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Microbial Degradation of Dibenzofuran, Fluorene, and Dibenzo-*p*-Dioxin by *Staphylococcus auriculans* DBF63

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***Staphylococcus auriculans* DBF63, which can grow on dibenzofuran (DBF) or fluorene (FN) as the sole source of carbon and energy, was isolated. Salicylic acid and gentisic acid accumulated in the culture broth of this strain when DBF was supplied as a growth substrate. Also, the formation of 9-fluorenol, 9-fluorenone, 4-hydroxy-9-fluorenone, and 1-hydroxy-9-fluorenone was demonstrated, and accumulation of 1,1a-dihydroxy-1-hydro-9-fluorenone was observed when this strain grew on FN. On the basis of these results, the degradation pathways of DBF and FN were proposed. The analogous oxidation products of dibenzo-*p*-dioxin were obtained by incubation with DBF-grown *S. auriculans* DBF63 cells.**

Polychlorinated derivatives of dibenzo-*p*-dioxin (DD) and dibenzofuran (DBF) are well known for their strong toxicity and mutagenicity. Contamination by these undesirable compounds has been one of the serious environmental problems, because these compounds are formed not only in the process of producing a variety of halogen-containing aromatics, such as herbicides, but also during combustion of dust or bleaching of pulp at paper mills. Microbial degradations of DD, DBF, and related compounds have been studied by several groups (1, 2, 4, 9, 10), but in those cases, oxidations were performed cometabolically by bacteria which can grow on other aromatic compounds.

Recently, some bacterial strains which can utilize DBF as the sole source of carbon and energy have been isolated. *Pseudomonas* sp. strain HH69 isolated by Fortnagel et al. (5–8), and *Brevibacterium* sp. strain DPO1361 isolated by Strubel et al. (13, 14) both converted DBF to 2,2',3-trihydroxybiphenyl and salicyclic acid. They proved the presence of novel dioxygenation at the 4,4a position of DBF (3).

Contamination by fluorene (FN) and other polycyclic aromatic hydrocarbons is also a great environmental concern because of their toxic, mutagenic, or carcinogenic properties. But information on the microbial degradation of polycyclic aromatic hydrocarbons is rare (15), and the catabolic pathways of these compounds have been completely unknown.

In this paper, the isolation and characterization of a gram-positive bacterial strain, *Staphylococcus auriculans* DBF63, which can grow on either DBF or FN as the sole source of carbon and energy, are described. Some catabolites from DBF and FN were isolated from culture broth prepared with this bacterium and were identified by the measurement of several physical and chemical properties.

The resting cell reaction of DD by this strain was also investigated. On the basis of these results, degradation pathways of DBF, FN, and DD were proposed.

MATERIALS AND METHODS

Organism. Strain DBF63 was isolated from soil in Japan on the basis of the ability to grow on DBF as the sole source of carbon and energy. The soil samples were collected from fields in various parts of the eastern area of Japan and were considered not to be contaminated by polycyclic aromatic hydrocarbons. Identification of this strain was carried out by the National Collections of Industrial Bacteria and Marine Bacteria, Torry Research Station, Aberdeen, Scotland.

Media and growth conditions. Minimal medium contained 2.2 g of Na_2HPO_4, 0.8 g of KH_2PO_4, 3.0 g of NH_4NO_3, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 2H_2O$, 0.01 g of $MgCl_2 \cdot 6H_2O$, 0.05 g of yeast extract, and 1 liter of deionized water. One gram per liter of DBF or FN was added as solids as a carbon source to the sterilized medium at inoculation. All cultivation was performed for 3 or 4 days at 30°C with rotary shaking.

Chemicals. DBF (Gold Label; +99% purity) and dibenzothiophenesulfone were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. 9-Fluorenone was obtained from Nacalai Tesque, Tokyo, Japan. Dibenzothiophene, FN, 9-fluorenol, and all other aromatic compounds except DD were purchased from Tokyo Kasei Kogyo, Tokyo, Japan. All of these chemicals were of the highest purity commercially available. DD was synthesized following the method described by Harms et al. (8). *N*-Methyl-*N*-trimethylsilyl trifluoroacetamide was purchased from Wako Pure Chemical Industries, Ltd., Tokyo, Japan.

