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Simultaneous Biodegradation of Chlorobenzene and Toluene by a *Pseudomonas* Strain

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***Pseudomonas* sp. strain JS6 grows on a wide range of chloro- and methylaromatic substrates. The simultaneous degradation of these compounds is prevented in most previously studied isolates because the catabolic pathways are incompatible. The purpose of this study was to determine whether strain JS6 could degrade mixtures of chloro- and methyl-substituted aromatic compounds. Strain JS6 was maintained in a chemostat on a minimal medium with toluene or chlorobenzene as the sole carbon source, supplied via a syringe pump. Strain JS6 contained an active catechol 2,3-dioxygenase when grown in the presence of chloroaromatic compounds; however, in cell extracts, this enzyme was strongly inhibited by 3-chlorocatechol. When cells grown to steady state on toluene were exposed to 50% toluene–50% chlorobenzene, 3-chlorocatechol and 3-methylcatechol accumulated in the medium and the cell density decreased. After 3 h, the enzyme activities of the modified *ortho* ring fission pathway were induced, the metabolites disappeared, and the cell density returned to previous levels. In cell extracts, 3-methylcatechol was degraded by both catechol 1,2- and catechol 2,3-dioxygenase. Strain JS62, a catechol 2,3-dioxygenase mutant of JS6, grew on toluene, and ring cleavage of 3-methylcatechol was catalyzed by catechol 1,2-dioxygenase. The transient metabolite 2-methyllactone was identified in chlorobenzene-grown JS6 cultures exposed to toluene. These results indicate that strain JS6 can degrade mixtures of chloro- and methylaromatic compounds by means of a modified *ortho* ring fission pathway.**

The pseudomonads have long been noted for their metabolic versatility (10, 23). The list of compounds that can be degraded by pseudomonads includes an increasing number of xenobiotic chemicals. One of the most versatile strains, *Pseudomonas* sp. strain JS6, was originally selected for its ability to grow on *p*-dichlorobenzene (22) and has subsequently been shown to biodegrade numerous other aromatic compounds (7, 21). This list includes both chloro- and methyl-substituted aromatic substrates that are generally considered not to be degraded simultaneously (11).

The simultaneous degradation of chloro- and methyl-substituted aromatic substrates is impossible for most bacteria because the catabolic pathways are incompatible. Methyl-substituted aromatic substrates are generally degraded via *meta* ring fission catalyzed by catechol 2,3-dioxygenase, whereas chloro-substituted aromatic substrates are degraded via modified *ortho*-ring fission catalyzed by catechol 1,2-dioxygenase (11, 2). The incompatibility of these pathways has been demonstrated at the biochemical level with the finding that the catechol 2,3-dioxygenase of the *meta* cleavage pathway is inactivated by chlorocatechols (1, 9). At the community level, the *meta* cleavage of aromatic substrates was selected against as mixed populations were exposed to haloaromatic compounds (19). Likewise, the construction of genetically engineered strains for degradation of haloaromatic compounds required the removal of the *meta* cleavage pathway (15). In the absence of the *meta* cleavage pathway, the methyl-substituted aromatic compounds are misrouted to the modified *ortho* cleavage pathway, which results in the accumulation of methylmucono-lactones (12). Strains that degrade methyl-substituted catechols produced from methylphenols and methylbenzoates via an alternative modified *ortho* cleavage pathway have recently been developed (17, 18, 24).

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The objective of this investigation was to determine whether the strain JS6 could be induced to degrade mixtures of chloro- and methyl-substituted aromatic compounds. In this report, we demonstrate the simultaneous degradation of chlorobenzene and toluene by JS6. Both catechol 2,3-dioxygenase and catechol 1,2-dioxygenase activities were detected when JS6 degraded mixtures of chlorobenzene and toluene. In addition, we report the ability of JS6 to degrade 3-methylcatechol via a *meta* or a modified *ortho* cleavage pathway.

MATERIALS AND METHODS

Isolation and growth of bacteria. *Pseudomonas* sp. strain JS6 has been previously described (22). Strain JS62, a spontaneous mutant lacking a functional catechol 2,3-dioxygenase, was isolated from a benzene-grown culture of JS6. The selection of strain JS62 was facilitated by its appearance among the JS6 colonies as a large white colony that did not produce a yellow metabolite when grown on benzene.

