Actinomycetes and Lignin Degradation

RALPH KIRBY

Department of Life Science, National Yang-Ming University, Taipei, Taiwan

I. Introduction

Actinomycetes are a group of eubacterial microorganisms that are commonly found in the soil, and which seem to be intimately involved in soil ecology. Many species of actinomycetes are myceliate in nature, and a large proportion of them are producers of a wide range of antibiotics and other antimicrobial compounds. The presence of actinomycetes in the soil is indisputable; what is not clear is their role in the ecology of the soil. Soil is a particularly difficult environment to study from the microbial/ecological point of view because of its complex structure and diverse and highly variable population of microorganisms. It is clear that for soil to function normally, there must be a mechanism by which lignocellulose is degraded. Lignocellulose is one of the major constituents of plant material because of its role as a structural material. It has a chemical structure that makes it very difficult to degrade, and degradation must occur for soil to function correctly. Much research has been done on the role of fungi, and the enzymes produced by fungi, on the breakdown of lignocellulose. The same cannot be said for the actinomycetes. It has been suggested that

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 58 0065-2164/06 $35.00

DOI: 10.1016/S0065-2164(05)58004-3

actinomycetes play a role in lignocellulose breakdown, but details of the scale and method by which such breakdown takes place are limited. However, there is a wide range of examples where *Streptomyces* and other actinomycetes have been identified as degrading lignin or lignocellulose. These strains come from a wide variety of sources, including a range of soils, high temperature environments, and termite guts. (Adhi *et al.*, 1989; Antai and Crawford, 1981; Ball and McCarthy, 1988; Ball *et al.*, 1989; Crawford, 1974, 1978, Crawford *et al.*, 1984; Iqbal *et al.*, 1994, Kukolya *et al.*, 2002, McCarthy, 1987, McCarthy and Williams, 1992; Pasti *et al.*, 1990; Paszczynski *et al.*, 1992, Rob *et al.*, 1996, 1997; Ruttiman *et al.*, 1998; Trigo and Ball, 1994; Tuncer and Ball, 2002; Watanabe *et al.*, 2003; Winter *et al.*, 1991).

II. The Actinomycetes

Phylogenetically and taxonomically, the actinomycetes form a coherent group within the Gram $+^{ve}$ bacteria characterized by a relatively high G+C% and distinct cell wall compositions. As can be seen by carrying out 16S small subunit ribosomal RNA gene sequence phylogenetic analysis of a range of typical actinomycetes with other gram-positive bacteria as an outgroup, the actinomycetes form a single clade that branches out into a number of distinct taxonomic classes. These classes can be subdivided into three for the purposes of examining their possible function in the soil. One group is made up of species that are not involved to any great extent in lignocellulose degradation, that usually do not undergo any complex morphological development, and that may be pathogens. Included in this group are *Mycobacteria*, *Corynebacteria*, and *Proprionobacteria*. A second group consists of the mesophilic actinomycetes, which undergo some morphological development, are generally considered to be soil microorganisms, and are rarely pathogenic; these include *Streptomyces* and *Kitasatosporia*. Finally, the third group consists of thermophilic actinomycetes, also from soil, also with some morphological development, and also considered to be nonpathogenic. Obviously, because the soil is a major place where lignocellulose is broken down, it is the latter two groups that potentially provide organisms to carry out this task, and it is likely that species from many of the major groups of soil-based actinomycetes may potentially be involved in lignocellulose degradation. Thus, the production of enzymes able to breakdown the chemical bonds holding lignin together might be fairly widely spread among these organisms. It should be noted that a significant number of actinomycetes have

complete genome sequences available, and that three of these, *Streptomyces coelicolor*, *Streptomyces avermitilis,* and *Thermobifidia fusca*, fall into categories that may be involved in lignocellulose breakdown.

III. The Enzymes Involved in Lignocellulose Breakdown

Cellulose is a major structural component of plants and is thus ubiquitously present across plants. This structure is normally quite stable, but a wide range of organisms produce enzymes that can cleave the various specific bonds within cellulose, acting as either ex- or endo-enzymes. Depending on the degree of crystallization of the cellulose, these enzymes vary in effectiveness, and each enzyme is specific to a particular chemical bond. This is in contrast with the situation for lignin and similar compounds.

Lignin has a highly complex and relatively random structure that provides this organic material with a high degree of resistance to degradation. Their wide varieties of chemical bonds make specific cleavage by the active site of an enzyme difficult, and would require a great many enzymes, each with a specific active site, for degradation. However, lignin is broken down, but unlike cellulose, this comes about using mechanisms than do not involve a specific interaction between an active site on an enzyme and a specific bond structure in the lignin. Instead, cleavage occurs via diffusible chemical processes produced by the lignin-degrading enzymes. There are two major groups of enzymes involved in this type of lignin degradation: peroxidases, including the lignin peroxidases, and phenol oxidases, including the polyphenol oxidases and laccases.

A. Peroxidases

Peroxidases (EC 1.11.1.7) are haem-containing enzymes that use hydrogen peroxide (H_2O_2) as the electron acceptor to catalyze a number of oxidative reactions and hydroxylations, utilizing both an oxidizing substrate and a reducing substrate. The oxidizing substrate is usually a peroxide or peroxy acid (Robinson, 1991). Typically, peroxidases can use hydrogen peroxide, methyl-hydrogen peroxide, and ethyl-hydrogen peroxide (Kermasha and Metche, 1988) as oxidizing agents in peroxidatic reactions, but most haem peroxidases follow the reaction mechanism shown in the following equations, using hydrogen peroxide as the oxidizing agent.

$$\text{Peroxidase } (Fe^{3+})\ \text{Porphyrin} + H_2O_2 \rightarrow \text{Peroxidase } (Fe^{4+}=O)\ \text{Porphyrin}^{+} + H_2O \quad (1)$$

$$\text{Peroxidase } (Fe^{4+}=O)\ \text{Porphyrin}^{+} + AH \rightarrow \text{Peroxidase } (Fe^{4+}=O)\ \text{Porphyrin} + A\bullet \quad (2)$$

$$\text{Peroxidase } (Fe^{4+}=O)\ \text{Porphyrin} + AH \rightarrow \text{Peroxidase } (Fe^{3+})\ \text{Porphyrin} + A\bullet + H_2O \quad (3)$$

The reaction mechanism of peroxidases summarized here is a multi-step reaction that starts with the enzyme reacting with one equivalent of hydrogen peroxide to give compound I a porphrin π-cation radical containing Fe^{IV}. This results in a distinctive absorption spectrum due to the haem prosthetic group exhibiting transient changes on mixing with hydrogen peroxide. This is a two-electron oxidation/reduction reaction where hydrogen peroxide is reduced to water and the enzyme is oxidized. One oxidizing equivalent resides on iron, giving the oxo-ferryl (Fe^{IV} =O) intermediate (Colonna *et al.*, 1999). This process is outlined in Fig. 1.

Compound I oxidizes an organic substrate to give a substrate radical (•AH). Compound I undergoes a second one-electron oxidation reaction yielding compound II, which contains an oxoferryl center coordinated to a normal (diamnionic) porphyrin ligand (Equation 2). Finally, compound II is reduced back to the native ferric state with concomitant one-electron substrate oxidation. The overall charge on the resting state form and compound I is +1, while compound II is neutral (Equation 3; Piontek *et al.*, 2001).

Peroxidases can be found in bacteria, fungi, plants, and animals. They have molecular weights ranging from 35,000–100,000 kd, and approximately 25% of this is carbohydrate (O'Brien, 2000). In terms of sequence similarity and structural divergence, fungal, plant, and bacterial peroxidases are viewed as members of a superfamily consisting of three major classes.

Class I These are intracellular peroxidases, and include: yeast cytochrome *c* peroxidase, a soluble protein found in the mitochondrial electron transport chain, where it probably protects against toxic peroxides; ascorbate peroxidase, the main enzyme responsible for hydrogen peroxide removal in chloroplasts and cytosol of higher plants; and bacterial catalase-peroxidases, exhibiting both peroxidase and catalase activities. It is thought that catalase-peroxidase provides protection to cells under oxidative stress (Robinson *et al.*, 1991).

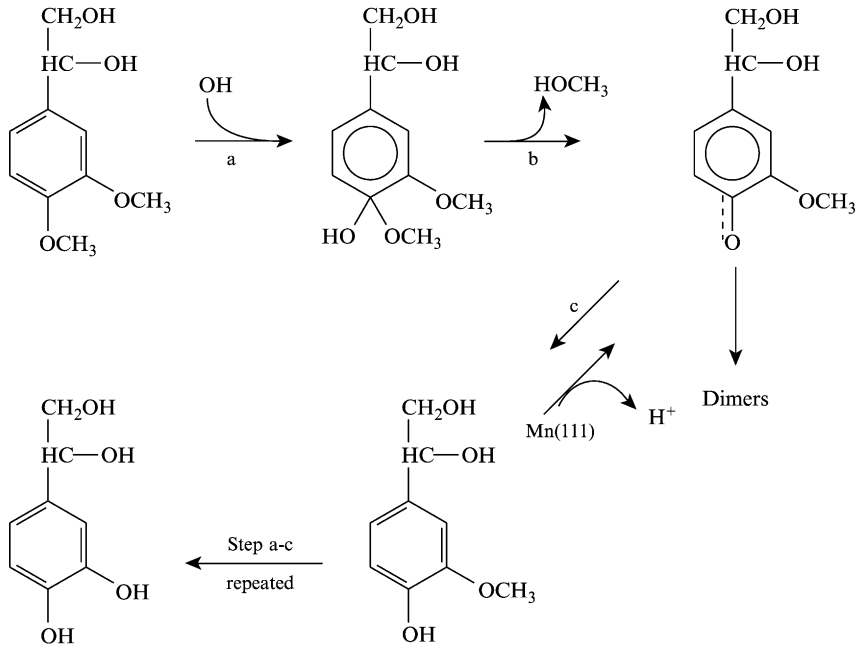


FIG. 1. Suggested reaction generated by peroxidase, for replacement of methoxy and hydroxyl groups on the model compound Veratryl glycol (Hildén *et al.*, 2000). Step a: a hydroxyl radical is attached and the free electron is delocalized on the ring; step b: a methanol leaves the VG; step c: a proton and an electron are donated (probably by an $FADH_2$ group). Steps a-c can then be repeated to generate another demeoxylation. In addition to this further demethoxylation, the peroxidase system can possibly generate dimmers after step c.

Class II These consist of secreted fungal peroxidases such as lignin peroxidases (LiP's) and manganese-dependent peroxidases (MnPs). These are monomeric glycoproteins involved in the degradation of lignin. In manganese peroxidase (MnP), Mn^{2+} serves as the reducing substrate (Robinson *et al.*, 1991).

Class III These consist of the secreted plant peroxidases, which have multiple tissue- specific functions, e.g., removal of hydrogen peroxide from chloroplasts and cytosol; oxidation of toxic compounds; biosynthesis of the cell wall; defense responses toward wounding; indole-3-acetic acid catabolism; and ethylene biosynthesis. Class III peroxidases are also monomeric glycoproteins. Class III includes the classic horseradish peroxidase isoenzyme and peanut peroxidase (Poulos *et al.*, 1993).

TABLE I

CLASSIFICATION OF PEROXIDASES SHOWING SUBSTRATES AND METHODS FOR PEROXIDASE ACTIVITY DETERMINATION (ADAPTED FROM ROBINSON, 1991)

Prosthetic group	Common name	Source	Potential substrates
Haem	peroxidases		Test substrates including guaiacol and *o*-dinisidine
	Ascorbic acid peroxidase	Plants	Ascorbic acid
	Cytochrome C peroxidase	Plants	Cytochrome C
	Lactoperoxidase	Animals	Thiocyanate
	Lignin peroxidase	Plants and fungal cell walls	Lignin precursors including ferulic and cinnamic acids; guaiacol and *o*-dinisidine
	Myeloperoxidase	Animals	Thiols
	Eosinophil peroxidase	Animals	Thiols
	Thyroid peroxidase	Animals	Thiols
Selenium	Glutathione peroxidase	Plants	Reduced glutathione
Vanadium	Bromoperoxidase	*Ascophyllum nodosum*	Halogens 2.4 DCP
Mangenese	Mn-haem peroxidase	*Phanerochaete chrysosporium*	Lignin ABTS
Flavin	Flavoperoxidase	Micro-organisms	—

Peroxidases are able to catalyze the oxidation of a large variety of substrates such as phenol, aromatic amines, and other compounds such as alkyl peroxides and aromatic peracids (Sjoblad and Bollag, 1981), using hydrogen peroxide (Table I). Several other peroxidases also exist that contain either metal ions or a flavin prosthetic group. Haem prosthetic-type peroxidases are the most commonly found. The specific biological function, the reduction potential of the iron, and the nature of the substrates, which can be oxidized, are determined largely by the structural features of the protein matrix around the prosthetic group (Table I). In microbial peroxidases such as lignin peroxidase and manganese peroxidases, the substrates include both phenolic and non-phenolic aromatic compounds; the phenolic substrates are oxidized to yield products similar to those of classical peroxidases such as HRP, while the oxidation of nonphenolic methoxybenzenes is unique to

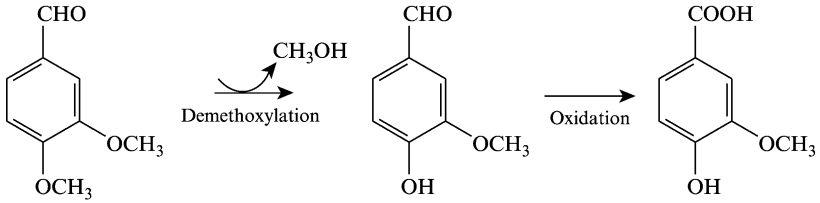


FIG. 2. Demethoxylation pathway of veratric aldehyde (Adapted from Lopretti *et al.*, 1998).

