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Propachlor Degradation by a Soil Bacterial Community

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Soil from a pesticide disposal site was used to enrich for microorganisms that degraded the acylanilide herbicide propachlor (2-chloro-*N*-isopropylacetanilide). After seven transfers of the enrichment, the culture contained about six strains. The highest yield of microbial biomass occurred if just two of these isolates, strains DAK3 and MAB2, were inoculated into a mineral salts medium containing propachlor. When only strain DAK3 was grown on propachlor, a metabolite (2-chloro-*N*-isopropylacetamide) was released into the medium. Strain MAB2 could grow on this metabolite. The results of morphological and physiological tests suggest that strains DAK3 and MAB2 most closely resemble species belonging to the genera *Moraxella* and *Xanthobacter*, respectively. Strain DAK3 can respire and grow on N-substituted acylanilides containing methyl, ethyl, or isopropyl substitutions, but is incapable of respiration or growth on acetanilide, aniline, or the acylanilide herbicides alachlor and metolachlor. Strain DAK3 appears to use the aromatic C atoms of propachlor for growth, as suggested by the growth yield on propachlor and the induction of catechol 2,3-oxygenase activity in acylanilide-grown cells.

Acylanilide herbicides are used as preemergence herbicides for selective control of grass and broadleaf weeds. Structurally, these compounds are related to the phenylcarbamate and phenylurea pesticides in that all of these chemicals have aniline linked to a carbonyl group via an amide bond. The two most widely used acylanilide herbicides are alachlor [2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide] and metolachlor [2-chloro-2′-ethyl-6′-methyl-*N*-(1-methyl-2-methoxyethyl)-acetanilide]; 37×10^6 and 22×10^6 kg, respectively, are used annually in the United States (8).

Microbial degradation is the primary mechanism of acylanilide dissipation from soil (36). However, these herbicides vary in their degrees of biodegradability. Typically, microbial metabolism of acylanilide herbicides is initiated by aryl acylamidases (6, 9, 12, 21, 29, 35), which cleave the pesticides to form an organic acid and an aniline derivative. The organic acid is readily metabolized by the microorganism, while often the aromatic portion is not mineralized but can remain in the soil and become covalently bound to soil organic matter (3, 7, 23), raising concerns of environmental persistence.

Acylanilides with N-alkyl substitutions appear to be less susceptible to enzymatic cleavage at the amide bond. Aryl acylamidase cleavage of the N-alkyl acylanilides alachlor and metolachlor has not been reported as an important reaction mechanism (17, 24, 26, 31), and microbial strains which mineralize these molecules have not been isolated. Novick et al. (28) have reported that propachlor does undergo aryl acylamidase cleavage with production of an intermediate aromatic compound, *N*-isopropylaniline. In this paper, we describe the isolation of two microbial species which metabolize propachlor. The initial attack on this herbicide occurs not by the typical amidase cleavage mechanism but by a novel mechanism. Furthermore, the organism which initially catabolizes propachlor appears to use the aromatic C atoms of the molecule as a carbon and energy source.

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MATERIALS AND METHODS

Isolation of bacteria. We prepared batch culture enrichments in media containing propachlor at a concentration of 150 ppm (150 μg/ml) by using an inoculum from a pesticide disposal site near West Lafayette, Ind., where spray tanks were rinsed after herbicide application for a number of years. This site has had a history of exposure to a wide variety of xenobiotic compounds, including propachlor. The bacteria were grown by using XBM mineral salts medium, which contained 10 mM phosphate buffer (pH 7), 0.25 mM $MgSO_4$, 10 mM NH_4Cl, 5 μM $CaCl_2$, 15 μM $FeCl_3$, 23 μM disodium EDTA, and 25 μM sodium citrate. In addition, XBM medium contained 1 ml of trace element solution SL7 (5) per liter and 10 ml of a vitamin solution supplement (32) per liter. Cultures were incubated in a shaking water bath at 30°C. Growth was monitored by measuring the optical density in a 1-cm cell at 600 nm with a model 250 spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio). Transfers consisted of a 1:10 dilution into fresh medium when chemical analysis showed that there was complete loss of the herbicide.

