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Mineralization of Sulfonated Azo Dyes and Sulfanilic Acid by *Phanerochaete chrysosporium* and *Streptomyces chromofuscus*†

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Five ^{14}C-radiolabeled azo dyes and sulfanilic acid were synthesized and used to examine the relationship between dye substitution patterns and biodegradability (mineralization to CO_2) by a white-rot fungus and an actinomycete. 4-Amino-[U-^{14}C]benzenesulfonic acid and 4-(3-sulfo-4-aminophenylazo)-[U-^{14}C]benzenesulfonic acid were used as representative compounds having sulfo groups or both sulfo and azo groups. Such compounds are not known to be present in the biosphere as natural products. The introduction of lignin-like fragments into the molecules of 4-amino-[U-^{14}C]benzenesulfonic acid and 4-(3-sulfo-4-aminophenylazo)-[U-^{14}C]benzenesulfonic acid by coupling reactions with guaiacol (2-methoxyphenol) resulted in the formation of the dyes 4-(3-methoxy-4-hydroxyphenylazo)-[U-^{14}C]benzenesulfonic acid and 4-(2-sulfo-3′-methoxy-4′-hydroxy-azobenzene-4-azo)-[U-^{14}C]benzenesulfonic acid, respectively. The synthesis of acid azo dyes 4-(2-hydroxy-1-naphthylazo)-[U-^{14}C]benzenesulfonic acid and 4-(4-hydroxy-1-naphthylazo)-[U-^{14}C]benzenesulfonic acid also allowed the abilities of these microorganisms to mineralize these commercially important compounds to be evaluated. *Phanerochaete chrysosporium* mineralized all of the sulfonated azo dyes, and the substitution pattern did not significantly influence the susceptibility of the dyes to degradation. In contrast, *Streptomyces chromofuscus* was unable to mineralize aromatics with sulfo groups and both sulfo and azo groups. However, it mediated the mineralization of modified dyes containing lignin-like substitution patterns. This work showed that lignocellulolytic fungi and bacteria can be used for the biodegradation of anionic azo dyes, which thus far have been considered among the xenobiotic compounds most resistant to biodegradation. Very specific structural changes in the azo dye molecules enhanced their biodegradability.

Azo dyes, the largest class of commercially produced dyes, are not readily degraded by microorganisms (34, 59). Acid azo dyes are characterized by the presence of a chromophoric azo group whose nitrogen atoms are linked to sp^2-hybridized carbon atoms of the aromatic ring, which in addition may carry a sulfonic acid group(s). These compounds are used for dyeing and printing natural and synthetic fibers, leather, furs, and paper and for other coloring purposes (26). Sulfo and azo groups are not naturally occurring, so sulfonated azo dyes are recalcitrant to oxidative biodegradation.

Biodegradation researchers have become increasingly interested in the versatile lignin-degrading white-rot fungus *Phanerochaete chrysosporium*. This fungus is able to mineralize, at least partially and sometimes completely, a variety of persistent environmental pollutants, including DDT [1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane]; 3,4,3′,4′-tetrachlorobiphenyl; 2,4,5,2′,4′,5′-hexachlorobiphenyl; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; lindane (1,2,3,4,5,6-hexachlorocyclohexane); 3,4-dichloroaniline; and dieldrin (9, 12, 36). In experiments with typical incubations of 30 days, the amount of mineralization of these compounds to CO_2 has been reported to vary from 1 to 15%. In all cases, a majority of the starting compound, if not mineralized, was transformed. For example, in an experiment in which 4% of an initial DDT substrate was mineralized by *P. chrysosporium*, more than 75% of the starting compound disappeared from the medium (10). This study also showed that the pathway of DDT degradation by *P. chrysosporium* differed from that described for bacteria. Additional work showed that lignin peroxidases were involved in DDT catabolism by *P. chrysosporium* (23).

Experiments with pyrene showed that pyrene-1,6-dione and pyrene-1,8-dione are the major products of pyrene oxidation by *P. chrysosporium* (25, 49). The quinone oxygen introduced into the pyrene molecule came from water, and a role for the extracellular ligninase of the fungus in the degradation of benzo[*a*]pyrene was confirmed. *P. chrysosporium* rapidly oxidized benzo[*a*]pyrene to CO_2, using its ligninolytic enzymes to mediate oxidation via an aryl cation radical mechanism. The oxidation of pyrene was enhanced in the presence of 3,4-dimethoxy benzyl alcohol, a fungal secondary metabolite.