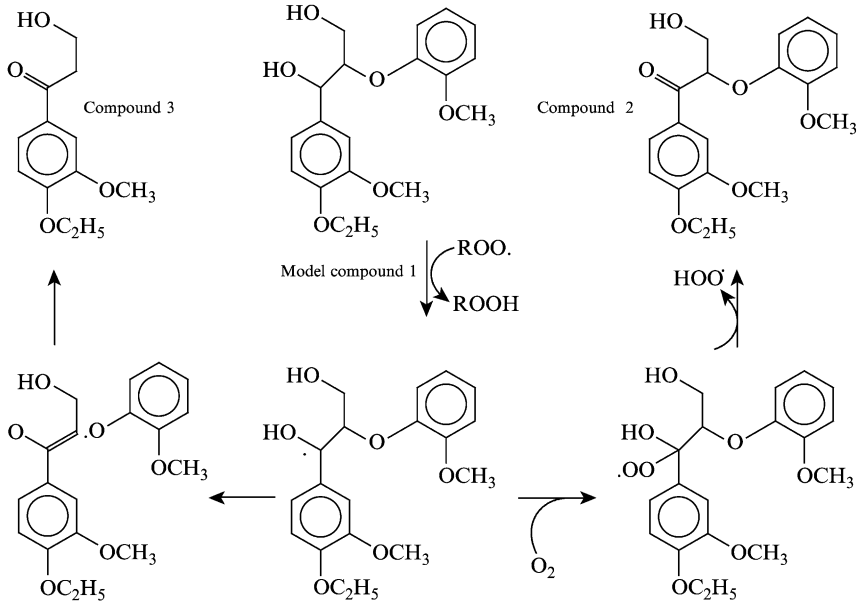


FIG. 3. Proposed pathway for the oxidation of model 1 to compounds 2 and 3 by peroxyl radicals (peroxidase) (Adapted from Kapich *et al.*, 1999).

lignin peroxidases. The oxidation of these substrates to yield aryl cation radicals can result in:

- Demethoxylation (Fig. 2)
- Cα – Cβ cleavage of lignin model compounds (Fig. 3)
- Benzylic alcohol oxidation (Fig. 3)
- Hydroxylation of aromatic rings and side chains (Figs. 2 and 4).

The substrate range is very broad, with the reactivity being largely determined by redox potentials; lignin peroxidase can catalyze the oxidation of substrates with a reduction potential greater that 1.3 volts.

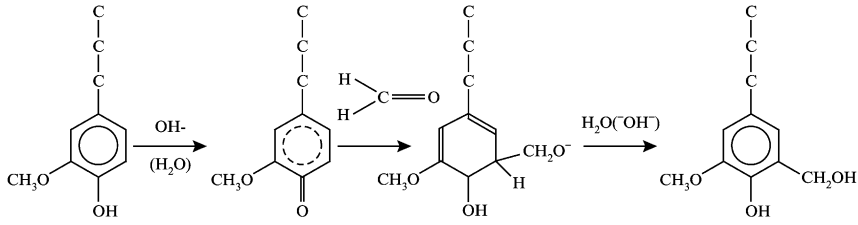


FIG. 4. Hydroxymethylation of the phenolic ring of a lignin unit (guaiacyl type; Gonçalves and Benar, 2001) catalysed by a peroxidase.

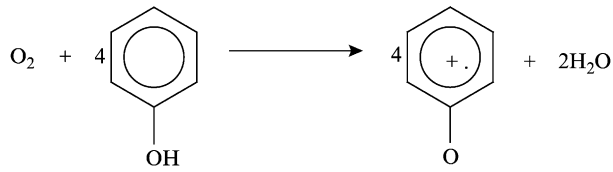


FIG. 5. Reaction example of the oxidizing power of oxygen on a phenolic compound (Adapted from Arcand and Archibald, 1991).

The enzyme has been shown to oxidize lignin monomers, dimers, and trimers as well as polycyclic aromatic compounds such as benzopyrene (Haemmerli *et al.*, 1986). The radicals (compounds I and II) cause side-chain fragmentation (C-C cleavage), leading to the breakdown of the lignin polymer (Evans and Fuchs, 1988). They can also catalyze lipid peroxidative pathways, which are capable of oxidizing substrates such as lignin-model dimers and polycyclic aromatic hydrocarbons.

B. PHENOL OXIDASES

In contrast to peroxidases, phenol oxidases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) utilize oxygen as an oxidant. These enzymes are ubiquitous in nature and are involved in the biosynthesis of a wide range of natural products including lignins, tannins, melanins, and alkaloids, as well as polymerization of naturally occurring phenols resulting in the formation of humic acid and humus in soil by oxidative coupling (Trigo and Ball, 1994). Oxidative coupling is a process by which phenolic compounds or aromatic amines are linked together after oxidation by an enzyme or a suitable chemical reagent. The initial step in the reaction is the production of an aryloxy radical from phenol by the removal of an electron and hydrogen ion from the hydroxyl group (Fig. 5). Polymerization of phenolic compounds by phenol

oxidase has been reported and may be related to lignin biosynthesis (Guerra *et al.*, 2000). The phenol oxidases can be split into laccases and polyphenol oxidases based on substrate specificity.

1. *Laccases*

Laccases (p-diphenol: dioxygen oxidoreductase; EC 1.10.3.2) are copper-containing proteins that need atmospheric oxygen to oxidize phenols, polyphenols, aromatic amines, and a range of nonphenolic substrates. They do this by one-electron transfer, and this activity results in the formation of reactive radicals. They seem to be involved in the morphogenesis of a number of microorganisms and in the breakdown/formation of complex recalcitrant organic compounds such as lignin and humic matter. However, this may not be the complete story, and other functions are possible as well. Laccases have been found to be produced by many eukaryotes such as fungi and plants, and it is in these organisms, and more specifically in the enzymes from the ligninolytic white-rot fungi, that most research has been carried out. (Dean and Erikkson, 1994; Leonowicz *et al.*, 2001; Mayer and Staples, 2002). The most detailed analysis of laccases has been carried out in the basidiomycetes *Trametes versicolor* and *Polyporus ostreatus*, and in the ascomycetes *Podospora anserine* and *Neurospora crassa*. These enzymes show similarity to the ascorbate oxidases of plants and the mammalian plasma protein ceruloplasmin (Thurston, 1994); together these have been grouped as the ''blue oxidases.'' The presence of a laccase gene in a prokaryote was first demonstrated by Givaudan *et al.* (1993) for *Azospirillum lipoferum*, and laccases have now been found in an increasingly wider range of gram-positive and gram-negative bacteria (Alexandre and Zhulin, 2000; Claus and Filip, 1997); they seem to be involved in cell pigmentation and metal oxidation, although this is not to say that other functions, such as lignin degradation, are not possible. These multinuclear blue copper-containing oxidases catalyze the four-electron reduction of oxygen to water. The enzymatic activity is due to four copper (II) ions, which are arranged in the three different sites, each characterized by unique spectroscopic peculiarities (Bonomo *et al.*, 2001). Laccases exploit the full oxidizing power of oxygen to perform the one-electron oxidation of a large number of organic compounds with a phenolic functional group, as well as inorganic compounds.

Sequence alignment of the fungal laccases shows that the copper-binding domains are highly conserved (the copper-coordination sites are His-Trp-His, His-Leu-His, and His-Cys-His), even though there may be low similarity throughout the rest of the protein primary structure

(Cullen, 1997; Dean and Erikkson, 1994). The diversity of laccase sequence outside the conserved copper-binding domains in the fungi suggests that the identification of prokaryote laccases by database searching of complete genome sequences may easily miss enzymes that do not conform to the norm of the fungal model. Therefore, the use of *in vivo* biochemical methods to identify the presence of prokaryote laccases remains important.

It is believed that laccases arose by domain duplication and divergence from small copper-containing electron-transfer proteins (Ryden and Hunt, 1993) between 3000 million and 1400 million years ago. Laccase-type enzymes have now been found in a range of prokaryotes (Claus, 2003), including five Actinomycetes: *Streptomyces antibioticus* (Freeman *et al.*, 1993), *Streptomyces griseus*, *Streptomyces coelicolor* (Endo *et al.*, 2003a,b), *Streptomyces cyaneus* (Arias *et al.*, 2003) and *Streptomyces lavendulae* (Suzuki *et al.*, 2003). However, it is not clear how widespread they are in the Actinomycetes because, although there is indirect evidence for the presence of laccases in the Actinomycetes, it is based on rather nonspecific substrate reactions (Sjoblad and Bollag, 1981). Thus, with the availability of 17 complete genome sequences and six partially completed genome sequences from the Actinobacteria, the possibility of identifying candidate laccases from the Actinomycetes is possible using a bioinformatics-based approach. Unfortunately, these sequenced genomes include only one thermophile, *Thermo bifida fusca*, which has not been completely annotated (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi; last accessed 4–01–2005).

This group of phenol oxidases uses oxygen as an electron acceptor to remove hydrogen from the phenolic hydroxyl group. This reaction leads to the formation of free radicals, which can be demonstrated with most lignin compounds, and these can undergo rearrangements leading to alkyl-aryl cleavage of side chains and aromatic rings. The range of substrates oxidized by laccase depends on the type of laccase, and the substrate specificity of laccases can be explained by their physiological functions. The classical action of laccases is oxidation (Fig. 6) of substrate by transferring electrons to oxygen in one-electron steps, resulting in polymerization of phenols and/or the formation of quinones (Majcherczyk *et al.*, 1998). Demethylation of phenolic and nonphenolic lignin substructures has been shown to happen, as has the oxidation of phenolic lignin-model compounds by Cα – Cβ cleavage of the side chain, which explains the enhancing role in lignin degradation. The action of laccase on dimeric lignin-model compounds in the presence of mediators such as ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid], phenol red, violuric acid, and 1-Hydroxybenzotriazole

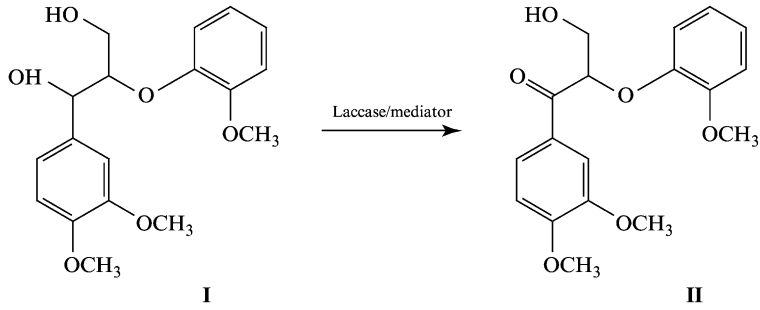


FIG. 6. Oxidation of dimer I using laccase plus mediator (Adapted from Li *et al.*, 1999).

expands the substrate spectrum of laccases to various benzoic acid derivatives (Li *et al.*, 1999). Typical reaction patterns catalyzed by microbial laccases are summarized in Table II.

In nature, laccases have various functions, which include participation in lignin biosynthesis, plant pathogenicity, degradation of plant cell walls, insect sclerolization, bacterial melanization, and melanin production in humans. Chemically, all the functions of laccases are related to oxidation of a range of aromatic substances, but the net effect of such oxidations can be very different and may work in opposite directions (Li *et al.*, 1999). Plant laccases are known to oxidize monolignols to form polymeric lignin, whereas microbial laccases degrade and depolymerize lignins.

2. *Polyphenol Oxidases*

Burton (1994) described polyphenol oxidase as a copper-containing monoxygenase that catalyses two reactions:

1. The *ortho*-hydroxylation of phenols to catechols (cresolase activity)
2. The subsequent oxidation of catechols to *ortho*-quinones (catecholase activity).

