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Degradation of 1,4-Dichlorobenzene by *Xanthobacter flavus* 14p1†

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***Xanthobacter flavus* 14p1 was isolated from sludge of the river Mulde by selective enrichment with 1,4-dichlorobenzene as the sole source of carbon and energy. The bacterium did not use other aromatic or chloroaromatic compounds as growth substrates. During growth on 1,4-dichlorobenzene, stoichiometric amounts of chloride ions were released. Degradation products of 1,4-dichlorobenzene were identified by gas chromatography-mass spectrometry analysis. 3,6-Dichloro-*cis*-1,2-dihydroxycyclohexa-3,5-diene and 3,6-dichlorocatechol were isolated from culture fluid. 2,5-Dichloromuconic acid and 2-chloromaleylacetic acid as well as the decarboxylation product 2-chloroacetoacrylic acid were identified after enzymatic conversion of 3,6-dichlorocatechol by cell extract. 1,4-Dichlorobenzene dioxygenase, dihydrodiol dehydrogenase, and catechol 1,2-dioxygenase activity were induced in cells grown on 1,4-dichlorobenzene. The results demonstrate that 1,4-dichlorobenzene degradation is initiated by dioxygenation and that ring opening proceeds via *ortho* cleavage.**

Over the past few decades, the extensive use of chlorinated benzenes has led to considerable release of these compounds into the environment (13). Since chlorinated benzenes are chemically stable in nature (19), biological degradation is the only process by which these compounds are eliminated.

Pure cultures of *Pseudomonas* (7, 16, 18) and *Alcaligenes* species (1, 12, 17) with long generation times of 5 to 15 h, which use dichlorobenzenes (DCBs) as the sole source of carbon and energy, are known. It has been proposed that *Pseudomonas* (7, 18) and *Alcaligenes* (1, 12) species attack DCBs by a dioxygenase and that after rearomatization, the resulting chlorocatechols are degraded via the modified *ortho* pathway. Even though a number of multicomponent dioxygenases catalyzing the NADH-dependent introduction of two adjacent hydroxyl groups on aromatic or haloaromatic rings have been purified (3), the purification of a DCB-attacking dioxygenase has not been reported to date. These multicomponent enzyme complexes are unstable, and only with *Pseudomonas* JS 100 growing on 1,2-DCB could an initial dioxygenase activity be demonstrated in cell extracts (7). Therefore, an attempt was made to isolate faster-growing organisms able to use DCBs, with DCB dioxygenase activity in cell extracts allowing the purification of the initial enzyme. In the present study, we report the isolation of a *Xanthobacter* species using 1,4-DCB as the sole source of carbon and energy. The first three enzyme activities catalyzing the conversion of 1,4-DCB to 2,5-dichloro-*cis*,*cis*-muconic acid were found in cell extract, and in addition, all intermediates of the degradation pathway to 2-chloromaleylacetic acid except 2-chloro-4-carboxymethylene-but-2-en-4-olide were isolated, showing unambiguously that bacterial degradation of 1,4-DCB occurs via dioxygenation and the modified *ortho* pathway.

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† Dedicated to F. Lingens on the occasion of his 70th birthday.

MATERIALS AND METHODS

Enrichment and isolation. Sludge (20 g) from the river Mulde near Bitterfeld, Germany, was suspended in 100 ml of mineral salt medium (MMY) containing (per liter of distilled water) 7 g of Na_2HPO_4, 2 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \times 7H_2O$, 10 mg of yeast extract, and 5 ml of a trace element solution (8) in which the chloride salts were replaced with equimolar concentrations of sulfates. This suspension served as the inoculum for enrichment cultures with MMY shaken at room temperature. A mixture of the three isomeric DCBs was supplied via the vapor phase (1). Subcultures, each with one of the three isomeric DCBs as the sole carbon source, were prepared in MMY. After several passages, dilutions were spread on MMY agar plates, and the plates were incubated in a sealed vessel at room temperature. The respective DCB as the carbon source was supplied in the lids of the petri dishes. Isolated colonies were inoculated into 20 ml of MMY with one of the DCBs as the sole carbon source. Strain 14p1 was isolated on 1,4-DCB and maintained on MMY agar plates with 1,4-DCB as the carbon source.