Analytical methods. Each culture broth was centrifuged (6,000 × *g*, 10 min) to remove the residual organic substrate and the cells. Preparation of crude extract from the supernatant by using a Sep-Pak C_{18} (Waters, Millipore Corp., Milford, Mass.) cartridge and isolation of each of the metabolites by silica gel column chromatography were performed as described in our previous paper (11) with the solvents stated. Another purification procedure using thin-layer chromatography (TLC) was performed as follows; TLC was developed on precoated silica gel plate (1 mm thick; Whatman, Clifton, N.J.) with a solvent system of benzene–dioxane–acetic acid (90:25:4, by volume). The desired band was scraped off and eluted with methanol. The resulting solutions were centrifuged (14,000 × *g*, 1 min) to remove silica gel powder before evaporation and served for analytical experiments.

Gas chromatograms were made on a Hitachi 163 gas chromatograph by using a glass column (2 mm by 1 m) with silicon OV-17 (Gasukuro Kogyo, Tokyo, Japan). Determination of mass spectra, proton and carbon nuclear magnetic

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resonance (NMR) spectra, and TLC were carried out as described previously (11). For trimethylsilyl derivatization of metabolites for gas chromatography-mass spectrometry (GC-MS) analysis, 20 μl of *N*-methyl-*N*-trimethysilyltrifluoroacetamide was added to 1 μg of dried sample in a sample tube and incubated at 80°C for 30 min. UV spectra were determined on Shimadzu UV 160A. Methanol was used as a solvent.

Estimation of enzyme activities. Bacteria were harvested at the late logarithmic phase by centrifugation at 14,000 × *g* for 1 min at 4°C and washed twice with 50 mM phosphate buffer, pH 7.6. Cells were suspended in the same buffer and broken by sonication with a Kubota Insonator 201M, at 200 W for 15 min, and the extract was centrifuged at 4°C for 15 min at 14,000 × *g* before being used for enzyme assays. The protein concentration of extracts was measured by a protein assay kit (Bio-Rad, Richmond, Calif.) using bovine serum albumin as standard.

Catechol-2,3-dioxygenase (EC 1.13.11.2) activity was determined by photometrically measuring the formation of the products of *meta*-fission reactions (13). The wavelength monitored and molar extinction coefficient used to calculate the reaction rate for each of the substrates are as follows: 2-hydroxymuconic semialdehyde produced from catechol, 375 nm and 33,400; 2-hydroxy-6-keto-2,4-heptadienoate produced from 3-methylcatechol, 388 nm and 13,800; 2-hydroxy-5-methyl-muconic semialdehyde produced from 4-methylcatechol, 382 nm and 28,100; and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid produced from 3-phenylcatechol, 432 nm and 11,100. One unit of enzyme activity was defined as the amount of enzyme converting 1 mmol of substrate per min.

RESULTS

Isolation of bacteria. Twenty strains of DBF-utilizing bacteria were isolated from 592 soil samples. The color of culture broths turned deep yellow when these strains grew on DBF. One of these strains, named DBF63, was selected and served for further investigation. Strain DBF63 was identified as *S. auriculans* by the National Collections of Industrial Bacteria and Marine Bacteria, Torry Research Station, Aberdeen, Scotland.

Growth of strain DBF63 with other aromatic compounds. Strain DBF63 was able to grow well on DBF, FN, and anthracene but grew weakly on phenoxathiin and phenanthrene. Figure 1 shows the growth of strain DBF63 on DBF and FN. The cooxidation of various polycyclic aromatic compounds was also investigated. Dibenzothiophene, carbazole, and naphthalene were cooxidized in the presence of succinate or DBF as a carbon source, but no reaction or growth was observed with dibenzothiophene sulfone, phenazine, *o*,*o*'-biphenol, 2,3-benzofuran, and DD.

Identification of DBF degradation products. TLC of crude extract showed four major spots at R_f 0.63 (DBF-P1), 0.54 (DBF-P2), 0.50 (DBF-P3), and 0.33 (DBF-P4). Products were isolated by silica gel column chromatography and eluted with chloroform, and recrystallization gave a major quantity of product DBF-P2. DBF-P2 was obtained as colorless needles and identified as salicylic acid by mass, UV, and ^{1}H-NMR spectra. Product DBF-P4 was obtained from TLC and identified as gentisic acid by mass and UV spectra. Cultivation of strain DBF63 in minimal medium supplemented with 10 mM salicylate or gentisate (pH 7.0) was performed. Strain DBF63 grew well on gentisate, but no growth was observed on salicylate at this concentration.

