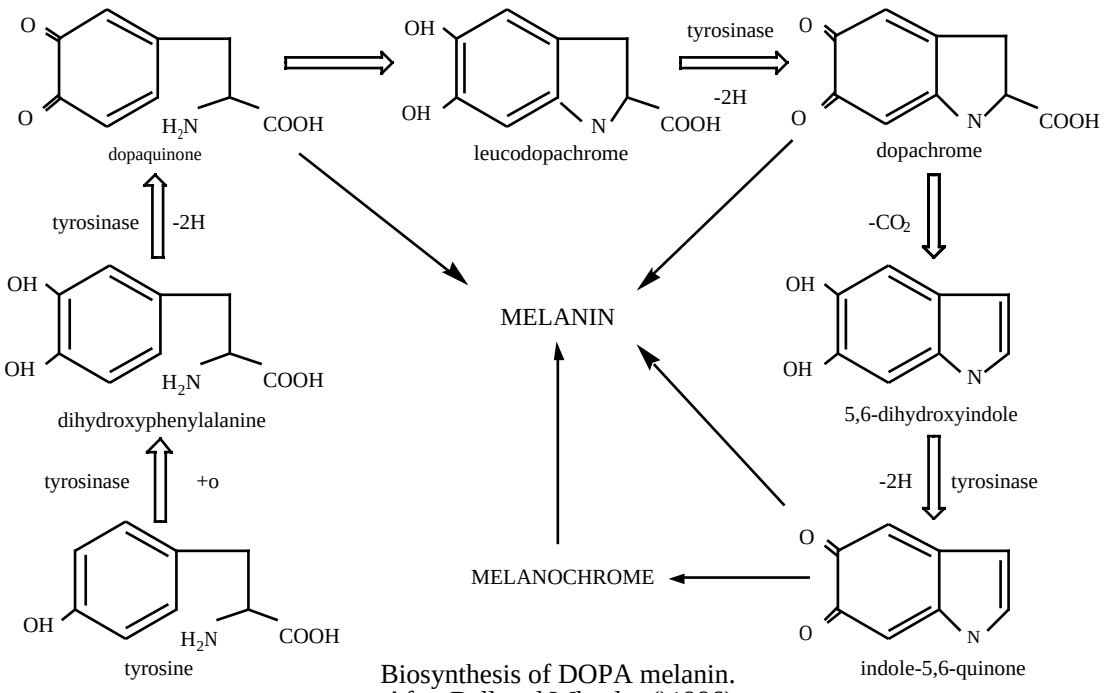
Griffith, G.W. (1994). Phenoloxidases. Chapter 28 in *Aspergillus nidulans*: 50 years on (Eds S.D. Martinelli and J.R. Kinghorn); Progress in Industrial Microbiology, Volume 29. Elsevier Science Publishers, Amsterdam. pp. 763-788.

28.1. Introduction.

Phenoloxidases represent a major group of enzymes involved in secondary metabolic activity, most commonly being associated with the production of melanins (see figure 1) and other pigments. The (usually) polymerizing reactions catalysed by these enzymes are also related to changes in the properties of cell walls (increased impermeability and hydrostatic strength), intercellular interactions (e.g. hyphal aggregation) and the removal/detoxification of certain secondary metabolites (Mayer, 1987; Mayer & Harel, 1979).

Research interest in developmental processes and to a lesser extent the structure and growth of hyphal cell walls has led to the identification of several phenoloxidase enzymes in *A. nidulans*. Detailed protein structure analysis has focussed on phenoloxidases from other fungi, notably *Agaricus bisporus* and *Neurospora crassa* but *A. nidulans* has provided a useful system for investigation of the developmental regulation and different cellular functions served by these enzymes.



Biosynthesis of DOPA melanin.
After Bell and Wheeler ()1986).

28.2. Classification of Phenoloxidases.

The generic terms phenoloxidase, phenolase or polyphenol oxidases are used to describe enzymes which catalyse the oxidation by molecular oxygen of aromatic compounds. Given the complexity and diversity of secondary metabolic pathways in nature, it is perhaps unsurprising that a wide range of substrates are used by these enzymes. This fact has led to confusion with respect to terminology and has also hampered the identification of the normal/natural substrates of particular enzymes.

Since molecular oxygen is also a phenoloxidase substrate, the kinetics of such oxidative reactions are influenced by the prevailing redox conditions. Under conditions of highly positive redox potential, the reactions catalysed by phenoloxidases may also occur spontaneously. In addition, the presence of enzymes such as catalase, peroxidase and superoxide dismutase which react with activated oxygen are also important in determining the stability of aromatics. Failure to take account of these complications has sometimes led to false identification of phenoloxidase activity (Mayer & Harel, 1979).

Phenoloxidases are split into two subgroups, laccases and catechol oxidases (tyrosinases). Both are characterized by the involvement of copper ligands and a role in the biosynthesis of melanins and other polyphenols. Cross-specificity exists between laccases and catechol oxidases with respect to the oxidation of ortho-diphenol substrates (e.g. Marr, 1979). However, laccases, unlike catechol oxidases, are insensitive to inhibition by carbon monoxide and phenylhydrazine, and are usually able to oxidize para-diphenol and

para-diamine compounds (Mayer, 1987). Non-copper containing hydroxylases, as exemplified by mammalian L-tyrosine hydroxylase, which catalyses a similar oxidative reaction using a pterine cofactor (Vanneste & Zuberbuhler, 1974), will not be considered in this review.