Growth conditions. Cultures (100 ml of MMY) of strain 14p1 were shaken in 500-ml Erlenmeyer flasks with 1,4-DCB or another volatile aromatic compound as the carbon and energy source supplied via the vapor phase (1). All other substrates tested were supplied as 5 mM solutions in MMY. Cells were collected at 4°C by centrifugation at an A_{580} of 0.9 to 1.1. Large-scale cultures (10 liters of MMY) were grown at 30°C and with an O_2 saturation of 90% in a Biostat E fermentor (Braun, Melsungen, Germany). Twenty grams of 1,4-DCB dissolved in 1 liter of 2,2,4,4,6,8,8-heptamethylnonane was added, resulting in a liquid two-phase system (10). Cells were collected at an A_{580} of 1.2 to 1.4.

For quantitative determination of chloride release, 500-ml serum bottles containing 30 ml of MMY were inoculated, and different amounts of 1,4-DCB in small glass tubes were added. The bottles were made gas-tight with viton stoppers.

Preparation of washed cell suspensions and cell extracts. Cells were harvested, washed with 50 mM Tris-HCl (pH 7.5), and resuspended in the same buffer. Crude cell extracts were prepared by passing cell suspensions twice through a chilled French pressure cell (Aminco, Silver Spring, Md.) at 90 MPa. After centrifugation at 25,000 × *g* for 30 min at 4°C, the clear supernatant was used as the cell extract.

Enzyme assays. All enzyme assays were performed at 25°C. Oxygen uptake rates were measured polarographically in an oxygen electrode unit type DW1 (Bachhofer, Reutlingen, Germany) with 1 ml of washed cell suspension with an A_{580} of 1.0 (0.165 mg of protein). The reaction was started by adding 30 μl of a saturated solution of 1,2-DCB (0.88 mM), 1,3-DCB (0.88 mM), or 1,4-DCB (0.27 mM) or of a 1.6 mM solution of catechol in water. The activity of the initial oxygenase in cell extracts was measured polarographically (4) in 50 mM ammonium acetate buffer (pH 6.8). Extracts were eluted from a Sephadex G-25 column (0.6 by 4 cm) to reduce endogenous oxygen consumption. Reaction mixtures contained 1.5 mg of protein, 0.7 μmol of NADH, 0.05 μmol of $(NH_4)_2Fe(SO_4)_2$, and 4 nmol of 1,4-DCB in a final volume of 500 μl. Dihydrodiol dehydrogenase activity was quantified by determining NAD^+ reduction at 340 nm (14), catechol 1,2-dioxygenase activity was quantified by measuring the formation of *cis,cis*-

muconic acids at 260 nm (2), and catechol 2,3-dioxygenase was measured by monitoring the formation of 2-hydroxymuconic semialdehyde at 375 nm (11).

Analytical methods. Chloride concentrations were measured by argentometric titration with a Methrom 665 dosimat (Metrohom, Herisau, Switzerland) and a silver-sensitive electrode (Ingold AG, Urdorf, Switzerland). Gas chromatography-mass spectrometry with electron impact ionization at 70 eV (GC-MS) was performed with a model 5890 series II gas chromatograph (Hewlett-Packard GmbH, Bad Homburg, Germany) coupled to a Hewlett-Packard model 5989 A mass spectrometer. Protein was measured by the method of Groves et al. (6).

Isolation and derivatization of metabolites. Dichlorocatechol and DCB dihydrodiol were extracted with dichloromethane and transformed to boronates with butyl- and phenylboronic acid as described by Kirsch and Stan (9). 3,6-Dichlorocatechol was converted with cell extract as follows: to 4 ml of 100 mM sodium phosphate buffer (pH 6.9), containing 1.8 mM 3,6-dichlorocatechol, was added 700 μl of cell extract (3.5 mg of protein in 50 mM Tris-HCl buffer [pH 7.5]). The reaction was stopped by adding cold methanol (−20°C). After centrifugation at 25,000 × *g* and 4°C for 20 min, the supernatant was acidified to pH 3.5 and saturated with NaCl. Products were extracted twice with 5 ml of ice-cold ethyl acetate. The organic phase was dried with anhydrous sodium sulfate, and after evaporation, the residues were dissolved in 100 μl of methanol. Diazomethane in diethylether was added until a slight yellow coloration became visible. The derivatized extracts were subjected to GC-MS analysis without further purification.

