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Degradation of 1,4-dichlorobenzene by enriched and constructed bacteria

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Summary. Three strains, RHO1, R3 and B1, tentatively identified as a *Pseudomonas* sp., an *Alcaligenes* sp. and a *Pseudomonas* sp. which were able to use 1,4-dichlorobenzene as the sole carbon and energy source were isolated from water of the Rhine river and from the sewage plant at Leverkusen-Bürrig. A hybrid strain, WR1313, which uses chlorobenzene as the growth substrate, was obtained by mating the benzene-growing *Pseudomonas putida* strain F1 with strain B13, a *Pseudomonas* sp. degrading chlorocatechols. Further selection of this strain for growth on 1,4-dichlorobenzene allowed the isolation of strain WR1323. During growth on 1,4-dichlorobenzene the strains released stoichiometric amounts of chloride. The affinity of the organisms to 1,4-dichlorobenzene was measured with strain R3 showing a K_s value of 1.2 mg/l. Respiration data and enzyme activities in cell extracts as well as the isolation of 3,6-dichlorocatechol from the culture fluid are consistent with the degradation of 1,4-dichlorobenzene via 3,6-dichlorocatechol, 2,5-dichloro-*cis,cis*-muconate, 2-chloro-4-carboxymethylenebut-2-en-4-olide.

Introduction

1,4-Dichlorobenzene (1,4DCB) has been used for several decades. Approximately 50% is used to deodorize and sanitize toilets and refuse containers, 40% to control moths, and 10% in other applications such as the synthesis of dyes and other chemicals. The total production in the world of 1,4DCB was estimated at 80000 t per year (Pearson 1982). Large amounts of 1,4DCB have entered the environment and has been detected in various sorts of waters, air, and biological tissues including those from man (Morita 1977; Morita and Ohi 1975; Pearson 1982).

Because of its high use information on its biodegradation is of great interest. Trace concentrations of 1,4DCB were cometabolized by acetate-supported biofilms under aerobic conditions with an acclimation period of 10 days (Bouwer and McCarty 1985). Kuhn et al. (1985) observed the degradation at low concentration (nM to μM) of 1,4DCB under aerobic conditions by using laboratory soil columns. Two field studies aimed at investigating the transport and fate of organic micropollutants, including 1,4DCB, during natural infiltration of river water to groundwater also indicated that it is biodegradable (Schwarzenbach et al. 1983). The first isotope study which employed ^{14}C-labelled dichlorobenzenes (a mixture of the isomers) was published by Haider et al. (1974). They reported that approximately 1—6% of the original organically bound ^{14}C was converted to $^{14}CO_2$ by benzene-growing pure strains and soil-samples within 10 weeks of incubation. In contrast, Bouwer and McCarty (1982) reported that about 98% of 1,4DCB bound ^{14}C was released as $^{14}CO_2$ when acetate-grown microbial populations were acclimated to the chlorinated compound.

The complete metabolism of 1,4DCB has not been fully elucidated to date. Erikson (1941) observed an increase in turbidity due to growth of strains classified as *Micromonospora* at the expense of 1,4DCB during a 6-week incubation. Recently, two strains of *Alcaligenes* sp. and a strain

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of *Pseudomonas* sp. were isolated which were able to use 1,4DCB as the growth substrate (DeBont et al. 1986; Schraa et al. 1986; Spain and Nishino 1987).

Here we describe the isolation of three 1,4DCB-growing strains from riverwater and sewage samples as well as the construction of a 1,4DCB-growing strain by mating a benzene-growing strain with one which degrades chlorocatechol.

Materials and methods

Bacterial strains. The strains used in this work are listed in Table 1.

Media and culture conditions. The media and culture conditions used were as previously described (Dorn et al. 1974).

Continuous culture conditions. The conditions used for growth in continuous culture were as previously described (Reineke and Knackmuss 1984).

