Poplar Lignin Decomposition by Gram-Negative Aerobic Bacteria

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Eleven gram-negative aerobic bacteria (Pseudomonadaceae and Neisseriaceae) out of 122 soil isolates were selected for their ability to assimilate poplar dioxane lignin without a cosubstrate. Dioxane lignin and milled wood lignin degradation rates ranged between 20 and 40% of initial content after 7 days in mineral medium, as determined by a loss of absorbance at 280 nm; 10 strains could degrade in situ lignin, as evidenced by the decrease of the acetyl bromide lignin content of microtome wood sections. No degradation of wood polysaccharides was detected. Lignin biodegradation by Pseudomonas 106 was confirmed by 14CO2 release from labeled poplar wood, although in lower yields compared with results obtained through chemical analysis based on acetyl bromide residual lignin determination.

The decomposition of lignin in nature has been considered for a long time to occur by the action of wood-rot fungi mostly of the Basidiomycete class. These microorganisms simultaneously decompose lignin and wood polysaccharides. Only recently, several reports brought strong evidence of the ability of certain bacteria to degrade lignins (6, 17, 20, 31).

We reported the isolation of several bacterial strains able to degrade and assimilate isolated lignins (22, 23). Milled wood lignins (MWL) are considered to possess a structure closely related to that of in situ lignin. They are also highly polymerized (1). However, they often contain high amounts of polysaccharides which make them difficult to use for microbiological studies. For this reason, dioxane lignin is prepared at ambient temperature as previously reported (23); dioxane lignin structure is less modified than those of other extracted lignins provided they are obtained by mild acid hydrolysis at ambient temperature with 0.2 N HCl in aqueous dioxane. Kraft lignins and lignosulfonates, which have sometimes been used for microbiological studies, are much more modified (15, 21). Wheat and poplar dioxane lignins contain very small amounts of carbohydrates (22). Nevertheless, results obtained with this material need confirmation, using less structurally modified lignins such as MWL or in situ lignins.

In this paper we report the isolation of bacteria able to use poplar dioxane lignin and MWL as sole carbon and energy sources. The degradation of lignin in situ in wood was also studied since incrustation of lignin in a lignin-carbohydrate complex can affect lignin biodegradability, especially by non-polysaccharide-degrading microorganisms.

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MATERIALS AND METHODS

Dioxane lignin and milled wood preparation. Dioxane lignin was extracted from wood at ambient temperature for 20 days and purified as previously reported (23). MWL was extracted by dioxane-water (9:1) from wood milled by the method of Brownell (4). Purification was performed by using the same method as for dioxane lignin (23).

Preparation of [14C]lignin from poplar (Populus euramerica Dode cultivar 1214). Poplar cut stems (20 to 25 internodes) were fed 25 μCi of L-[14C]phenylalanine, using an adaptation of the Freudenberg and Neish stem infusion method (13) according to Alibert and Boudet (2), taking care to always hold the cut end of the stem in the nutrient solution during aging. The labeled wood was first sliced into blocks (about 5 mm3). Then, to remove water and organic soluble components, it was extracted by the method of Crawford (7) to which a proteinase digestion was added, as later suggested by Crawford et al. (10) and Reid (26); i.e., after water extraction at 80°C, wood was submitted to pronase digestion (100 mg/liter) in 0.01 M phosphate buffer (pH 7) for 1 h at 30°C.

Specific activity of extracted wood was 31,064 dpm/mg, as determined by combustion of a sample to 14CO2, using an Oxymat Intertechnique apparatus. Klason lignin was obtained after 72% sulfuric acid hydrolysis
Xanthomonas, of aerobic rods identified under aerobic conditions. Poplar dioxane was tested by hydrolysis (7).

Barnoud et al. (27) found that 5% sulfuric acid dissolved dioxane at 30°C in air. They used this method to isolate dioxane lignin degradation by inoculating 10 ml of mineral medium (23) containing 0.1% dioxane lignin. After 21 days of incubation at 30°C in stationary-phase culture, tubes were examined for growth microscopically and by nutrient agar inoculation. Positive cultures were subcultured on the same medium by the same technique. Only strains showing growth after three transfers were considered positive.

**Cultivation and degradation procedures.** A total of 20 mg of dioxane lignin or MWL sterilized by ethyl ether (22) was added to 50 ml of mineral medium (23) in 150-ml flasks which were inoculated and incubated at 30°C with agitation. Residual lignin was determined by using absorbancy at 280 nm (A_{280}) as previously described (23).