Cultures were routinely grown at 30°C in the minimal salts medium described by Stanier et al. (23). Volatile aromatic substrates were supplied to small liquid cultures and agar plates as described previously (7). Larger cultures were grown in a 580-ml airlift bioreactor (model 880600; Kontes, Vineland, N.J.) with an airflow rate of 1 liter h^{-1}. Modifications to this bioreactor included the replacement of polycarbonate baffles with stainless steel, replacement of the stainless steel air sparger with fritted glass, and incorporation of a gas flowmeter (model 600; Matheson Co., Inc., E. Rutherford, N.J.). The bioreactor was operated as a chemostat (dilution rate, 0.05 h^{-1}) with minimal salts medium fed by a peristaltic pump (Harvard Apparatus, South Natick, Mass.). Aromatic compounds were added directly to the downcurrent side of the airlift bioreactor (flow rate, 42 μl h^{-1}) by a syringe pump (model 975; Harvard Apparatus). Concentrations of the aromatic solvents were measured in the

effluent air by gas chromatography (GC). Concentrations of water-soluble metabolites were measured by high-performance liquid chromatography (HPLC). Growth of the cultures was monitored spectrophotometrically at 600 nm.

Enzyme assays. Culture samples (50 ml each) were centrifuged, and cell pellets were stored at −70°C for less than 24 h before being washed and suspended in 0.02 M phosphate buffer (pH 7.0) at 4°C. Cells were broken by two passages through a French pressure cell (SLM Instruments, Inc., Urbana, Ill.; 20,000 lb/in^2, 2°C), and the exudate was centrifuged at 16,000 × *g* for 30 min. The pellet was discarded, and the supernatant fluid was stored on ice and used as a crude cell extract.

Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase were measured as previously described (22). Molar extinction coefficients of substituted muconic acids were obtained from the work of Spain and Nishino (22) and Dorn and Knackmuss (3). Molar extinction coefficients for *meta* cleavage products were obtained from the work of Klečka and Gibson (9). Specific activities are expressed as micromoles of substrate transformed per minute per milligram of protein at 25°C. Protein was measured by the method of Smith et al. (20). UV and visible-spectrum absorbance changes were measured with a Cary model 219 spectrophotometer (Varian, Sunnyvale, Calif.).

Isolation of metabolites. Chlorobenzene-grown cells of JS6 were harvested by centrifugation and suspended in 50 ml of minimal salts medium (A_{600} = 0.5). Toluene was added in the vapor phase, and the accumulation of UV-absorbing metabolites was monitored by HPLC. The medium was clarified by centrifugation after 30 min of incubation in the presence of toluene, acidified to pH 4.0, and extracted twice with 100 ml of ethyl acetate. The extract was dried over anhydrous sodium sulfate, and the ethyl acetate was removed under vacuum by rotary evaporation at 40°C. Metabolites were then characterized by GC-mass spectral (MS) analysis.

Analytical methods. HPLC was performed on a μBondapak C_{18} column (3.9 mm by 30 cm; Waters Associates, Inc., Milford, Mass.) with methanol-water-phosphoric acid (370:630:1) as the mobile phase at a flow rate of 1.5 ml min^{-1}. Compounds were detected by the UV A_{210}, A_{254}, and A_{280} with a model 1040A diode array detector (Hewlett-Packard Co., Palo Alto, Calif.). GC was done on a 10-ft (304.8-cm) stainless steel packed column (10% SP-1000, 80/100 Supelcoport; Supelco, Inc., Bellefonte, Penn.) in a Hewlett-Packard 5890 GC equipped with a photoionization detector (HNU Systems, Inc., Newton, Mass.). The carrier gas was high-purity helium maintained at a flow rate of 30 ml/min. The injection temperature was 220°C, oven temperature was held at 75°C for 3 min and then increased at a rate of 15°C/min to a final temperature of 220°C, and the detector temperature was maintained at 200°C. Capillary column GC-MS analyses were performed with a Hewlett-Packard 5987 GC-MS equipped with an SE/54 fused-silica capillary column and electron impact ionization.

