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Fungal Metabolism of *n*-Alkylbenzenes

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Isolates of *Paecilomyces*, *Verticillium*, *Beauveria*, and *Penicillium* species were tested for ability to metabolize a variety of *n*-alkylbenzenes. Minimum side chain lengths were required for metabolism of these substrates. These were C_4 for the *Paecilomyces* sp., C_8 for the *Verticillium* sp., and C_9 for the other two isolates. Growth on dodecylbenzene yielded benzoic and phenylacetic acids as transient intermediates, and these acids supported growth of the isolates.

Numerous studies have reported the ability of fungi to metabolize a variety of aromatic hydrocarbons (3, 5, 6, 8, 10, 17). Typically, metabolites are hydroxylated derivatives of the aromatic substrates. Transformations of alkyl-substituted aromatic compounds, such as methylnaphthalenes (7) and 7-methylbenz[a]anthracene (4), also have been reported. No evidence of ring cleavage was reported in any of these studies.

In this study, four *n*-alkane-degrading fungi isolated from the marine environment (11) were grown in the presence of *n*-alkylbenzenes to determine the effect of side chain length (C_1 to C_{12}) on substrate utilization. Metabolites of dodecylbenzene (DB) were identified in batch cultures.

Isolates of *Paecilomyces*, *Beauveria*, *Penicillium*, and *Verticillium* species were streaked onto six slants of basal marine agar containing N and P (11) and grown for 7 days at 28°C. Sterile 0.1-ml portions of hexylbenzene, octylbenzene, nonylbenzene, decylbenzene, and DB were then added to individual cultures. The sixth replicate of each culture received 0.3 ml of 970 mM sodium benzoate. Equal volumes of each substrate were added to sterile slants to serve as controls. After a further 28 days of incubation, each slant was extracted with 5 ml of *n*-pentane, and the amount of substrate remaining was determined by gas chromatography (GC).

Growth of these isolates on volatile substrates (benzene, toluene, ethylbenzene, butylbenzene, and hexylbenzene) was tested by using a well flask method (13). Dry weights of mycelium (2) were determined after 30 days of incubation.

The data in Table 1 summarize the percentage of substrate lost from slants due to fungal metabolism. The well flask experiments showed that the vapors from benzene and toluene inhibited the growth of all four isolates, and only the *Paecilomyces* sp. isolate could grow on butylbenzene and hexylbenzene. These results clearly show that there is a minimum *n*-alkyl side chain length required for the growth of these fungi. The *Paecilomyces* sp. will grow if a C_4 side chain is present, the *Verticillium* sp. requires a C_8 side chain, and the *Beauveria* sp. and *Penicillium* sp. require a C_9 side chain.

The metabolites of DB degradation were identified by growing the *Paecilomyces* sp. culture in 200 ml of a liquid basal marine medium (BMM) (11) containing 2 ml of NP solution (12) and 0.5 ml of sterile DB. This culture was incubated without shaking, and after 8 days it was acidified with 2 ml of 6 M HCl. The fungal mat was recovered by filtration (Whatman no. 1 paper) and washed with 25 ml of methylene chloride. The filtrate was extracted with methylene chloride, and the organic phase was extracted with 0.1 M NaOH to separate acidic from neutral compounds. Both the neutral and the acid extracts were analyzed by GC. Methyl esters of the carboxylic acids in the acid extract were formed (13) and these were analyzed by GC and GC-mass spectroscopy (GC-MS).

A DB-5 fused silica column (30 m by 0.25 mm) was used for the GC analyses (14) of the *n*-alkylbenzenes, culture extracts, and methylesters of the metabolites from DB. GC-MS analyses were performed using a 30-m DB-1 capillary column in a Varian Vista 6000 GC coupled to a VG7070E mass spectrometer and a VG11/250 data system. The effluent end of the GC column was inserted directly into the ion source of the MS. Scans were acquired at a rate of 1/s from mass 600 to 50 at resolution 1,000 at 70 eV.

