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Recent advances in studies on the bacterial degradation of lignin

Avances recientes en el estudio de la degradación bacteriana de lignina

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Bacterial degradation of lignin has been recently reviewed by one of us (1). Evidence reported in that work showed that bacteria are much less efficient than fungi in degrading lignin. To date it is not clear whether non-filamentous bacteria secrete ligninolytic enzymes outside of the cell. The low degree of lignin mineralization observed with these microorganisms (2-4) could be explained by intracellular metabolism of small molecular weight lignin fragments that are released abiotically from the polymer (5). This capacity to degrade and even grow on lignin substructures strongly suggests that bacteria play an important role in the turnover of lignin in the biosphere. Actinomycetes also exhibit a poor lignin mineralization capacity (6, 7). However, filamentous bacteria attack grass lignocellulose producing lignin-rich soluble fragments called APPL (for acid precipitable polymeric lignin, MW > 5,000). Slow progress is being made in the understanding of the mechanism involved in this process (see below).

In this article we discuss metabolic pathways for the degradation of lignin dimers in various bacterial strains and the enzymes catalyzing cleavage of intermonomeric linkages. Attention is also given to bacterial action of polymeric lignin.

Metabolism of dimeric structures

There are several reports dealing with bacterial degradation of dimeric lignin-model compounds. These include aerobic strains from genera as diverse as *Pseudomonas, Corynebacterium, Streptomyces, Arthrobacter* and *Agrobacterium,* as well as anaerobic bacteria (1). In most instances, partial catabolic pathways have been elucidated.

Catabolism of arylglycerol-β-aryl ether (β-O-4) compounds

These dimers are frequently used as lignin models because they contain the intermonomeric linkage most frequently found in lignin (8). Ether cleavage has been observed in *in vivo* studies with four different Pseudomonas strains: *P. paucimobilis* TMY 1009 (9), *P. acidovorans* D_3 (10), *P. fluorescens* biovar I (11) and *P. paucimobilis* SYK-6 (12). With guaiacylglycerol-β-guaiacyl ether as growing substrate, guaiacol and β-hydroxypropiovanillone are detected in the cultures, the latter being the only C_6-C_3 compound identified (Fig. 1A). In turn, the non-phenolic counterpart of this dimer gives rise to β-hydroxypropioveratrone as metabolic intermediate. On the other hand, dimers lacking the C_γ hydroxymethyl group produce the C_6-C_2 intermediates acetovanillone or acetoveratrone. All this evidence points to a reductive type of cleavage, although the possibility that the reduced intermediates are not formed directly after ether cleavage can not be discarded.

In spite of intensive efforts made by several groups to isolate the enzyme responsible for this reaction, preliminary evidence of such activity was reported only in 1989 (12). Crude extracts prepared from the membrane fraction of *P. paucimobilis* SYK-6 cleave guaiacylglycerol-β-guaiacyl ether to yield β-hydroxypropiovanillone and guaiacol. The activity is enhanced by NADH and is unstable in the absence of the detergent MEGA-8. Our group has been

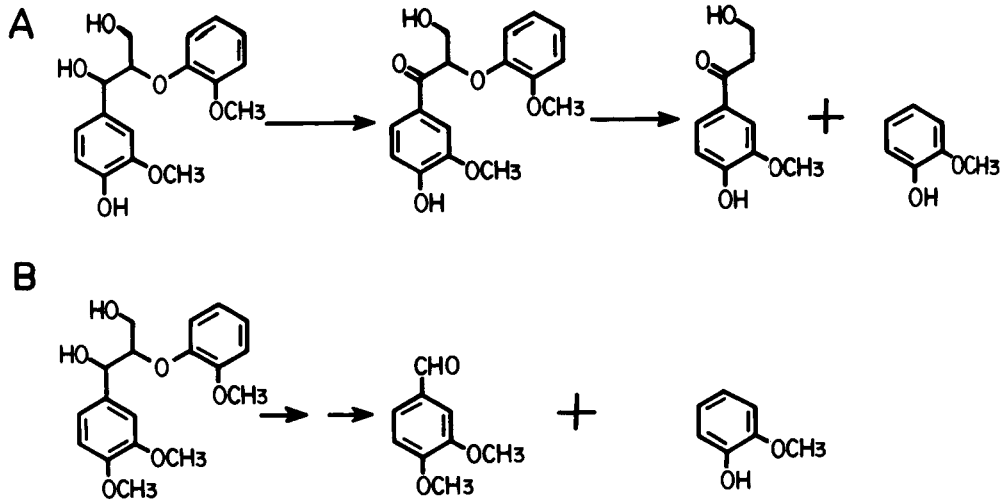

Fig. 1: Initial steps in the catabolism of arylglycerol-β-arylether compounds by bacterial strains. A) Ether bond cleavage; B) C_α-C_β bond cleavage.