28.2.1. Laccase

Laccases (benzenediol: oxygen oxidoreductase [EC 1.10.3.2] or p-diphenol oxidase) represent a distinct group of enzymes which oxidize a broad range of o-, m- and p-diphenols to quinones (see figure 2). Although first identified in plants (the Japanese lacquer tree, *Rhus* spp; Yoshida, 1883), laccases are far more prevalent among the higher fungi (Leatham & Stahmann, 1981). There is some evidence, based on the inability of all plant and some fungal laccases to oxidize para-cresol, that two classes of this enzyme may exist. Along with ascorbate oxidase and ceruloplasmin, laccases belong to the blue multicopper oxidase family of enzymes, which are characterized by the binding of four or more divalent copper cations in several conformations (types 1 to 4; Frieden, 1981). It has been proposed that these oxidases originally served to protect against the toxic effects of oxygen (Dawson *et al.*, 1975). In the scheme of Dawson *et al*, laccases are considered primitive, since the other blue multicopper oxidases have evolved more specialized roles in copper transport and as terminal oxidases in animals and higher plants respectively.

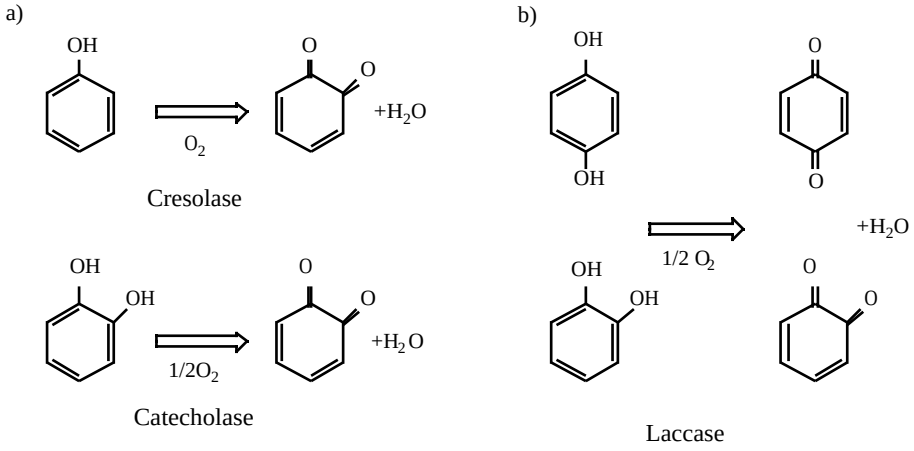


Figure 2. Tyrosinase and Laccase Activities

A number of fungal laccases have now been studied in detail, including those of *Neurospora crassa* and *Podospora anserina* (Germann & Lerch, 1986; Germann *et al.*, 1988; Prillinger & Esser, 1977). Most appear to be extracellular and heavily glycosylated (15-41% carbohydrate), although there have been occasional reports of intracellular activity (e.g. Ross, 1982). Catalysis involves the four-electron reduction of O_2 to water by direct

interaction with the reoxidizable type 1 (blue) copper. The other copper atoms are believed to play a role in substrate binding (Urbach, 1981).

28.2.2. Catechol Oxidase (Tyrosinase)

Catechol oxidases, in contrast to laccases, are nearly ubiquitous in their taxonomic distribution. Although detailed investigations of the tyrosinases from *Agaricus bisporus* and *Neurospora crassa* have been made, the enzyme is most commonly associated with melanization in mammals and cuticle tanning in insects. Confusion with regard to nomenclature has arisen, largely due to the occurrence of two distinct catalytic activities, which may occur to different degrees with different tyrosinases. IUBAC, has recently redefined the two catalytic activities: (i) EC 1.14.18.1 (monophenol monooxygenase; also called cresolase or monophenolase) which catalyzes the ortho-hydroxylation of monophenols to para-diphenol, usually followed by oxidation to the corresponding quinone; (ii) EC 1.10.3.1 (o-diphenol oxidase, also called catecholase or diphenolase) which catalyzes the oxidation to quinone of para-diphenol compounds (see figure 2). For historical reasons, the term tyrosinase is frequently used as a generic term for catechol oxidases, although tyrosine itself is a poor substrate for many of these enzymes.

The dual catalytic activity of tyrosinases has led to disagreement in the past as to whether both reactions occur at the same active site (Mayer, 1987; Mayer & Harel, 1979). However, recent studies have established that both types of substrate bind to a single active site, although monophenols bind to only one of the two divalent copper ions present at this site (Lerch, 1981). The reaction mechanism is similar to that found in the mollusc/arthropod oxygen-carrier haemocyanin (Urbach, 1981).

Tyrosinases represent a heterogeneous group of enzymes which differ considerably with respect to substrate specificity and subunit composition. Multiple isoenzymes are frequently encountered, arising both from post-translational modifications and isolation artefacts (Lerch, 1981; Mayer, 1987). The ratio of cresolase/catecholase activity of tyrosinases have also been found to be variable in several cases. Cresolase activity is increased by the presence of catalytic amounts of o-diphenols and tends to be less stable. A model for tyrosinase activity has been proposed which accounts for these observations (Lerch, 1981). This model also accounts for reaction inactivation (tanning) associated with catecholase activity, which is believed to occur as a result of attack by o-quinone, resulting in loss of copper from the active site.

28.3 Developmental Regulation of Phenoloxidase Activity.

A number of studies of both basidiomycetes and ascomycetes have shown that production of phenoloxidases is developmentally regulated. In the hyperparasitic basidiomycete *Phanerochaete magnoliae*, reciprocal distribution of laccase and tyrosinase activity is correlated with hyphal septation and the occurrence of non-assimilative aerial mycelia

(Ainsworth & Rayner, 1991). In other species, the onset of phenoloxidase production is correlated with the depletion of nutrients from the culture medium and the cessation of mycelial growth (DeVries *et al.*, 1986; Leatham & Stahmann, 1981). Similar conditions are associated with the onset of both developmental differentiation and the production of secondary metabolites (Vining, 1990). The fact that aromatic compounds produced by the latter processes represent the substrates for pigments associated with the former has not gone unnoticed.

A widespread phenomenon among the higher fungi is pigment production associated with the expression of somatic (heterokaryon) incompatibility responses between genetically distinct mycelia (Caten, 1971; Rayner & Coates, 1987). Although poorly understood, phenoloxidase production is known to be associated with such zones of pigment in some species (Li, 1981; Stenlid & Rayner, 1989). However, it is unclear whether pigment production is due to the induction of phenoloxidase activity or the production of particular substrates.