Measurement of growth. Growth of the cultures was monitored turbidimetrically at 546 nm with a UV-240 spectrophotometer (Shimadzu, Kyoto, Japan) or a colorimeter (Klett-Summerson, New York, USA) equipped with a 520- to 580-nm filter (10 Klett-units corresponds to 0.063 $OD_{546\,nm}$).

Enrichment. The bacteria from 1 l of water taken from the Rhine river at Leverkusen were concentrated by filtration and then washed from the filters into 50 ml mineral medium (Dorn et al. 1974) in closed Erlenmeyer flasks. 1,4DCB was introduced into the side arm. After shaking the flasks for 4 days at 30° C the culture was turbid. Subcultures were inoculated every fourth day for 7 passages. The culture was then plated on mineral agar plates which were incubated with or without 1,4DCB vapor. After eight days colorless to slightly yellow colonies approximately 1—2 mm in diameter appeared on the plates with 1,4DCB. No colonies were observed on plates lacking 1,4DCB. After several transfers on solid medium with 1,4DCB as the only growth substrate, strain RHO1 was isolated. By using the API 20E-system we have tentatively classified the strain as *Pseudomonas aeruginosa*. The strains R3 and B1, which were independently isolated from the Rhine river and a sewage sample from Leverkusen, were identified as *Alcaligenes* sp. and *Pseudomonas* sp.

Construction. Some details of the construction of strain WR1313 have been previously described (Weisshaar et al. 1987). *P. putida* strain F1 and *Pseudomonas* sp. strain B13 were grown overnight together on L-broth agar. A suspension was then spread on mineral agar plates placed in the presence of vapor of chlorobenzene (660 ppmv). Colonies appearing after 3 weeks were purified by several transfers on solid medium in the presence of chlorobenzene. One clone, strain WR1313, was then inoculated into closed Erlenmeyer flasks with 1,4DCB as the only carbon source present in the side arm. When turbidity was observed the culture was subcultured. After 18 months and 5 transfers we were able to isolate strain WR1323 which can grow in the presence of 1,4DCB as the sole carbon and energy source.

Preparation of cell-free extracts. Cells were grown and harvested and extracts were prepared as previously described (Reineke and Knackmuss 1984).

Protein estimation. The protein content of extracts was determined by the method of Bradford (1976), using bovine serum albumin as the standard.

Enzyme assays. Enzymes were assayed by using previously described procedures: catechol 1,2-dioxygenase (C120, E.C. 1.13.11.1), catechol and chlorosubstituted analogs (Dorn and Knackmuss 1978); catechol 2,3-dioxygenase (C230, E.C. 1.13.11.2), catechol (Nozaki 1970); muconate cycloisomerase (E.C. 5.5.1.1.), muconate and 2-chloromuconate (Schmidt and Knackmuss 1980); 4-carboxymethylenebut-2-en-4-olide hydrolase (DLH, E.C. 3.1.1.45), *trans*-4-carboxymethylenebut-2-en-4-olide (Schmidt and Knackmuss 1980); maleylacetate reductase (E.C. 1.3.1.32), maleylacetate (Gaal and Neujahr 1980).

Dioxygenation of benzene and chlorosubstituted derivatives was assayed indirectly with whole cells. The stimulation of uptake of oxygen by the addition of substrate was measured by Warburg respirometry as previously described (Reineke and Knackmuss 1984).