Chemicals. Chlorobenzene and toluene were obtained from Fisher Scientific Co. (Fairlawn, N.J.). Catechol was purchased from Eastman Organic Chemicals (Rochester, N.Y.), and 3-methylcatechol (3-MC) was purchased from Pfaltz and Bauer, Inc. (Waterbury, Conn.). 3-Chlorocatechol (3-CC) was prepared biologically from chlorobenzene by the action of *Pseudomonas putida* F1 (6). Catechols were examined for purity by HPLC and GC-MS analysis and were further purified by HPLC and vacuum sublimation when necessary. 4-Carboxymethyl-2-methylbut-2-en-1,4-olide (2-methyllactone [2-ML]) was a generous gift from H.-J. Knackmuss, University of Stuttgart.

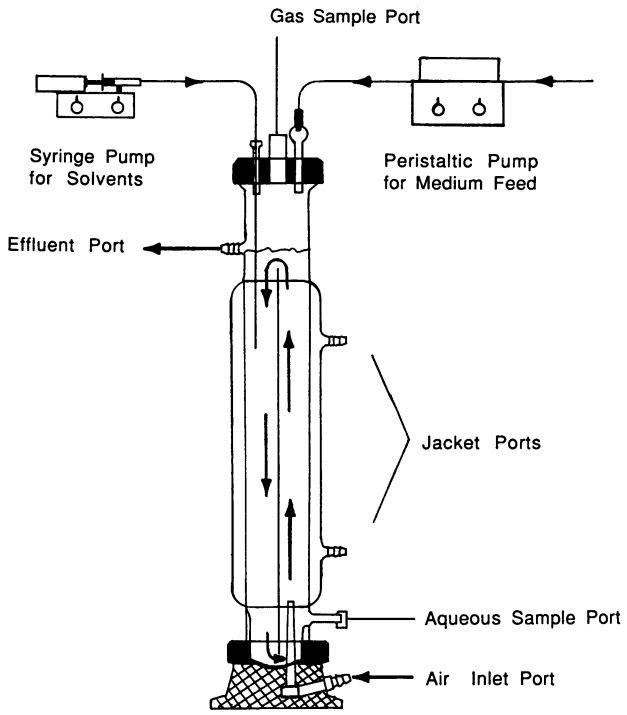

FIG. 1. Schematic diagram of the airlift bioreactor used for chemostat growth of JS6 with direct addition of solvents.

RESULTS

Degradation of chlorobenzene and toluene. Several strains of bacteria have been shown to grow on chlorobenzene or toluene as the sole source of carbon and energy (5, 7, 16, 22). These previous studies have relied on vapor-phase addition of these substrates because they are toxic at high concentrations. It is difficult to provide defined mixtures of volatile substrates in the vapor phase; therefore, we added liquid solvent mixtures directly to an airlift bioreactor (Fig. 1). The substrates were added via a syringe pump, and air stripping prevented the accumulation of excess solvent. JS6 readily grew on benzene, toluene, or chlorobenzene when the chemical was added directly to the airlift bioreactor.

The bioreactor was operated as a chemostat, and JS6 was grown to steady state on chlorobenzene, toluene, and mixtures of chlorobenzene and toluene (Fig. 2). The effluent gas from the airlift bioreactor contained 15 to 25% of the added substrate. The density of JS6 cultures grown on 100% chlorobenzene decreased slightly when the substrate feed was changed to 50% chlorobenzene-50% toluene but increased following a change to 100% toluene (Fig. 2). These results provided the first evidence that strain JS6 could grow on chlorobenzene and toluene simultaneously. The decrease in cell density following a decrease in substrate addition rate demonstrated that cultures growing in the airlift bioreactor were substrate limited as would be expected under chemostat conditions.