In basidiomycetes, phenoloxidases have been implicated in both lignocellulose degradation and basidiocarp formation. Mutants of *Sporotrichum pulverulentum* and *Phanerochaete chrysosporium* which lack phenoloxidase activity are unable to degrade lignin (Ander & Eriksson, 1976; Liwicki *et al.*, 1985). However, further studies have suggested that the oxidative depolymerization of lignin by basidiomycetes can be catalyzed by peroxidases (manganese-dependent peroxidase and ligninase) acting either alone or in combination with laccase (Kirk & Farrell, 1987; Kojima *et al.*, 1990; Szklarz *et al.*, 1989). There is also evidence that laccases involved in lignin degradation may contain the prosthetic group pyrroloquinoline quinone (PQQ) (Saloheimo *et al.*, 1991).

Laccase activity is associated with the early stages of basidiocarp formation in several species (DeVries *et al.*, 1986; Leatham & Stahmann, 1981; Leonard & Phillips, 1973). Ross (1982) has suggested that the membrane-bound laccase of *Coprinus congregatus* is involved in the initial light-requiring induction process rather than in primordium formation. In other species, extracellular laccase activity is correlated with fruit body enlargement (Wood & Goodenough, 1977) or stipe/pileus pigmentation (Leatham & Stahmann, 1981). Mutants of *Schizophyllum commune* defective in basidiocarp production have also been shown to be defective in phenoloxidase activity (Leonard, 1972).

Developmental changes associated with host infection in a number of plant pathogens have suggested a critical role for phenoloxidase enzymes Laccase induction by plant metabolites such as pectin and host phenolics has been demonstrated in *Botrytis cinerea* (Marbach *et al.*, 1985), while phenoloxidase-mediated melanization is a prerequisite for appressorial penetration in *Magnaporthe* (*Pyricularia*) and *Colletotrichum* spp. (Kubo *et al.*, 1989). Appressorial melanization is required to generate sufficient intracellular

hydrostatic pressure to penetrate the host cuticle (Howard *et al.*, 1991). A reduction of laccase activity is also associated with dsRNA-associated hypovirulence in *Cryphonectria parasitica* (Rigling & Van Alfen, 1991).

28.4. Phenoloxidases of *A. nidulans*

A total of four phenoloxidase enzymes have hitherto been identified in *A. nidulans*. A summary of the properties of these enzymes is presented in table 1.

	Molecular Weight (kDa)	Glycosylation	Extracellular	Natural Substrate	Details
Hyphal Tyrosinase	130 x 4 (tetramer)[1]	NK	+[1]	DOPA?[1]	Endogenous protein inhibitor (150kDa)[1] Cresolase activity is unstable[1]
Conidiophore Cresolase	48/50[2] (349 a.a.[3])	+[2]	+[4]	N-acetyl-6-hydroxytryptophan[5]	No catecholase activity[4] Zn^{2+} cofactor[4]
Conidial Laccase (I)	36-80[8][6,7] (609 a.a.[8])	+[7]	+[9]	polyketide?[10]	Enzyme size is affected by interaction with its substrate[7]
Cleistothecial Laccase (II)	110[11]	+[11]	?[11]	polyketide?[11]	Localized in the inner wall of the cleistothecium[11]

Table 1. Summary of the properties of the four known phenoloxidases of *Aspegillus nidulans.*
NK - Not known.
[1]. Bull & Carter (1973); [2]. Birse & Clutterbuck, 1990; [3]. Griffith, unpublished data; [4]. Clutterbuck, 1977; [5]. McCorkindale *et al.*, 1983; [6]. Law & Timberlake, 1980; [7]. Kurtz & Champe, 1982; [8]. Aromayo & Timberlake, 1990; [9]. Clutterbuck, 1972; [10]. Mayorga & Timberlake, 1992; [11]. Hermann *et al.* 1983

28.4.1. Conidial Laccase (Laccase I)

Conidia of *A.nidulans* contain a green pigment of unknown but probably polyketide composition, which is believed to confer resistance to ultraviolet radiation and may be important for survival in nature (Wright & Pateman, 1970). Yellow spore colour mutation mapping to the *yA* locus are the most commonly used of a number of spore colour mutants employed as easily detectable markers in *A. nidulans* genetics (Pontecorvo *et al.*, 1953). The involvement of an extracellular factor was suggested by crossfeeding experiments in which conidia of yellow-spored (*yA*) mutant colonies adjacent to green (y^+) or white-spored (*wA* mutant) colonies became greenish (Holt & MacDonald, 1967). Yellow spored mutants are known in other green and black-spored *Aspergillus* species (Raper & Fennel, 1965). The green pigment of *A. nidulans* may be related to these.

Clutterbuck (1972) showed that conidiating cultures of green-spored strains contained a laccase ('p-diphenol oxidase'), absent from colonies of the *yA* mutant. Crude extracts from green or white-spored strains were able to induce greening of spores when added to wells near conidiating *yA* strains. This effect was enhanced by low pH and the use of nutrient rich media. A temperature-sensitive mutant (*yA31*) which produced green conidia at 22 ^{0}C but not at 37 ^{0}C and contained a thermolabile laccase, suggested that the *yA* locus encodes at least a component of the laccase enzyme.

The absence of laccase activity in *brlA* mutants but its presence in *abaA* mutants suggested that production of the enzyme is developmentally regulated and is induced at the phialide stage of conidiation (Clutterbuck, 1972; see Timberlake & Clutterbuck chapter 16). Law & Timberlake (1980) used inhibitors of RNA and protein synthesis to show that developmental regulation of the *yA* gene occurred at the level of transcription. Yelton *et al.* (1985) have cloned the *yA* gene and have confirmed that expression is regulated at the level of transcript accumulation (O'Hara & Timberlake, 1989). Transcription and translation of the gene appears to occur in the phialides rather than in the conidia.