unable to reproduce these results with *P. acidovorans* D_3 and *P. fluorescens* biovar I. It is possible that the enzime *in vivo* catalyzes the reductive cleavage in an anaerobic microenvironment and that once homogeneized in crude extracts it becomes highly unstable. The stabilizing effect of the detergent points in this direction. This hypothesis could explain, at least partially, the difficulties encountered in the isolation of this enzyme. A similar situation may be occurring in the reductive dehalogenation of chlorinated aromatics. Crude extracts prepared from cells grown in aerobic conditions either lack the enzymatic activity (13) or lose it unless ascorbate is added (14).

All C_6-C_3 and C_6-C_2 intermediates identified so far possess a C_α in the keto form. Furthermore, β-O-4 dimers with an hydroxyl group at the C_α position undergo C_α oxidation prior to cleavage (9, 10, 15). This requirement has also been observed with lignin tetramers (16). Pelmont *et al.* (17) were the first to describe a dehydrogenase, isolated from *Pseudomonas* sp GU5, responsible for this reaction. Two dehydrogenases (DH-I and DH-II) with different substrate specificity have been found in *Pseudomonas* sp TMY1009 (18, 19). Both enzymes catalyze redox reactions with β-O-4 and 1,2-diarylpropane (β-1) models, being DH-I more active with the latter and *viceversa.*

C_α-C_β bond cleavage, a reaction commonly catalyzed by fungal lignin peroxidase (LiP), has been observed *in vivo* with the actinomycete *Corynebacterium equi* ATCC 6939 (20) (Fig. 1B). The identification of an aromatic aldehyde and guaiacol during growth of *Streptomyces cianeus* on a β-O-4 dimer also suggests C_α-C_β cleavage (21). Ramachandra *et al.* recently described the extracellular production of a lignocellulose-induced peroxidase by *Streptomyces viridosporus* T7A (22). The enzyme, called ALiPx for actinomycete lignin peroxidase, is present in the form of five isoenzymes (23). It oxidises several phenolic substrates and it is active also with β-1 dimers. The authors propose that this peroxidase catalyzes C_α-C_β cleavage, although the products identified by GC-MS analysis, *i.e.*, vanillin and guaiacol, are insufficient to support this proposition. The C_α-reduced dimers, whose formation could not be explained by the peroxidase action, are also found in the enzymatic assays. The formation of ALiPx in large amounts seems to be related with the use of larchwood xylan as an inducer (23). The failure of our group to detect *in vivo* modification of β-O-4 dimers by *S. viridosporus* T7A may be related to the absence of an appropiate inducer in the cultures (24). Further work is necessary to assess the role of this peroxidase in APPL production.

C_α-C_β cleavage of a β-O-4 dimer has also been found with *Pseudomonas cepacia* 122 (25) and with an anaerobic consortium (26), as was discussed previously (1).

Catabolism of 1,2-diarylpropane (ethane) and phenylcoumaran compounds

Lignin substructures of the 1,2-diarylpropane type possess the so called β-1 linkage, whose frequency in natural lignin is about 5-10% (8). Studies on bacterial degradation of these compounds are scarce, perhaps due to the difficulties in finding strains with the required enzymatic machinery (1). Only in 1989, Habu *et al.* (27) reported for the first time the catabolism of the 1,2-diarylpropane dimer erythro-1,2-bis (4-hydroxy-3-methoxyphenyl)-1,3-propanediol by an isolated strains, namely *Pseudomonas* sp. TMY1009. The pathway starts with the oxidation of the C_γ hydroxymethyl to a carboxyl group, followed by the double elimination of this group and the hydroxyl linked to C_α to give a stilbene intermediate. This molecule then undergoes oxidative C_α-C_β cleavage producing two molecules of vanillin (Fig. 2A). In close connection with this degradative pathway is the one reported for the metabolism of the phenylcoumaran structure dehydrodiconiferyl alcohol by the same strain (28) (Fig. 2B). In this case, cleavage of the C_α-oxygen bond of the cyclic ether results in a phenolic stilbene that is oxidatively cleaved between C_α and C_β.