Chemicals. 2,2,4,4,6,8,8-Heptamethylnonane, 3- and 4-methylcatechol, and 1,2-dihydroxycyclohexa-3,5-diene were purchased from Aldrich Chemical Co., Milwaukee, Wis. 1,2-Dihydro-3-fluorocatechol and 1,2-dihydro-3-methylcatechol were products of Lancaster Synthesis GmbH, Mühlheim, Germany. 3-Chlorocatechol, 4-chlorocatechol, and 3,4-, 3,5-, 3,6-, and 4,5-dichlorocatechol were a generous gift from Nils Kirsch, Technische Universität Berlin.

3,6-dichloro- and 3,5-dichloro-1,2-dihydroxycyclohexa-3,5-diene (3,6- and 3,5-DCB dihydrodiol) were obtained from 1,4- and 1,3-DCB by use of the mutant strain *Pseudomonas putida* F39/D as described by Gibson et al. (5). All other chemicals obtained were of the highest purity grade commercially available.

RESULTS AND DISCUSSION

Isolation of a pure bacterial culture with 1,4-DCB as the sole source of carbon and energy. Starting with sludge from the river Mulde near Bitterfeld, Germany, a gram-negative, catalase- and oxidase-positive, pleomorphic bacterium which forms tiny yellow colonies growing on 1,4-DCB as the sole source of carbon and energy was isolated. Glucose, fructose, sucrose, maltose, succinate, acetate, citrate, lactate, pyruvate, glutamate, methanol, ethanol, *n*-propanol, and *n*-butanol also supported growth. No growth occurred with tartrate, ribose, and histidine.

The organism was classified as *Xanthobacter* sp. on the basis of *Bergey's Manual of Systematic Bacteriology* (21). Fatty acid and rRNA-analyses performed by the Deutsche Sammlung für Mikroorganismen (Braunschweig, Germany) allowed the unambiguous identification of the isolated strain 14p1 as *Xanthobacter flavus*. On 1,4-DCB, the organism grew with a doubling time of 8 h, which is similar to those reported for other DCB-degrading *Pseudomonas* or *Alcaligenes* species (1, 7, 12, 17, 18), and the stoichiometric amount of two chloride ions per molecule of 1,4-DCB was released. At concentrations higher than 0.1 mM 1,4-DCB, bacterial growth was inhibited.

X. flavus 14p1 is a potent 1,4-DCB-degrading bacterium with an extremely limited substrate range: no aromatic or chloroaromatic compound other than 1,4-DCB supported growth. The organism failed to grow with benzene, chlorobenzene, 1,2- and 1,3-DCB, all isomeric tri- and tetrachlorobenzenes, penta- and hexachlorobenzene, phenol, 2- and 4-chlorophenol, 2,3-, 2,4-, 2,5-, and 3,4-dichlorophenol, toluene, 1,4-xylene, naphthalene, 4-chlorobenzoate, 2,4-dichlorophenoxyacetic acid, dichloromethane, 2-chloroethanol, 1,3-dichloropropane, and 1,4-dibromobenzene.

Metabolites. 3,6-dichloro-1,2-dihydroxycyclohexa-3,5-diene (3,6-DCB dihydrodiol) and 3,6-dichlorocatechol were extracted from spent fermentor medium and identified by GC-MS analysis. The presence of 3,6-DCB dihydrodiol demonstrates that degradation of 1,4-DCB is initiated by a DCB dioxygenase. Since the organism does not grow on 2,5-dichlorophenol, a monooxygenase system seems to be absent. 3,6-Dichlorocatechol was converted by cell extract of *X. flavus* 14p1. Incubation for 20 min resulted in one major peak (16.8 min) in the total ion chromatogram besides that of unconverted catechol. The mass spectrum is identical with the spectrum of the dimethylated 2,5-dichloromuconic acid described by Sander (15).