Wood sections (50 μm thick, about 4 mg) extracted with methanol-water (1:1, vol/vol) were solvent exchanged against ethanol, acetone, and ethyl ether successively, and then weighed with a Cahn electrobalance (±0.1 μg) and sterilized by ethyl ether in 5-ml vials. Sterile mineral medium (4 ml) was then added aseptically. Cultures were inoculated by titrated suspensions of cells and incubated in static conditions in closed containers to prevent drying. After incubation, wood sections were removed from the culture medium with forceps, washed gently in water, and extracted by water, ethanol, acetone, and ethyl ether successively. Dried wood sections were weighed and residual lignin was determined by the method of Johnson et al. (19).

**Radiorespirometry.** For radiorespirometry, labeled wood, sterilized by ethyl ether, was added to 10 ml of mineral medium in 25-ml flasks equipped for flushing and ¹⁴C CO₂ determination, as described by Crawford (7).

**Total wood carbohydrate analysis.** Wood was analyzed for total carbohydrates by gas-liquid chromatography of alditol acetate derivatives after acid hydrolysis by the method of Albersheim as described by Barnoud and Joseleau (3).

### RESULTS

Eleven bacterial strains out of 122 soil isolates tested were found to be able to grow by using poplar dioxane lignin as the sole carbon and energy source in mineral medium in aerobic conditions. The strains consisted of Gram-negative aerobic rods identified as *Pseudomonas*, *Xanthomonas*, and *Acinetobacter*, by Bergey's *Manual of Determinative Bacteriology* (5). Dioxane lignin biodegradation rates ranged between 20 and 40% of initial content after 7 days, estimated on the basis of A_{280} of residual lignin. MWL gives results very similar to those of dioxane lignins (Table 1). *Acinetobacter 121* was isolated by an enrichment procedure with dioxane lignin and gave the best degradation rate with both dioxane lignin and MWL (Table 1).

All of these strains, except for *Acinetobacter 88*, are able to attack lignin when grown on poplar wood sections (Table 2). Acetyl bromide determination of residual lignin shows an important decrease of A_{280}, meaning that lignin structure had been substantially modified by these strains. This decrease does not necessarily imply extensive degradation to CO₂ but could be sim-

**Table 1. Degradation of poplar dioxane lignin and MWL by bacterial strains in mineral medium (50 ml) after 7 days in agitated cultures (30°C)**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lignin degradation (%)</th>
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<tbody>
<tr>
<td></td>
<td>Dioxane lignin</td>
</tr>
<tr>
<td><em>Acinetobacter 121</em></td>
<td>39.5</td>
</tr>
<tr>
<td><em>Pseudomonas 122</em></td>
<td>39.0</td>
</tr>
<tr>
<td><em>Pseudomonas 106</em></td>
<td>35.0</td>
</tr>
<tr>
<td><em>Xanthomonas 14</em></td>
<td>32.1</td>
</tr>
<tr>
<td><em>Xanthomonas 53</em></td>
<td>27.9</td>
</tr>
<tr>
<td><em>Pseudomonas 19</em></td>
<td>29.9</td>
</tr>
<tr>
<td><em>Acinetobacter 88</em></td>
<td>25.0</td>
</tr>
<tr>
<td><em>Acinetobacter 15</em></td>
<td>24.6</td>
</tr>
<tr>
<td><em>Pseudomonas 27</em></td>
<td>24.6</td>
</tr>
<tr>
<td><em>Acinetobacter 2</em></td>
<td>23.2</td>
</tr>
<tr>
<td><em>Pseudomonas 3</em></td>
<td>20.9</td>
</tr>
</tbody>
</table>

*Lignin content: 0.4 g/liter. Degradation rates are estimated on the basis of A_{280} of residual lignin in dioxane-water (1:1).*

**Table 2. Lignin degradation of microtome sections of poplar wood, (P. euramericana) by bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lignin degradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter 121</em></td>
<td>57.3 ± 7</td>
</tr>
<tr>
<td><em>Pseudomonas 106</em></td>
<td>52.7 ± 6</td>
</tr>
<tr>
<td><em>Pseudomonas 19</em></td>
<td>52.0 ± 10</td>
</tr>
<tr>
<td><em>Acinetobacter 15</em></td>
<td>51.2 ± 7</td>
</tr>
<tr>
<td><em>Pseudomonas 27</em></td>
<td>49.7 ± 6</td>
</tr>
<tr>
<td><em>Xanthomonas 53</em></td>
<td>48.1 ± 7</td>
</tr>
<tr>
<td><em>Acinetobacter 2</em></td>
<td>47.4 ± 8</td>
</tr>
<tr>
<td><em>Pseudomonas 122</em></td>
<td>44.3 ± 4</td>
</tr>
<tr>
<td><em>Pseudomonas 3</em></td>
<td>40.5 ± 11</td>
</tr>
<tr>
<td><em>Xanthomonas 14</em></td>
<td>39.5 ± 14</td>
</tr>
</tbody>
</table>

*Degradation was determined after 1 month of incubation at 30°C in static cultures (4 ml). Residual lignin was determined by the acetyl bromide method. Percent degradation was estimated by comparison with uninoculated controls. Means and standard deviation were estimated from the results of four tests. Standard deviation of controls was 9.3%.*
ply due to structural modifications of the aromatic chromophore of lignin. Again, *Acinetobacter* 121 gave the highest degradation rate.