A novel dioxygenase has been isolated thereafter from this strain and shown to catalyze the oxidative cleavage of the double bond (29). Apparently, it requires no cofactor. Isotope experiments have confirmed the participation of molecular oxygen in the generation of the two aldehyde molecules. This enzyme, named lignostilbene α,β-dioxygenase, has four isoenzymes. One of them, LSD-I, has been purified to homogeneity. It is a dimer of MW 94,000 and contains 1 g atom of iron per mol of enzyme (30). Crude preparations of α,β-dioxygenase convert natural stilbenes to aromatic aldehydes (31).

A completely different pathway allows *P. fluorescens* biovar I to metabolize diarylethane ketols (32). The acyloin linkage in dimers such as anisoin or benzoin is cleaved by a highly specific thiamine pyrophosphate requiring lyase, yielding two molecules of the corresponding aromatic aldehyde (33) (Fig. 2C).

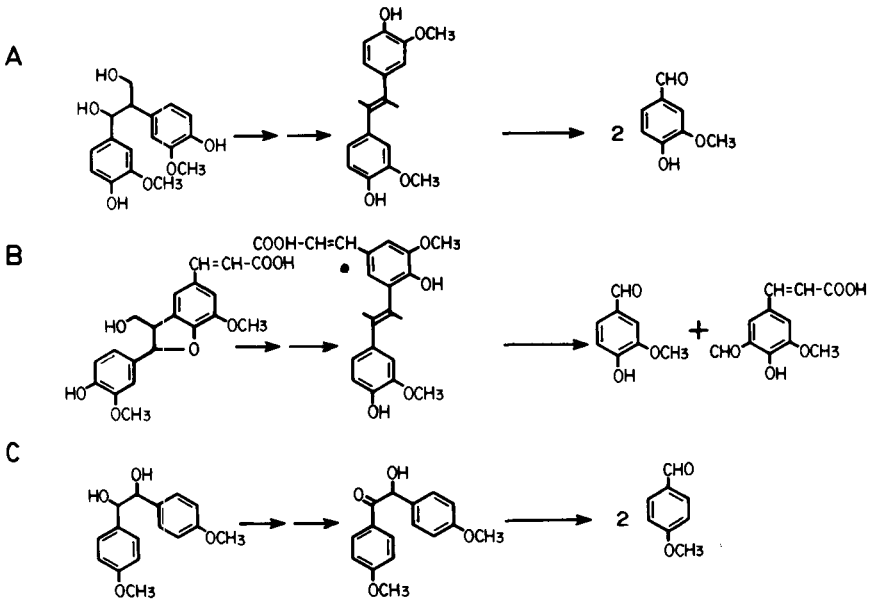

Fig. 2: Initial steps in the catabolism of: A) a 1,2-diarylpropane compound by *Pseudomonas* sp. TMY 1009; B) a phenylcoumaran compound by the same strain, and C) a 1,2-diarylethane compound by *P. fluorescens* biovar I.

Catabolism of biphenyl structures

About 10% of the lignin residues are linked through biphenyl bonds (8). Therefore, biphenyl structures have also been considered lignin model compounds (1). The pathway usually starts with a dioxygenase that forms a catechol, followed by ring fission catalyzed by a second dioxygenase. The C-C bond that constitutes the biphenyl linkage of the original molecule is cleaved much later in the pathway, after the second ring has also undergone fission. Breakdown of dehydrodivanillin in *P. paucimobilis* SYK-6 (34) seems to follow the same pathway found with an anaerobic consortium (35), which includes dehydrodivanillic acid, 5'-carboxyvanillic acid, vanillin and vanillic acid as intermediates. However, with crude extracts prepared from a recombinant component of the consortium, only vanillin was detected (36). Some of the genes that code for the enzymes involved in biphenyl degradation have been cloned (37-39).

Effect of bacteria on high MW lignin preparations

Since the action of bacteria on either natural or synthetic lignins is almost negligible (1), studies have focused on two areas: bacterial modification of industrial lignins and lignocellulose solubilization by actinomycetes.