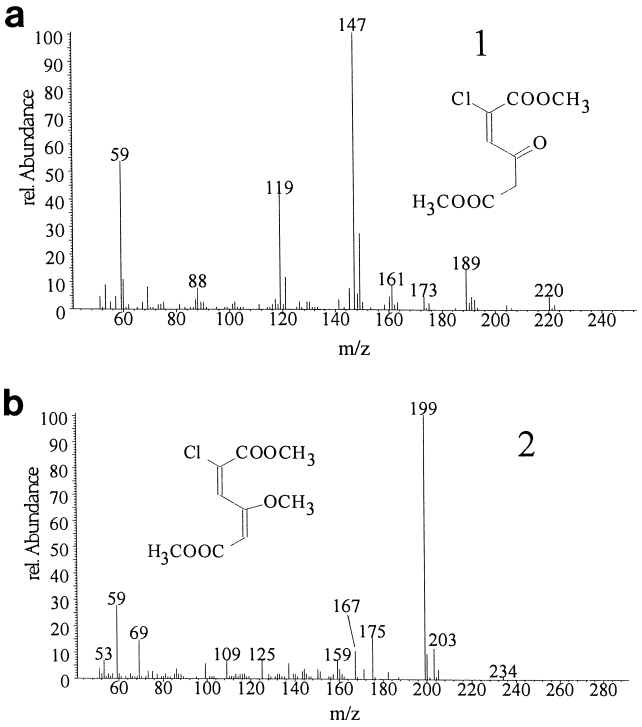

FIG. 1. Conversion of 3,6-dichlorocatechol by cell extract of *X. flavus* 14p1 for 80 min. The mixture was extracted and methylated as described in Materials and Methods. (a) MS of dimethylated 2-chloromaleylacetic acid (compound 1); (b) MS of trimethylated 2-chloro-4-hydroxymuconic acid (compound 2).

Enzymatic conversion of 3,6-dichlorocatechol for 80 min showed a more complex total ion chromatogram. 2,5-Dichloromuconic acid dimethylester was present only in small amounts, but two other chlorinated compounds were found. The spectrum of compound 1 (12.6 min) was interpreted as deriving from dimethylated 2-chloromaleylacetic acid (Fig. 1a). The molecular ion is visible with low intensity at *m/z* 220. Loss of one methoxy group yields the fragment with *m/z* 189, whereas elimination of a carboxymethoxy group from the molecular ion explains the fragment with *m/z* 161. The base peak of the spectrum is *m/z* 147, which could originate from α-cleavage of the molecular ion releasing methylacetate $\cdot CH_2COOCH_3$. The ion at *m/z* 119 can be explained by loss of CO from *m/z* 147; *m/z* 88 can be rationalized by loss of the second carbomethoxy group from *m/z* 147. The isotopic patterns of all of these ions show a ratio of 3:1, indicating monochlorinated compounds. The presence of the carbomethoxy group is also indicated by an intense ion at *m/z* 59.

The spectrum of compound 2 (15.7 min) was interpreted as deriving from the tautomeric form of 2-chloromaleylacetic acid, the trimethylated product of 2-chloro-4-hydroxymuconic acid (Fig. 1b). The intensity of the molecular ion at *m/z* 234 is very low, but the spectrometer detects the ions at *m/z* 234 and *m/z* 236 in an abundance ratio of 339:102, indicating a monochlorinated compound. Further characteristic ions with the

same isotopic cluster are at *m/z* 203 after elimination of a methoxy group and *m/z* 175 due to loss of a carboxymethoxy group from the molecular ion. The base peak at *m/z* 199 has a mass difference of 35 atomic mass units to the molecular ion and is lacking the isotopic chlorine pattern, proving the elimination of the chlorine atom. Further evidence is given by the ^{13}C-isotopic peak at *m/z* 200 with an intensity of 10% of that at *m/z* 199, indicating the nine carbon atoms of the postulated compound. The ion at *m/z* 167 can be explained by elimination of HCl from *m/z* 203, and *m/z* 59 represents the carboxymethoxy group. The other ions are present in intensities lower than 10% of the base peak.