Lignin degradation and bacterial growth kinetics were studied for the most active strains with respect to in situ lignin degradation ability: *Pseudomonas* 106, *Pseudomonas* 122, and *Acinetobacter* 121 (Table 3). Cell counts do not account for bacterial cells fixed to wood cells.

In no case did attacked wood sections show a statistically significant decrease in glucose, xylose, arabinose, and galactose contents. *Xanthomonas* 14 can grow at the expense of carboxymethyl cellulose, but not crystalline cellulose. All other strains are negative with respect to carboxymethyl cellulose and crystalline cellulose utilization. It can therefore be concluded that the strains studied are devoid of cellulolytic and hemicellulolytic activities.

*Pseudomonas* 106 generated $^{14}$CO$_2$ from $^{14}$C-lignin-labeled poplar wood, although in low amounts. The degradation rate is much higher in the case of ultramilled wood as compared with that for wood meal (Fig. 1). The rate of metabolic activity was fairly constant with wood meal for about 150 days (3,600 h), after which 2\% of radioactivity was recovered as $^{14}$CO$_2$. Ultramilled wood gave a much higher rate of metabolic activity which decreased after 200 h.

To check that $^{14}$CO$_2$ evolution accounted for lignin and not phenylalanine- or tyrosine-labeled protein degradation, 10 mg of labeled wood sample (3,137,447 dpm) was hydrolyzed by 6 N HCl for 18 h. Of the total $^{14}$CO$_2$, 5.2\% was solubilized. Total hydrolysate (16,527 dpm) was spotted on a cellulose plate and analyzed by thin-layer electrophoresis, using a formic acid-acetic acid-water buffer (17:57:1,000, vol/vol).

Phenylalanine and tyrosine were run simultaneously as references. Zones corresponding to phenylalanine and tyrosine were scraped off. Radioactivity measured by scintillation accounted for 0.9\% of hydrolyzable components and 0.05\% of total labeled wood. The efficiency of the pronase treatment used in the extraction procedure (see above) was tested by comparing the phenylalanine contents of pronase-digested and control extracted woods; protein hydrolysis by pronase caused a decrease of phenylalanine in extracted wood from 0.05 to 0.07\% to less than 0.01\% of the total dry weight.

All strains studied were able to assimilate simple phenols such as vanillic acid, ferulic acid, protocatechuic acid, syringic acid, $p$-hydroxybenzoic acid, $p$-methoxylated phenols, such as veratic acid where utilized, and $m$-methoxylated phenols, such as vanillic acid.

**DISCUSSION**

Using dioxane lignin as a model lignin preparation, bacterial strains able to assimilate extracted lignins such as dioxane lignins or MWL could be screened. No other substrate is needed for lignin degradation by any of these strains, opposed to strains of white-rot fungi which require carbohydrates as cosubstrates (8).

We previously reported the isolation of gram-negative aerobic bacteria able to degrade wheat dioxane lignin in similar conditions (22).

Dioxane lignin was then prepared by treatment of wood in aqueous dioxane with 0.2 N HCl under reflux which might have resulted in significant modifications, such as rearrangement and depolymerization or condensation reactions. This time, extraction took place at ambient temperature for 20 days. The resulting dioxane lignin gave lower degradation rates than those for dioxane lignin obtained by refluxing, but very similar to the rates for MWL. Thus, ambient temperature-obtained dioxane lignins are a convenient model substrate for the isolation and study of lignin-degrading microorganisms. Determination of residual lignin after microbial attack is a difficult problem which has not yet been satisfactorily resolved: all lignin determination methods are based on the measurement