Cell density decreased dramatically when the substrate feed was changed to 50% chlorobenzene–50% toluene in toluene-grown (Fig. 3) and pyruvate-grown (Fig. 4) cultures. The decrease in cell density coincided with the accumulation

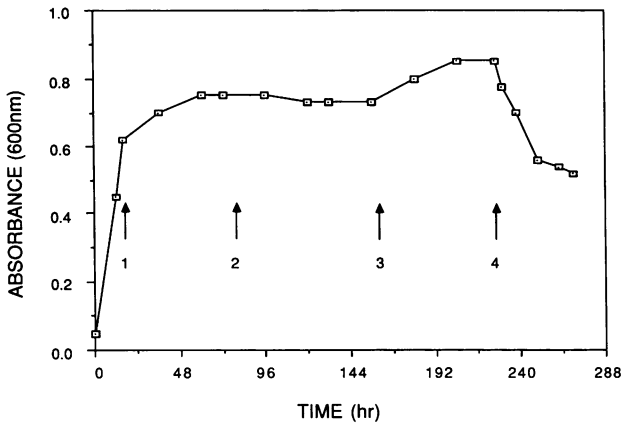

FIG. 2. Chemostat cultivation of JS6 with 100% chlorobenzene (arrow 1), 50% chlorobenzene–50% toluene (arrow 2), and 100% toluene (arrow 3). The dilution rate was maintained at 0.05 h^{-1}, airflow was held constant at 1 liter h^{-1}, and the temperature was maintained at 30°C throughout the experiment. The solvent feed rate was 42 μl h^{-1} for 226 h at which point it was changed to 21 μl h^{-1} (arrow 4).

of 3-CC and 3-MC in the culture fluid (Fig. 3 and 4). After 3 h, 3-CC and 3-MC disappeared and the cell density in the JS6 cultures began to return to previous levels. These results suggest that induction of the enzymes for degradation of the chlorocatechols required several hours and that transient accumulation of 3-CC inactivated the catechol 2,3-dioxygenase.

Enzyme activities of the various catechol oxygenases in cell extracts of JS6 cultures exposed to chlorobenzene and toluene simultaneously were determined (Table 1). Catechol 2,3-dioxygenase activity was induced in cells grown on toluene but not in those grown on pyruvate. Following the change in substrate feed from 100% toluene to 50% toluene–50% chlorobenzene, the activity of the catechol 2,3-dioxygenase decreased by an order of magnitude but was maintained at detectable levels. Likewise, the catechol 2,3-dioxygenase activity was induced and maintained at low levels in pyruvate-grown JS6 cells that were switched to growth on toluene and chlorobenzene. Induction of catechol 1,2-dioxygenase activity (Table 1) was concomitant with the removal of 3-MC and 3-CC (Fig. 3 and 4). Cell extracts from toluene- and pyruvate-grown cultures did not contain detectable levels of catechol 1,2-dioxygenase.

Misrouting of substituted catechols. Our results suggested that the *meta* cleavage pathway was blocked because of the inactivation of catechol 2,3-dioxygenase by 3-CC produced from chlorobenzene (1, 9). Therefore, we investigated whether 3-MC could be degraded by the modified *ortho* cleavage pathway. We also examined a putative mutant strain, designated JS62, which appeared identical to the wild-type strain JS6 in all respects except for the absence of a functional catechol 2,3-dioxygenase. In chemostat studies, JS6 cultures achieved a lower cell density at steady state than JS62 cultures growing on chlorobenzene (Fig. 5). These results suggest that the presence of catechol 2,3-dioxygenase reduces the efficiency of JS6 during growth on chlorobenzene. In contrast, JS6 grew to a higher cell density than JS62 on toluene (Fig. 5). Cell extracts prepared from the toluene-grown JS6 culture contained catechol 2,3-dioxygenase but not catechol 1,2-dioxygenase (Table 2). Similar extracts prepared from toluene-grown JS62 contained only catechol 1,2-dioxygenase. Toluene-grown cells of strain JS62 readily degraded 2-ML when the chemical was added to the culture fluid. These results indicated that JS62 metabolized 3-MC via a modified *ortho* cleavage pathway that allowed for growth on toluene but was less efficient than the *meta* cleavage degradative pathway used by the wild-type strain JS6.