Both reactions utilize molecular oxygen (Fig. 7). The quinones are readily condensed to produce relatively insoluble high-molecular-weight brown polymers known as melanins (Burton, 1994; Rodríguez-López *et al.*, 2001). *Ortho*-quinones are unstable, reactive species that may polymerize and oxidize other substrates or undergo nucleophilic attack by various substances including water, proteins, or polyphenols. The *o*-hydroxylation activity of polyphenol oxidase distinguishes it

TABLE II

REACTION PATTERNS CATALYZED BY MICROBIAL LACCASES
(ADAPTED FROM H.-D. YOUN *ET AL.*, 1995)

Compound	Reaction pattern	Major products
acetosyringone	Single electron transfer	Acetosyringone phenoxy radical
Vanillic acid	Polymerization	2-meoxy-6-(2-meoxy-4-carboxyphenyl)-1, 4-benzoquione
1-(3,5-Demethoxy-4-hydroxyphenyl)-2-(3,5-dimethoxy-4-ethoxyphenyl) propane-1,3-diol; β-1 lignin model compound	Depolymerization	
	Cα oxidation	1-(3,5-Dimeoxy-4-hydroxyphenyl)-2-(3,5-dimethooxy-4-ethoxyphenyl)-3-hydroxypropanone
	Alkyl-aryl cleavage	2,6-Dimeoxy-*p*-hydroquinone and 1-(3,5-dimethoxy-4-ethoxyphenyl)-3-hydroxypropanal
	Cα – Cβ cleavage	Syringaldehyde and 1-(3,5-dimethoxy-4-ethoxyphenyl)-2-hydroxyethanone
1- (3,5-Dimethoxy-4-hydroxyphenyl)-2-(2-methoxyphenyl)-1,3-dihy droxypropane; β-*O*-4 lignin model compound	Depolymerization	
	Cα oxidation	1-(3,5-Dimethoxy-4-hydroxyphenyl)-2-(2-methoxyphenyl)-1-oxo-3-hydroypropane
	alkyl-aryl cleavage	2-(2-Methoxyphenyl)-3-hydroxypropanl and 2,6-dimethoxybenzoquinone
	Cα – Cβ cleavage Ring cleavage	Guaiacol and syringic acid 2,4-Di (*tert*-butyl)-4-(methoxycarbonylmethyl)-2-buten-4-olide
4,6-Di (*tert*-butyl) guaiacol; aromatic ring compound	C – C cleavage (+ABTS)	Veratraldehyde and benzaldehyde
1-(3,4-Dimethoxyphenyl)-2-phenoxy-ethane-1, 2-diol; non-phenolic β-1 ligin model compound		

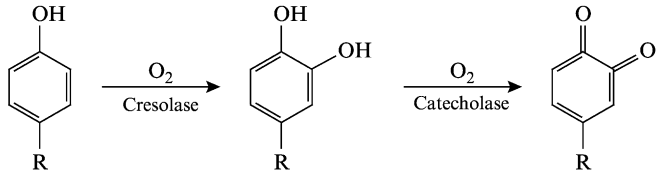


FIG. 7. Reactions catalysed by polyphenol oxidase.

from other phenol-oxidizing enzymes such as laccase and peroxidases (Sánchez-Ferrer *et al.*, 1995). Polyphenol oxidase is an unusual monooxygenase in that it does not require a reducing cofactor such as NADH (Aitken *et al.*, 1993). A wide variety of *o*-dihydroxy phenols, such a caffeic acid derivatives, can also act as good substrates for polyphenol oxidase (Cheynior and Mountounet, 1992). Polyphenol oxidase oxidation of monophenols and *o*-diphenols has been studied extensively, even though some of these substrates are not natural compounds (Jimenez *et al.*, 1999).

In vitro studies have suggested that polyphenol oxidase participates in the degradation of natural phenols with more complex structures such as anthocyanins and flavanols (Finger, 1994). These compounds are not directly oxidized by the enzymes but by the quinones formed by polyphenol oxidase from catechol and catechin (Jiménez *et al.*, 1999).

IV. Thermophilic Actinomycetes and Lignin Breakdown

The genera of thermophilic Actinomycetes isolated from compost include *Nocardia, Streptomyces, Thermoactinomycetes*, and *Micromonospora* (Strom, 1985), and Actinomycetes have also been isolated from soil, mud, marine sediments, sand, and compost (Cross, 1968). In their natural habitat, they are able to degrade cellulose and solubilize lignin, and are known to tolerate a higher temperature and pH in comparison to fungi. An ideal environment to isolate thermophilic Actinomycetes is at a composting site. Actinomycetes are known to be involved in the process of composting due to their ability to break down lignocellulose degradation during peak heating in the composting process.

Among the factors that contribute to the development of microbial populations in compost are oxygen and nutrient availability and the temperature increase, which rises to between 50 °C and 80 °C, resulting in a transition from mesophilic to thermophilic microbial communities,

which also results in a decrease in the microbial diversity (Blanc *et al.*, 1999). For isolation purposes, it is crucial to select soil samples from specific regions of the composting site where the temperature is known to be highest.

Composting at temperatures above ambient is a common process. The presence of thermophilic Actinomycetes in the compost at temperatures up to 70 °C is well documented. The problem is that it is not clear what these Actinomycetes are doing during the composting process. During composting, cellulose and lignin are broken down and may be eventually mineralized to some degree. However, the microorganisms that have been found to produce the most active enzymes that successfully breakdown lignin are fungi, and in particular, fungi that specialize in growing on high density wood, such as the bracket fungi. Members of the group *Tremetes* and *Pleurotus* produce a suit of lignin-degrading enzymes under inducing conditions, and these enzymes have been well studied. In contrast, only a limited amount of research has been carried out on the potential lignin-degrading enzymes from the Actinomycetes. Although a number of candidate enzymes have been identified from the Actinomycetes that may degrade lignin, it is far from clear whether this is the enzymes' primary role. This problem arises because, by definition, the enzymes that degrade lignin have a broad chemical-bond target range and thus an oxidase, even if it might not have a primary or even a secondary role in lignin breakdown, might still be able to attack the bonds that are present in lignin.

The most well studied fungi that produce large amounts of enzymes capable of degrading lignin are *mesophiles*. These species do not optimally grow at temperatures, generally found in compost heaps (50–70 °C). Thus, there is a significant probability that thermophilic Actinomycetes, under the correct induction conditions, may be capable of producing enzymes able to degrade lignin. The identification of such enzymes can be approached in a number of ways. First, screening, enzyme assay, and induction experiments can be carried out. Secondly, a molecular genetic approach can be used. Finally, a bioinformatics approach, in conjunction with whole genome sequencing, can be used to find appropriate candidate open-reading frames for further *in vivo* analysis. Obviously, all three approaches can also be used on mesophilic as well as thermophilic Actinomycetes. An integration of the analysis of the two groups of Actinomycetes will have advantages until more work is carried out on the thermophiles.

All three approaches have limitations. The screening and assay approaches are limited by three factors. First, the isolation of organisms from an environment is well known to be selective, even under the

most nonselective growth conditions. The presence of uncultivatable microorganisms in environmental samples always leaves open the question as to whether the isolated and cultivated organisms are representative. Secondly, the normal screening approaches for lignin-degrading enzymes, which will be described later, were developed for the three known classes of such enzymes and for the fungal versions of these enzymes. It is conceivable, though unlikely because of the broad range of bonds attacked by these enzymes, that these assays might not be optimal for prokaryote lignin-degrading enzymes. Finally, it is clear from the detailed studies carried out on fungal lignin-degrading systems that specific conditions may be required to induce these enzymes. Most of the enzyme systems studied in fungi are from species that attack wood directly, and not those involved in the soil degradation of lignin. The induction conditions and possible chemicals involved in induction for high-temperature soil-based lignin degradation may be quite different.

The second approach, which involves molecular genetics, has been limited due to the lack of heterologous probes available from the genomes of Actinomycetes. Only a few known and published sequences of prokaryote enzymes possibly involved in lignin degradation are available. These will be discussed later in this article, but inherently, without homologues from the same Kingdom to work from, this approach has been restricted. Added to this is the problem that the function of the potential prokaryote lignin-degrading enzymes is not well established in some cases.

The third approach is a bioinformatics-based analysis of the three full genome sequences available for Actinomycetes that may possibly encode lignin-degrading enzymes. These are two mesophilic Actinomycetes, *Streptomyces coelicolor* A3(2) and *Streptomyces avermitilis*, and one thermophilic Actinomycete, *Thermobifida fusca*. *T. fusca* is still not completely annotated, which does pose somewhat of a limitation. The first two species are typical soil-dwelling *Streptomyces* and thus may or may not contain genes encoding lignin-degrading enzymes as part of their suite of enzymes. *T. fusca*, on the other hand, is known as a cellulose-degrading Actinomycete, and therefore is much more likely to contain such enzymes as part of its metabolic repertoire. All three organisms after annotation have been found to contain many open-reading frames that are highly likely to be transcribed and translated based on their structure, but which have an unknown function. Thus, the potential for identification of novel lignin-degrading enzymes within these three databases using bioinformatics is high.

A. Selective Isolation of Thermophilic Lignin-Degrading Actinomycetes

Classical enrichment procedures select for bacterial strains with the highest growth rates under specified high-nutrient and environmental conditions, which are often not representative of *in situ* soil communities; therefore, direct isolation often gives a greater diversity. Incubation temperature selection is also crucial in terms of selecting for thermophilic Actinomycetes. All samples need to be incubated at 50 °C at least, although optimal temperature can be determined later. The choice of growth media is open and depends on whether a specific group of Actinomycete is being targeted. This approach does have its limitations, however, including insufficient information on cultivation requirements of many novel bacteria and the inability to recover starving, dormant, and viable and nonculturable cells. Using microscopic techniques, Torsvik *et al.* (1998) demonstrated that while the majority of bacteria in soil samples are actively respiring, only about 1% of the total number of cells could be cultured on laboratory media. In addition, less than 1% of bacterial species have been isolated and characterized using enrichment techniques (McCain *et al.*, 2001). However, this technique, in combination with molecular-based analysis, proves to be a useful method of identification.

B. Screening Enzymatic Assay Methods for the Identification of Lignin-Degrading Actinomycetes

The degradation of phenolic residues requires the concerted action of enzymes that vary in substrate specificity. It is beneficial to screen microorganisms for their ability to produce oxidoreductase enzymes under a range of environmental conditions. Dye decolorization has been proposed as a quick, reproducible screening method to determine lignolytic enzymes, which in turn are also capable of degrading phenolic residues (Gold and Glenn, 1988). Dyes such as poly R478, blue dextran, crystal violet, bromophenol blue, and cresol red have proven useful for screening and degradative studies. Simple plate tests for direct visualization of biological lignin degradation can be done to identify the presence of lignolytic activity in cultures (Tekere *et al.*, 2001). It should be noted, however, that dye decolorization does not always match lignolytic results. This should not invalidate dye-screening methods, but it does indicate the need to confirm preliminary results by conducting enzyme assays and PAGE activity stains to determine which enzymes are being produced.

Initial screening experiments should involve the use of the dye Poly R478, a red synthetic lignin-model compound that is known to be decolorized in the presence of lignin-degrading enzymes (Gold and Glenn, 1988). The experimental set-up involves placing cut-out agar sections containing the isolates onto empty Petri dishes and surrounding the sections with agar containing 10% agar and Poly R478. A positive result produces a distinct, expanding clear halo as the enzyme(s) produced by the Actinomycete diffuses outward. The reaction is reversible, however, and as a result, the clearing halos disappear after approximately 48 hours. Other dyes have been used, including Poly B411, a blue dye, as well as indicator dyes with aromatic compounds incorporated into the growth media, where color changes result from the utilization of the aromatic compound because of a pH. These techniques seem to be less consistent than when Poly R478 is used.

After initial screening, *in vitro* enzyme assays and gel assays are needed to confirm the presence of oxidoreductive enzymes capable of breaking down lignin. These include peroxidase activity, lignin peroxidase activity, polyphenol oxidase activity, and laccase activity. Figure 8 shows the SSU rRNA phylogenetic tree of the three Actinomycetes isolated from compost in our laboratory: RU-A01, RU-A03, and RU-A06 (Mhlanga, 2001). The first two species clearly fall with the Streptomyces, while RU-A06 falls outside of the well known groups. The morphological characteristics are outlined in Fig. 9.

Once potential peroxidase- or polyoxidase-producing strains have been identified, it is necessary to specifically assay for the enzymes involved. This can involve either *in vitro* enzyme activity measurement or activity gel enzyme detection.

Techniques to determine peroxidase activity are based on the use of chromogenic substrates, particularly guaiacol and pyrogallol. Other substrates include phenol-containing compounds such as 2,7-diaminoflourene (DAF), *o*-dianisine, ABTS, ferulic acid, and catechol. 2,4–Dichlorophenol (DCP) is a common substrate used to detect peroxidases in the *Streptomyces* group (Mercer *et al.*, 1996: Ramachandra *et al.*, 1987, 1988). Analysis of substrate specificity of novel peroxidases is usually carried out under the optimal conditions determined for horseradish peroxidase (HRP). However, the optimal conditions for catalysis by different peroxidases are not identical, and hence react differently to different substrates. Hydrogen peroxide also affects enzyme determination. If the concentration of hydrogen peroxide is too high, inactivation of the peroxidase can occur (Sakhorov *et al.*, 2001).