The conidial laccase from a white-spored (*wA*) mutant has been purified and characterized by Kurtz & Champe (1982). It exists as several glycosylated isoenzymes ranging in molecular weight from 36,000 to 80,000. The latter size is consistent with the findings of another study (Law & Timberlake, 1980) and the smaller forms may be degradation products of the larger. Law & Timberlake (1980) have suggested that conidial laccase is active as a monomer. The sequence of the *yA* gene has now been published (Aramayo & Timberlake, 1990). The gene encodes a putative polypeptide of 609 amino acids which has several predicted copper binding sites. Although the blue colour in concentrated solutions of conidial laccase and its requirement for copper ions have been shown, the metal ion content of the enzyme has not been investigated. Computer searches indicate homology in the predicted copper binding domains to several laccases and other blue copper oxidases.

Several other mutants defective in conidial pigmentation have been identified (Dorn, 1967). The effect of *ygA* and *yB* mutants is discussed below. Clutterbuck (1972) found slightly reduced laccase activity in chartreuse (*chaA3*) mutants but normal levels in fawn (*fwA1*) strains. *wA* mutants are epistatic to all other spore colour mutants (with the exception of wet-white, *wetA*; (see Timberlake & Clutterbuck, chapter 16). Therefore, it has suggested that mutants at this locus are defective in the synthesis of a precursor of the conidial pigment (Clutterbuck, 1990).

The *wA* gene has been cloned and its transcript appears to accumulate slightly later than that of *yA*. (Mayorga & Timberlake, 1990). It encodes a predicted polypeptide of 1986 amino acids, whose sequence suggests that it is involved in polyketide synthesis (Mayorga & Timberlake, in press*). Differences in both stability and electrophoretic mobility were found between the enzyme from green and white-spored strains (Kurtz & Champe, 1982). The correlation of these differences with the higher level of laccase activity found in *wA* mutants, suggests that they are due to the interaction of the enzyme with its natural substrate.

A rodlet layer which lies just outside the pigmented layer of mature conidia (Claverie-Martin *et al.*, 1986; Oliver, 1972) is similar in appearance under the electron microscope to the surface of spores and aerial mycelia of several fungi and *Streptomyces* spp. (Chater,

1991). A mutant at the recently defined *rodA* locus (Stringer *et al.*, 1991) lacks the spore rodlet layer. This gene is predicted to encode a hydrophobic and cysteine-rich polypeptide which has homology with a family of hydrophobins from *Schizophyllum commune* (Schuren & Wessels, 1990; Wessels *et al.*, 1991b). The fact that the outer layer of dark (*drkA*) mutant conidia detaches readily in water (Clutterbuck, 1969) suggests that the rodlet layer may interact with the spore pigment layer. Evidence for possible interactions between hydrophobins and phenoloxidases is discussed below.

28.4.2. Cleistothecial Laccase (Laccase II)

A second developmentally regulated laccase associated with cleistothecial production has been identified by Hermann *et al.* (Hermann *et al.*, 1983). In cultures incubated for at least 60 hours at 37 ^{o}C, foci representing cleistothecial initials can be visualised using 4-amino-2,6-dibromophenol as a substrate. This stain showed cross-reactivity with conidial laccase (laccase I) but enzyme activity could be assayed quantitatively in yellow-spored (*yA*) mutants. The electrophoretic mobility of laccase II differed from that of laccase I and antibodies raised against laccase II did not cross-react with laccase I. Staining was more intense in hülle cells and cleistothecial primordia than mature cleistothecia. Only the enzyme from hülle cells appeared to be readily extractable, although physical disruption of mature cleistothecia prior to staining revealed the presence of stellate crystals of the stain on the spongy inner surface of the cleistothecial wall.

Three non-allelic cleistothecial pigmentation mutants *blA3,* (blue) *clA4* and *clB1* (colourless) (Apirion, 1963) had higher levels of laccase II activity than the wild-type (Hermann *et al.*, 1983). This is reminiscent of the high levels of conidial laccase in white (*wA*) mutants. However, four other *Aspergillus* species, which had light-coloured (yellow/white) cleistothecia but no hülle cells, showed no laccase activity. The hypothesis that hülle cells represent the primary source of laccase II is supported by the observation that normal activity was found in the hülle cells of three acleistothecial *Aspergillus* species and the hülle cells of an acleistothecial *medA* (medusa) mutant. It remains unclear whether laccase II is involved in the synthesis of the red asperthecin pigment of the cleistothecia (Neelakantan *et al.*, 1957).

Champe and his co-workers have identified an endogenous inducer of sexual development which accumulates in three non-allelic aconidial (*aco*) mutants blocked in both sexual and asexual sporulation (Butnick *et al.*, 1984a; Butnick *et al.*, 1984b; see Champe chapter). These mutants overproduce the Psi factor (precocious sexual inducer) along with a number of phenolic metabolites, some of which may be derived from the polyketide precursor orsellinic acid (Ballantine *et al.*, 1971; Champe & El-Zayat, 1989; Champe *et al.*, 1987; see Champe, chapter 17). The fact that laccase II is produced during the early stages

of cleistothecial production suggests that it may play a role in this polyketide synthesis pathway.