In order to determine whether 3-MC was also degraded via a modified *ortho* cleavage pathway in chlorobenzene-grown JS6 cultures exposed to toluene, HPLC analysis of polar metabolites was performed. A transient metabolite was detected in chlorobenzene-grown JS6 cultures following the addition of toluene. This metabolite had a UV maximum at A_{210} and cochromatographed with 2-ML (retention time, 2.4 min). This metabolite was identical to authentic 2-ML by GC-MS analysis (Fig. 6). The rapid disappearance of 2-ML suggests that it was further metabolized, but subsequent metabolites were not characterized. JS6 cells grown on 50% chlorobenzene–50% toluene also readily degraded 2-ML.

DISCUSSION

The simultaneous biodegradation of organic compounds is of interest because relatively little is known about the influence of one organic compound on the removal of

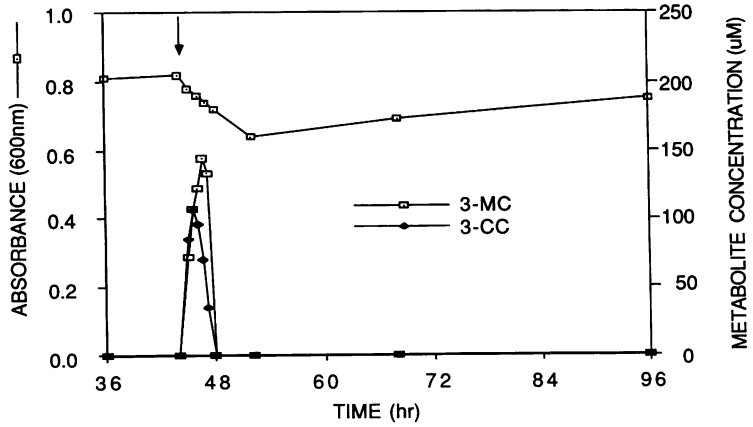

FIG. 3. Chemostat cultivation of JS6 with an initial substrate feed of 100% toluene followed by a switch to 50% toluene–50% chlorobenzene at 44 h (arrow). Conditions were as described in the legend to Fig. 2. Concentrations of metabolites were determined by HPLC.

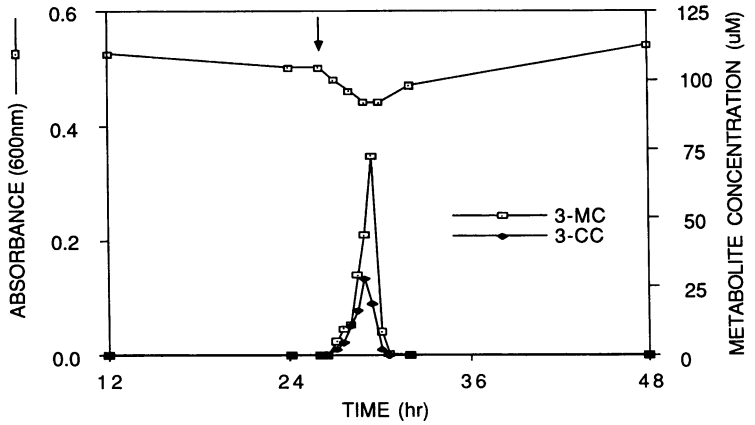

FIG. 4. Chemostat cultivation of JS6 on 0.1% (wt/vol) pyruvate followed by a switch to 50% toluene-50% chlorobenzene after 26 h (arrow). Conditions were as described in the legend to Fig. 2. Concentrations of metabolites were determined by HPLC.

another, especially for bacteria growing at low specific growth rates (13). In the present study, we have shown that *Pseudomonas* sp. strain JS6 can grow on toluene and chlorobenzene simultaneously. The initial conversion of toluene and chlorobenzene to the corresponding substituted catechols by JS6 is mediated by dioxygenase and dihydrodiol dehydrogenase enzymes (7, 22). These reactions are very similar to the well-characterized pathway for degradation of toluene by *P. putida* F1 (21). In contrast to *P. putida* F1, however, strain JS6 is also capable of inducing a modified *ortho* cleavage pathway. Previous studies with JS6 cultures have revealed the presence of a *meta* ring fission enzyme in cells grown on toluene (7) and a *meta* and a modified *ortho* ring fission enzyme in cells grown on chlorobenzene (22). Catechol 2,3-dioxygenase activity is coordinately induced with the initial toluene dioxygenase and dihydrodiol dehydrogenase in *P. putida* F1 (4). A similar control mechanism in JS6 would account for the presence of catechol 2,3-dioxygenase in cells grown on chlorobenzene.