The peroxide-dependant oxidation of 2,4-DCP gives the best combination of high activity and reproducibility (Tables III and IV). It has also

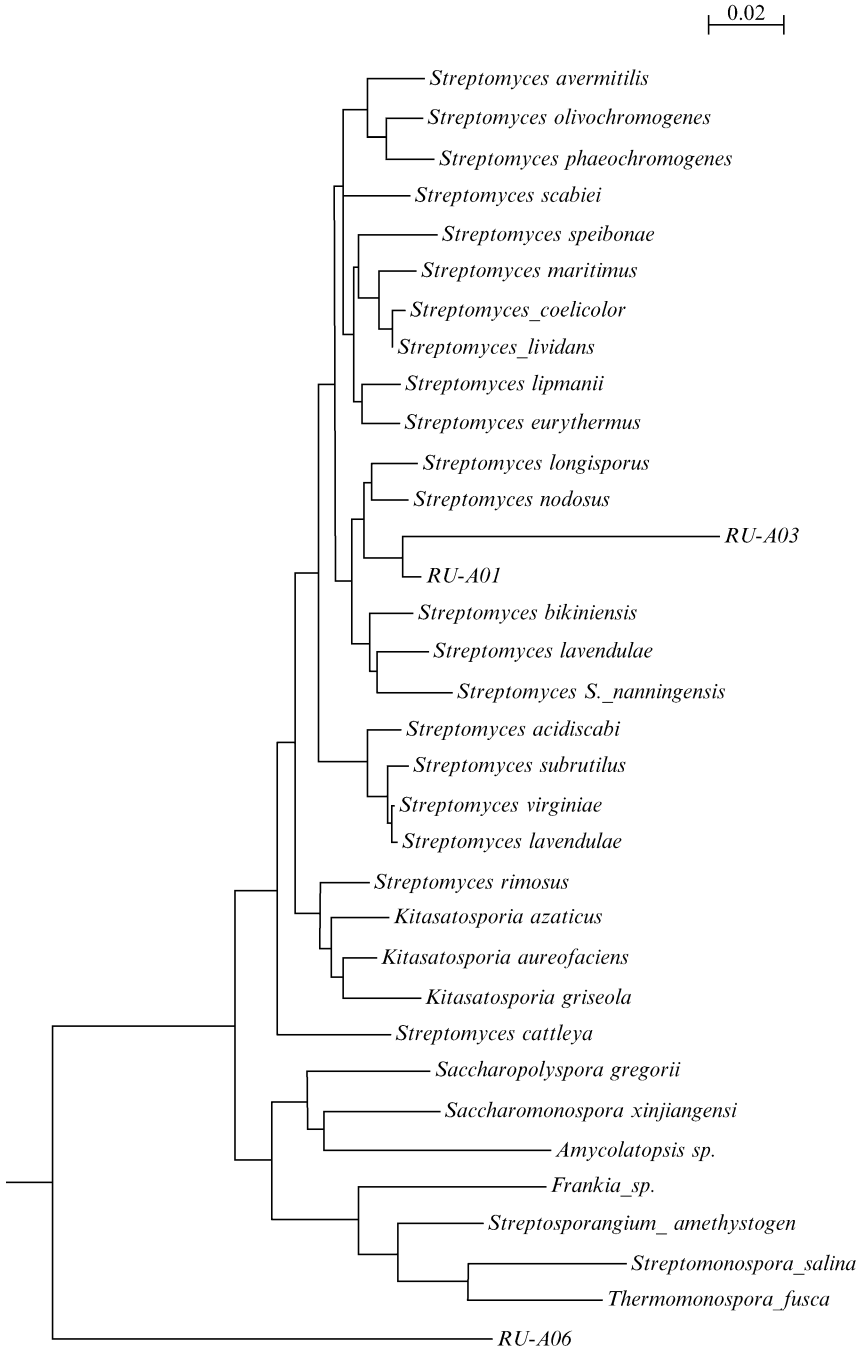


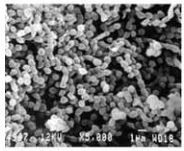
FIG. 8. Phylogenetic tree of the SSU RNA sequences of three thermophilic lignin degrading actinomycales.

MORPHOLOGICAL CHARACTERISTICS RU A01

Colour of aerial mycelium	Greyish
Spore shape	Coccus
Spore surface	Smooth

PHYSIOLOGICAL CHARACTERISTICS

Melanin formation	Positive
Temperature	40°C - 50°C, optimum 47°C
Phenolic acid utilization	Positive

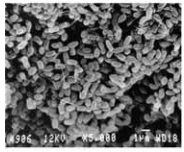


MORPHOLOGICAL CHARACTERISTICS RU A02

Colour of aerial mycelium	Greyish/brown
Spore shape	Oblong
Spore surface	Smooth

PHYSIOLOGICAL CHARACTERISTICS

Melanin formation	Negative
Temperature	40°C - 50°C, optimum 45°C
Phenolic acid utilization	Positive



MORPHOLOGICAL CHARACTERISTICS RU A06

Colour of aerial mycelium	Grey
Spore shape	Rods
Spore surface	Smooth

PHYSIOLOGICAL CHARACTERISTICS

Melanin formation	Positive
Temperature	40°C - 50°C, optimum 50°C
Phenolic acid utilization	Positive

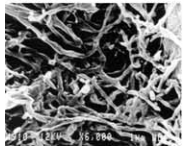


FIG. 9. Characteristics of three novel thermophilic lignin degrading actinomycales.

TABLE III

PEROXIDASE AND PHENOL OXIDASE REACTIVITY TOWARD DIFFERENT SUBSTRATES AS DETERMINED BY ENZYME ACTIVITY FROM THE ISOLATES RU-A01, RU-A03, AND RU-A06

		Activity* μmols/mL/min					
		RU-A01		RU-A03		RU-A06	
Substrate	Enzyme	Intracellular	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular
2,4 DCP + H_2O_2	Peroxidase	2.8000	3.5000	1.3600	0.9700	6.3000	4.6000
Guaiacol + H_2O_2	Peroxidase	0.0000	0.0000	0.0014	0.0000	0.0000	0.0014
o-dianisidine + H_2O_2	Peroxidase	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Veratryl alcohol + H_2O_2	Lignin peroxidase	0.4200	6.5000	0.4200	0.0000	1.2000	0.6000
Catechol	Polyphenol oxidase	0.6500	0.1300	0.8000	0.6000	0.3700	0.2000
L-DOPA	Polyphenol oxidase	0.0010	0.0050	0.0020	0.0000	0.0180	0.0120
ABTS	Laccase	0.0000	0.0000	0.0000	0.00000	0.0000	0.0000

*Activity values are based on samples taken during the course of growth of the isolates when activity was known to be at its maximum. Units are defined as the amount of enzyme required for an increase of 1.0 absorbance unit min^{-1} using each substrate.

TABLE IV

SPECIFIC ACTIVITY OF INTRACELLULAR, AND EXTRACELLULAR PROTEIN FROM RU-A01, RU-A03, AND RU-A06 USING 7 DIFFERENT SUBSTRATES TO DETERMINE PEROXIDASE AND PHENOL OXIDASE ACTIVITY

		Specific activity μmols/min/mg					
		RU-A01		RU-A03		RU-A06	
Substrate	Enzyme	Intracellular	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular
2,4 DCP + H_2O_2	Peroxidase	23.300	35.000	6.8000	9.700	21.000	46.000
Guaiacol + H_2O_2	Peroxidase	0.005	0.0000	0.0070	0.0000	0.0020	0.014
o-dianisidine + H_2O_2	Peroxidase	0.000	0.0000	0.0010	0.0000	0.0010	0.0000
Veratryl alcohol + H_2O_2	Lignin peroxidase	3.5000	65.0000	2.1000	0.0000	4.0000	6.0000
Catechol	Polyphenol oxidase	5.4000	1.3000	4.0000	6.0000	1.2000	2.0000
L-DOPA	Polyphenol oxidase	0.0050	0.0500	0.0100	0.0000	0.0600	0.1200
ABTS	laccase	0.0080	0.0000	0.0200	0.0010	0.0000	0.000

been found that the substrate and assay procedure are stable even at high temperatures (50 °C), and therefore we recommend 50 °C for the determination of peroxidase activity of thermophilic Actinomycetes. This substrate has been widely used to determine peroxidase activity amongst Actinomycetes (Antonopoulos *et al.*, 2001; Ramachandra *et al.*, 1987, 1988; Manguson and Crawford, 1992; Mliki and Zimmermann, 1992). This assay is not specific, however, and can be termed a general peroxidase assay, as it does not appear to distinguish between lignin peroxidase and non-haem-based peroxidases as shown by the specific activity differences between 2,4-DCP and veratryl alcohol as substrates (Table IV).

Previous assumptions that positive peroxidase activities in Actinomycetes are due to lignin peroxidase have led to the use of a specific substrate for lignin peroxidase activity determination. The peroxide-dependant oxidation of veratryl alcohol has been frequently used as the procedure for lignin peroxidase determination in white-rot fungi, (Tien and Kirk, 1983). When applied to three thermophilic Actinomycetes isolated from compost, activity was detected in all three isolates but was most pronounced in RU-A01 in the extracellular protein fraction (Tables III and IV), giving a specific activity of 65 umols/mg/min. Previous studies have shown that few of the peroxidases produced by Actinomycetes species are due to lignin peroxidases except for *Streptomyces viridosporus* and *Streptomyces albus*. Hence, these results imply a wider distribution of lignin peroxidases than previously thought. Most peroxidases produced by Actinomycetes species are often non-haem peroxidases, in particular the intracellular peroxidases, and commonly belong to the haloperoxidases and catalase-peroxidase groups. In general, it is believed that extracellular peroxidases would be expected to have improved stability over their intracellular counterparts, particularly if they are from thermophiles. In addition, it is thought that the role of intracellular peroxidases are involved in cellular processes and that it is very possible that the extracellular peroxidases are involved in the degradation of complex organic compounds in the Actinomycetes' environment. The present results involving guaiacol as a peroxidase substrate indicate that guaiacol is unsuitable for the determination of Actinomycetes peroxidases. This was also found to be true by Mercer and colleagues (1996), who found guaiacol inadequate for the assessment of peroxidases in *Streptomyces* species.

The substrate reactivity of intra- and extracellular crude samples of RU-A01, RU-A03, and RU-A06 toward *o*-dianisidine was very low and in some cases, no activity was detected (Table III). This made

o-dianisidine most unsuitable for quantitative determination of peroxidases in RU-A01, RU-A03, and RU-A06. However, in a previous study conducted by Mercer *et al.* (1996), *o*-dianisidine gave the second highest peroxidase activity out of 10 substrates used to determine peroxidase activity in *Streptomyces thermoviolaceus* and *Streptomyces* strain EC22. Specific activities, however, were not quoted in that study.

For polyphenol oxidases, L-3, 4-dihydroxyphenylalanine (L-DOPA) displayed relatively low reactivity towards intra- and extracellular proteins of isolates RU-AO1, RU-A03, and RU-A06 (Table III). Previous studies using L-DOPA has found the reproducibility of L-DOPA for the determination of polyphenol oxidases relatively poor due to its extreme light sensitivity, as well as its interference due to nonspecific oxidation of the substrate (Winter *et al.*, 1991). Catechol proved to be a better substrate for polyphenol-oxidase determination as it was more stable than L-DOPA and the results were reproducible and less affected by light exposure. However, the results in Table IV indicate the presence of a higher proportion of peroxide-dependant enzymes, i.e., peroxidases, than polyphenol oxidases.

When the thermophilic Actinomycete isolates were assayed, no laccase was detected using 2,2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate, although this is the substrate of choice. According to published reports, very few bacteria produce laccases, and screening of laccase-producing microbes has been focused mainly on white-rot fungi such as *Trametes versicolor, Trametes pubescens, Rhus vernicifera*, and *Neurospora crassa*.

Simple methods have been developed in the form of PAGE activity gels, which enable the researcher to determine the number of isozymes present in the sample that are commonly found in Actinomycetes species, and spectrophotometric assays, which quantify the enzymes concerned and normally take a few minutes to run. Catechol, L-DOPA and ABTS can be used to determine phenol oxidase activity. Catechol gave the highest activity as a substrate, whereas L-DOPA gave the lowest activity (Table III). L-DOPA has been the substrate of choice when determining activity of tyrosinases and polyphenol oxidases in fungi such as the mushrooms *Agaricus bisporus* and *Neurospora crassa* (Boshoff, 2001; Luke and Burton, 2001; Russell, 1999). However, relatively low enzyme activity can be found with this substrate for Actinomycetes, and if this is true, then catechol can be used as a substrate to determine polyphenol oxidase activity. Catechol is light–sensitive, and darkens when exposed to light; thus it was important to use freshly prepared catechol with each assay. Enzyme samples can be loaded onto gels containing no SDS (4% stacking and 10% resolving gel) and the

proteins separated by electrophoresis for approximately three hours at 100 V. Peroxidase bands may be detected by incubating the gels in 10 mM *o*-dianisidine in the presence of 1.6 mM hydrogen peroxide for 30 minutes. Polyphenol oxidase may be detected by incubating for 30 minutes in a 10 mM solution of L-DOPA dissolved in 0.1 M phosphate buffer.

C. Comparison of the Various Lignin-Degrading Enzymes from Mesophilic and Thermophilic Actinomycetes

Peroxidase and polyphenol oxidase activity has been compared between the thermophilic *Streptomyces* isolates and mesophilic *Streptomyces* (Mhlanga, 2001). Polyphenol oxidase activity between the two groups was comparable, and peroxidase activity was significantly higher for the thermophiles than for the mesophiles (Table V). RU-A01, RU-A03, and RU-A06 also have an advantage over the mesophilic *Streptomyces* strains in that they produce lignin peroxidases also found amongst some of the white-rot fungi and are known to play a role in lignin solubilization (Ball *et al.*, 1989). Peroxidase and polyphenol oxidase extracts from the thermophilic isolates react the same as the peroxidases and polyphenol oxidases from the mesophilic *Streptomyces* spp toward the various substrates.

TABLE V

Comparisons Between Mesophillic and Thermophillic *Streptomyces* Polyphenol Oxidase and Peroxidase Activity[a]

Species	Activity (μmols/mlL/min)		
	Polyphenol oxidase[b]	Lignin peroxidase	Peroxidase[c]
Streptomyces antibioticus	0.69	0	0.51
Streptomyces hygroscopicus	0.85	0	0.62
Streptomyces sp. strain 7	1.32	0	0.79
RU-A01	0.60	6.5	3.50
RU-A03	0.80	0.42	1.36
RU-A06	0.37	1.2	5.28

[a]Optimum activity values were selected for each species.
[b]Catechol was used as substrate for polyphenol oxidase activity.
[c]2,4 DCP was used as substrate for peroxidase activity.