Decarboxylation of 2-chloromaleylacetic acid under acid extraction conditions is described by several groups (15, 18). The resulting 2-chloroacetoacrylic acid was also detected in our enzymatic conversion reactions of 3,6-dichlorocatechol (data not shown), confirming the interpretation of the mass spectra of compounds 1 and 2.

The conversion of 3,6-dichlorocatechol by cell extract to 2,5-dichloro-*cis,cis*-muconic acid and 2-chloromaleylacetic acid shows that *X. flavus* 14p1 uses the modified *ortho* pathway as it has been proposed for *Pseudomonas* and *Alcaligenes* species (12, 17, 18). All degradation intermediates of 1,4-DCB shown in Fig. 2 except 2-chloro-4-carboxymethylene-but-2-en-4-olide could be isolated and identified by GC-MS with *X. flavus* 14p1, providing definitive proof for the bacterial degradation of 1,4-DCB by dioxygenation, ring opening by *ortho* cleavage, and conversion to 2-chloromaleylacetic acid.

Enzyme activities after growth on different carbon sources. An initial oxygenase attacking DCBs and a catechol 1,2-dioxygenase were present in intact cells grown on 1,4-DCB. No activity was found in cells grown on succinate (Table 1). Activity of the initial NADH- and Fe^{2+}-dependent oxygenase was also detected in cell extracts when acetate buffer was used. The enzyme, however, was very unstable, and the activities varied from cell batch to cell batch. No activity of catechol 2,3-dioxygenase was detected. Dihydrodiol dehydrogenase activity was found in cells grown on 1,4-DCB as well as in cells grown on succinate. Upon growth on 1,4-DCB, the activity was induced twofold. The specific activities reported in Tables 1 and 2 are from parallel-grown cultures. The specific activities of identical but non-parallel-grown cultures varied by ±15%.

In all of the initial multicomponent dioxygenase gene clusters known, the genes for the dioxygenase are followed by a gene encoding dihydrodiol dehydrogenase (20). Induction of both enzymes has also been reported for other DCB-degrading bacteria (7, 18); however, in *Pseudomonas* sp. strain PS12, degrading 1,2,4-trichlorobenzene, both enzymes are expressed constitutively (16). The catechol 1,2-dioxygenase is induced on 1,4-DCB in all DCB-degrading bacteria studied to date (1, 7, 12, 16, 18).

The initial dioxygenation appears to be the rate-limiting step in 1,4-DCB degradation by *X. flavus* 14p1 (Tables 1 and 2). This conclusion, however, is not in agreement with the accumulation of 3,6-dichlorocatechol in the culture fluid. Assuming that the activity of 1,4-DCB dioxygenase is significantly underestimated because of its instability, the accumulation of 3,6-dichlorocatechol is readily explained by the exceptional high activity of the dihydrodiol dehydrogenase compared with that of chlorocatechol 1,2-dioxygenase (Table 2). The limited potential of *X. flavus* to use chlorinated benzenes is not due to the limited substrate specificity of the initial dioxygenase, since it attacks 1,3-DCB (Table 1). The dihydrodiol dehydrogenase also oxidizes with high activity 3,5-DCB dihydrodiol, the product of the dioxygenation of 1,3-DCB, and catechol 1,2-dioxygenase accepts a number of substituted chloro- and methylcatechols as shown in Table 2. Either 1,3-DCB does not induce the degradation pathway or the chloromuconate cycloisomerase and/or the dienelactone hydrolase is not able to accept as substrates the respective metabolites from the degradation of 1,3-DCB.