Kraft lignin seems to be a rather good substrate for anaerobic bacteria, as measured either by mineralization (40, 41) or depolymerization (42). This has been an unexpected finding, not only because mineralization rates are the highest ever found with bacteria, but also because native lignin mineralization does not take place in anoxic environments (1, 43). Certainly, the biochemistry of this process deserves further study. On the other hand, aerobic bacteria do not affect Kraft lignin significantly (1), although strains can be isolated that grow on purified, low and high MW Kraft lignins as the only source of carbon and energy (44). A strain-specific consumption of aromatic acids present in the former has been reported (45), whereas the nature of chemical modifications brought about by bacteria on the latter has not been unraveled (Merino, A., personal communication). Since depolymerization has not been found, it is conceivable that bacteria may grow at the expense of abiotically released fragments. In constrast to Kraft lignin, the industry derived chlorolignins have not been found to support bacterial growth (46).

The APPL-producing actinomycetes *Termomonospora mesophila* (47), *Streptomyces badius* (47, 48) and *S. viridosporus* (48) can also incorporate carbon from Kraft lignin into biomass. However, this property is not shared by other filamentous bacteria that are also able to produce APPL from grass lignocellulose (47). Another report concerning the effect of *S. viridosporus* T7A on two Kraft lignin fractions showed that this microorganism was not only unable to proliferate on the high MW fraction, but that growth on yeast extract was inhibited by the low MW fraction (49). The different behaviour of both *S. viridosporus* strains may be related to the source of Kraft lignin or to the fractionation-purification procedure of this substrate.

As stated above, the enzymes involved in APPL formation by actinomycetes have not been identified with certainty. According to preliminary evidence, cellulases but not xylanases could play a role in this process (see ref. 1). ALiPx, which is also produced by *S. badius* (50), represents a good candidate for this function, but this has still to be determined. On the other hand, Mason *et al.* (51) have described a lignocellulosic solubilizing enzymatic activity, which has not yet being characterized.

It has been shown that the aromatic moiety of APPL resist further attack by the strains that produce them (52, 53) and even by fungi (54). To date, no attention has been given to the action of non-filamentous bacteria on APPL, with the exception of the work performed by Seelenfreund *et al.* (55). These authors isolated natural strains in enrichment cultures containing APPL derived from wheat straw as the only source of carbon

and energy. Three cultures were selected by these means: *Pseudomonas* E_{21}, *Pseudomonas* B_{23} and a consortium composed of a Pseudomonas and an Enterobacter strain. Monosaccharide and thioacidolysis analyses after bacterial growth showed that these strains do not modify the APPL structure extensively. However, it was determined that *Pseudomonas* B_{23}, which does not grow on lignin dimers, developed at the expense of guaiacyl and syringyl residues of the substrate. In contrast, *Pseudomonas* E_{21} and the consortium metabolized a fraction of the sugar moieties without modifying the aromatic residues of APPL. On the other hand, *P. fluorescens* biovar I and *P. acidovorans* D_3, both able to cleave the β-O-4 linkage in lignin dimers, did not attack the guaiacyl and syringyl residues of APPL. These results show that there is no direct correlation between consumption of small lignin-related compounds and ability to degrade polymeric lignin.

Concluding remarks

Current knowledge indicates that bacteria seem to lack a non-specific system able to carry out lignin combustion. However, it is not uncommon to find strains that can simultaneously degrade lignin dimers of different types. Good examples are *Pseudomonas* sp TMY1009 (β-O-4, β-1 and phenylcoumaran structures) (9, 27, 28), *P. fluorescens* biovar I (β-O-4 and diarylethane structures) (11, 32), *P. paucimobilis* SYK-6 (β-O-4 and biphenyls) (12, 34) and *P. cruciviae* S93B1 (diarylethane and biphenyls) (56). Specific, intracellular enzymes are in charge of the metabolism of each type of dimer. Alternatively, lignin oligomers possessing different linkages can be degraded by mixed cultures (57, 58). ALiPx might resemble fungal lignin peroxidase, although evidence reported to date is too preliminary to draw any conclusions in this respect. In turn, enzymes that modify APPL or Kraft lignin, or those that allow bacteria to erode the secondary layer of the cell wall (59) remain to be discovered and characterized.

NOTE ADDED IN PROOF

While this manuscript was in press, a review on the subject came out from Zimmerman, W. J. Biotech. (1990), 13: 119-130.

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