Table 1. Bacterial strains used in this work

Strain	Relevant phenotypes	Source
B13 (*Pseudomonas* sp.)	$3CBA^+$	Dorn et al. (1974)
F1 (*P. putida*)*	B^+, T^+, E^+	Gibson et al. (1968), Finette et al. (1984)
WR1313 (*P. putida*)	B^+, T^+, CB^+	Mating: B13 × F1, selection for growth with CB (Weisshaar et al. 1987)
WR1323 (*P. putida*)	B^+, T^+, CB^+, $1,4DCB^+$	Adaptation of WR1313 when selected for growth with 1,4DCB
RHO1 (*Pseudomonas* sp.)	B^-, T^-, CB^+, $1,4DCB^+$, $1,3DCB^+$	isolated from the Rhine river
R3 (*Alcaligenes* sp.)	B^+, T^+, CB^+, $1,4DCB^+$	isolated from the Rhine river
B1 (*Pseudomonas* sp.)	B^+, T^+, CB^+, $1,4DCB^+$	isolated from samples of the sewage plant at Leverkusen-Bürrig

$3CBA^+$, B^+, T^+, E^+, CB^+, $1,4DCB^+$, $1,3DCB^+$ denote the ability to grow on 3-chlorobenzoate, benzene, toluene, ethylbenzene, chlorobenzene, 1,4-dichlorobenzene, and 1,3-dichlorobenzene, respectively, as the sole source of carbon and energy

* formerly designated in our laboratory as strain DTG (Weisshaar et al. 1987)

Analytical methods. Chloride ion concentrations were measured with an ion selective combination chloride electrode (Model 96/17, Orion Research, Inc., Cambridge, Mass.), which was calibrated with NaCl (0.1 up to 50 mM) in mineral medium before each measurement.

Substrates and metabolites in culture media were analysed by high-pressure liquid chromatography on a LiChrospher RP8 column from Bischoff Analysentechnik, Leonberg, FRG. The mobile phase consisted of 10 mM H_3PO_4, 0.5% (v/v) 2-propanol and 56.5% (v/v) MeOH.

Chemicals. 3-Chloro-, 4-chloro-, 3,5-dichlorocatechol, and *trans*-4-carboxymethylenebut-2-en-4-olide were prepared by previously described biological and chemical procedures (Reineke and Knackmuss 1980; 1984). 3,6-Dichlorocatechol was a generous gift from Juha Knuutinen, University of Jyväskylä, Finland, while *cis,cis*-muconate and 2-chloro-*cis,cis*-muconate were kindly provided by E. Schmidt, Wuppertal.

All other chemicals were from commercial sources.

Results

Isolation of 1,4-dichlorobenzene-growing strains

Organisms able to grow upon 1,4-dichlorobenzene were isolated using two different procedures. Three organisms were isolated by using an enrichment technique. One organism was constructed by mating two known pure cultures. The enrichment procedure is described in detail for strain RHO1 in "Materials and methods". Strain WR1323 was obtained by mating *Pseudomonas putida* strain F1 with *Pseudomonas* sp. strain B13 and by selecting for a chlorobenzene clone which could grow with 1,4DCB as the sole carbon and energy source. *P. putida* strain F1 does not contain any detectable plasmid. In addition, conjugal transfer of the toluene dioxygenase genes could not be demonstrated (B. A. Finette, PhD dissertation, University of Texas at Austin, 1984). Therefore genes responsible for the degradation of toluene and related compounds appear to be located on the chromosome. Although plasmid isolation techniques have failed in our hands to indicate the presence of an extrachromosomal element in strain B13, Chatterjee and Chakrabarty (1983) reported the successful isolation of a 111 kb plasmid from this strain. The conjugal transfer of the genes coding the degradation of chlorocatechols from strain B13 has previously been shown (Reineke et al. 1982b). One chlorobenzene-positive clone from the mating, designated WR1313, was further investigated. By comparison of characteristic nutritional properties (growth at 41°C, growth with nicotinate and geraniol) of this hybrid strain with those of the parent organisms, it became evident that strain WR1313 had the genetic background of *Pseudomonas putida* strain F1. Probably this organism has acquired the capability of chlorocatechol degradation from *Pseudomonas* sp. strain B13. We were recently successful in isolating cccDNA from this clone (Weisshaar et al. 1987) by use of the method of Hansen and Olsen (1978). Strain WR1323, which was able to grow upon 1,4DCB as its sole carbon and energy source was derived from strain WR1313 after growth for 18 months in the presence of 1,4DCB vapor in batch culture.