P. chrysosporium degraded (decolorized) triphenylmethane dyes, including crystal violet (*N*,*N*,*N*′,*N*′,*N*″,*N*″-hexamethylpararosaniline), pararosaniline, cresol red, bromophenol blue, ethyl violet, malachite green, and brilliant green (11). Three metabolites of crystal violet were identified. The three compounds *N*,*N*,*N*′,*N*′,*N*″-pentamethylpararosaniline, *N*,*N*,*N*′,*N*′-tetramethylpararosaniline, and *N*,*N*′,*N*″-trimetpararosaniline were formed by sequential N demethylations of the parent compound. From this evidence, it was suggested that fungal ligninase may catalyze N-demethylation reactions. However, since a nitrogen-rich medium was used, crystal violet degradation by *P. chrysosporium* may have involved a nonligninolytic enzyme system. The ligninolytic system of this fungus is repressed in media containing significant amounts of nitrogen (29).

Oxidative dechlorinations catalyzed by lignin peroxidase

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were first postulated by Hammel and Tardone (24). They performed several experiments at pH 3, at which chlorinated phenols are insoluble, by emulsifying the substrates with Tween 80. On the basis of this research, they proposed a mechanism for 2,4,6-trichlorophenol oxidation by fungal lignin peroxidase, and they observed the dechlorination of pentachlorophenol by crude fungal ligninase preparations. Others have had difficulty reproducing these results because of the poor solubility of such substrates at pH 3, the near-optimal pH for fungal ligninase activity (43). Recently, Vali and Gold (55) proposed a pathway for the degradation of 2,4-dichlorophenol by *P. chrysosporium*. The suggested pathway involved the oxidative dechlorination of 2,4-dichlorophenol to yield 1,2,3,4-terahydroxybenzene, which was then cleaved to produce, after subsequent oxidation, malonic acid.

Other toxic and hazardous compounds known to be degraded or partially degraded by *P. chrysosporium* (8) include aromatic, polycyclic aromatic, chlorinated aromatic, polycyclic chlorinated aromatic, and chlorinated nonaromatic compounds; native and modified biopolymers; the complex mixture of polycyclic aromatic hydrocarbons present in anthracene oil (7, 8); and 2,4,6,-trinitrotoluene (16). Arjmand and Sandermann (3, 4) demonstrated that *P. chrysosporium* will degrade not only pure lignin but also, simultaneously, chloroaniline bound to the lignin. The metabolites formed from wheat plant lignin containing bound 4-chloroaniline and 3,4-dichloroaniline were mineralized as readily as noncontaminated control lignins, suggesting that the ligninolytic system of this fungus recognized and degraded both natural lignins and those modified by incorporation of xenobiotic molecules.

Haider and Martin (21) investigated the degradation of humic acid polymers by *P. chrysosporium*. Turnover times in soil for humic acids are estimated to range from hundreds to thousands of years (53). Haider and Martin, however, found that *P. chrysosporium* degraded humic acids as well as xenobiotic molecules bound to humic acids and that the favored culture conditions for degradation were similar to those required for lignin degradation. In recent experiments with *P. chrysosporium* in vivo (35), manganese peroxidase has been shown to play a major role in the initial breakdown and decolorization of high-molecular-weight chlorolignin in bleach plant effluents.

P. chrysosporium may also be able to transform other naturally occurring polymers such as lignite and subbituminous coals (58). Incubation of a water-soluble fraction of subbituminous or lignite coal with a partly purified preparation of ligninase and manganese peroxidase resulted in substantial depolymerization of the coal, although no releases of monomeric compounds were observed.

A recent report by Cripps et al. (15) has added an entirely new family of dyes to the long list of organic compounds attacked by *P. chrysosporium*. The dyes include Azure B [3-(dimethylamino)-7-(methylamino)phenothiazin-5-ium chloride], Tropaeoline O, {4-[(2,4-dihydroxyphenyl)azo]benzenesulfonic acid}, Orange II {4-[(2-hydroxy-1-naphthyl) azo]benzenesulfonic acid}, and Congo Red {3,3'-[[1,1'biphenyl]-4,4'diylbis-(azo)]bis[4-amino-1-naphthalenesulfonic acid]}.

The genus *Streptomyces* has been reported to degrade benzene derivatives via classic aromatic catabolism pathways (2, 54). Little research has been devoted to determining whether actinomycetes efficiently degrade condensed polycyclic aromatics, although some strains are able to metabolize naphthalene derivatives (14). *Streptomyces* spp. degrade some recalcitrant compounds such as carbamates (18, 51), diazinon (20), and bromoxylin (32). Actinomycetes were recently shown to degrade organochlorine compounds in spent sulfite bleach plant effluent (57). Recently, a correlation between the abilities of streptomycetes to decolorize anthron-type dyes and the degradation of lignocellulose has been examined (39). Some evidence indicates that streptomycetes can act synergistically with other soil microorganisms to degrade recalcitrant compounds (20, 32).