D. Molecular Analysis of Potential Candidate Genes for Lignin-Degrading Enzymes from Actinomycetes

One lignin peroxidase gene has been identified, cloned, and well characterized; this gene is from *Streptomyces viridosporus* T7A (Burke and Crawford, 1998; Wang *et al.*, 1990). The gene has been heterologously cloned into a number of species including *Streptomyces lividans*; the enzyme was shown to increase mineralization of organic carbon in soil (Crawford *et al.*, 1993) and in *Pichia pastoros*, where it was shown to be on the same 4.1 kb piece of DNA as an endoglucanase (Thomas and Crawford, 1998). However, the nucleotide sequence of this gene has never been published. Thus, it cannot be used for *in vitro*, *in vivo*, or *in silico* studies of other Actinomycetes that might allow the identification of other similar genes. Some doubt has been placed on the existence of lignin peroxidases from Actinomycetes, specifically *T. fusca* BD25 (Mason *et al.*, 2001). Notwithstanding the lack of a gene sequence for this lignin peroxidase (which is very unusual, as this is now insisted upon for publication by most journals), the molecular evidence for its existence is quite strong, and therefore this type of enzyme would seem to exist in the Actinomycetes. The availability of a DNA microarray that covers the complete genome of *S. coelicolor* has opened up the possibility of identifying conserved genes involved in lignin degradation. The technique involves the use of comparative genomics, where chromosomal DNA from a known related strain or species is labeled and hybridized to the microarray of the standard strain parallel to the DNA from the standard strain. An analysis of the relative signals for the two DNA samples reveals whether the test genome contains genes able to hybridize to the DNA from a particular gene on the array. Thus, it is possible to score for gene presence or absence. This type of data can be approached in two ways. First, by scoring known genes that may be involved in lignin degradation, the presence or absence of such genes can be estimated. Secondly, genes classified as potential lignin-degrading genes based on annotation of the genome of *S. coelicolor* can be screened for genes that seem to be conserved across many Actinomycetes, and particularly across species that have a high probability of being involved in lignin degradation.

There are two known genes involved in the first category present on the genome of *S. coelicolor*: phenol oxidase, or tyrosinase, and *epoA*. Tyrosinase is usually associated with the conversion of tyrosine to the black pigment melanin, and was originally identified in a number of *Streptomyces* species that produce large quantities of melanin such as *Streptomyces glaucescens* and *Streptomyces antibioticus*. The genes

were cloned and expressed in non-melanin-producing *Streptomyces* such as *Streptomyces lividans* and *S. coelicolor*. Therefore, it was surprising that genes encoding tyrosinase were found in both *S. coelicolor* and *S. avermitilis* when their genomes were sequenced. There are three possible explanations for this: first, that the genes are inactive, and thus are pseudo-genes; secondly, that melanin is needed only under rather special conditions in these species and is normally highly repressed; and finally, that the mono phenol oxidase enzyme identified as tyrosinase makes melanin as a secondary effect, and that the enzyme's primary function is not yet clearly identified, and therefore the conditions for induction are unknown.

The genetic structure for the tyrosinases from the two sequenced *Streptomyces* makes the first hypothesis unlikely, because of the genes' codon usage, GC Frame Plot, and high similarity to other functional tyrosinases. There is no well established function for melanin, and loss of melanin production does not cause any obvious detriment in melanin-producing *Streptomyces* species; therefore, the second hypothesis is not well supported. The most likely candidate for the process to be involved, if the third hypothesis is correct, is degradation of lignin or related chemicals. The third hypothesis is also supported by the microarray data shown in Table VI, where tyrosinase is shown to be conserved in DNA terms across at least six Actinomycetes, suggesting an important role for both tyrosinase and the co-factor gene for tyrosinase.

EpoA gives a contrasting result in Table VI for the microarray data. This gene is not conserved in *S. avermitilis*, *Streptomyces rimosus*, *Kitasatosporia aureofaciens*, or *T. fusca*, but seems to be conserved in two non-*Streptomyces* species. *EpoA* is a laccase based on the chemical reaction that it catalyzes and on the conserved-copper coordinating regions found in its primary sequence as shown in Fig. 10. This suggests that laccases do occur in Actinomycetes. Even within the fungi, laccases show high sequence diversity outside of the copper-coordinating regions and a similar diversity in *Streptomyces* is not that unexpected. The alignment of the two known *EpoA* protein sequences (no similar sequence is found in *S. avermitilis*, which confirms the microarray data in Table VI) with various eukaryote laccases allows the identification of four conserved regions that were used for further genome screening for laccases.

Two other genes in *Streptomyces* are known to encode laccases. These are the gene encoding the thermostable laccase enzyme STSL from *S. lavendulae* (Suzuki *et al.*, 2003) and the phenoxazinone synthase from *Streptomyces antibioticus* (Hsieh and Jones, 1995).

TABLE VI

MICROARRAY HYBRIZATION DATA FOR VARIOUS GENES PRODUCED BY A *S. COELICOLOR* MICROARRAY PROBED WITH VARIOUS ACTINOMYCETE CHROMOSOMAL DNAs

	S. lividans	**S. cattleya**	**S. avermitilis**	**S. rimous**	**K. aureofaciens**	**St. roseum**	**T. fusca**	**Sa. viridis**
melC2. SCO2700. tyrosinase	**−0.136**	**−0.083**	**0.362**	**−0.087**	−0.662	**0.292**	**0.081**	−1.048
melC1. SCO2701. tyrosinase co-factor	**0.118**	**0.963**	**0.901**	**0.382**	**0.785**	**0.138**	**0.398**	**0.888**
epoA SCO6712. copper oxidase	−0.564	−0.558	−0.681	− 1.257	−0.92	**0.102**	−0.992	**−0.487**

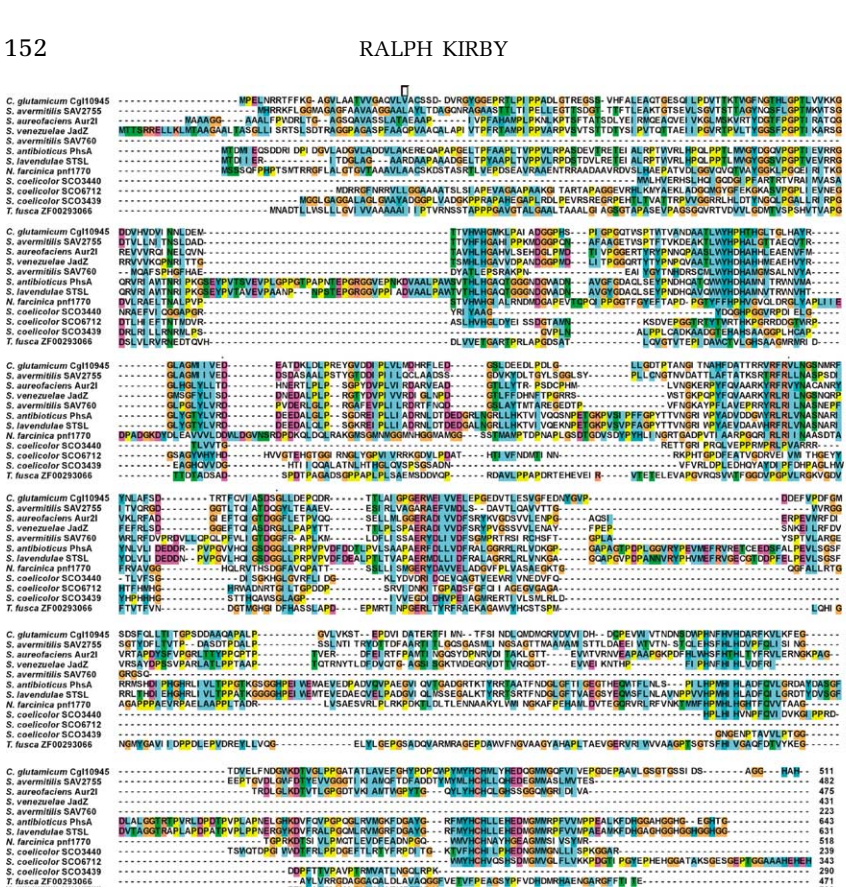


FIG. 10. Laccase type enzymes identified in Actinomycetes using STSL from *S. lavendulae* and EpoA from *S. griseus* with the Eukaryote enzymes as a related outgroup and aligned using Clustalx.

These genes are similar on alignment and were used in a BLAST search of all Actinobacteria (http://avermitilis.ls.kitasato-u.ac.jp/blast_other/) using a criterion of 1.0e-10. The proteins identified using this approach were aligned using Clustalx, and are also shown in Fig. 10.

Phylogenetic analysis of these enzymes using a *Nocardia farcinica* enzyme as an outgroup yields two major clades. One of the clades was split into two subgroups (Fig. 11). One clade, which includes JadZ and PhsA, may have a significant role in antibiotic production based on the original identification of these genes, although this does not exclude a role in lignin degradation due to the wide-ranging activity of the radicals produced by laccase-type enzymes. The other clade, which

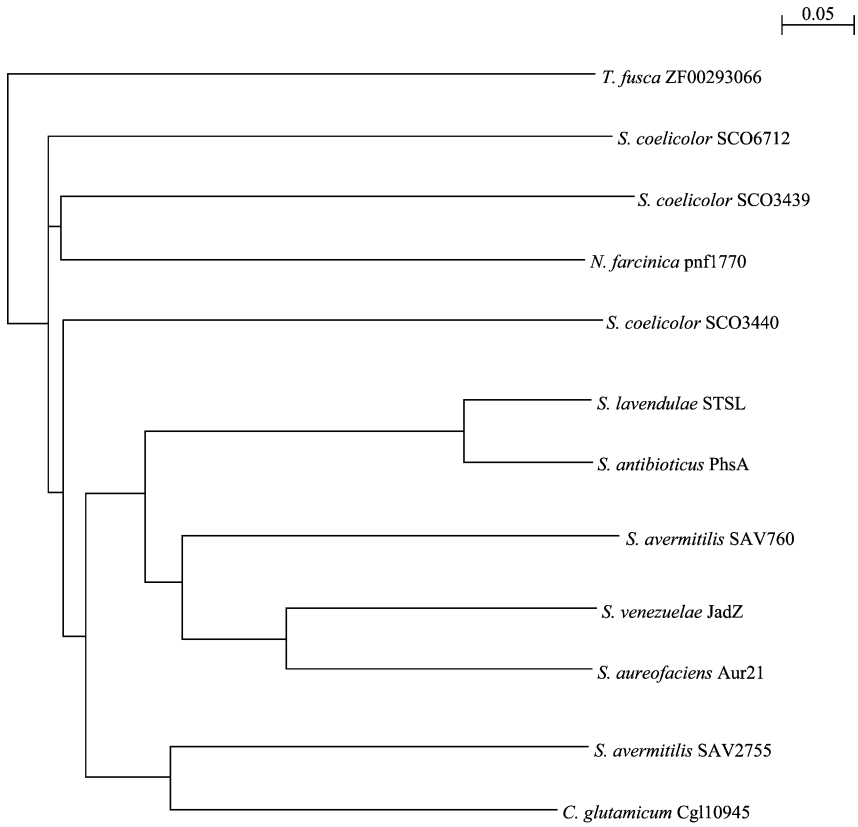


FIG. 11. Phylogenetic tree of laccase type enzymes identified using STSL from *S. lavendulae* and phenoxazinone synthase from *S. antibioticus* using NJ tree.

includes the two *Corynebacterium* enzymes and SAV2755, does not seem to be involved in antibiotic production as such. The homologue of SAV2755 in *S. coelicolor* with a BLAST score of 3.1e-11 is SCO3440, a hypothetical protein that seems also to be present in *T. fusca* (according to the microarray data in Table VII), may be Genbank sequence ZF00293066. Also identified by this approach are SCO3439, SCO6712, and SAV760. The fact that SO3439 and SCO3440 are side by side, have the characteristics of laccases, and are in the central core region of the *S. coelicolor* genome, suggests that these genes might have an important role. No other laccase-like enzymes were identified by this

TABLE VII

PUTATIVE OXIDASES AND SIMILAR PROTEINS FROM *S. COELICOLOR*

	S. lividans	**S. cattleya**	**S. avermitilis**	**S. rimous**	**K. aureofaciens**	**St. roseum**	**T. fusca**	**Sa. viridis**
SCO0122, putative secreted monooxygenase	−0.37	−0.149	0.096	0.128	0.158	0.048	−0.536	0.052
SCO0249, putative monooxygenase	0.171	−0.229	−0.113	−0.696	−0.696	0.144	−0.09	−1.186
SCO0300, putative monooxygenase	−1.088	−0.024	−0.133	−0.655	0.461	0.452	0.447	0.717
SCO0333, putative secreted dioxygenase	0.338	0.232	−0.059	−1.098		0.762	−0.178	0.919
SCO0484, putative monooxygenase	−0.129	0.302	0.662	1.071	−0.002	−0.737	−0.945	−0.371
SCO0486, putative monooxygenase	0.314	−0.318	0.407	−0.205	−0.436	0.184	−0.083	−1.451
SCO0902, hypothetical protein	0.177	−0.096	−0.33	−1.564	−0.043	0.261	−0.151	0.504
SCO0917, putative oxygenase	0.297	−0.162	−0.305	0.108	0.341	−0.05	−1.94	0.241
SCO1338, putative secreted monooxygenase	0.165	−0.565	−0.286	0.587	0.42	−0.322	−0.193	−1.294
SCO1715, putative 1,2-dioxygenase	0.346		0.304	0.408	0.057	0.189	−0.521	0.571
SCO1923, putative dioxygenase	−0.34	0.044	−1.932	0.517	0.123	0.023	0.37	0.182
SCO1970, putative dioxygenase	−0.442	0.843	0.377	−0.164	0.723	0.179	−0.181	1.218