Pure cultures were obtained by inoculating agar plates containing 150 ppm of propachlor as the sole carbon source or tryptic soy (TS) medium (Difco Laboratories, Detroit, Mich.). Isolates were characterized by using standard morphological and physiological criteria (11). Growth on organic substrates was tested at 30°C and at concentrations that provided 25 mmol of C per liter, unless indicated otherwise. Cell dry mass was determined by growing a culture to early stationary phase and then centrifuging it at $10,000 \times g$ for 10 min. The cells were washed in 10 mM phosphate buffer (pH 7) and recentrifuged, after which the cell material was dried at 105°C for 48 h before it was weighed. Proteins were analyzed by using the method of Lowry et al. (25), with bovine serum albumin as the standard; 1 M NaOH extracts of cells that were heated to 100°C for 10 min were used in this procedure.

Analytical methods. Acylanilides and the resulting metabolites were assayed by capillary gas chromatography, using a Supelco type SPB-1 column (30 m by 0.25 mm [inner diameter]) and a Varian model 3700 gas chromatograph

equipped with a flame ionization detector. The flow rate of N_2 carrier gas was 4 ml/min, and gas flow rates to the detector were 40 ml of N_2 (as makeup gas) per min, 34 ml of H_2 per min, and 300 ml of air per min. The injector and detector temperatures were 140 and 190°C, respectively. The column was operated at 70°C for 1 min, and then there was a temperature increase of 20°C/min up to a temperature of 190°C. Aqueous samples from the experimental cultures were extracted 1:1 with ethyl acetate, and 2-μl portions of the ethyl acetate extracts were injected into the column. Aqueous solutions of acylanilide standards were also extracted 1:1 with ethyl acetate in order to quantify the experimental amounts.

A gas chromatography-mass spectral analysis was performed by using a Hewlett-Packard model 5890 gas chromatograph and a 5970 Mass Selective Detector operated in a full scan acquisition mode with a Supelco type SPB-5 capillary column (30 m by 0.25 mm [inner diameter]). The helium carrier gas flow rate was 1 ml/min. The injector and detector interface temperatures were 170 and 280°C, respectively. The temperature program and extraction method were identical to the program and method used during the Varian gas chromatography analysis.

A high-pressure liquid chromatography (HPLC) analysis was performed by using a Varian model 5000 liquid chromatograph equipped with a Gilson Holochrome UV detector set at 210 nm. A 20-μl portion of a filtered culture solution was injected onto a Supelcosil LC-PAH column (Supelco) by using an isocratic mobile phase consisting of 40% acetonitrile in water at a flow rate of 0.5 ml/min.

Enzyme assays. Catechol oxygenase activities were measured by using cell extracts of log-phase cells. The cells were harvested by centrifugation, washed in 10 mM phosphate buffer (pH 7), and resuspended in the same buffer. The cells were broken by sonication (model W185 Sonifier cell disruptor; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). Six 30-s pulses were used, and the pulses were separated by 30-s intervals. Samples were kept in an ice water bath during the treatment. The extract was centrifuged at 25,000 × *g* for 15 min, and the supernatant was used for enzyme assays. All procedures prior to the enzyme assay were conducted at <5°C. Catechol 1,2-oxygenase and catechol 2,3-oxygenase activities were measured by performing a spectrophotometric assay of the products of these enzymes (13, 30).

Respirometry. Oxygen consumption by washed resting cell suspensions or suspensions of sonicated cells was measured by using a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio). A 1.6-ml portion of a cell suspension was incubated at 30°C, and the rate of endogenous respiration was monitored. The test substrate was then added to a final concentration of 1 mM, and the rate of oxygen consumption was monitored. The rates were corrected for endogenous respiration. In those cases in which the solubilities of the test substrates did not permit a final concentration of 1 mM, a 100-μl injection of a saturated solution was used.

Chemicals. Propachlor (purity, 99%), alachlor (99.5%), and metolachlor (98%) were obtained from Chem-Service, West Chester, Pa., and were used as is. *N*-Ethylacetanilide was obtained from Alfred Bader Rare Chemicals (Aldrich) and was originally assayed at 97% purity. *N*-Methylacetanilide (purity, 99%) was purchased from Lancaster Synthesis, Windham, N.H.