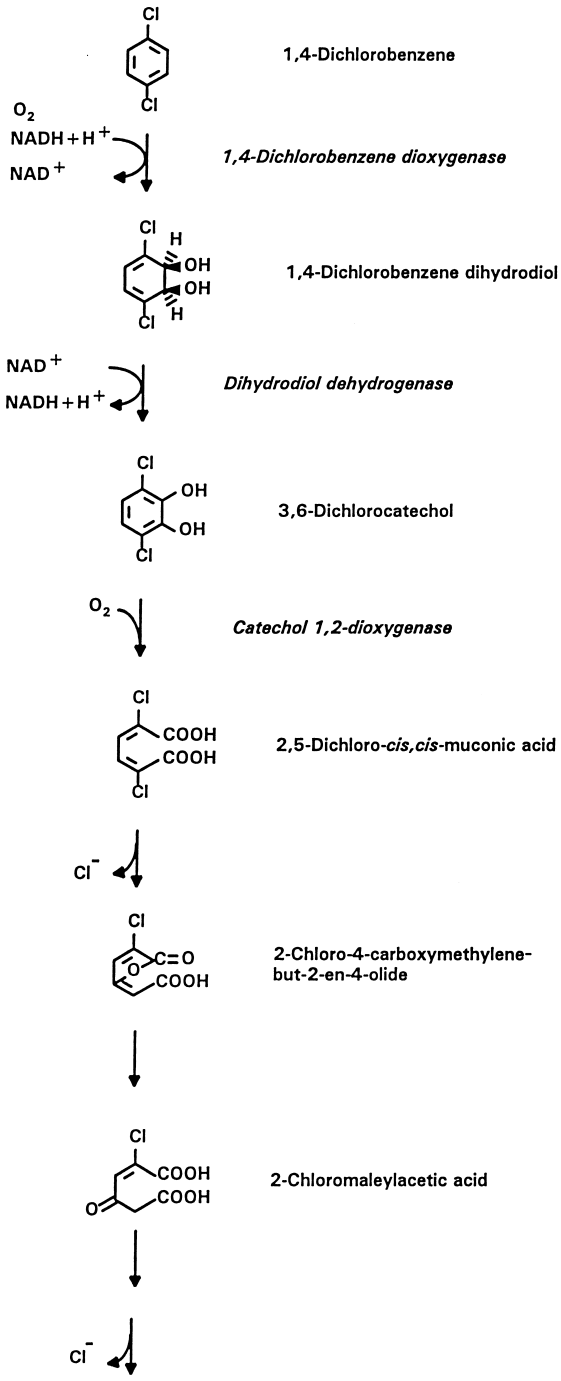

FIG. 2. Degradation pathway of 1,4-DCB as found in *X. flavus* 14p1. The metabolites shown have been isolated from culture fluid and enzymatic conversion mixtures (except 2-chloro-4-carboxymethylene-but-2-en-olide). The first three enzymes have been found in cell extracts.

Batch fermentation of *X. flavus* 14p1 on 1,4-DCB. Reliable and reproducible growth of *X. flavus* 14p1 in a 10-liter batch fermentation with 1,4-DCB as the sole carbon and energy

TABLE 1. Oxygen consumption by washed cells of *X. flavus* 14p1

Test substrate	Rate of oxygen consumption[a] after growth on:	
	1,4-DCB	Succinate
Benzene	<1	<1
1,4-DCB	24	<1
1,3-DCB	13	<1
1,2-DCB	<1	<1
Catechol	460	<1

[a] Results are from parallel-grown shaking cultures. Rates of oxygen consumption are expressed as nanomoles per minute per milligram of protein. Triplicate measurements were taken, and the standard deviation was ±10%.