SCO2016, putative monooxygenase	0.012	0.264	0.277	0.575	0.07	0.474	1.673	−0.169
SCO2267, probable heme oxygenase	0.451	0.125	0.406	0.873	0.507	0.627	−0.09	1.473
SCO2693, putative secreted oxygenase	0.136	0.245	0.808	0.662	0.305	0.22	1.652	−0.029
SCO2783, putative monooxygenase	0.599	0.249	0.616	0.024	0.2	0.725	−0.09	0.43
SCO3172, putative monooxygenase	0.114	−1.161		0.428	−0.609		0.554	0.518
SCO3229, putative dioxygenase	0.324	0.565	−0.609	0.157	0.133	−0.107	−0.722	−0.654
SCO3440 hypothetical protein	−2.569			−2.09	−0.333	−0.138	1.374	
SCO3245, putative secreted hydroxylase	−0.056		−0.806	−1.169	0.052	0.371	−0.035	0.568
SCO4416, putative monooxygenase	−0.265	0.277	−0.312	0.307	−0.066	−0.282	0.884	0.256
SCO4870, putative monooxygenase	−0.226	0.258	−1.178	−1.76	−0.898		0.684	−1.626
SCO5293, putative oxygenase subunit	−3.511	0.034	−0.455	−2.629	−0.779	−0.207	−0.165	−1.844
SCO5390, putative alkanal monooxygenase	−0.315	0.815	−1.634	−1.651	−0.263	−0.122	−0.558	−0.029
SCO5773, possible monooxygenase	−0.038	0.16	−0.908	−1.884	−0.364	0.473	−0.182	0.275

(*continued*)

TABLE VII (*Continued*)

	S. lividans	S. cattleya	S. avermitilis	S. rimous	K. aureofaciens	St. roseum	T. fusca	Sa. viridis
SCO5980, putative bifunctional oxidoreductase	−0.037			−1.526	0.74	0.978	0.189	0.164
SCO6276, putative secreted protein	−0.217	0.634	0.327	−0.103	0.708	0.18	0.419	0.781
SCO6699, 3,4-dioxygenase alpha subunit	−0.507	0.275	−0.209	−2.478	−0.441	0.091	−0.155	−0.847
SCO6700, 3,4-dioxygenase beta subunit	−0.087	0.271		−0.722	1.194	0.904	0.187	0.774
SCO6828, putative secreted protein	−0.402			−1.515			4.597	0.831
SCO6838, putative monooxygenase	0.135	−0.183	−0.11	−2.562	−0.503	−0.41	−1.013	−1.251
SCO7180, hypothetical protein	−0.37	−0.149	0.096	0.128	0.158	0.048	−0.536	0.052
SCO7223, putative monooxygenase	0.171	−0.229	−0.113	−0.696	−0.696	0.144	−0.09	−1.186

SCO7357, probable dioxygenase	−1.088	−0.024	−0.133	−0.655	0.461	0.452	0.447	0.717
SCO7468, putative monooxygenase	0.338	0.232	−0.059	−1.098		0.762	−0.178	0.919
SCO7507, putative dioxygenase	−0.129	0.302	0.662	1.071	−0.002	−0.737	−0.945	−0.371
SCO7625, putative monooxygenase	0.314	−0.318	0.407	−0.205	−0.436	0.184	−0.083	−1.451
SCO7626, putative monooxygenase	0.01	0.341	0.846	0.813	0.031	−0.181	−0.134	0.225
SCO7659, putative oxidoreductase	0.434	0.654	0.349	0.766	0.647	−0.062	−0.651	0.848
SCO7669, putative oxidoreductase	0.071	−0.939	−0.421	−1.353	−0.413	−0.412	−5.27	−0.522
SCO7791, putative secreted oxidoreductase	0.177	−0.096	−0.33	−1.564	−0.043	0.261	−0.151	0.504

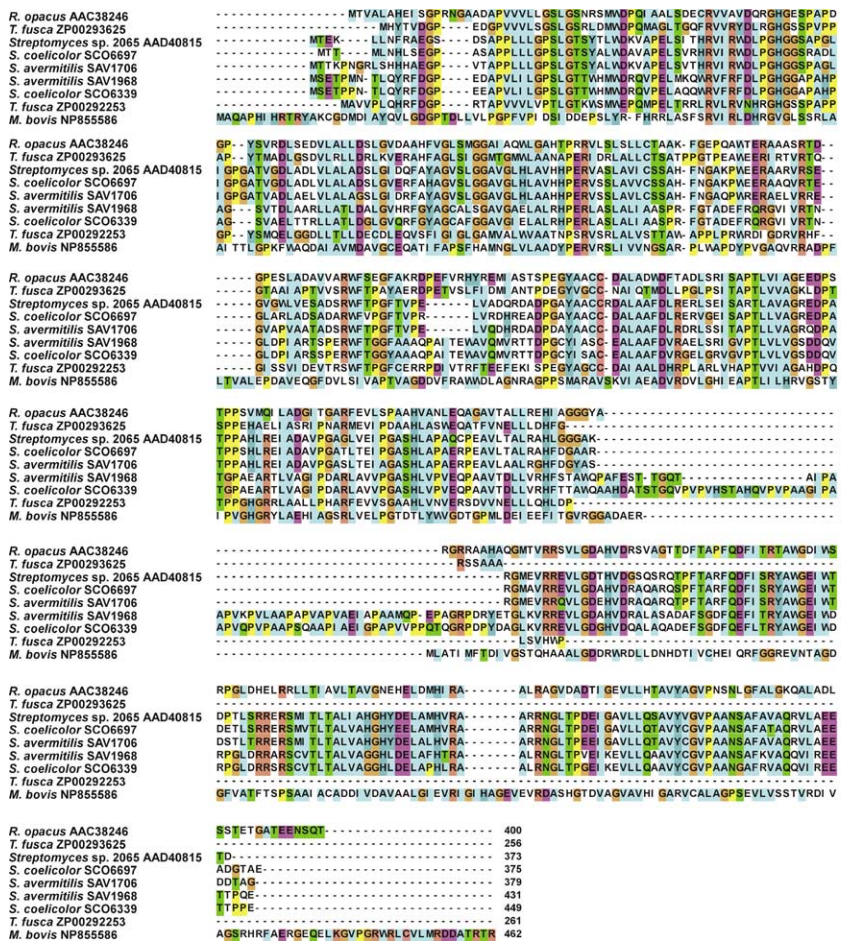


FIG. 12. Potential lignin peroxidases identified based on the *Mycobacteriun bovis* lignin peroxidase.

approach in *T. fusca*. However, the classes identified here for nonthermophilic Actinomycetes should simplify identification of such enzymes in future genomic analysis of thermophiles.

One lignin peroxidase has been identified from the Actinomycete *Mycobacterium bovis*. This was used in a similar approach to that used for the *Streptomyces* enzymes with a BLAST criterion of 1.0e-10. The results were aligned and are shown in Fig. 12. The potential lignin

peroxidases include two from *T. fusca*, one from *Rhodococcus opacus*, two from *S. coelicolor*, two from *S. avermitilis*, and one from an unknown *Streptomyces* species. The gene from the *Streptomyces* sp. 2065 was described by Iwagami *et al.* (2000) as a 4-carboxymuconolactone decarboxylase/3-oxoadipate enol-lactone hydrolase, PcaL. The genes from the two *Streptomyces* species would seem to be homologues and in syntheny, and both are annotated as a 3-oxoadipate enol-lactone hydrolase or 4-carboxymuconolactone decarboxylase. ZP00293625 and ZP_00292253 from *T. fusca* are predicted as a member of the alpha/beta hydrolase and are also possible homologues of the *Streptomyces* enzymes. Thus, it is unlikely that these two homologues are lignin peroxidases, but this needs to be tested biochemically. The presence of two homologues within three genomes does suggest that there might be functional diversity between the two homologues.

One catalase-peroxidase has been identified and sequenced in *S. coelicolor* (Hahn *et al.*, 2000). No homologue is present in either *S. avermitilis* or *T. fusca*, while one is present in *Streptomyces reticuli* (CAA74698), in *Nocardia farcinica* (BAD57797) and probably in *Streptomyces scabies* based on a preliminary BLAST against the unfinished genome (http://www.sanger.ac.uk/Projects/S_scabies/). The enzyme would seem to be limited to the cytosol in *S. coelicolor* and therefore is an unlikely candidate for involvement in lignin degradation, although it could possibly act on some of the soluble breakdown products of lignin degradation in a detoxification role. Its position in the terminal region of *S. coelicolor* also suggests that the gene's distribution will be variable across the Actinomycetes, and this is confirmed by its absence from *S. avermitilis* and *T. fusca*.

One final approach at the molecular level is to identify genes involved in the downstream processing of lignin degradation products such as lignostilbene, vanillin, and similar compounds (Pometto and Crawford, 1983; Sutherland *et al.*, 1983). Three such enzymes have been identified: lignostilbene dioxygenase, vanillate O-demethylase oxygenase subunit A, and vanillate O-demethylase oxygenase subunit B. The results of a BLAST search are shown in Fig. 13 and in Fig. 14. For lignostilbene dioxygenase, one *S. coelicolor* enzyme (SCO0333), one *Streptomyces antibioticus* enzyme, and one *Nocardia farcinica* enzyme are identified. SCO0333 is in the terminal region of the genome, and there does not seem to be a homologue in *S. avermitilis*. No oxygenases that may be putative laccases or peroxidase enzymes are near these genes. The situation is similar for vanillate O-demethylase oxygenase subunit A and vanillate O-demethylase oxygenase subunit B.

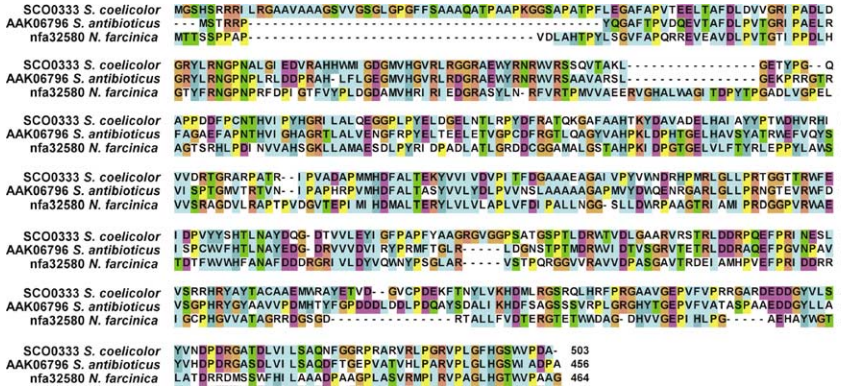


FIG. 13. Alignment of three lignostilbene dioxygenase.

E. BIOINFORMATICS APPROACH TO IDENTIFICATION OF POTENTIAL LIGNIN DEGRADING GENES

Table VII lists the potential oxygenase enzymes annotated in the *S. coelicolor* genome sequence. Because lignin degradation should be a conserved characteristic across the Actinomycetes, these genes are shown with their signal from hybridization of an *S. coelicolor* microarray with a range of DNA from *Streptomyces* and other Actinomycetes, including *T. fusca*. Twenty-nine of the genes give a positive signal for *T. fusca* (>-0.5) and twelve give a strong signal (>0.0). Of these twelve, five genes, namely, SCO1923, SCO2016, SCO2693, SCO4416, and SCO6276, show significant conservation across all the Actinomycetes tested. SCO2693 and SCO6276 are putative secreted oxygenases and therefore have potential as lignin-degrading enzymes. Obviously, other candidates can be identified based on other criteria. For these two, SCO2693 is similar to clavimate synthase enzyme from *Streptomyces clavuligerus*, and SCO6276 is similar to a squalene monooxygenase from *Streptomyces globisporus*. Therefore, there is no clear linkage that these enzymes are involved in lignin degradation, and in both cases, no similar enzymes were found in the thermophile *T. fusca*.