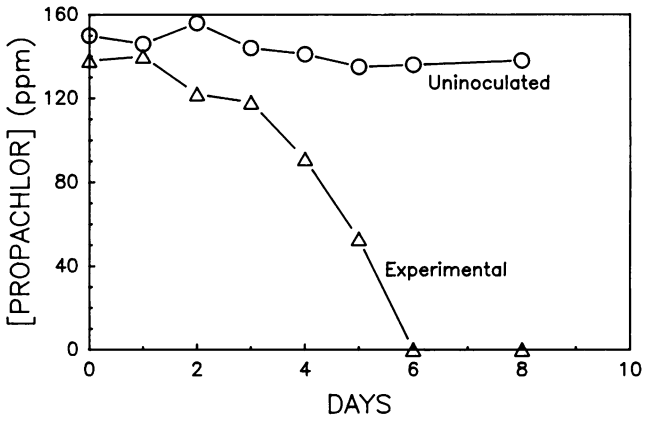

FIG. 1. Loss of propachlor in an enrichment culture of soil obtained from a pesticide disposal site (△) or in an uninoculated control (○). Propachlor at a concentration of 150 ppm was used as the carbon and energy source in XBM mineral salts medium.

RESULTS

Enrichment cultures and strain isolation. Transfers of the enrichment culture consistently showed that there was a complete loss of propachlor within 2 weeks (Fig. 1). After seven transfers, samples were streaked onto TS or propachlor-XBM agar plates in order to isolate pure cultures. Six isolates were obtained from the TS agar plates, and three isolates were obtained from the plates containing propachlor-XBM medium. The strains were inoculated individually into propachlor-XBM broth medium to determine whether any single isolate could degrade the herbicide. We found one isolate, strain DAK3, that degraded propachlor and formed a metabolite (Fig. 2). The strains were also inoculated into propachlor-XBM medium in pairs. The combination of strain DAK3 and a second isolate, strain MAB2, resulted in no accumulation of metabolite and increased biomass when these organisms were grown on propachlor. The growth yields for DAK3 alone on the herbicide (per mol of propachlor) were 43.5 g of protein and 77.6 g of dry weight. The yields for a DAK3-MAB2 mixed culture (per mol of propachlor) were 61.6 g of protein and 94.0 g of dry weight.

To more clearly elucidate the relationship between DAK3 and MAB2 in propachlor metabolism, DAK3 was inoculated

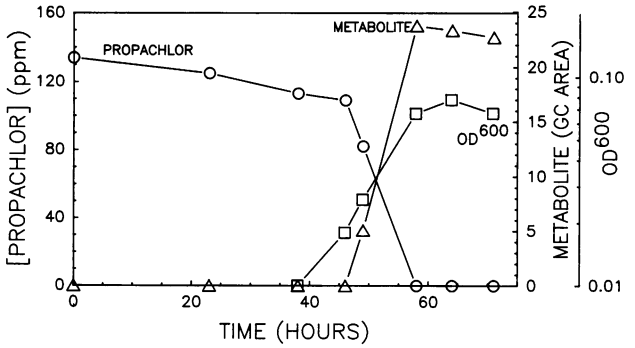

FIG. 2. Changes in biomass (□), propachlor concentration (○) and metabolite level (△) in a culture of strain DAK3 inoculated into XBM mineral salts minimal medium containing 150 ppm of propachlor. GC, gas chromatography; OD^{600}, optical density at 600 nm.

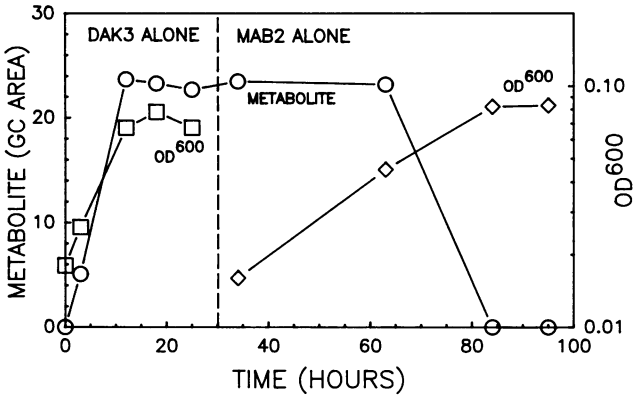

FIG. 3. Formation of the metabolite (○) produced from propachlor by growth of strain DAK3 (□) and growth of strain MAB2 (◇) by metabolism of this metabolite. GC, gas chromatography; OD^{600}, optical density at 600 nm.