We have shown that the susceptibility of azo dyes to degradation by *P. chrysosporium* and *Streptomyces* species can be increased by attaching a naturally occurring guaiacyl structure found in lignin (47). We have also shown that both organisms are able to degrade azo dyes in concentrations of up to 300 ppm for *P. chrysosporium* (46) and 50 ppm for *Streptomyces* spp. (41) and that degradability depends on the substitution pattern of the aromatic ring. Pasti et al. (40) showed that lignocellulolytic *Streptomyces* spp. are also able to partly mineralize azo dyes.

Paszczynski and Crawford (44) found that veratryl alcohol was involved in the oxidation of azo dyes by lignin peroxidase. Lignin peroxidase compound I oxidized azo dyes. Lignin peroxidase compound II was formed and then reduced by veratryl alcohol to help complete the catalytic cycle of the enzyme.

In this work, we examined the degradability of sulfanilic acid and aromatic azo dyes with different substitution patterns on their benzene or naphthalene rings. *P. chrysosporium* at least partly mineralized each of the compounds examined. Derivatives with lignin-like substitution patterns were mineralized by *Streptomyces chromofuscus*. Dyes with naphthalene rings were also degraded, and those with hydroxyl and azo groups in the 1,2 position were degraded rapidly by the fungus. In contrast, naphthalene dyes with the hydroxyl group in the *para* position relative to the azo linkage were degraded by *S. chromofuscus*. We show that azo dyes are not only decolorized but also mineralized to CO_2.

MATERIALS AND METHODS

Organism and culture conditions. *P. chrysosporium* Burds BKM-1667 (ATCC 24725) was used in these experiments. The fungus was grown in 125-ml flasks containing 25 ml of defined medium (31, 47). Filter-sterilized concentrated stock solutions were used to prepare the final medium. The mineral salt stock solution contained 10 g of L-asparagine, 5 g of NH_4NO_3, 20 g of KH_2PO_4, 5 g of $MgSO_4 \cdot 7H_2O$, 1 g of $CaCl_2 \cdot 2H_2O$, 0.05 g of thiamine, 100 ml of trace elements (6), and water to 1,000 ml. The other stock solutions consisted of 20% glucose, 1 M sodium 2,2'-dimethylsuccinate (pH 4.5), and 0.75 g of phenylalanine and 0.275 g of adenine in 1 liter of distilled water.

The final culture medium was prepared by mixing 10 ml of mineral salt stock solution, 50 ml of glucose solution, 10 ml of 1 M sodium dimethylsuccinate, 100 ml of phenylalanine-adenine solution, 15 ml of spore suspension containing 2×10^8 spores per ml, and 815 ml of sterile water. This medium was used for mineralization experiments.

S. chromofuscus A11 (ATCC 55184) was selected from 20 strains isolated from higher termites in Kenya (38) because of its ability to decolorize modified azo dyes 3 and 19 (Fig. 1) (47). The streptomycetes were grown in 250-ml flasks containing 25 ml of medium. The medium contained, per liter, 20 mM Tris-HCl buffer (pH 7.6), 2 g of D-glucose, 1 g of vitamin-free Casamino Acids (Difco), 0.1 mg of thiamine,