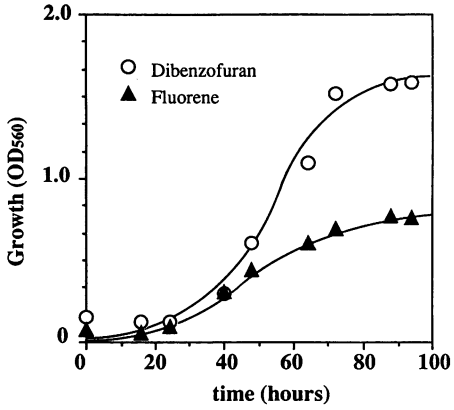

FIG. 1. Growth of *Staphylococcus auriculans* DBF63 on DBF and FN as the sole source of carbon and energy. OD_{560}, optical density at 560 nm.

Additional experiments showed that strain DBF63 could grow on salicylate at 1.0 mM or lower concentrations. Thus, salicylate is considered to be a metabolic intermediate of DBF degradation, which would undergo further degradation by this strain and could support its growth.

Identification of FN degradation products. TLC of crude extract showed five major spots at R_f 0.68 (FN-P1), 0.64 (FN-P2), 0.58 (FN-P3), 0.47 (FN-P4), and 0.34 (FN-P5) and several minor ones. Products FN-P1, FN-P2, FN-P3, and FN-P4 were isolated by silica gel column chromatography and eluted by benzene, and FN-P5 was obtained from TLC.

Product FN-P1 was obtained as yellow needles with a melting point of 81.5 to 82.5°C. GC-MS analysis showed that the molecular weight of FN-P1 is 180, and UV and mass spectra were superimposable with those of authentic 9-fluorenone. On the basis of these data, product FN-P1 was identified as 9-fluorenone.

Product FN-P3 was obtained as colorless crystals with a melting point of 153 to 157°C. GC-MS analysis of FN-P3 showed the parental ion at *m/z* = 182. UV, mass, and ^{1}H-NMR spectra were superimposable with those of authentic 9-fluorenol. These data identified FN-P3 as 9-fluorenol.

Product FN-P4 was an orange powder with a melting point of 249 to 251°C. The molecular weight of the TMS derivative of P4 was 268, indicating that the molecular weight of FN-P4 was 196 (268 − 72). ^{1}H-NMR and ^{13}C-NMR showed the presence of 7 protons and 13 carbons in the aromatic area, and no singlet was observed in ^{1}H-NMR spectra, showing there is no isolated proton. These results suggested that FN-P4 is a 1-hydroxy or 4-hydroxy derivative of 9-fluorenone. Product FN-P4 was identified as 4-hydroxy-9-fluorenone by comparing the UV spectrum of FN-P4 with that of authentic 4-hydroxy-9-fluorenone.

Product FN-P5 gave the largest spot on TLC, but it was difficult to isolate this metabolite stably. Conversion of FN-P5 into FN-P2 gradually occurred in the process of TLC development, elution, and evaporation. This reaction was promoted by acidification or heating, suggesting spontaneous dehydration of dihydrodiol. The parental ion of trimethylsilyl derivative of FN-P5 was observed at *m/z* = 358 on mass spectra, and the molecular weight of FN-P5 was supposed to be 214 [358 − (72 × 2)]. The UV spectrum of FN-P5 measured in methanol showed a characteristic maximum at 314 nm.