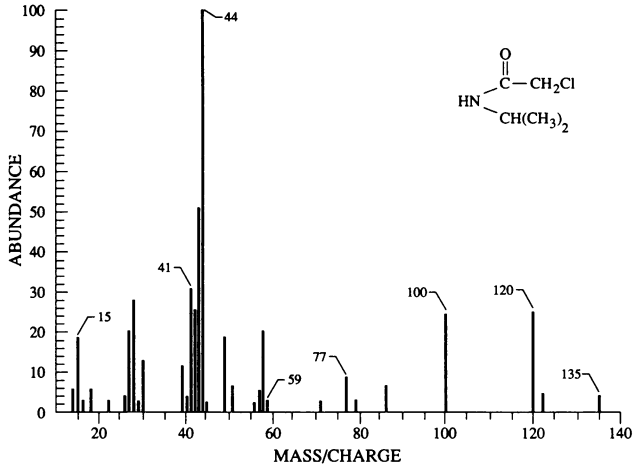

FIG. 4. Gas chromatography-mass spectrometry of the metabolite produced from propachlor by strain DAK3. The resulting mass spectrum was consistent with the structure of 2-chloro-*N*-isopropylacetamide. Major fragment *m/z* 44 was due to fragmentation of the molecular ion (*m/z* 135) by loss of the CH_2Cl radical to give *m/z* 86 [$(H_3C)_2$-NH-C≡O^+], which fragmented further to $^+NH_2$=C=O (*m/z* 44) and also to *m/z* 58 by loss of CO. Fragment ions *m/z* 77 and *m/z* 79 corresponded to Cl-CH_2-C≡O^+, which fragmented to *m/z* 41 by loss of the CO moiety. The ions at *m/z* 120/122 and *m/z* 100 corresponded to fragmentation of the molecular ion by loss of a methyl radical and a chlorine radical, respectively.

into XBM medium containing 150 ppm of propachlor. By 12.5 h, the propachlor was completely metabolized (Fig. 3). After the culture had reached stationary phase, the DAK3 cells were removed by filtration with a polycarbonate membrane filter (pore size, 0.45 μm; Nuclepore Corp.), and 0.25 ml of washed, stationary-phase, TS medium-grown MAB2 cells was inoculated into the culture fluid. The metabolite which accumulated from DAK3 metabolism of propachlor disappeared from the medium upon growth of MAB2.

The results of morphological and physiological tests suggested that DAK3 most closely resembles species belonging to the genus *Moraxella*, while MAB2 appears to belong to the genus *Xanthobacter*. DAK3 typically occurred as gram-negative, short, plump rods in pairs and short chains in nonpigmented colonies. DAK3 was oxidase positive, catalase negative, nonmotile, able to denitrify, and highly sensitive to penicillin. MAB2 occurred as gram-variable, rod-shaped cells that were nonmotile and formed characteristic yellow colonies. This strain was catalase positive, oxidase negative, obligately aerobic, able to grow on methanol and ethanol, and able to fix N_2.

Metabolite identification. The metabolite produced by strain DAK3 from propachlor was analyzed by gas chromatography-mass spectroscopy and produced a spectrum (Fig. 4) that corresponded to that of 2-chloro-*N*-isopropylacetamide. Authentic 2-chloro-*N*-isopropylacetamide was not commercially available for comparison. However, DAK3 could also catabolize *N*-ethylacetanilide (NEACT) and produced a metabolite that was extracted with ethyl acetate. Mass spectral analysis of this extract showed the presence of the corresponding acetamide, *N*-ethylacetamide. The metabolite produced during growth on NEACT comigrated with authentic *N*-ethylacetamide during gas chromatography analysis and gave an identical mass spectrum.

A high-pressure liquid chromatography analysis of the culture supernatants was also performed to detect other metabolites. The DAK3 culture grown on medium containing 150 ppm of propachlor showed a complete loss of the herbicide and the formation of two metabolite peaks, one of which had a retention time identical to that of authentic *N*-isopropylacetamide. HPLC analysis also showed that supernatants of NEACT-grown DAK3 cells contained one metabolite which comigrated with authentic *N*-ethylacetamide. We observed stoichiometric formation of 1 mol of *N*-ethylacetamide per mol of NEACT metabolized.