28.4.3. Conidiophore Cresolase (AHTase).

The conidiophore vesicles, metulae and phialides of *A. nidulans* and several other *Aspergillus* species are known to have grey-brown pigmented conidiophores (Raper & Fennel, 1965), although this pigment is normally masked by pigmented conidia. The melanic pigment is present in the outer (secondary) cell wall (Oliver, 1972). Mutants defective in the early stages of conidiation (*abaA* and leaky *brlA* mutants) display conidiophore pigment more clearly (Clutterbuck, 1969) so that it was possible to obtain mutants lacking this pigment (Ivory) from *brlA* or *abaA* mutant backgrounds. Such mutations were found to map to two loci, *ivoA* and *ivoB*. The *ivoB* mutants were found to lack a specific phenol oxidase (Clutterbuck, 1974; Clutterbuck, 1977). The isolation of a temperature sensitive mutant (*ivoB192*), containing a thermolabile enzyme, suggested the *ivoB* gene encodes a structural component of the enzyme.

ivoB mutants accumulate a soluble metabolite which has been identified as N-acetyl-6-hydroxytryptophan (AHT) (McCorkindale *et al.*, 1983). AHT accumulation in *ivoB* mutants can be quantified with a stain specific for 6-hydroxyindoles (Jepson *et al.*, 1962). The failure of *ivoA* mutants to accumulate AHT and the ability of crude extracts of *ivo*$^{+}$ strains (but not *ivoB* strains) to rapidly oxidize purified AHT, suggests that this gene functions in the synthesis of this pigment precursor.

The phenoloxidase involved in conidiophore pigmentation is an extracellular, substrate-specific cresolase (Clutterbuck, 1977). Birse & Clutterbuck (1990) purified AHTase and found it to be a glycosylated protein. It is present in at least two isoforms (48 and 50 kDa) with identical N-terminal residues which may differ in the degree of glycosylation. Analysis of its metal ion content suggested that each molecule contains two copper and one zinc atom. The occurrence of zinc in an enzyme with phenoloxidase activity is highly unusual and is reminiscent of cuprozinc superoxide dismutase and cytochrome oxidase.

Although the enzyme shows susceptibility to inhibitors typical of cresolase activity in catechol oxidases, it failed to oxidize a number of standard substrates and showed only limited activity with 6-hydroxytryptamine and AHT methyl ester. There was also evidence that the enzyme may contribute to further oxidation reactions leading to melanin formation. The predicted polypeptide product of the *ivoB* gene has copper binding domains and shows homology to several microbial tyrosinases (G.W. Griffith, unpublished). As with conidial laccase (see above 28.4.1.), AHTase activity is 2-fold higher in the absence of its natural substrate (i.e. in *ivoA* strains), although this factor did not

alter its electrophoretic properties (Clutterbuck, 1990). It is possible that the enzyme is inactivated by incorporation into a melanic complex.

The conidiophore pigmentation pathway represents an accurate means of investigating the early events in conidiation. Levels of AHTase and AHT accumulation correlate well with the degree of morphological development in a range of leaky mutants at the *brlA* locus. The cloning of the *ivoA* and *ivoB* genes has shown that their expression is regulated at the level of transcription and has confirmed that expression of the *ivoB* gene occurs slightly earlier than *ivoA* (Birse & Clutterbuck, 1991; G.W. Griffith, unpublished). The implication of these results is that *ivoB* is more closely regulated by *brlA* than *ivoA*. Promoter deletions of the *ivoB* gene have identified potential *BRLA* and *ABAA* responder elements (G.W. Griffith, unpublished).

It seems unlikely that the role of pigmentation is to provide protection from the damaging effects of light, since the conidiophores are not normally exposed. The *ivo* mutants do not appear to have any serious structural deficiency, although Clutterbuck (1990) observed that pigmented bristles of *brlA* mutants were more rigid than unpigmented bristles. *ivo* mutants have yet to be examined in a 'true' wild-type (i.e. ve^{+}) background. Among the pleiotropic effects of the *veA* mutation is the production of shorter conidiophores, which may not require the additional strengthening or insulation provided by the deposition of melanin in the cell wall.

Exogenous tryptophan or N-acetyltryptophan reduces AHT accumulation in *ivoB* mutants (Clutterbuck, 1990; G.W. Griffith, unpublished), while the amount of exogenous tryptophan required for normal levels of conidiation in tryptophan auxotrophs is much higher than that required for hyphal growth (Yelton *et al.*, 1983). These and other lines of evidence suggest that tryptophan may play an important role in conidiophore morphogenesis (see figure 3).

The predicted polypeptide encoded by the *ivoA* gene (G.W. Griffith, unpublished) shows strong homology to the ACV synthetases of both *A. nidulans* (MacCabe *et al.*, 1991) and *Penicillium chrysogenum*, (Smith *et al.*, 1990), as well as other proteins involved in the formation of non-peptide bonds via the ATP-dependent binding of AMP to their substrate. ACV synthetase is involved in the first step of penicillin synthesis in which a tripeptide is synthesized using amino acid thiolester intermediates. It is therefore likely that the

product of the *ivoA* gene mediates the N-acetylation of its substrate, although it is not known whether it also acts as a hydroxylase, nor even if tryptophan is directly involved in this pathway. The synthesis of indole-3-acetic acid without tryptophan as an intermediate has been demonstrated in maize (Wright *et al.*, 1991). All mutations giving an *ivo* phenotype (Clutterbuck, 1977) map to either *ivoA*, *ivoB* or *ygA* (see 28.4.4). It is possible that other genes are involved in this pathway and that mutants at these loci are aconidial.

The *ivo* pathway, involving puzzling interactions with tryptophan metabolism, combined with the use of unusual indolic precursors in melanin formation and a cuprozinc phenoloxidase is possibly unique and suggests that it may serve to detoxify some active metabolite or that it has a role in the oxygen metabolism of the conidiophore.

28.4.4. Copper Metabolism Mutants

Among the mutants defective in conidial pigmentation (Clutterbuck, 1969; Dorn, 1967) are two, *ygA* (yellow-green) and *yB*, which appear to be defective in copper metabolism. The pleiotropic effects of both are related in part to the role of copper as an essential cofactor of phenoloxidase enzymes (Clutterbuck, 1972; Kurtz & Champe, 1981; Mayer, 1987).

Holt & MacDonald (1967) observed that green but not yellow spore pigmentation was dependent on the availability of copper in cultures growing on media lacking certain trace elements. Clutterbuck (1972) found that *ygA* mutants possessed reduced laccase activity, particularly when grown on low pH media and appeared to be slightly less susceptible to toxic levels of copper salts in the medium. Addition of 20 mM copper (II) salts to YG medium remedied the mutant phenotype (Kurtz & Champe, 1981). Dialysis of extracts of a *ygA* mutant against a copper (I or II) chloride solution partially restored laccase activity (Clutterbuck, 1972), although Kurtz & Champe (1981) were unable to repeat this observation.