A slightly different approach was used to see if it was possible to identify the lignin peroxidase from *S. viridosporus* T7A. It is believed that the gene sequence might have been withheld for intellectual property reasons. However, at the time of publication of Thomas and Crawford (1998), two endoglucanase sequences were deposited in Genbank. As the lignin peroxidase gene is stated to be on the same 4.1 kb piece of DNA as an endoglucanase, it can be suggested that one

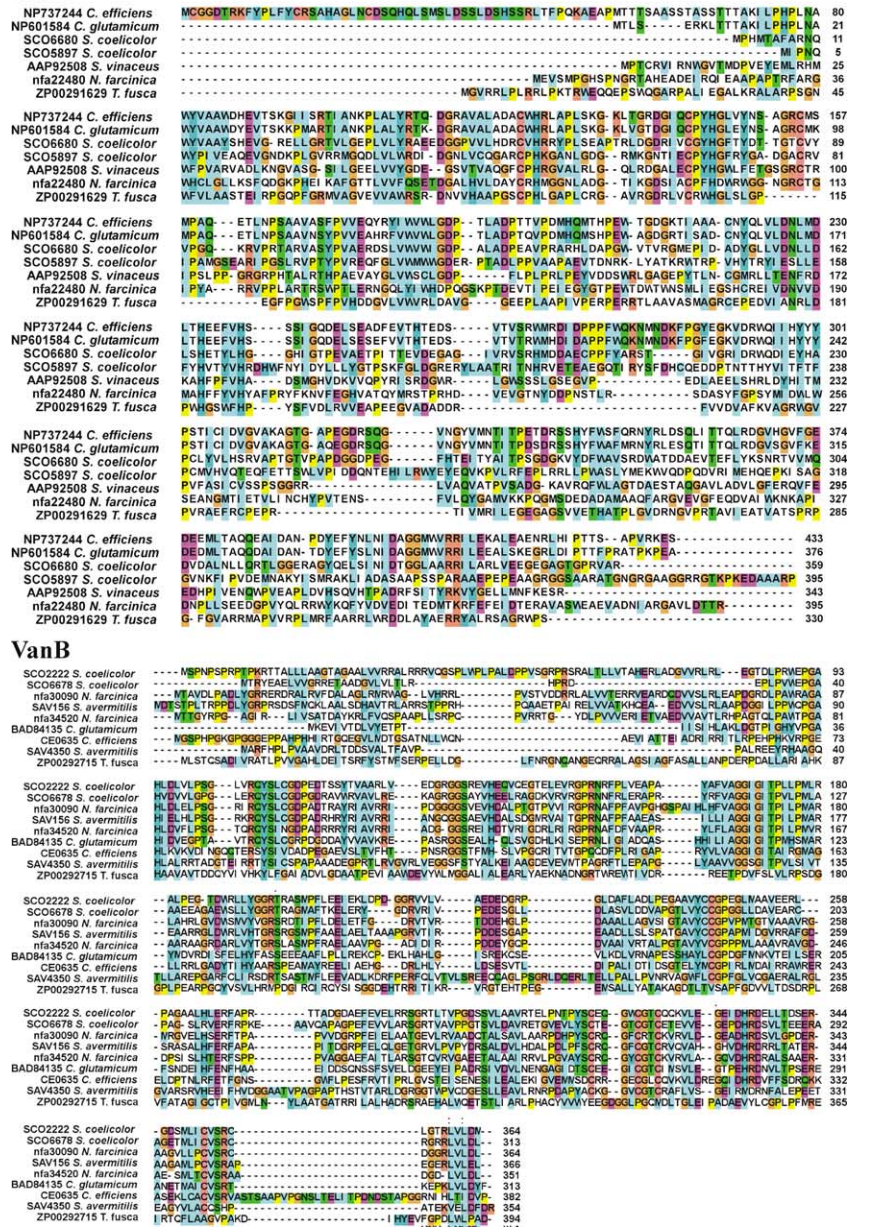


FIG. 14. Alignment of homologues of vanillin response proteins VanA (vanillate o-demethylase oxygenase subunit A) and VanB (vanillate o-demethylase oxygenase subunit B).

of these might be the linked endoglucanase. The genes have homologues in *S. coelicolor*, namely SCO1188 and SCO2292. The regions round these genes were examined for possible candidate oxygenases within the 4.1 kb range that were conserved in a range of Actinomycetes based on microarray data. Unfortunately, no possible genes could be pinpointed. Three other genes from *S. viridosporus* have been identified as involved in a response to peroxide, and therefore possibly involved in the control of lignin peroxidase; these genes are *ahpC*, *ahpX*, and *oxyR* (Ramachandran *et al.*, 2000). The homologues of these genes in *S. coelicolor* are SCO5031, SCO5032, and SCO5033. There is one candidate oxidoreductase close to these genes in *S. coelicolor*, namely, SCO5024. If this protein is processed through BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against *T. fusca*, four quite similar candidate genes can be identified. *S. avermitilis* contains a single homologue, SAV3243. The genes in *S. coelicolor* are in syntheny with those present in *S. avermitilis*. Figure 15 shows the alignment of the four *T. fusca* genes with the two *Streptomyces* genes. The presence of

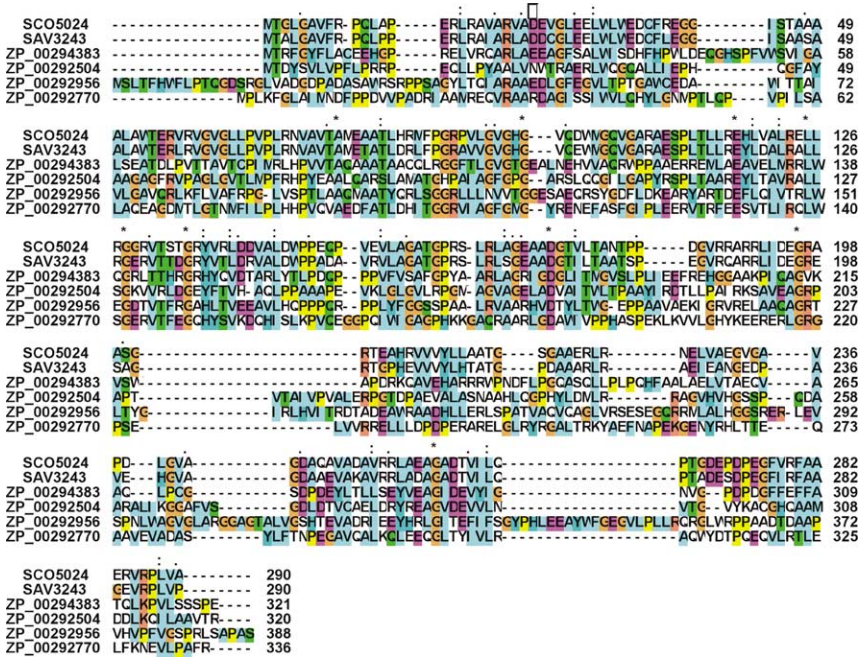


FIG. 15. Alignment of potential lignin oxygenases from *S. coelicolor*, *S. avermitilis* and *T. fusca*.

The presence of four and perhaps more similar genes in *T. fusca*, a thermophilic lignocellulose-degrading Actinomycete, suggests that this might be an important class of oxygenases.

V. Conclusion

The enzymes involved in lignin degradation by the Actinomycetes, and the thermophilic Actinomycetes in particular, still remain elusive. In this review we have described the various approaches available to find these genes and identified a series of candidate genes, which are tabulated with a description in Table VIII. Further complete genome sequences from Actinomycetes, particularly thermophilic species, will allow better analysis of these candidates. Of the genes identified in Table VIII, only SCO0333 does not seem to have a homologue in the partial genome sequence of *S. scabies* supporting their significance.

There are three main reasons for the difficulty in identifying these genes and pathways. First, the chemical structure of lignin requires that the enzymes involved be highly versatile and that they use free radicals as a means of attack on lignin. Therefore, they also must be able to breakdown a very wide range of other compounds. Secondly, the conditions for induction of lignin degradation in Actinomycetes are not well understood, and therefore, obtaining consistent enzyme production is not easy.

TABLE VIII

CANDIDATE GENES FROM ACTINOMYCE (?) THAT MAY BE INVOLVED IN LIGNIN DEGRADATION

Gene in *S. coelicolor*	Gene in *S. avermitilis*	Gene in *T. fusca*	Description
SCO2700			Tyrosinase
SCO6712; SCO3429	SAV760; SAV2755	ZF00293066	Laccase type ulticopper oxidase
SCO6687; SCO6338	SAV1706; SAV1968	ZP00293625; ZP00293353	oxygenase
SCO0333			Lignostilbene dioxygenase
SCO6680; SCO6887		ZP00291629	Vanillate oxygenase subunit A
SCO2222; SCO6678	SAV156; SAV4350	ZP00292715	Vanillate oxygenase sununit B
SCO5024	SAV3243	ZP00294383; ZP00292504; ZP00292956; ZP00292779	oxygenase

Finally, the enzymes involved in lignin degradation in Actinomycetes do not seem to closely resemble those of the better understood fungi; this has limited the use of bioinformatics in the identification of potential lignin-degrading enzymes. It is clear that a systematic approach in terms of complete and well annotated genome sequencing of a thermophilic Actinomycete that can be easily genetically manipulated, followed by in-depth investigation of candidate genes, is probably the best approach to understanding lignin degradation in this useful group of microorganisms.

References

Adhi, T. P., Korus, R. A., and Crawford, D. L. (1989). Production of major extracellular enzymes during lignocellulose degradation by two *Streptomyces* in agitated submerged culture. *Appl. Environ. Microbiol.* **55,** 1165–1168.

Aitken, M. D. (1993). Waste treatment applications of enzymes: Opportunities and obstacles. *J. Chem. Eng.* **52,** 49–58.

Alexandre, G., and Zhulin, I. B. (2000). Laccases are widespread in bacteria. *Trends Biotechnol.* **18,** 41–42.

Antai, S. P., and Crawford, D. L. (1981). Degradation of softwood, hardwood and grass lignocellulose by two *Streptomyces* strains. *Appl. Environ. Microbiol.* **42,** 378–380.

Antonopoulos, V. T., Rob, A., Ball, A. S., and Wilson, M. T. (2001). Dechlorination of chlorophenols using extracellular peroxidases produced by *Streptomyces albus* ATCC 3005. *Enzyme. Microb. Tech.* **29,** 62–69.

Arias, M. E., Arenas, M., Rodriguez, J., Soliveri, J., Ball, A. S., and Hernandez, M. (2003). Kraft pulp biobleaching and mediated oxidation of a nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335. *Appl. Environ. Microbiol.* **69,** 1953–1958.

Ball, A. S., and McCarthy, A. J. (1988). Saccharification of straw by *Actinomycete* enzymes. *J. Gen. Microbiol.* **134,** 2139–2147.

Ball, A., Betts, W., and McCarthy, A. (1989). Degradation of lignin-related compounds by *Actinomycetes*. *Appl. Environ. Microbiol.* **55,** 1642–1644.

Blanc, M., Manilley, L., Beffer, T., and Arogno, M. (1999). Thermophilic bacterial communities in hot compost as revealed by most probable number counts and molecular 16S rDNA methods. *FEMS. Micro. Ecol.* **28,** 141–149.

Boshoff, A. (2001). The biotransformation of phenolic pollutants using polyphenol oxidase. PhD Thesis, Rhodes University.

Bonomo, R. P., Cennamo, G., Purrello, R., Santoro, A. M., and Zappalà, S. R. (2001). Comparison of three fungal laccases from *Rigidoporus lignosus* and *Pleurotis ostreatus*: Correlation between conformational changes and catalytic activity. *J. Inorg. Biochem.* **83,** 67–75.

Burke, N. S., and Crawford, D. L. (1998). Use of azo dye ligand chromatography for the partial purification of a novel extracellular peroxidase from *Streptomyces viridosporus* T7A. *Appl. Microbiol. Biotechnol.* **49,** 523–530.

Burton, S. G. (1994). Biocatalysis with polyphenol oxidases. A review. *Catalysis Today* **22,** 459–487.

Claus, H., and Filip, Z. (1997). The evidence of a laccase-like activity in a *Bacillus sphaericus* strain. *Microbiol. Res.* **152,** 209–215.

Colonna, S., Gaggero, N., Richelmi, C., and Pasta, P. (1999). Recent biotechnological developments in the use of peroxidases. *Tibtech.* **171,** 163–168.

Crawford, D. L. (1974). Growth of *Thermonospora fusca* on lignocellulase pulps of varying lignin content. *Can. J. Microbiol.* **20,** 1069–1072.

Crawford, D. L. (1978). Lignocellulose decomposition by selected *Streptomyces* strains. *Appl. Environ. Microbiol.* **35,** 1041–1045.

Crawford, D. L., Pometto, III, A. L., and Crawford, R. L. (1984). Production of useful modified lignin polymers by bioconversion of lignocellulose with *Streptomyces*. *Biotechnol. Adv.* **2,** 217–232.

Crawford, D. L., Doyle, J. D., Wang, Z., Hendricks, C. W., Bentjen, S. A., Bolton, H., Jr., Fredrickson, J. K., and Bleakley, B. H. (1993). Effects of a lignin peroxidase-expressing recombinant, *Streptomyces lividans* TH23.1, on biogeochemical cycling and the numbers and activities of microorganisms in soil. *Appl. Environ. Microbiol.* **59,** 508–518.

Cross, T. (1968). Thermophilic *Actinomycetes*. *J. Appl. Bacteriol.* **31,** 36–53.

Cullen, D. (1997). Recent advances on the molecular genetics of ligninolytic fungi. *J. Biotechnol.* **53,** 273–289.

Dean, J. F. D., and Eriksson, K. E. L. (1994). Laccase and the deposition of lignin in vascular plants. *Holzforschung* **48,** 21–33.

Endo, K., Hayashi, Y., Hibi, T., Hosana, K., Beppu, T., and Ueda, K. (2003a). Enzymological characterization of EpoA, a laccase-like phenol oxidase produced by *Streptomyces griseus*. *J. Biochem.* **133,** 671–677.

Endo, K., Hosono, K., Beppu, T., and Ueda, K. (2003b). A novel extracytoplasmic phenol oxidase of *Streptomyces*: Its possible involvement in the onset of morphogenesis. *Microbiology.* **148,** 1767–1776.

Evans, W. C., and Fuchs, G. (1988). Anaerobic degradation of aromatic compounds. *Ann. Rev. Microbiol.* **42,** 289–317.

Freeman, J. C., Nayar, P. G.., Begley, T. P., and Villafranca, J. J. (1993). Stoichiometry and spectroscopic identity of copper centers in phenoxazonine synthase, a new addition for the blue copper oxidase family. *Biochemistry* **32,** 4826–4830.