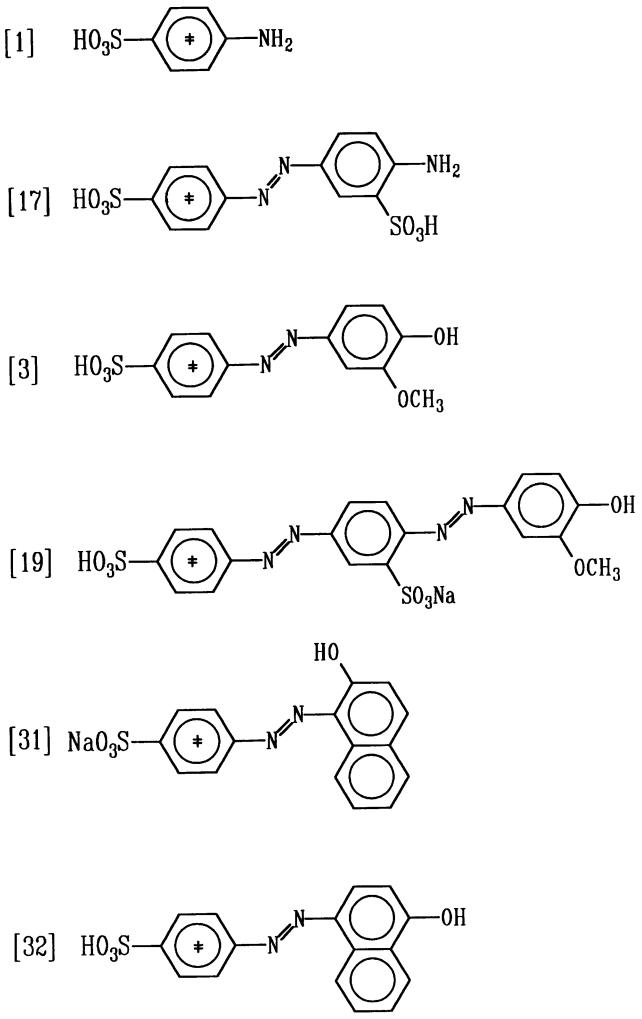

FIG. 1. Structures of radiolabeled compounds used during these investigations. 1, [U-^{14}C]sulfanilic acid; 3, 4-(3-methoxy-4-hydroxyphenylazo)-[U-^{14}C]benzenesulfonic acid; 17, 4-(3-sulfo-4-aminophenylazo)-[U-^{14}C]benzenesulfonic acid; 19, 4-(2-sulfo-3′-methoxy-4-hydroxyazobenzene-4-azo)-[U-^{14}C]benzenesulfonic acid monosodium salt; 31, 4-(2-hydroxynaphthylazo)-[U-^{14}C]benzenesulfonic acid sodium salt; 32, 4-(4-hydroxynaphthylazo)-[U-^{14}C]benzenesulfonic acid. ‡, aromatic rings uniformly labeled with ^{14}C.

and 0.1 mg of biotin. Glucose and vitamins were filter sterilized and added to the autoclaved medium. The stock solutions of dyes (5 mg/ml) were filter sterilized by passage through a 0.2-μm-pore-size Acrodisc (Gelman Science, Ann Arbor, Mich.) and added to the medium.

Measurement of the mineralization of ^{14}C-labeled dyes. Agitated cultures containing 100,000 to 150,000 dpm of azo dye at a concentration of 200 mg/liter for *P. chrysosporium* and 50 mg/liter for *S. chromofuscus* were incubated at 37°C for 20 days with shaking at 200 rpm. For each mineralization experiment, six replicates per culture were run for *P. chrysosporium* and three replicates per culture were run for *S. chromofuscus*. Each culture flask contained a CO_2 trap consisting of a small glass cup connected to the bottom of the rubber stopper. The cup contained 1 ml of 1 N NaOH (22). Samples were taken every 2 days, and after each sampling, cultures were flushed with 100% oxygen for 30 s. At the sampling time, the stopper was removed, the NaOH containing trapped CO_2 was transferred to a 20-ml scintillation vial, and the cup was then rinsed twice with 1 ml of water. Two milliliters of rinse water was also transferred to the vial. Finally, 10 ml of Ecolite scintillation cocktail (ICN Biochemicals Inc., Irvine, Calif.) was added to each vial. After solutions were mixed, vials were stored in the dark for 24 h. The counts per minute of trapped radioactivity were then determined in a Packard Tri-Carb liquid scintillation analyzer, model 1500, by using the ^{14}C protocol. Counts per minute were converted to disintegrations per minute by using an efficiency plot for known ^{14}C quench standards and SIS (spectral index of the sample) numbers.

Measurement of organic volatile compounds. Either a charcoal-containing polyurethane sponge (Bio-Rad Laboratories, Richmond, Calif.) or 2-methoxyethanol was used for trapping volatile organic compounds that might be released during mineralization experiments. The sponge (1 cm^3) replaced the 1 N NaOH solution in the small glass cap attached to the rubber stopper; other conditions were as described above for the $^{14}CO_2$ experiments. For counting, the charcoal sponges were dissolved in 1 ml of hyamine hydroxide contained in scintillation vials (1 M solution in methanol; Rohm and Hass, Inc.; distributed by NEN Research Products, Boston, Mass.) held at 55°C for 2 h. Ecolite scintillation cocktail (10 ml) was then added, and the counts per minute were measured (48).

In the second procedure, cultures with CO_2 traps were flushed with oxygen for 10 min, and the organic volatiles present were trapped in 10 ml of 2-methoxyethanol mixed with 10 ml of Ecolite as described elsewhere (1).

Radioactivity in the medium and biomass. The removal of radioactivity from the medium was determined by measuring the amount of ^{14}C label in 0.1 ml of the medium at time 0 and 21 days for each culture. Uninoculated cultures were used as evaporation controls. At the end of each mineralization experiment, culture broths were acidified with 5 ml of 1 M H_3PO_4, fresh 1 N NaOH was added to the trap, and the cultures were shaken overnight. No additional releases of $^{14}CO_2$ were observed. The radioactivity assimilated by the cells was measured by solubilizing biomass harvested from each 21-day-old culture in an equal volume of hyamine hydroxide solution. Before solubilization, cells were washed three times with 50 ml of distilled water to remove adsorbed dyes. Ecolite (10 ml) was added to the mixture, which was vortexed vigorously. To increase counting efficiency, the solution of biomass-hyamine-scintillation cocktail was further diluted 10 to 20 times with fresh Ecolite before counting.