Conidiophore pigmentation is also affected by the *ygA* mutation and some leaky *ivo* mutants were found to map to this locus. Addition of copper (I or II) salts to a *brlA42; ygA6* strain increased the AHTase activity of extracts, while both *ivoA* and *ivoB* mutants partially suppressed the spore-colour phenotype of *ygA* (Clutterbuck, 1990). AHTase activity in *ygA* strains was also affected by medium pH. Suppression by *ivoA* mutants (which have higher levels of AHTase activity than the wild-type ; see 28.4.3.) suggests that chelation of copper occurs when the conidiophore melanin is synthesized.

The effects of mutations at the *yB* locus are semi-dominant and pleiotropic, causing reduced growth rate (due to delayed conidial germination), delayed conidiation and the production of reduced numbers of small cleistothecia (Kurtz & Champe, 1981). However, addition of low levels (5 mM) of copper sulphate or high levels (100 mM) of NaCl remedied the mutant phenotypes. Antibodies prepared against conidial laccase showed no cross-

reactivity with extracts of *yB* or *ygA* strains grown on low copper media, suggesting that the enzyme was either not synthesized or rapidly degraded. Cleistothecial laccase activity was also absent in these mutants. The fact that both *yB* and *ygA* mutants are defective in sexual development but do produce hülle cells provides additional evidence that activity of laccase II may be necessary for cleistothecial production.

A number of extragenic suppressors of the *yB* mutation have been identified (Kurtz & Champe, 1981; Kurtz *et al.*, 1984). Two of these (r6 and r25) produced a smaller number of pale green conidia and large numbers of small cleistothecia, while a third (r9) produced normal levels of yellow-green conidia but hardly any cleistothecia. When crossed into a yB^+ background, the suppressors exhibited normal conidiation but maintained the abnormal patterns of sexual reproduction. All the revertants,whether in *yB* or yB^+ backgrounds also produced increased amounts of aerial mycelium. The *yB* mutation was also suppressed in ve^+ and *ivoA* strains.

The nature of the interaction of *yB* with the various suppressors suggests that remediation of the spore-colour phenotype is achieved by reallocation of limited amounts of copper either by reduction of conidial density (ve^+, r6, r25) or by reduced competition for copper by other enzymes (r9, *ivoA*). Kurtz *et al.* (1984) have suggested that the *yB* locus may encode a metallothionein and that *yB* mutants chelate copper with great efficiency hence reducing its availability to other cellular components.

28.4.5 Hyphal tyrosinase

The impetus for studying the phenoloxidase involved in conidial and cleistothecial developmental has primarily been to elucidate their regulation and function during cellular differentiation. However, more limited studies aimed at understanding the physiology of hyphal growth and cell wall structure have implicated the importance of melanin and phenoloxidases in these processes.

Hyphal melanin in *A. nidulans* is laid down in a microfibrillar form on the exterior of the fungal hyphae (Pirt & Rowley, 1969) and comprises 16-18% of the cell wall (Bull, 1970a). Some less polymerized melanin is released into the medium in liquid culture (Rowley & Pirt, 1972). Mutants defective in the production of melanin in hyphal cell walls were characterized by Bull & Faulkner (1965). Some of these accumulated pink and purple pigments, identified as dopachrome (indole 5,6-quinone) and melanochrome, which are intermediates in the DOPA melanin pathway (Bell & Wheeler, 1986). Although DOPA was not detected as an intermediate, the indolic nature of the melanin has been confirmed (Bull, 1970a; Pirt & Rowley, 1969).

The phenoloxidase which mediates the formation of this melanin has been shown to be a tyrosinase based on its substrate specificity and susceptibility to inhibitors (Bull & Carter,

1973; Martinelli & Bainbridge, 1974). Bull & Carter found the enzyme to be intracellular and to exist in monomer-tetramer equilibrium, although four isoenzymes were resolved in electrophoretic studies. The ratio of cresolase/catecholase varied according to the method of purification (cresolase activity was unstable). The affinity of the *Aspergillus* enzyme for DL-DOPA, in common with two other fungal tyrosinases, is lower than that observed for plant and animal tyrosinases. The enzyme was non-competitively inhibited by an endogenous protein (1.5 x 10^5 MW). Although the effect of this inhibitor may be artefactual, there is evidence that such protein inhibitors are widespread among the fungi (Madhosingh, 1975).

In a separate study, Martinelli & Bainbridge (1974) identified at least three phenoloxidase isoenzymes (DOPA substrate) in laboratory wild-type strains, including a thermolabile band which appeared during early log phase growth. Direct comparisons with the tyrosinase isoenzymes studied by Bull & Carter (1973) were not made but its is likely that at least some of the isoenzymes are the same, since they occurred in the same cell fractions. The observed differences, including the failure to detect cresolase activity may have resulted from differences in sample preparation.

Analysis of phenoloxidases from two non-allelic and recessive melanin-production mutants, one an overproducer (*melA^{x}1*) and one melanin-less (*melB^{o}2*), as well as several wild-type strains from diverse origins and two *ivo*$^-$ mutants revealed differences in isoenzyme profiles. The overproducer mutant *melA^{x}1* lacked the thermolabile isoenzyme while the melanin-less *melB^{o}1* mutant showed two additional smaller bands not normally seen in other strains unless non-frozen samples were analysed. Non-laboratory wild-type strains also lacked one of the constitutive isoenzyme bands. Natural polymorphisms with respect to fungal phenoloxidase isoenzymes are known (Kerrigan & Ross, 1988; Prillinger & Molitoris, 1979). However, the isoenzyme polymorphism observed by Martinelli & Bainbridge (1974) may correlate with the widespread occurrence of the *veA1* mutation among laboratory "wild-type " strains (Kafer, 1965). The pleiotropic effects of the *veA1* mutation on developmental differentiation have only recently been appreciated (see 28.4.4; Mooney & Yager, 1990).