Aromatic and acetamide substrate range. The range of substrates used by strains DAK3 and MAB2 was tested in the following two ways: (i) the substrates were provided as sole sources of carbon and energy for growth in XBM medium; and (ii) the substrates were added to washed cell suspensions or suspensions of sonicated cells to determine whether they stimulated respiration. DAK3 grew on propachlor up to a concentration of 350 ppm (1.65 mM). It also grew on *N*-methylacetanilide (NMACT) up to a concentration of 8 mM and on NEACT up to a concentration of 6 mM after prolonged incubation. DAK3 grew faster on propachlor or NEACT than it did on acetate or lactate (doubling times, 6.3, 6.6, 14.0, and 19.1 h, respectively). The generation time for growth on NMACT was 12.2 h. DAK3 neither grew on nor cometabolized (when it was grown on propachlor) the following compounds: alachlor, metolachlor, propanil, aniline, *N*,*N*-dimethylaniline, *N*-isopropylaniline, benzene, phenol, and acetanilide. None of these compounds stimulated respiration in cells grown on NEACT. DAK3 did not grow on acetamides as carbon and energy sources, nor did acetamides stimulate respiration in cells grown on any of the N-alkyl acylanilides.

DAK3 was able to respire propachlor, NMACT, and NEACT when it was grown on propachlor or NEACT (Table 1). Some respiration of these compounds was detected when cells were grown on nonaromatic carbon sources, such as acetate, or when DAK3 was grown on one-third-strength TS broth (Table 1), but the respiration rates were substantially higher for cells grown on the acylanilide substrates.

Strain MAB2 did not grow on propachlor, NMACT, NEACT, catchol, phenol, or aniline. However, it did grow on several acylanilides, including acetanilide and 2-, 3-, and 4-methylacetanilides, as well as 2-chloroacetanilide, with the corresponding anilines accumulating in the medium. MAB2 also grew on acetamide, *N*-ethylacetamide, 2-chloro-*N*-eth-

TABLE 1. Respiration rates for strain DAK3 grown on N-alkyl acylanilides

Substrate	Respiration rate for DAK3 grown on[a]:			
	TS medium[b]	Acetate	*N*-Ethylacetanilide	Propachlor
Propachlor	15	14	61	26
NMACT	ND[c]	13	58	26
NEACT	19	19	70	30
TS medium	9	ND	ND	ND
Acetate	ND	15	0	3

[a] Respiration rates are expressed as micromoles of O_2 produced per minute per milligram of protein.
[b] Strain DAK3 was grown in one-third-strength TS broth.
[c] ND, not determined.

ylacetamide, and *N*-isopropylacetamide, and it grew in the filter-sterilized supernatants of stationary-phase DAK3 cells grown on propachlor, NEACT, and NMACT but not lactate. Cells grown on any one acetamide were simultaneously adapted for the other acetamides tested during respirometry experiments (Table 2).

Catechol oxygenase. Aerobic metabolism of aromatic compounds generally involves ring cleavage of an aromatic diol, such as catechol. Strain DAK3 grown on propachlor and DAK3 grown on NEACT respired catechol at a rates of 14 and 39 μmol of O_2 per min per mg of protein, respectively. These cells also respired methylated catechols. Cells grown on propachlor respired 3-methylcatechol and 4-methylcatechol at rates of 12 and 5 μmol of O_2 per min per mg of protein, respectively. NEACT-grown cells also showed activity toward these two compounds, with oxygen uptake rates of 34 and 15 μmol of O_2 per min per mg of protein, respectively. Acetate-grown cells also showed some activity toward catechol but at a reduced rate (4 μmol of O_2 per min per mg of protein).

Enzyme assays for aromatic ring-cleaving dioxygenases were conducted by using DAK3 cells grown on propachlor, NEACT, or acetate. Catechol 2,3-oxygenase activity was detected in extracts of cells grown on each of these substrates. However, the specific activity was significantly higher in cells grown on the N-alkyl acetanilides (Table 3), especially NEACT. Catechol 1,2-oxygenase activity was not found in extracts of DAK3 cells grown on any of the substrates.

TABLE 3. Catechol 2,3-oxygenase activity of strain DAK3

Substrate	Enzyme activity of DAK3 grown on[a]:		
	Propachlor	NEACT	Acetate
Catechol	5.6	26.2	1.9
3-Methylcatechol	5.4	19.3	ND[b]
4-Methylcatechol	0.9	3.8	ND

[a] Enzyme activity is expressed as micromoles of product produced per minute per milligram of protein.
[b] ND, not determined.