Chemicals. [U-^{14}C]aniline-hemisulfate and 2,2′-dimethylsuccinic acid were purchased from Sigma Chemical Co. (St. Louis, Mo.). Aniline, guaiacol, 1-naphthol, 2-naphthol, *p*-toluenesulfonyl chloride, and 2-methoxyethanol of the highest purity available were purchased from Aldrich Chemical Co. (Milwaukee, Wis.).

The following radiolabeled compounds were prepared in our laboratory as described below: [U-^{14}C]sulfanilic acid, 4-sulfobenzene diazonium betaine, 4-[4-(*p*-tolylsulfamino)phenyl azo]-[U-^{14}C]benzenesulfonic acid sodium salt, 4-(4-aminophenylazo)-[U-^{14}C]benzenesulfonic acid, *p*-toluenesulfonanilide, and five dyes: 4-(3-methoxy-4-hydroxyphenylazo)-[U-^{14}C]benzenesulfonic acid, 4-(3-sulfo-4-aminophenylazo)-[U-^{14}C]benzenesulfonic acid (Acid Yellow 9; Colour Index [C.I.] 13015), 4-(2-sulfo-3′-methoxy-4′-hydroxyazobenzene-4-azo)-[U-^{14}C] benzenesulfonic acid, 4-(2-hydroxynaphthylazo)-[U-^{14}C] benzenesulfonic acid (Orange II [C.I. 15510]), and 4-(4-hydroxy-

naphthylazo)-[U-^{14}C]benzenesulfonic acid (Orange I [C.I. 14600]).

Synthesis and structure of ^{14}C-labeled azo dyes. Figure 1 shows the structures of the compounds used in the degradation experiments. The bracketed numbers are used as abbreviations for the names of the compounds throughout this paper. All ^{14}C-labeled dyes were of 99% or greater radiochemical purity as determined by high-pressure liquid chromatography and/or thin-layer chromatography coupled with counting of the radioactivity associated with dye peaks.

Preparative procedures. (i) [U-^{14}C]sulfanilic acid (Fig. 1, structure 1). [U-^{14}C]aniline (0.93 g, 10 mmol, 25 μCi/mmol) was sulfonated as described elsewhere (p. 912 in reference 56) except that the product was not recrystallized. The crude product was dissolved in 1% sodium hydroxide solution, treated with decolorizing carbon, filtered, and precipitated with dilute hydrochloric acid. The product was colorless crystals (1.40 g, 8.1 mmol), the specific activity was 3.2×10^5 dpm/mg, and the yield was 81%.

(ii) [U-^{14}C]4-sulfobenzene diazonium-betaine. [U-^{14}C]sulfanilic acid (0.17 g, 1 mmol, 25 μCi) was diazotized under standard conditions (p. 951 in reference 56), and the crystalline suspension was used immediately in a coupling reaction.

(iii) 4-(3-Methoxy-4-hydroxyphenylazo)-[U-^{14}C]benzenesulfonic acid (Fig. 1, structure 3). 4-Sulfobenzenediazonium betaine (prepared from 1 mmol of [U-^{14}C]sulfanilic acid, 3.2×10^5 dpm/mg) was coupled with guaiacol according to a procedure given elsewhere (28). The product was metallic-green needles (mono hydrate; 0.30 g, 0.92 mmol, 23 μCi), the specific activity was 1.7×10^5 dpm/mg, and the yield was 92%.

(iv) 4-(4-Hydroxynaphthylazo)-[U-^{14}C]benzenesulfonic acid (Fig. 1, structure 32). 1-Naphthol (0.15 g, 1.05 mmol) was coupled with 4-sulfobenzenediazonium betaine prepared from 1 mmol of sulfanilic acid in an ethanol-water solution according to a method given in reference 52. The crude product (0.29 g, 88%) was purified by recrystallization from water and an ethanol-acetone mixture. The product was black-purple needles (0.20 g, 0.61 mmol, 15.2 μCi), the specific activity was 1.7×10^5 dpm/mg, and the yield was 61%.

(v) 4-(2-Hydroxynaphthylazo)-[U-^{14}C]benzenesulfonic acid sodium salt (Fig. 1, structure 31). 2-Naphthol (0.15 g, 1.05 mmol) was coupled in an alkaline medium with a water suspension of 4-sulfobenzenediazonium betaine prepared from 1.0 mmol of [U-^{14}C]sulfanilic acid as described previously (p. 950 in reference 56). The product was orange leaflets (dihydrate; 0.30 g, 0.78 mmol, 19.3 μCi), the specific activity was 1.4×10^5 dpm/mg, and the yield was 78%.

(vi) *p*-Toluenesulfonanilide. Aniline (5 g, 54 mmol) was reacted with *p*-toluenesulfochloride (15 g, 78 mmol) in 10% sodium hydroxide solution under standard conditions (p. 1275 in reference 56). The product (8.9 g, 36 mmol) was twice recrystallized from ethanol, the melting point was 102 to 103°C, and the yield was 67%.