Finger, A. (1994). *In vitro* studies on the effect of polyphenol oxidase and peroxidase on the formation of polyphenolics black tea constituents. *J. Sci. Food Agric.* **66,** 293–305.

Givaudan, A., Effosse, A., Faure, D., Potier, P., Bouillant, M. L., and Bally, R. (1993). Polyphenol oxidase in *Azospirillum lipoferum* isolated from rice rhizosphere: Evidence for laccase activity in non-motile strains of *Azospirillum lipoferum*. *FEMS Microbiol. Lett.* **108,** 205–210.

Gonçalves, A. R., and Benar, P. (2001). Hydroxymethylation and oxidation of Organosolv lignins and utilization of the products. *Bioresource Tech.* **79,** 103–111.

Guerra, A., Feraz, A., Catrim, A. R., and Da Silva, F. T. (2000). Polyermization of lignin fragments contained in a model effluent by polyphenoloxidases and horseradish peroxidase/hydrogen peroxide system. *Enzyme Microbial. Technol.* **26,** 315–323.

Haemmerli, S. D., Leisola, M. S., Sanglard, D., and Fiechter, A. (1986). Oxidation of benzo (a)pyrene by extracellular ligninases of *Phanerochaete chrysosporium*. Veratryl alcohol and stability of ligninase,. *J. Biol. Chem.* **261,** 6900–6903.

Hahn, J.-S., Oh, S.-Y., and Roe, J.-R. (2000). Regulation of the fur A and CatC operon, encoding a ferric uptake regulator homologue and catalase-peroxidase, respectively in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **182,** 3767–3774.

Hildén, L., Johansson, G., Pettersson, G., Li, J., Ljungquist, P., and Henriksson, G. (2000). Do the extracellular enzymes cellobiose dehydrogenase and manganese peroxidase form a pathway in lignin biodegradation? *FEBS Letts.* **477,** 79–83.

Hsieh, C. J., and Jones, G. H. (1995). Nucleotide sequence, transcriptional analysis, and glucose regulation of the phenoxazinone synthase gene (phsA) from *Streptomyces antibioticus*. *J. Bacteriol.* **177,** 5740–5747.

Iqbal, M., Mercer, D. K., Miller, P. G. G., and McCarthy, A. J. (1994). Thermostable extracellular peroxidases from *Streptomyces thermoviolaceus*. *Microbiology* **140,** 1457–1465.

Iwagami, S. G., Yang, K., and Davies, J. (2000). Characterization of the protocatechuic acid catabolic gene cluster from *Streptomyces* sp. strain 2065. *Appl. Environ. Microbiol.* **66,** 1499–1508.

Jiménez, M., and García-Carmona, F. (1999). Oxidation of the flavonol quercetin by polyphenol oxidase. *J. Agric. Food Chem.* **47,** 56–60.

Kapich, A. N., Jensen, K. A., and Hammel, K. E. (1999). Peroxyl radicals are potential agents of lignin biodegradation. *FEBS Letts.* **461,** 115–119.

Kermasha, S., and Metche, M. (1988). Studies on seed peroxidase *Phaseoulus vulgaris* cv. Haricot. *J. Food Sci.* **53,** 247–252.

Kukolya, J., Nagy, I., Láday, M., Tóth, E., Oravecz, O., Márialigeti, K., and Hornok, L. (2002). *Thermobifida cellulolytica* sp. nov., a novel lignocellulose-decomposing Actinomycete. *Int. J. Sys. Evol. Microbiol.* **52,** 1193–1199.

Leonowicz, A., Cho, N. S., Luterek, J., Wilkolazka, A., Wojtas-Wasilewska, M., Matuszewska, A., Hofrichter, M., Wesenberg, D., and Rogalski, J. (2001). Fungal laccase, properties and activity on lignin. *J. Basic Microbiol.* **41,** 185–227.

Li, K., Xu, F., and Eriksson, K. E. (1999). Comparison of fungal laccases and redox mediators in oxidation of a nonphenolic lignin model compound. *Appl. Environ. Microbiol.* **65,** 2654–2660.

Lopretti, M., Cabella, D., Morais, J., and Rodrigues, A. (1998). Demethoxylation of lignin-model compounds with enzyme extracts from *Gloeophilum trabeum*. *Process Biochemistry* **33,** 657–661.

Luke, A. K., and Burton, S. G. (2001). A novel application for *Neurospora crassa*, progress from batch culture to a membrane bioreactor for the bioremediation of phenols. *Enzyme Microb. Technol.* **29,** 348–356.

Majcherczyk, A., Johannes, C., and Hüttermann, A. (1998). Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. *Enzyme Microb. Tech.* **22,** 335–341.

Manguson, T., and Crawford, D. L. (1992). Comparison of extracellular peroxidase and esterase deficient mutants of *Streptomyces viridosporus* T7A. *Appl. Environ. Microbiol.* **58,** 1070–1072.

Mason, M. G., Ball, A. S., Reeder, B. J., Silkstone, G., Nicholls, P., and Wilson, M. T. (2001). Extracellular heme peroxidases in actinomycetes, a case of mistaken identity. *Appl. Environ. Microbiol.* **10,** 4512–4519.

Mayer, A. M., and Staples, R. C. (2002). Laccases, new functions for an old enzyme. *Phytochemistry* **60,** 551–565.

McCarthy, A. J. (1987). Lignocellulose-degrading *Actinomycetes*. *FEMS Microbiol. Rev.* **46,** 145–163.

McCarthy, A. J., and Williams, S. T. (1992). *Actinomycetes* as agents of biodegradation in the environment – a review. *Gene* **115,** 189–192.

Mercer, D. K., Iqbal, P. G., Miller, G., and McCarthy, A. J. (1996). Screening *Actinomycetes* for extracellular peroxidase activity. *Appl. Environ. Microbiol.* **62,** 2186–2190.

Mhlanga, C. Y. L. (2001). Thermophilic lignin degrading enzymes from Actinomycetes for biotechnological applications. MSc Thesis, Rhodes University.

Mliki, A., and Zimmermann, W. (1992). Purification and characterisation of an intracellular peroxidase from *Streptomyces cyaneus*. *Appl. Environ. Microbiol.* **58,** 916–919.

O' Brien, P. J. (2000). Peroxidases. *Chem. Biol. Interactions* **129,** 113–139.

Pasti, M. B., Pometto, A. L., III, Nuti, M. P., and Crawford, D. L. (1990). Lignin-solubilizing ability of actinomycetes isolated from termite (*Termitidae*) gut. *Appl. Environ. Microbiol.* **56,** 2213–2218.

Paszczynski, A., Pasti-Grigsby, M. B., Goszczynski, S., Crawford, R. L., and Crawford, D. L. (1992). Mineralization of sulphonated azo dyes and sulfanilic acid by *Phanerochaete chrysosporium* and *Streptomyces chromofuscus*. *Appl. Environ. Microbiol.* **58,** 3598–3604.

Piontek, K., Smith, A. T., and Blodig, W. (2001). Lignin peroxidase structure and function. *Biochem. Soc. Trans.* **29,** 111–116.

Pometto, A. L., and Crawford, D. L. (1983). Whole-cell bioconversion of vanillin to vanillic acid by *Streptomyces viridosporus*. *Appl. Environ. Microbiol.* **45,** 1582–1585.

Poulos, T. L., Edwards, S. L., Wariishi, H., and Godl, M. H. (1993). Crystallographic refinement of lignin peroxidase at 2 Å. *J. Biol. Chem.* **268,** 4429–4440.

Ramachandra, M., Crawford, D. L., and Pometto, A. L. (1987). Extracellular enzyme activities during lignocellulase degradation by *Streptomyces* spp, A comparative study of wild type and genetically manipulated strains. *Appl. Environ. Microbiol.* **53,** 2754–2760.

Ramachandra, M., Crawford, D. L., and Hertel, G. (1988). Characterisation of an extracellular lignin peroxidase of the lignocellulolytic *Actinomycete Streptomyces viridosporus*. *Appl. Environ. Microbiol.* **54,** 3057–3063.

Ramachandran, S., Magnuson, T. S., and Crawford, D. L. (2000). Isolation and analysis of three peroxide sensor regulatory gene homologs ahpC, ahpX and oxyR in *Streptomyces viridosporus* T7A–a lignocellulose degrading actinomycete. *DNA Seq.* **11,** 51–60.

Rob, A., Ball, A. S., Tuncer, M., and Wilson, M. T. (1996). Thermostable novel non-haem extracellular glycosylated peroxidase from *Thermomonospora fusca* BD25. *Appl. Biochem.* **24,** 161–170.

Rob, A., Hernandez, M., Ball, A. S., Tuncer, M., Arias, M. E., and Wilson, M. T. (1997). Production and partial characterization of extracellular peroxidases produced by *Streptomyces avermitilis* UAH30. *Appl. Biochem. Biotechnol.* **62,** 159–174.

Robinson, D. S. (1991). Peroxidases and catalases in food. *In* ''Oxidative Enzymes in Foods'' (D. S. Robinson and A. M. Eskin, eds.), pp. 1–47. Elsevier Science Publishers Ltd, Essex, England.

Rodríguez-López, J., Fenoll, L. G., Peñalver, M. J., García-Ruiz, P. A., Varón, R., Martínez-Ortíz, F., García-Cánovas, F., and Tudela, J. (2001). Tyrosinase action on monophenols, evidence for direct enzymatic release of *o*-diphenol. *Biochim. Biophy. Acta* **1548,** 238–256.

Russell, I. M. (1999). Development of an immobilized enzyme bioprobe for the detection of phenolic pollutants in water. Masters Thesis, Rhodes University.

Ruttiman, G. B., Vicuna, R., Sapag, C., and Seelenfreund, D. (1998). Biochemical and genetic studies of bacteria metabolising lignin-related compounds. *Arch. Biol. Med. Exp.* **21,** 247–255.

Ryden, L. G., and Hunt, L. T. (1993). Evolution of protein complexity, the blue copper-containing oxidases and related proteins. *J. Mol. Evol.* **36,** 41–66.

Sánchez-Ferrer, A., Rodríguez-López, J. N., and García-Cánovas, F. (1995). Tyrosinase, a comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1247,** 1–11.

Sjoblad, R. D., and Bollag, J. M. (1981). Oxidative coupling of aromatic pesticide intermediates by a fungal phenol oxidase. *Appl. Environ. Microbiol.* **33,** 906–910.

Strom, P. F. (1985). Identification of thermophilic bacteria in solid-waste composting. *Appl. Environ. Microbiol.* **50,** 906–913.

Sutherland, J. B., Crawford, D. L., and Pometto, III, A. L. (1983). Metabolism of cinnaminc, *p*-coumaric and ferulic acids by *Streptomyces setonii*. *Can. J. Microbiol.* **29,** 1253–1257.

Suzuki, T., Endo, K., Ito, M., Tsujibo, H., Miyamoto, K., and Inamori, Y. (2003). A thermostable laccase from *Streptomyces lavendulae* REN-7: Purification, characterization, nucleotide sequence, and expression. *Biosci. Biotechnol. Biochem.* **67,** 2167–2175.

Tekere, M., Mswaka, A. Y., Zvauya, R., and Read, J. S. (2001). Growth, dye degradation and lignolytic activity studies in Zimbabwean white rot fungi. *Enzyme Microbial. Tech.* **28,** 420–426.

Thomas, L., and Crawford, D. L. (1998). Cloning of clustered *Streptomyces viridosporus* T7A lignocellulose catabolism genes encoding peroxidase and endoglucanses and their extracellular expression in *Pichia pastoros*. *Can. J. Microbiol.* **44,** 364–372.

Thurston, C. F. (1994). The structure and function of fungal laccases. *Microbiology* **140,** 19–26.

Tien, M., and Kirk, T. K. (1983). Lignin-degrading enzyme from the Basidiomycete *Phanerochaete chrysosporium*. Burds. *Science* **221,** 661–663.

Torsvik, V., Daae, F. L., Sandaa, R. A., and Ovreas, L. (1998). Novel techniques for analysing microbial diversity in natural and perturbed environments. *J. Biotechnol.* **64,** 53–62.

Trigo, C., and Ball, A. S. (1994). Is the solubilization product from the degradation of lignocellulose by *Actinomycetes* a precusor of humic substances? *Microbiology* **140,** 3145–3152.

Tuncer, M., and Ball, A. S. (2002). Degradation of lignocellulose by extracellular enzymes produced by *Thermomonospora fusca* BD25. *Appl. Microbiol. Biotechnol.* **58,** 608–611.

Wang, Z., Bleakley, B. H., Crawford, D. L., and Hertel, G. (1990). Cloning and expression of a lignin peroxidase gene from *Streptomyces viridosporus* in *Streptomyces lividans*. *J. Biotechnol.* **13,** 131–144.

Watanabe, Y., Shinzato, N., and Fukatsu, T. (2003). Isolation of actinomycetes from termites' guts. *Biosci. Biotechnol. Biochem.* **67,** 1797–1801.

Winter, B., Fiechter, A., and Zimmerman, W. (1991). Degradation of organochlorine compounds in spent sulfite bleach plant effluents by *Actinomycetes*. *Appl. Environ. Microbiol.* **57,** 2858–2863.

Youn, H. D., Yim, Y. I., Kim, K., Hah, Y. C., and Kang, S. O. (1995). Spectral characterization and chemical modification of catalase-peroxidase from *Streptomyces* sp. *J. Biol. Chem.* **270,** 13740–13747.