DISCUSSION

The proposed pathway of propachlor degradation by strains DAK3 and MAB2 is shown in Fig. 5. The results of our study provide evidence for a novel mechanism for the degradation of an acylanilide herbicide. Initial cleavage of propachlor occurs not at the amide bond but at the bond between the N atom and the aromatic ring, with subsequent metabolism of the aromatic carbons by DAK3. This mechanism of degradation contrasts with the mechanism previously found for propachlor in microorganisms, plants (19), and animals (1, 18).

Microorganisms in soil (22), in other environments (27, 33), or in isolated cultures (16) have been reported to metabolize or cometabolize propachlor, but aromatic metabolites were formed. Novick et al. (28) described a microbial consortium that did mineralize propachlor, and these authors identified *N*-isopropylaniline as an intermediate after initial cleavage at the amide bond. Two strains were necessary for any propachlor mineralization to occur, but the specific roles of each were not elucidated. As reported above, DAK3 is solely responsible for the novel cleavage of the herbicide and can use it as a sole source of carbon and

TABLE 2. Growth and respiration of strain MAB2 grown on acetamides[a]

Substrate	Growth	Respiration rate for MAB2 grown on[b]:		
		N-Ethylacetamide	Acetamide	Acetanilide
Propachlor	−	0	0	0
NEACT	−	0	0	0
NEACT supernatant[c]	+	114	127	37
Propachlor supernatant[d]	+	16	26	ND[e]
NMACT supernatant[f]	+	ND	143	ND
Lactate supernatant[g]	−	1	ND	ND
Acetamide	+	117	146	76
N-Ethylacetamide	+	110	ND	28
N-Isopropylacetamide	+	31	ND	ND
2-Cl-*N*-ethylacetamide	+	20	ND	6
Acetanilide	+	ND	162	171

[a] Substrates were tested as sole carbon and energy sources in XBM minimal medium containing a vitamin solution. Substrates were also tested for their ability to stimulate oxygen consumption by MAB2 cells grown on 5 mM *N*-ethylacetamide, 8 mM acetamide, or 5 mM acetanilide.
[b] Respiration rates are expressed as micromoles of O_2 produced per minute per milligram of protein.
[c] Supernatant from a DAK3 culture grown on NEACT.
[d] Supernatant from a DAK3 culture grown on propachlor.
[e] ND, not determined.
[f] Supernatant from a DAK3 culture grown on NMACT.
[g] Supernatant from a DAK3 culture grown on lactate.

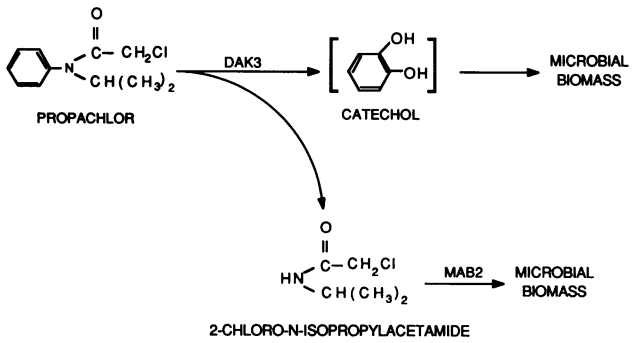

FIG. 5. Proposed pathway of propachlor degradation by strains DAK3 and MAB2.

energy. *N*-Isopropylaniline is not an intermediate; DAK3 neither grows on nor respires this compound.

The relationship between the metabolic activities of strains DAK3 and MAB2 is shown in Fig. 3. DAK3 initiates herbicide degradation and does not metabolize the acetamide produced by cleavage of propachlor. MAB2 can metabolize and grow on this acetamide. The lack of availability of radioactively labeled propachlor prohibited radiotracer studies, but the yields of protein and dry weight which we obtained are consistent with this two-step mineralization of the molecule. DAK3 metabolizes only the aromatic moiety and produces yields that are consistent with utilization of the six carbons (77.6 g [dry weight] per mol of propachlor). For comparison, yields of bacteria grown on aniline have been reported to be 65 and 78 g (dry weight) per mol (14, 34), and yields of bacteria grown on phenol have been reported to be 67 g/mol (14). Increased yields on propachlor were found in cocultures of DAK3 and MAB2, presumably because of the growth of MAB2 on 2-chloro-*N*-isopropylacetamide. Aerobic catabolism of aromatic xenobiotic compounds usually involves ring cleavage of an aromatic diol by an oxygenase (4). From structural considerations, we predicted that DAK3 would use a catechol oxygenase for ring cleavage. This organism uses the *meta* pathway of aromatic metabolism, because catechol 2,3-oxygenase but not catechol 1,2-oxygenase activity was found.