(vii) 4-[4-(*p*-Tolylsulfamino)phenylazo]-[U-^{14}C]benezenesulfonic acid sodium salt. *p*-Toluenesulfonanilide (0.5 g, 2 mmol) was dissolved in 5% sodium hydroxide (1.6 ml, 2.1 mmol), ice (3 g) was added, and a suspension of [U-^{14}C]4-sulfobenzenediazonium betaine (prepared from 2.0 mmol of [U-^{14}C]sulfanilic acid) was added over a period of 5 min while the reaction mixture was mechanically stirred in a salt-ice bath. After 4 h, the yellow crystalline reaction mixture was filtered off, and the product was washed on the filter with ice-cold water and air dried. The crude product (0.75 g; yield, 83%) was taken to the next step.

(viii) 4-(4-Aminophenylazo)-[U-^{14}C]benzenesulfonic acid. 4-[4-(*p*-Tolylsulfamino) phenylazo]-[U-^{14}C]benzenesulfonic acid (0.75 g, 1.6 mmol, 40 μCi) was dissolved in 90% sulfuric acid (2 ml, 3.6 g, 33 mmol), warmed to 40°C, and kept at this temperature for 4 h. The reaction mixture was poured onto ice, and the precipitated product was filtered, washed with water, and dried at room temperature. The product was cherry red crystals (0.34 g; 1.2 mmol; 30 μCi; yield, 75%).

(ix) 4-(3-Sulfo-4-aminophenylazo)-[U-^{14}C]benzenesulfonic acid (Fig. 1, structure 17). 4-(4-Aminophenylazo)-[U-^{14}C] benzenesulfonic acid (0.34 g, 1.2 mmol, 30 μCi) was dissolved in fuming (20% SO_3) sulfuric acid (1.0 ml, 1.93 g, 20.6 mmol), warmed to 60°C, and kept at this temperature for 7 h. After being cooled to room temperature, the reaction mixture was treated with crushed ice (5 g), and the precipitated product was filtered off. The crude product was dissolved in 5% sodium hydrogen carbonate (2 to 2.5 ml) to reach approximately pH 4, and a small amount of undissolved solid material was filtered off and discarded. The filtrate was treated with concentrated hydrochloric acid (10 ml), and the purified product was filtered and washed with ethanol and ether. The product was metallic glittering dark-purple needles (0.32 g, 0.90 mmol, 22.5 μCi), the specific activity was 1.6×10^5 dpm/mg, and the yield was 75%.

(x) 4-(2-Sulfo-3′-methoxy-4′-hydroxyazobenzene-4-azo)-[U-^{14}C]benzenesulfonic acid monosodium salt (Fig. 1, structure 19). 4-(3-Sulfo-4-aminophenylazo)-[U-^{14}C]benzenesulfonic acid (0.18 g, 0.5 mmol, 12.5 μCi) was dissolved in 2% sodium hydroxide solution (2 ml). Sodium nitrite solution (1 N, 0.5 ml, 0.5 mmol) was added, and the mixture was poured onto ice (3 g) and treated with concentrated HCl (0.2 ml). The thick crystalline suspension was kept in an ice-water bath, with occasional mixing, for 15 min. Guaiacol (65 mg, 0.52 mmol) was dissolved in 2% sodium hydroxide solution (1 ml), and diazonium salt suspension was added portion-wise over 10 min while the reaction mixture was mechanically stirred in a salt-ice bath. The thick crystalline paste was kept at 0°C for 3 h. The reaction product was filtered off, washed with acetone, and dried. The product was yellow crystals (0.185 g, 0.36 mmol, 9.0 μCi), the specific activity was 1.1×10^5 dpm/mg, and the yield was 72%.

RESULTS AND DISCUSSION

Two lignin-degrading organisms, *P. chrysosporium* and *S. chromofuscus*, were chosen for this study of the mineralization of sulfonated azo dyes. *P. chrysosporium* is the most-studied white-rot fungus, and *S. chromofuscus* is a lignocellulolytic microorganism (42) belonging to an abundant soil actinomycete group. Actinomycetes, which are widely distributed in soil, composts, water, and other environments, are recognized primarily for their abilities to produce antibiotics (37). They include a wide range of mesophilic and thermophilic strains that decompose lignocellulosic plant residues, including agricultural and urban waste (13). Such residues are often contaminated with xenobiotic molecules (17). Actinomycetes can catalyze hydroxylations; O, N, and S oxidations; and O- and N-dealkylation reactions against various xenobiotic compounds (50). Bacterial cytochrome P-450 is believed to catalyze most of these reactions (5). Volatilization has been shown to represent a major route of substrate removal for certain pesticides during the composting processes (17).