The presence of catechol 2,3-dioxygenase resulted in a decreased cell yield for JS6 growing on chlorobenzene compared with JS62 cultures growing under the same conditions. The absence of active catechol 2,3-dioxygenase in strain JS62 prevented such misrouting. Our results were consistent with those of Jeenes et al. (8), who reported that mutations in the structural gene of catechol 2,3-dioxygenase prevented unproductive channelling to the *meta* cleavage pathway in strain WR211 grown on 4-chlorobenzoate.

During the simultaneous degradation of toluene and chlorobenzene, JS6 cultures can potentially degrade 3-MC by either a *meta* or a modified *ortho* cleavage pathway. The ability to degrade 3-MC by the modified *ortho* cleavage pathway was demonstrated by the growth of JS62 on toluene and by the appearance and removal of 2-ML in chlorobenzene-grown cultures of JS6 exposed to toluene. Reduced cell yields for strain JS62 compared with yields for JS6 during growth on toluene indicates that the modified *ortho* cleavage pathway is less efficient than the *meta* cleavage pathway for degradation of 3-MC. Taeger et al. (24) also reported that *ortho* cleavage resulted in lower growth rates than *meta* cleavage in bacteria grown on 3-methylbenzoate. The proportion of 3-MC that was degraded by the modified *ortho* cleavage pathway during the simultaneous degradation of toluene and chlorobenzene by JS6 cultures was not directly determined. However, the modified catechol 1,2-dioxygenase exhibited higher activity for 3-MC than for 3-CC (Table 2). While this observation is in keeping with the broad substrate specificity associated with the modified catechol 1,2-dioxygenase, it also suggests that 3-CC may not be the primary substrate for this enzyme.

The presence of *meta*, *ortho*, and modified *ortho* cleavage pathways in one organism appears to be rare. The well-

TABLE 1. Enzyme activities in cell extracts of JS6

Enzyme assayed and assay substrate	Sp act (μmol/min per mg of protein) after growth with 50% toluene–50% chlorobenzene									
	100% Toluene→toluene-chlorobenzene[a]					0.1% Pyruvate→toluene-chlorobenzene[b]				
	Steady state (100% toluene)	2 h	4 h	24 h	Steady state[c]	Steady state (0.1% pyruvate)	3 h	6 h	24 h	Steady state[c]
Catechol 1,2-dioxygenase										
Catechol	0.004	0.003	0.013	0.058	0.050	<0.001	0.016	0.038	0.033	0.038
3-CC	<0.001	0.006	0.014	0.068	0.057	<0.001	0.017	0.042	0.035	0.046
Catechol 2,3-dioxygenase										
Catechol	0.123	0.082	0.007	0.013	0.018	<0.001	0.002	0.005	0.017	0.013
3-MC	0.300	0.172	0.024	0.040	0.048	<0.001	0.005	0.015	0.044	0.052

[a] Data correspond to those in Fig. 3; times are sample times following substrate change to toluene-chlorobenzene.
[b] Data correspond to those in Fig. 4; times are sample times following substrate change to toluene-chlorobenzene.
[c] 50% toluene–50% chlorobenzene.

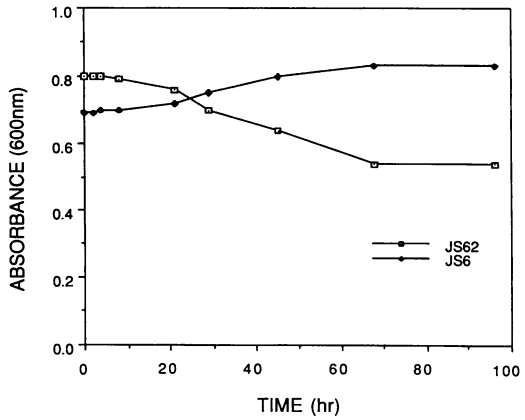

FIG. 5. Chemostat cultivation of JS6 and JS62 with toluene. Cultures were grown to steady state on chlorobenzene, and then the substrate feed was changed to toluene at time zero. Conditions were as described in the legend to Fig. 2.