Product FN-P2 was obtained as bright yellow needles with a melting point of 110 to 120°C. GC-MS analysis showed that the molecular weight of FN-P2 is 196, and this compound was considered to be another monohydroxy derivative of 9-fluorenone. Because ^1H-^1H correlated spectroscopy (COSY) NMR of FN-P2 (Fig. 2A) showed that every proton except one of the hydroxyl group shows ortho coupling(s), proving the presence of no isolated proton on the aromatic rings, the position of the hydroxyl group should be 1 or 4, and according to the identification of FN-P4, product FN-P2 was found to be 1-hydroxy-9-fluorenone, which suggests that the chemical structure of predehydration product FN-P5 is 1-hydro-1,1a-dihydroxy-9-fluorenone or 1,2-dihydroxy-9-fluorenone. But the former is favored more because of two reasons: first, all amounts of FN-P5 converted into P2 with no formation of a 2-hydroxy derivative; second, the analogous 1,1a-dioxygenation of FN is possible according to the 4,4a-dioxygenation of DBF by DBF degraders reported by Fortnagel et al. (5–8) and Strubel et al. (13, 14). The UV spectrum of FN-P2 measured in methanol showed a characteristic maximum at 251 nm. The mass spectrum of P2 is shown in Fig. 2B.

The accumulation of FN degradation products was monitored by GC. After 168 h of cultivation, approximately 3 mM 9-fluorenol together with 9-fluorenone had accumulated and 0.4 mM 4-hydroxy-9-fluorenone and 0.1 mM 1-hydroxy-9-fluorenone were detected.

Oxidation of DD by DBF-grown DBF63 cells. DBF-grown DBF63 cells were prepared as follows. Culture broth of DBF63 was spread on plates of minimal medium and cultivated for 4 days at 30°C with DBF crystals on the lid. The plates were placed upside down so the bacteria could utilize the vapor of DBF and the cells could be collected without contamination by DBF crystals. Collected cells were washed once, suspended in fresh minimal medium, and incubated with 500 mg of DD per liter at 30°C for 3 days with rotary shaking.

The major metabilites DD-P1 (R_f, 0.72) and DD-P2 (R_f, 0.52) were isolated from TLC, but DD-P2 was converted to DD-P1 in the process of purification. DD-P1 was obtained as colorless needles with a melting point of 194 to 201°C. The UV spectrum of DD-P1 measured in methanol showed a characteristic maximum at 261.5 nm. Determination of the chemical structure of DD-P1 and DD-P2 is in progress.

Metapyrocatechase activities of the DBF- or Luria-Bertani medium-grown DBF63 cells. Metapyrocatechase activities (in milliunits per milligram of protein) of cell extract of DBF63 grown on DBF were estimated with the following substrates: catechol, 240 (14%); 3-methylcatechol, 518 (30%); 4-methylcatechol, 70 (4%); and 3-phenylcatechol, 1,722 (100%). Cell extract of Luria-Bertani medium-grown DBF63 cells showed no activity (<1 mU/g of protein) of metapyrocatechase for these catechols. These results suggest that the expression of DBF-oxidizing enzymes is inductive and that at least one *meta*-fission reaction is involved in the degradation of DBF by this strain.

Accumulation of catechol-type product in the presence of metapyrocatechase inhibitor. Strain DBF63 was grown in 1 liter of minimal medium supplemented with 1 g of DBF to mid-log phase, and 50 mg of pyrogallol was added to the culture. Pyrogallol is an established inhibitor of metapyrocatechase (12). Incubation was continued with rotary shaking in the dark for 4 h. The culture broth was centrifuged to remove cells and substrate residues, acidified to pH 2 with HCl, and extracted with ethylacetate. One catechol-type compound different from pyrogallol was obtained, and UV spectra of this metabolite measured in methanol showed the characteristic maximum at 284 nm, suggesting that this product was 2,2',3-trihydroxybiphenyl (13).

DISCUSSION

In the present study, the isolation and characterization of *S. auriculans* DBF63, which can utilize DBF and FN as the sole source of carbon and energy, were described. The chemical structures of some of the degradation products of DBF and FN were also determined, and the degradation pathways of these compounds by strain DBF63 were proposed. Strain DBF63 growing on DBF as the sole source of carbon and energy accumulated salicylic acid and gentisic acid. This strain accumulated large amounts of 2,2',3-trihydroxybiphenyl in the presence of the inhibitor of metapyrocatechase, pyrogallol. These results suggest that strain DBF63 has a degradation pathway analogous to those of the DBF degraders *Pseudomonas* sp. strain HH69 (5–8) and *Brevibacterium* sp. strain DPO1361 (13, 14); The degradation starts with 4,4a-dioxygenation of DBF, which is then followed by spontaneous fission of the hemiacetal bond, *meta* fission, and hydrolysis to produce salicylic acid (Fig. 3). Whether gentisic acid is formed by hydroxylation of salicylic acid or from other metabolites remains to be determined.