source could be achieved by using a liquid two-phase system. 2,2,4,4,6,8,8-Heptamethylnonane was chosen because it is nontoxic, nondegradable, insoluble in water, and has a high solubility for hydrophobic substrates (10). In this way, a relatively constant level of 1,4-DCB, supporting a reasonable growth rate and reproducible fermentation conditions, is ensured. Feeding of the chloroaromatic compound via the gaseous phase needs expensive analytical and regulatory equipment to avoid toxic effects (14, 18). Exponential growth was observed with a doubling time of 8 h, and the concentration of chloride released increased exponentially as well (Fig. 3). A decrease in pH from 7.1 to 6.0 had no inhibitory effect because of the broad pH optimum of *X. flavus* (21). The specific activity of the initial dioxygenase was essentially constant, that of dihydrodiol dehydrogenase increased significantly with time, while catechol 1,2-dioxygenase showed a slight maximum at an A_{580} of 1.2 to 1.3. The specific activities varied from fermentation to fermentation by ±15%. The reason why the specific activities of dihydrodiol dehydrogenase and catechol 1,2-dioxygenase in cells from shaking cultures (Table 2) were consistently higher by a factor of two than the activities of cells grown in the two-phase system (Fig. 3) is not known.

TABLE 2. Specific catabolic enzyme activities in cell extracts of *X. flavus* 14p1

Enzyme	Test substrate	Enzyme activity[a] after growth on:	
		1,4-DCB	Succinate
Dihydrodiol dehydrogenase	Benzene dihydrodiol	2.3 (100)	1.3 (100)
	3-Methylbenzene dihydrodiol	5.0 (220)	2.8 (220)
	3-Fluorobenzene dihydrodiol	3.3 (140)	1.8 (140)
	3,5-DCB dihydrodiol	4.4 (190)	2.4 (185)
	3,6-DCB dihydrodiol	2.0 (85)	0.79 (65)
Catechol 1,2-dioxygenase	Catechol	0.28	<0.01
	3-Chlorocatechol	0.26	<0.01
	4-Chlorocatechol	0.18	<0.01
	3,4-Dichlorocatechol	0.015	<0.01
	3,5-Dichlorocatechol	0.22	<0.01
	3,6-Dichlorocatechol	0.12	<0.01
	4,5-Dichlorocatechol	<0.005	<0.01
	3-Methylcatechol	0.67	<0.01
	4-Methylcatechol	0.47	<0.01
Catechol 2,3-dioxygenase	Catechol	<0.01	<0.01
	3-Methylcatechol	<0.01	<0.01
	4-Methylcatechol	<0.01	<0.01

[a] Results from parallel-grown shaking cultures. Enzyme activities are expressed in micromoles per minute per milligram of protein and, for dihydrodiol dehydrogenase, also as percentages (in parentheses) of that found with benzene dihydrodiol as the substrate. Triplicate measurements were taken, and the standard deviations for dihydrodiol dehydrogenase and catechol 1,2-dioxygenase were ±5 and ±7%, respectively.

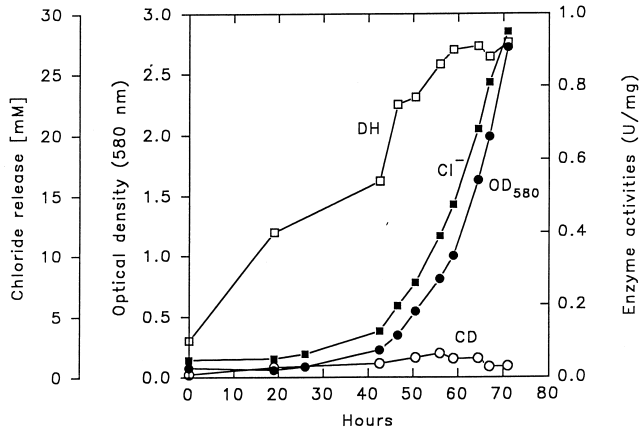

FIG. 3. Growth of *X. flavus* 14p1 on 1,4-DCB. The liquid two-phase system described in Materials and Methods was used in a 10-liter batch fermentation. Symbols: ●, optical density at 580 nm; ■, chloride release; □, dihydrodiol dehydrogenase activity with 3,6-dichlorobenzene dihydrodiol as the substrate (triplicate measurements, ±5% standard deviation); ○, catechol 1,2-dioxygenase activity with 3,6-dichlorocatechol as the substrate (triplicate measurements, ±7% standard deviation).

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Elke Spiess and Claudia Sommer contributed equally to this work.

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