Among the mutants obtained by Bull & Faulkner (1965) was one (13.1.OL) which was both colourless and acleistothecial. This mutant possessed catecholase but not cresolase activity, although it was established that this defect was not due either to an endogenous inhibitor or the failure to synthesize an essential cofactor. Kuo & Alexander (1967) found that this mutant was more susceptible to hyphal lysis by microbial enzymes. This result was confirmed by Bull (1970b), who found that both β-1,3-glucanase and chitinase were non-competitively inhibited by melanin and that melanin was covalently bound to chitin, probably via amido groups. Correlation between melanization and resistance to hyphal

lysis has also been observed in several other ascomycete species (Bloomfield & Alexander, 1967; Potgieter & Alexander, 1966).

The production of hyphal melanin in liquid batch cultures occurs after the cessation of exponential growth (Rowley & Bull, 1978; Rowley & Pirt, 1972). Melanin production in chemostat cultures is favoured by carbon rather than nitrogen limitation and by higher pH (up to 7.9) and low dissolved oxygen tension (up to 30 mm Hg). The non-enzymatic oxidation of DOPA was also found to increase under these pH and oxygen conditions. Carter & Bull (1971) observed that tyrosinase activity increased both in carbon-limited chemostat cultures at high dilution rates and fast-growing batch cultures. The corresponding decrease in mitochondrial metabolism associated with changes in branching frequency led them to suggest that the tyrosinase may be involved in the reoxidation of dinucleotide coenzymes. They cite as evidence for this hypothesis the low K_m of tyrosinase for O_2, the reduction in oxygen uptake caused by a specific inhibitor of tyrosinase and the localisation of the enzyme in both soluble and particulate fractions.

The possibility that phenoloxidase enzymes may act as terminal oxidases involved in respiration has been suggested but not demonstrated in other systems (Frese & Stahl, 1990; Hill, 1992; Mayer, 1987; Rayner & Ross, 1991). This hypothesis appears to be consistent with the recent model of Hansberg & Aguirre (Hansberg & Aguirre, 1990) in which cellular differentiation is proposed to be triggered by an unstable hyperoxidant state. Melanin production by phenoloxidases and the concomitant reduction in cell wall permeability may also be important in this process. The passage of extracellular enzymes occurs primarily through apical cell wall (Chang & Trevithick, 1974; Wosten *et al.*, 1991), a fact which may be due to the progressive melanization, and hence impermeability, of the walls of older hyphae.

Hyphal tyrosinase may be an indispensable enzyme, since non-producer mutants have not been reported. It is possible that the complete absence of this enzyme is lethal at an early stage of growth. A number of mutants defective in hyphal melanin synthesis have been identified. Few have been genetically mapped, since the absence of melanin is correlated with defective sexual reproduction. The *melB*^{o}*2* (strain number SM16; Martinelli & Bainbridge, 1974), 13.1.OL (Potgieter & Alexander, 1966) and *acl* (acleistothecial; Zonneveld, 1974) mutants have been examined further and appear to have similar pleiotropic effects with respect to reduced levels of cell wall melanin (phenoloxidase activities were not compared) and α-(1,3)-glucan, as well as decreased cleistothecium production (Polacheck & Rosenberger, 1977). Polacheck & Rosenberger suggested that in log phase cultures the α-(1,3)-glucan, like melanin in later stages of growth, could confer resistance to lytic enzymes by sterically hindering their access to β-glucanase and chitinase substrates. They further proposed that the primary target of these mutations was glucan synthesis, since Zonneveld (1974) has provided evidence that

α-(1,3)-glucan may be the endogenous carbon source for biosynthetic processes during stationary growth.

However, the conidial walls of white-spored (*wA*) mutants lack both melanin and α-(1,3)-glucan as well as the spore pigment (Claverie-Martin *et al.*, 1988). Since *wA* mutants are defective in the pigment/melanin synthesis pathway (28.4.1.) but also fail to accumulate α-(1,3)-glucan in their conidial walls, it is possible that the one or more of the hyphal melanin mutants may have a similar defect in the hyphal melanin synthesis pathway.

28.5. Conclusions

Four distinct phenoloxidase enzymes have been identified in *A, nidulans*. Although the two conidiation-specific enzymes (laccase I and AHTase) appear to be at least partly dispensable, there is evidence that hyphal tyrosinase and laccase II are necessary for normal development. A range of mutants defective in sexual and/or asexual sporulation are also defective in laccase II production or hyphal pigmentation. Correlations between laccase activity and sexual sporulation have been observed in a number of ascomycete and basidiomycete species (Esser & Minuth, 1970; Leatham & Stahmann, 1981; Lerch, 1981).

Extensive studies of *Podospora anserina* by Esser and co-workers have shown that mutants defective in hyphal pigmentation, mycelial growth rate and perithecial production are also defective in laccase activity (Esser, 1968; Prillinger & Esser, 1977). Several forms of laccase enzyme have been purified from mutant strains and it is possible that more than one structural locus for laccase is involved.

Unlike *A. nidulans* and *P. anserina*, tyrosinase production in *N.crassa* is associated with sexual differentiation but not vegetative growth. Tyrosinase activity in *N.crassa* is responsible for perithecial melanization, while laccase is excreted into the culture medium by non-conidiating cultures after induction by phenolics. It would appear that contrasting patterns of tyrosinase and laccase production exist in these species, although no explanation has been proposed to account for these differences. In *Phanerochaete magnoliae*, laccase and tyrosinase activity were observed at different developmental stages (Ainsworth & Rayner, 1991).. Similar patterns of phenoloxidase activity also occur in other basidiomycetes (Stenlid & Rayner, 1989; Griffith *et al.*, 1994) with tyrosinase activity being more commonly associated with aerial (emergent) structures and laccase with submerged hyphae.