<table>
<thead>
<tr>
<th>Strain</th>
<th>0 time</th>
<th>7 days</th>
<th>15 days</th>
<th>1 mo</th>
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<tbody>
<tr>
<td></td>
<td>Lignin degradation (%)</td>
<td>Cell counts (ml$^{-1}$)</td>
<td>Lignin degradation (%)</td>
<td>Cell counts (ml$^{-1}$)</td>
</tr>
<tr>
<td><em>Pseudomonas</em> 106</td>
<td>0</td>
<td>3 $\times$ 10$^2$</td>
<td>30.4 $\pm$ 1.5</td>
<td>2.5 $\times$ 10$^6$</td>
</tr>
<tr>
<td><em>Acinetobacter</em> 121</td>
<td>0</td>
<td>3 $\times$ 10$^2$</td>
<td>25.7 $\pm$ 4.3</td>
<td>1.06 $\times$ 10$^6$</td>
</tr>
<tr>
<td><em>Pseudomonas</em> 122</td>
<td>0</td>
<td>10$^3$</td>
<td>31.4 $\pm$ 10</td>
<td>1.6 $\times$ 10$^7$</td>
</tr>
</tbody>
</table>

*a* Residual lignin was determined by the acetyl bromide method. Percent degradation was estimated by comparison with uninoculated controls. Means and standard deviation were estimated from the results of four tests. For each strain results are statistically different, except for the following: *Pseudomonas* 106, 15 to 7 days; *Acinetobacter* 121, 15 to 7 days; and *Pseudomonas* 122, 15 to 7 days.
of physicochemical properties of lignin which can be modified after microbial attack. For example, the assay based on methoxyl determination cannot differentiate extensive degradation to CO$_2$ and simple demethylation (10). Here we used $E^{10}_	ext{cm}$ (280 nm) of lignin dissolved in aqueous dioxane. In this case, modifications of aromatic structures and their conjugated chromophores caused by microbial attack may affect $E_{280}$ and, hence, interfere with lignin determination. However, in previous studies we used this method and obtained results consistent with assays based on methoxyl determination (23).

The ability of the strains studied to degrade in situ lignin (protolignin) in pure culture is more limited, especially when considering results of radiorespirometric experiments. Crawford (6), Haider et al. (17), and Trojanowski et al. (31) reported similar results in which actinomycetes able to degrade isolated lignins had a low in situ lignin-degrading ability. Only recently, several *Streptomyces* strains were isolated which can degrade in situ lignin to CO$_2$ with relatively high degradation rates (9, 24).

Observations by electron microscopy reported elsewhere confirmed the decrease of lignin in microtome wood section cell walls, showing also that delignification results in an unmasking of polysaccharides which become more accessible to vicinal glycol contrasting reagent (B. Monties, E. Odier, G. Janin, and Y. Czaninski, Holzforschung, in press).

Of particular interest is the observation that wood ultrastructure considerably affects lignin biodegradability, as shown by the much higher degradation rate obtained with ultramilled wood as compared with ordinary 60-mesh wood meal (11). It is suggested that milling increases biodegradability through better substrate accessibility.

The discrepancy in lignin degradation rates between the microtome wood sections method and radiorespirometry is difficult to explain. We cannot exclude the possibility that, in microtome wood sections, part of the lignin was attacked but not completely degraded to CO$_2$ in such a way that attacked lignin was extracted by solvents and not accounted for in the acetyl bromide determination. Acetyl bromide lignin determination would then lead to an overestimation of the lignin biodegradation to CO$_2$. This assay also suffers from possible variations of lignin absorbancy as described above (12, 28).

Determination of $^{14}$CO$_2$ generally provides an underestimation of lignin degradation since it does not account for incorporation of $^{14}$C into the cell mass and water-soluble compounds. In this case, however, we could not detect any
radioactivity in the culture medium after incubation of cultures.

Evolution rates for \(^{14}\text{C} \text{CO}_2\) from \(^{14}\text{C}\)-labeled lignin wood cultures seem to depend strongly on the labeling and extraction procedures. Depending on whether L-[\(^{14}\text{C}\)]phenylalanine or [\(^{14}\text{C}\)]ferulic acid is used, \(^{14}\text{C}\) will be incorporated in guaiacyl, syringyl, and \(p\)-coumaric units or in guaiacyl and syringyl units only. It has been shown that the degree of incorporation in poplar tissue culture of \(^{14}\text{C}\) in guaiacyl and syringyl subunits of lignin depends on the photoperiod and stage of development of the tissue (16). Labeling may also be cytologically heterogeneous depending on whether lignifying cells are depositing the middle lamella, the primary layer, or the secondary layers (14). Recently, Phelan et al. (24) briefly described the effects of labeling conditions on lignin biodegradation rates (Crawford, personal communication; 14).

Still, a high specific activity of labeled wood (31,064 dpm/mg) and very good labeling in lignin were obtained with poplar (Populus euramericana Dode cultivar I214). Reid (26) reported similar results, using trembling aspen (Populus tremuloides sp.). These results show that plant species belonging to the Populus genus seem very appropriate for preparation of \(^{14}\text{C}\)-labeled lignin wood, probably because they are fast-growing, well-lignified plants.

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LITERATURE CITED