These analyses showed that benzoic, phenylacetic, phenylpropionic, and cinnamic acids were produced during the growth of *Paecilomyces* sp. on DB. The GC retention times of authentic standards of the methylesters of these compounds matched those of the methylated compounds in the culture extract. No carboxylic acids with alkyl chain lengths greater than C_4 were detected. Since the side chain metabolism leads to the production of benzoic and phenylacetic acids from DB, it is likely that both α- and β-oxidations occurred, as was observed with *Nocardia salmonicolor* grown on DB (16). GC-MS analyses of the neutral fractions gave no evidence of ketones or secondary alcohols which would arise from subterminal oxidation of the alkyl side chain (1, 15).

Two other well-resolved peaks, with molecular ions of *m/z* 176 and fragments at 117 (base peak) and 91, were observed in the GC-MS chromatogram of the acidic fraction. These correspond to the methyl esters of isomers of 4-phenylbutenoic acid. Sariaslani et al. (16) have identified 4-phenylbut-3-enoic acid and tentatively identified 4-phenylbut-2-enoic acid in a culture supernatant from *N. salmonicolor* grown on DB.

Benzoic and phenylacetic acids were always the most abundant metabolites found in these fungal cultures. To monitor the changes in their concentrations as a function of time, each of the four isolates was inoculated into several flasks containing 200 ml of BMM, 2 ml of NP solution, and 0.2 ml of DB. At various incubation times a culture of each isolate was removed and analyzed by GC for DB metabolites, using sodium laurate as an internal standard.

The concentrations of DB, benzoic acid, and phenylacetic acid in cultures of *Paecilomyces* and *Penicillium* species are shown in Fig. 1a and b, respectively. The results obtained from the *Beauveria* and *Verticillium* sp. cultures were similar

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TABLE 1. Extent of substrate metabolism after fungal cultures had been incubated for 28 days in the presence of benzene derivatives[a]

Isolate	% of substrate metabolized					
	Benzoic acid	Hexyl-benzene	Octyl-benzene	Nonyl-benzene	Decyl-benzene	DB
Penicillium sp.	100	0	26	54	48	99
Beauveria sp.	100	0	0	90	75	99
Paecilomyces sp.	100	55	100	100	99	99
Verticillium sp.	100	0	100	100	100	98

[a] Amounts of substrate recovered from fungal cultures compared with amounts recovered from sterile controls.

TABLE 2. Dry weights of fungal mycelium from cultures grown in 30 ml of BMM and 30 ml of BMM supplemented with 200 mg of benzoic or phenylacetic acid

Isolate	Avg dry wt ± 1 SD (mg)[a]		
	Control (BMM)	Benzoic acid	Phenylacetic acid
Penicillium sp.	25.5 ± 2.5	95.6 ± 4.6	109 ± 15
Beauveria sp.	23.2 ± 1.8	37.6 ± 2.5	55.9 ± 24.5
Paecilomyces sp.	33.9 ± 3.6	146 ± 55	129 ± 44
Verticillium sp.	29.0 ± 4.1	73.2 ± 21.1	79.5 ± 4.5

[a] Three replicate cultures incubated for 14 days.

to those shown in Fig. 1a. Initially the *Paecilomyces* and *Penicillium* sp. cultures contained 4.5 and 4.0 mM DB, respectively. After a 3-day lag, the concentration dropped to near 0.3 mM and remained constant. In Fig. 1a, the phenylacetic and benzoic acid concentrations in the *Paecilomyces* sp. culture filtrate reached maximum levels on days 4 and 5, respectively, and then declined. On day 5 (Fig. 1a) the concentrations of the three compounds were 0.4 mM DB, 0.09 mM phenylacetic acid, and 0.11 mM benzoic acid. These values totalled 0.6 mM and indicated that concentrations of phenyl-substituted compounds had decreased to 15% of the initial concentration of DB. If only the *n*-alkyl side chain of DB was being metabolized, the total concentration of aromatic acid end products would have reached a value near 4.5 mM and remained constant. The transient accumulation of these aromatic acids and their subsequent decrease on incubation suggests that the *Paecilomyces* sp. isolate initially attacked the *n*-alkyl side chain but also metabolized the aromatic ring. The rates of metabolism of the aromatic acids were not as fast as the rate of metabolism of the *n*-alkyl side chain.