The process of melanization in a number of fungal and non-fungal systems is known to involve complex oxidative reactions, not only between aromatic precursors but also with protein and carbohydrate moieties (Hill, 1992). The role of cysteine in the formation of reddish phaeomleanins (as opposed to black eumelanins) via cysteinyldopa is recognized in animal systems (Hack & Helmy, 1983). Mushroom tyrosinase is able to oxidize tyrosine residues *in vitro* and the resulting dopaquinone residue can react with the sulfhydryl groups of cysteine residues (Ito *et al.*, 1984). Leatham *et al.* (1980) have also demonstrated the oxidative polymerization of proteins *in vitro* by quinones or free radicals (e.g. semiquinones) generated by phenoloxidases or peroxidases.

The role of sulfhydryl groups in fungal pigmentation systems has received scant attention (Bell & Wheeler, 1986). Prade & Terenzi (1982) have shown an inverse correlation between tyrosinase activity and sulfhydryl concentration in *N. crassa* and have suggested a link with γ-glutamyl cycle enzymes. The distribution of free sulfhydryl

groups in conidiating cultures of *A. nidulans* was examined by Oliver (1974). He found that sulfhydryl groups were localized in conidiophore foot cell, stalks and vesicles just prior to pigment formation in the wild-type. The sulfhydryl groups persisted in the unpigmented regions of *ivoA* mutants but were absent in the unpigmented bristles of a *brlA1* mutant. This suggests that sulfhydryl groups are oxidized during pigmentation although they are absent in undifferentiated foot cells.

Although the distribution of hydrophobins in *A. nidulans* has only been partially investigated (Stringer *et al.*, 1991), it is possible that a family of these peptides exists, as has recently been shown to be the case in *Schizophyllum commune* (Wessels *et al.*, 1991a,b). These cysteine-rich molecules are necessary for the production of aerial mycelia and basidiocarps in the latter species, probably being involved in oxidative cross-linking reactions. Protein oxidation has been shown to occur during the aggregation of aerial mycelia in *N. crassa* (Toledo & Hansberg, 1990). The potential role of phenoloxidases in such oxidative cross-linking is unclear, since the oxidation of sulfhydryl groups can also occur in the presence of other oxidases (e.g. peroxidases) or spontaneously under certain redox conditions. Evidence of the involvement of phenoloxidases is largely circumstantial. For instance, phenoloxidase (probably laccase I) is present in the hydrophobin-rich rodlet layer of wild-type *A. nidulans* conidia which contains 40% melanin (Claverie-Martin *et al.*, 1986).

Another widespread characteristic of phenoloxidases, which has also been observed in the *A. nidulans* enzymes, is their catalytic inactivation *in vivo* which is associated with modification of the enzyme and the occurrence of multiple isoenzymes (see 28.4.). This phenomenon is thought to result from the cross-linking of the enzymes into the cell wall matrix and the loss of copper from the active site (Lerch, 1981). A similar process of autotanning has been shown to occur during the tanning of insect cuticles (Anderson, 1985). In the case of hyphal tyrosinase, a small protein inhibitor was identified (Bull & Carter, 1973). Similar inhibition of *N. crassa* tyrosinase has been associated with a low molecular weight sulfhydryl metabolite (Prade & Terenzi, 1982). It is possible that thiol-rich compounds, such as hydrophobins or metallothioneins (Ecker *et al.*, 1989; Lerch, 1980) may play a role in phenoloxidase inactivation by chelating copper from the active site.

Most fungal phenoloxidases, including those of *A. nidulans*, are involved in cell wall pigmentation. The production of melanins and related pigments along with cell wall thickening provides increase rigidity and confers resistance to microbial lysis, as well as protection against damaging radiation. A further consequence of melanization is hyphal insulation, such that nutrient uptake is no longer possible. This in turn requires that the melanized hyphae must either make use of endogenous reserves (α-glucan in the case of *A. nidulans*) and/or be supplied with nutrients from adjoining hyphae (i.e. a source/sink relationship). Evidence of localized nutrient transfer (e.g. between a conidiophore foot

cell and neighbouring hyphae) is lacking, although the fact that misscheduled expression of *brlA* results in the inability of undifferentiated hyphae to utilize exogenous nutrients (Adams & Timberlake, 1990) does suggest that differentiating cells are non-assimilative. It is unclear whether cell wall melanization is directly involved in this process or whether phenoloxidases become involved in mitochondrial metabolism, as suggested by Carter & Bull (1971) and others (see 28.4.5.).

There is clearly no consensus regarding the role of phenoloxidases in developmental differentiation. Other metabolic changes, including changes in intracellular cAMP levels (Feldman & Thayer, 1974), the mobilization of carbohydrate reserves (Zonneveld, 1977) and the cessation of exponential growth (Martinelli & Bainbridge, 1974) are also associated with these processes in a number of microbial systems (Chater, 1991; Firtel, 1991) and a unifying hypothesis linking these observations is eagerly awaited. Phenoloxidases also likely to be involved in the antagonistic interactions between somatically incompatible mycelia (Croft & Dales, 1984; Croft & Jinks, 1977). Significant changes in polypeptide synthesis, including the intracellular accumulation of a normally extracellular laccase are associated with somatic incompatibility in *P. anserina* (Boucherie *et al.*, 1981).

Most of the investigations dealing with the physiological function of phenoloxidases in *A. nidulans* were conducted before the tools of modern molecular biology were available. The cloning of hyphal tyrosinase and laccase II is now a feasible task (for instance by antibody screening of expression libraries or the use of suitable degenerate oligonucleotides based on protein sequence or evolutionarily conserved regions) and there may also be further phenoloxidases in *A.nidulans*. Such progress should be accompanied by further physiological investigations of the role of these enzymes and characterization of their natural substrates.

I would like to express my thanks to John Clutterbuck, Alan Rayner, Gwyn Griffith and Professor David Wood for helpful discussion during the writing of this review.

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