Strain DBF63 could grow on FN and accumulated 9-fluorenol, 9-fluorenone, 1-hydroxy-9-fluorenone, and 1-hydro-1,1a-dihydroxy-9-fluorenone. According to these results, this strain first oxygenates the 9th carbon by monooxygenase(s) to produce 9-fluorenol and 9-fluorenone, and then 1,1a-dioxygenation, analogous to 4,4a-dioxygenation of DBF, occurs, producing 1-hydro-1,1a-dihydroxy-9-fluorenone.

But this compound could not be metabolized further and accumulated in the medium because this metabolite does not have a hemiacetal bond and is chemically stable. This compound received abiotic dehydration and produced 1-hydroxy-9-fluorenone. Because this strain could utilize FN as the sole source of carbon and energy, another dioxygenation to produce catechol-type intermediate 3,4-dioxygenation might be possible because of the accumulation of 4-hydroxy-9-fluorenone; enzymatic rearomatization and spontaneous dehydration of 3,4-dihydrodiol of 9-fluorenone might occur competitively to produce 3,4-diol and 4-ol (and probably 3-ol—not isolated so far), and the former would be immediately metabolized and utilized for the growth. Further investigation is needed to determine how this bacterium metabolizes FN to utilize it as a carbon source.

This strain produced 9-fluorenol and 9-fluorenone from FN and could grow well on these compounds, accumulating the same metabolite, 1-hydro-1,1a-dihydroxy-9-fluorenone. But it remains unclear whether this 9-oxygenation of FN is necessary for this bacterium to utilize FN as a carbon source.

Although strain DBF63 could not grow on DD, DBF-grown DBF63 cells oxygenated this compound and produced dihydrodiol and monohydroxy derivatives (the former changed into the latter by acidification and heating). Degradation of DD by a DBF degrader, *Pseudomonas* sp. strain HH69, as performed by Harms et al. (8), and accumulation of the 2-phenyl derivative of muconic acid were reported. Because 1-hydro-1,1a-dihydroxy-DD is considered to be chemically unstable and spontaneous conversion into a catechol-type compound can occur, whereas DBF-grown DBF63 cells have high levels of metapyrocatechase activity