The fungus demonstrated a greater ability than the actinomycete to mineralize the azo dyes we investigated. The ability of *S. chromofuscus* to mineralize certain of the dyes

TABLE 1. Percentage of radioactivity incorporated into CO_2 and biomass and removed from medium after 21 days of growth by *P. chrysosporium* and *S. chromofuscus*

Azo dye	% Radioactivity in:					
	P. chrysosporium			*S. chromofuscus*		
	Medium[a]	CO_2[b]	Cells	Medium	CO_2	Cells
3	31.7	25.7	5.2	19.0	3.6	4.6
17 (Acid Yellow 9)	33.0	26.9	2.3	0.0	0.0	0.0
19	28.0	23.2	3.6	17.9	1.3	3.9
31 (Orange II)	41.6	34.8	2.3	0.0	0.0	0.0
32 (Orange I)	29.6	19.7	4.0	22.0	1.1	7.0
Sulfanilic acid	25.0	17.2	1.3	0.0	0.0	0.0

[a] ^{14}C not accounted for as CO_2 or biomass was lost by absorption to cell material and could be removed by washing with distilled water.

[b] In addition to CO_2, a small amount (0.1 to 0.5%) of organic volatiles was detected.

was significant, since azo dyes are resistant to aerobic degradation by most other soil bacteria (33, 34, 59). About 19% of the radioactivity from guaiacol-substituted azo dye 3 was removed from the medium after 21 days of growth by *S. chromofuscus*. Of this amount, almost 4% of the ^{14}C was mineralized to CO_2, and 4.6% was assimilated by the cells of the actinomycete. Since our dyes were at least 99% radiochemically pure, the recovered ^{14}C in CO_2 and biomass must represent mineralized dye molecules. The remaining 9% of the radioactivity was accounted for by dye absorbed to the cells. Dyes 19 and 32 showed similar patterns of degradation, although mineralization was less efficient. *P. chrysosporium* assimilated similar ranges of degraded dyes and showed smaller amounts of absorption to cell mass, with the exception of sulfanilic acid (Table 1).

To detect the production of organic volatiles, we used two methods (1, 48). The use of polyurethane foam containing activated charcoal as a trap appeared to be less sensitive than the 2-methoxyethanol purging method. After solubilization, the foam released activated charcoal, which caused quench problems during the counting of radioactivity. By using 2-methoxyethanol as the trapping agent, we were able to detect small but measurable amounts of ^{14}C released by the cultures of *P. chrysosporium*.

For this work, we radiolabeled the most recalcitrant part of the dyes, the benzene ring that is attached directly to the azo linkage and has a sulfonic group in the *para* position. Since this moiety was common to all the investigated compounds, the results of the degradation and mineralization experiments were comparable. *S. chromofuscus* showed mineralization only when the lignin-like guaiacol structure was attached to the sulfonated aromatic ring. The results of the mineralization experiments with *P. chrysosporium* (Table 1) showed less of a pattern. Our observations therefore suggested that the bacterial enzymatic systems responsible for degradation are more selective of lignin-like structures than is the enzymatic system(s) of the fungus. That *P. chrysosporium* was able to transform all the synthesized radiolabeled dyes and the sulfanilic acid was not surprising, since this fungus can oxidize a very wide range of aromatic compounds (27, 45).

Mineralization experiments were conducted with both organisms to determine how structural variations in the second ring of the azo dyes affected degradation rates over a 21-day incubation period. We observed a progressive mineralization of each azo dye (Fig. 2 through 4). With *P.*

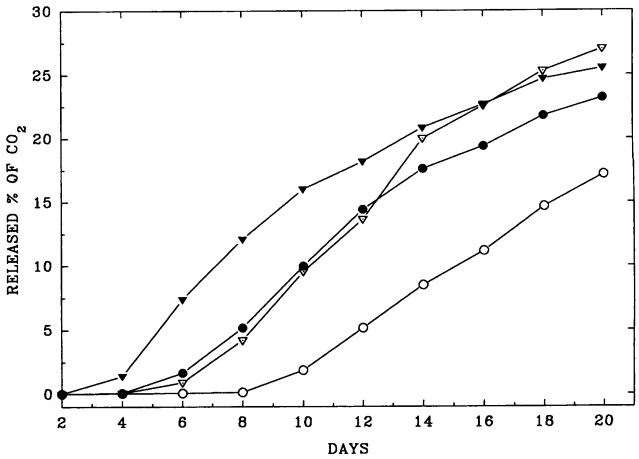

FIG. 2. Mineralization of sulfanilic acid and dyes 3, 17, and 19 by shaken cultures of *P. chrysosporium*. Cumulative releases of $^{14}CO_2$ from radiolabeled compounds were measured over a period of 20 days by using 25-ml cultures. Datum points are the means for six cultures. Symbols: ▽, dye 17; ▼, dye 3; ●, dye 19; ○, sulfanilic acid. The means of the datum points for day 20 are statistically different for sulfanilic acid but not for dyes 3, 17, and 19, as indicated by *t*-test results at the 0.05 level. Sigma Plot software, version 5.1, was used for graphical and statistical analysis.