characterized *Alcaligenes eutrophus* JMP 134 has also been shown to use all three of these pathways (14). In contrast to JS6, however, cells of strain JMP 134 synthesize only the enzymes of the modified *ortho* ring fission pathway during growth on chloroaromatic compounds (14). Therefore, there is no misrouting of chlorocatechols to the *meta* cleavage pathway in JMP 134 grown on chloroaromatic compounds, but the simultaneous exposure to methyl- and chloroaromatic compounds results in the induction of catechol 1,2- and 2,3-dioxygenases (14). In this respect, strain JS6 is similar to the isolate KTB4, described by Taeger et al. (24), in which both the *meta* and the modified *ortho* cleavage enzymes were induced simultaneously during growth on chloroaromatic compounds. Strain KTB4 was also able to degrade 3-CC, 3-MC, and 2-ML concurrently, but 3-CC partially inactivated catechol 2,3-dioxygenase (24). Although there are numerous reports that the *meta* and modified *ortho* cleavage pathways are incompatible, the results obtained with strains JS6 and KTB4 indicate that the coexistence of these pathways is possible. Furthermore, the induction of the *meta* cleavage pathway may provide a selective advantage to cells in environments where methylaromatic compounds predominate over chloroaromatic compounds. The potential application of JS6 and its derivatives to the biodegradation of more complex mixtures is currently under investigation.

TABLE 2. Enzyme activities in cell extracts of JS62 and JS6

Enzyme assayed and assay substrate	Sp act (μmol/min per mg of protein) at steady state[a]			
	Toluene		Chlorobenzene	
	JS62	JS6	JS62	JS6
Catechol 1,2-dioxygenase				
Catechol	0.039	0.003	0.067	0.042
3-MC	0.094	ND	ND	ND
3-CC	0.044	<0.001	0.074	0.048
Catechol 2,3-dioxygenase				
Catechol	<0.001	0.142	<0.001	0.016
3-MC	<0.001	0.349	<0.001	0.034

[a] Data correspond to those in Fig. 5. ND, Not done.

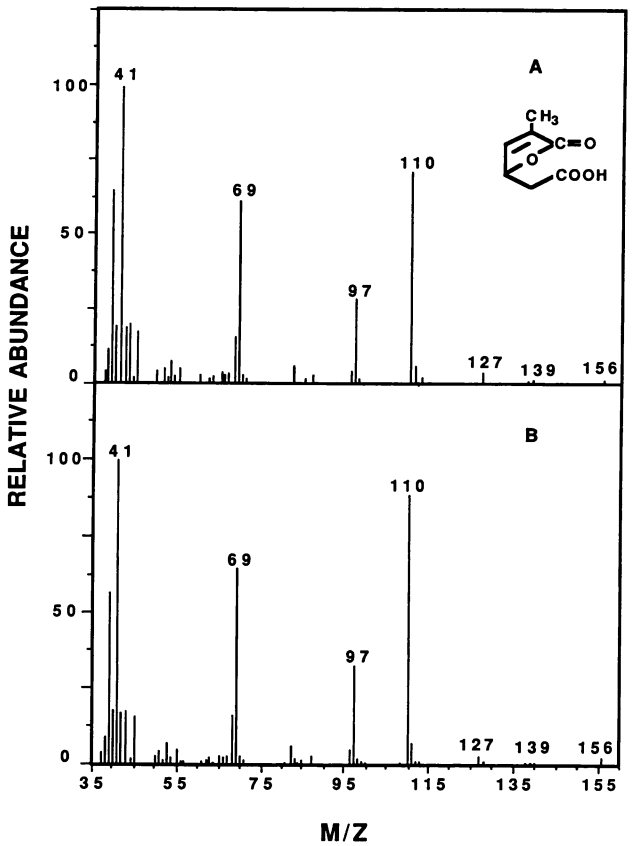

FIG. 6. (A) Mass spectrum of authentic 2-ML (obtained from H.-J. Knackmuss). (B) Mass spectrum of metabolite obtained from culture fluids of chlorobenzene-grown JS6 exposed to toluene. Experimental details are described in Materials and Methods.

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