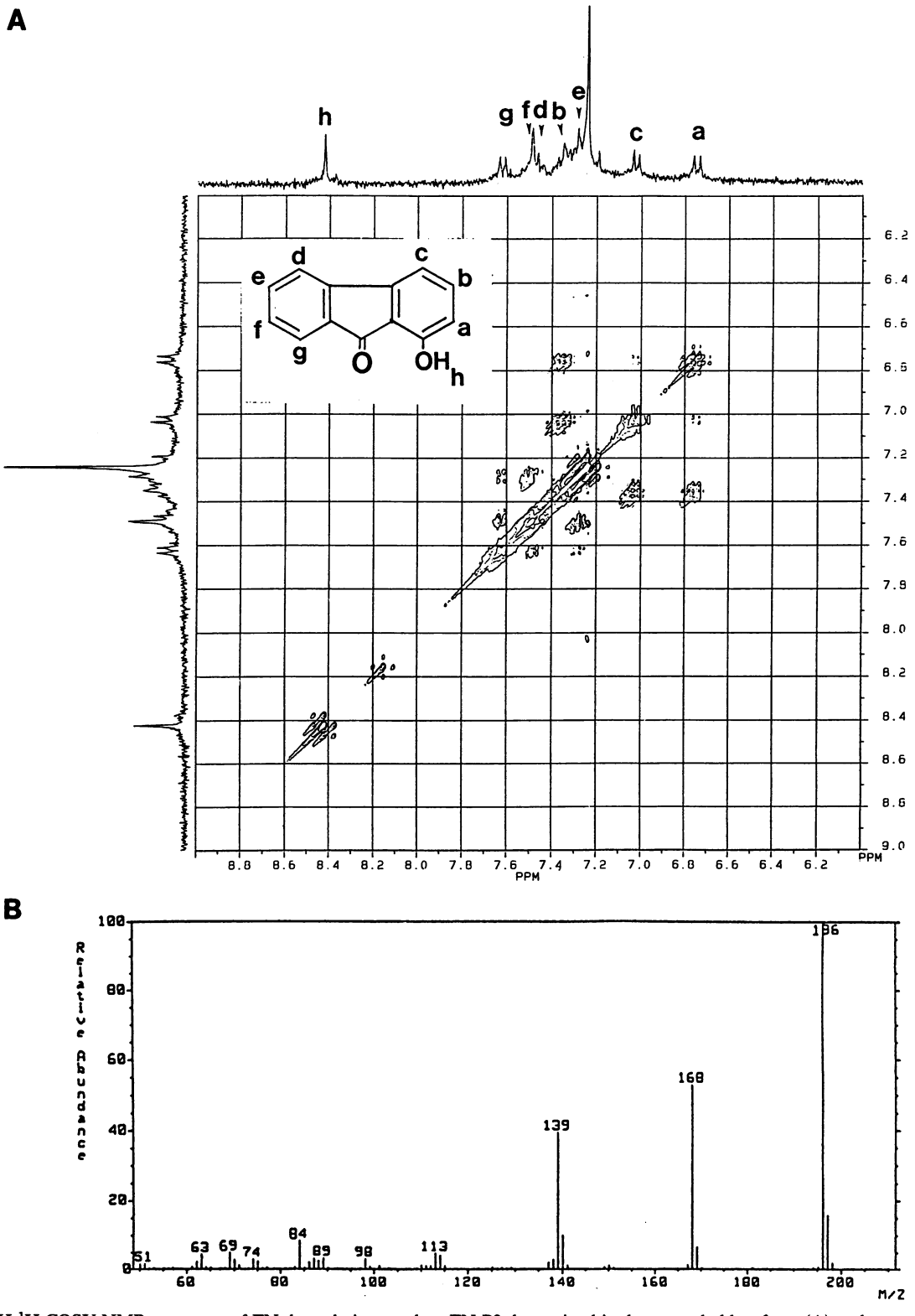

FIG. 2. ^{1}H-^{1}H COSY NMR spectrum of FN degradation product FN-P2 determined in deuterated chloroform (A) and mass spectrum of FN-P2 (B).

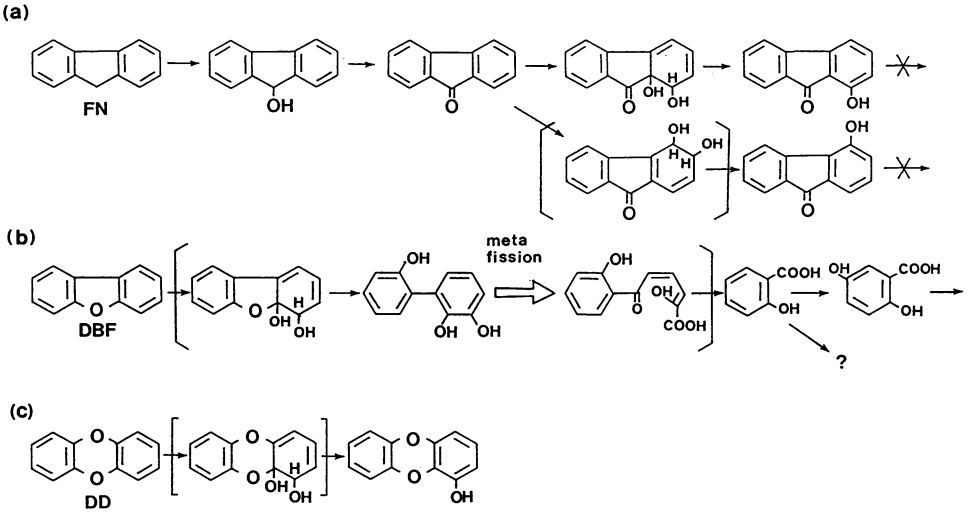

FIG. 3. Proposed pathways of degradation of FN (a), DBF (b), and DD (c) by *S. auriculans* DBF63.

for various catechols, further degradation of DD would be possible under more suitable conditions.

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