chrysosporium, the degradation rate of dye 31 was 2.5 times greater than that of dye 32, and degradation began 2 days earlier. Dyes 3, 17, and 19 were mineralized to a similar extent, although the mineralization rate of dye 3 was twice as fast during the first 10 days of incubation. The streptomycetes mineralized only the guaiacol derivatives of dyes 3 and 19. A significant increase in the mineralization rate of dye 3 could be observed between days 6 and 9. The mineralization of sulfanilic acid (dye 1) (Fig. 2) began on the 10th day of the fungal culture and reached only 17%. During mineralization, a brown oxidation product accumulated at the beginning of degradation (14 days). This product was subsequently

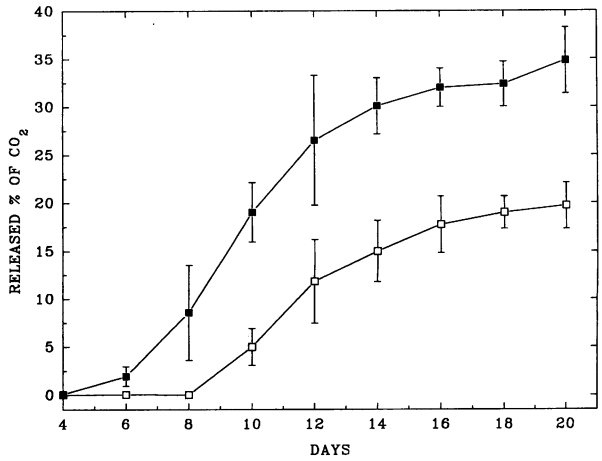

FIG. 3. Mineralization of dyes 31 and 32, which contain naphthalene, by shaken cultures of *P. chrysosporium*. Cumulative releases of $^{14}CO_2$ from labeled dyes were measured over a period of 20 days by using 25-ml cultures. Datum points are the means for six cultures. Symbols: ■, dye 31; □, dye 32. Error bars represent standard deviations of datum points.

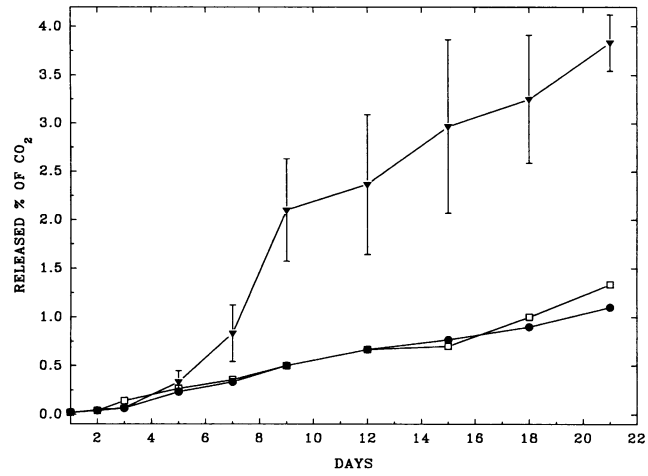

FIG. 4. Mineralization of dyes 3, 19, and 32 by shaken cultures of *S. chromofuscus*. Cumulative releases of $^{14}CO_2$ from labeled dyes were measured over a period of 21 days by using 25-ml cultures. Datum points are the means for three cultures. Symbols: ▼, dye 3; □, dye 32; ●, dye 19. Error bars represent standard deviations of datum points.

bleached by the culture. Accumulation of this brown material, which was probably a polymerization product of the oxidative coupling of catechol or aminophenol derivatives, was postulated by others (33) for bacterial systems.

Our results confirmed that both of the microorganisms investigated can mineralize sulfonated azo dyes, including naphthol derivatives, which make up the bulk of commercial dyes. The potential application of the powerful ligninolytic systems of these microorganisms is still not completely recognized. New structures that *P. chrysosporium* is able to degrade are continually being found. The degradation of methylene blue by a crude extracellular medium of *P. chrysosporium* was recently demonstrated (30), as was the aerobic biodegradation of the exotic dye Rose Bengal (tetrachloro-tetraiodo-fluorescein) (19).

Since actinomycetes and fungi are able to decolorize and mineralize azo dyes, it appears that such compounds might be used as assay compounds to isolate superior catabolic microbial strains from natural environments. Peroxidases seem to perform an essential role in azo dye transformations. More information, however, is needed to completely understand the mechanisms of azo dye oxidation by ligninolytic peroxidases, particularly those from the lesser-studied actinomycetes. The discovery of the synergistic effect of veratryl alcohol on the oxidation of azo dyes by fungal ligninase (44) resulted in part from investigations in this area.

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