Strain DAK3 has been assigned to the genus *Moraxella* although members of this genus are typically catalase positive and DAK3 is catalase negative. Because of the catalase reaction, DAK3 conforms to the description of the genus *Kingella*. However, *Kingella* species are nutritionally fastidious, and their natural habitat is typically the upper respiratory tract of humans. *Moraxella* species that degrade xenobiotic compounds have been described previously (37); therefore, it seems more appropriate to place DAK3 in this genus.

The aromatic substrate range of the DAK3 enzyme involved in propachlor cleavage is limited. The substrates are tertiary amides containing a methyl, ethyl, or isopropyl group as a substituent on the nitrogen atom. Metabolism does not occur with a secondary amide (acetanilide) or tertiary amine (*N*,*N*-dimethylaniline) as the substrate or with a tertiary amide which does not contain a benzene ring (*N*,*N*-dimethylacetamide). Cells grown on one of the N-substituted acyanilides are simultaneously adapted for respiration of the other two (Table 1). The presence of the alkyl substitution on the nitrogen atom of several acylanilide herbicides has been suggested as a reason for the apparent recalcitrance to microbial mineralization exhibited by these compounds (2, 15). In DAK3, the N substitution appears to be necessary for catabolism.

The novel mechanism of propachlor metabolism by strain DAK3 has several implications for the fate of acylanilide herbicides in the environment. A general problem with these molecules is that the aromatic portion persists in the environment. For example, propanil (3',4'-dichloropropionanilide) is readily metabolized by soil microorganisms via an aryl acylamidase (20, 29). However, the aromatic metabolite 3,4-dichloroaniline persists in the soil. In our system, metabolism of propachlor is accomplished by degradation of the aromatic carbons by the bacterial isolate which initially attacks propachlor; the resulting nonaromatic metabolite is subsequently degraded by a second isolate.

The acylanilides alachlor and metolachlor have been shown to be recalcitrant to mineralization in soil. A series of transformations, including dealkylations and dechlorinations, have been reported to occur, and the relevant studies have been reviewed by Chesters et al. (8). Our data on propachlor metabolism suggest that the N-alkyl substitutions of alachlor and metolachlor are not solely responsible for the recalcitrance of these compounds. It has been hypothesized that alkyl substitutions at the *ortho* positions of the aromatic ring prevent enzymes from attacking these substrates (15, 31). Our data are consistent with this hypothesis because strain DAK3 was unable to metabolize alachlor or metolachlor when it was grown on propachlor. An alternative hypothesis is that DAK3 may not be able to transport the herbicide to the cellular location of the enzyme. We are currently attempting to address this issue by purifying the enzyme from DAK3.

If it were possible to extend the substrate range of DAK3 to attack substrates such as alachlor or metolachlor, it is unclear whether the alkylated aromatic product would be metabolized by the *meta* cleavage pathway found in this organism. Catechols containing single methyl groups were substrates for the catechol dioxygenase found in DAK3, although the rates of activity were markedly reduced on 4-methylcatechol. The putative product of the initial attack on alachlor by DAK3 is 2,6-diethylphenol. It seems unlikely that an aromatic diol appropriate for ring cleavage would be produced from this compound in a single step. The metabolism of 2,6-dimethylphenol (i.e., 2,6-xylenol) via 2,6-dimethyl-3-hydroxy-hydroquinone by *Mycobacterium* sp. strain DM1 (10) provides an example of how metabolism of a dialkylated phenol might occur. In this organism, hydroxylations occur at *meta* and *para* positions to the existing hydroxyl group. Ring cleavage next occurs between the *meta* and *para* substituents.

Acylanilide substrates in soil or waste materials are present together with other organic substrates. The propachlor-cleaving enzyme and catechol oxygenase of DAK3 were synthesized constitutively (although higher levels were found in the presence of an N-alkyl acetanilide). A constitutive xenobiotic compound-degrading system would allow DAK3 to cleave the herbicide in soil while also using more abundant natural organic compounds in the soil environment.

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