During the growth of the *Penicillium* sp. on DB (Fig. 1b), benzoic, phenylacetic, phenylpropionic, and cinnamic acids were detected in the medium. However, these did not accumulate in the same manner as was observed with the *Paecilomyces* sp. isolate (Fig. 1a), indicating that the aromatic moiety was metabolized at a rate similar to that of the *n*-alkyl side chain.

Each of the four isolates was tested for its ability to grow on benzoic and phenylacetic acids. Volumes of 30 ml of BMM plus 0.3 ml of NP solution were supplemented with 200 mg of each acid (as their sodium salts). Control flasks containing only BMM and NP solution were prepared for each isolate. Equal volumes of homogenized inocula were added to each flask and these were incubated for 14 days, after which dry weight of the mycelium was determined (Table 2). Based on the Student *t* test ($P < 0.05$), the cell yields of all four isolates were greater in media supplemented with benzoic or phenylacetic acid than in the control me-

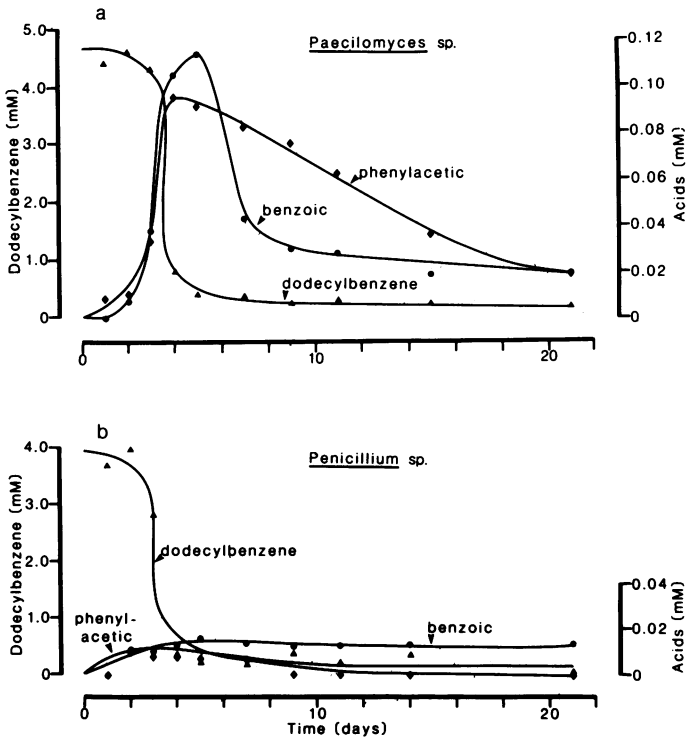

FIG. 1. Loss of DB and production of aromatic acids in liquid cultures of (a) *Paecilomyces* sp. and (b) *Penicillium* sp.

dium, thus directly confirming that the four fungi metabolize these acids.

Similar studies with *N. salmonicolor* (16) growing on DB have also demonstrated the transient appearance of aromatic acids. Our observations are in contrast to those of Davis and Raymond (9), who found that phenylacetic acid accumulated in cultures of several *Nocardia* sp. strains grown on DB.

In summary, this study has shown that the ability of four fungal isolates to metabolize *n*-alkylbenzenes is dependent upon the length of the alkyl portion. If the side chain is long enough, the *n*-alkyl portion is first attacked, yielding benzoic and phenylacetic acids as intermediates. These aromatic acids then also serve as growth substrates.

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