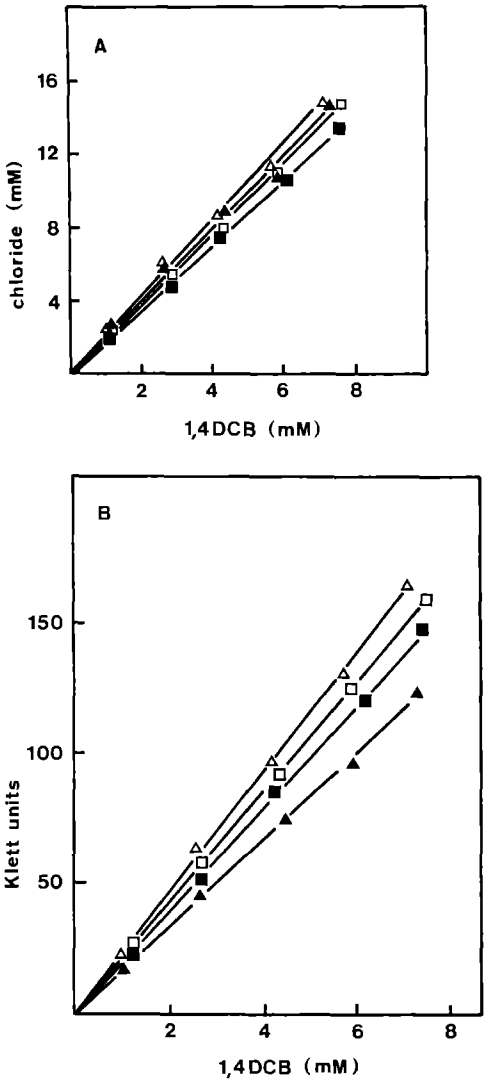

Fig. 1A. 1,4DCB consumed and chloride released during growth of strain RHO1 (△), WR1323 (▲), R3 (■), and B1 (□). Each strain was grown in five separate cultures in 250 ml mineral medium in sealed 3 l Erlenmeyer flasks. Solid 1,4DCB was added to a side arm in small portions over a period of 2 weeks. The concentration of the substrate was calculated as if it was being totally absorbed by the medium. **B.** Increase in turbidity versus 1,4DCB consumption in culture (for legend see 1A)

Growth characteristics

We determined the effect of various concentrations of 1,4DCB upon growth and release of chloride in liquid culture. The level of growth was directly proportional to the amount of 1,4DCB added to the cultures (Fig. 1). Chloride recovery was nearly stoichiometric indicating almost total degradation of 1,4DCB. Strains RHO1, R3 and B1 grew on 1,4DCB with generation times of 7.2, 7.9, and 7.8 h, respectively, while strain WR1323 needed 9.9 h for one doubling.

During the growth upon 1,4DCB a violet coloration of the medium occurred which changed to brown-black (shown in Figure 2 for strains WR1323 and RHO1). This is due to the accumulation of a compound we have identified as 3,6-dichlorocatechol. Strains WR1323, R3 and B1 differ from strain RHO1 by accumulating relatively high levels of 3,6-dichlorocatechol. With strain WR1323 the excretion of 3,6-dichlorocatechol into the medium could be initiated by the addition of reagents such as sodium dodecyl sulfate or cetyltrimethylammoniumbromide leading to a violet coloration of the medium. Strain RHO1 excreted only a constant, low amount of 3,6-dichlorocatechol into the culture fluid that did not discolor the medium.

By use of a chemostat, the K_s for 1,4DCB as well as the maximum growth rate of strain R3 was determined. Figure 3 shows the steady state relationship in the continuous culture. A K_s value of 1.2 mg/l was estimated according to Monod (1950) and Novick and Szilard (1950). The concentration of 1,4DCB in the fermentor fluid was estimated from the concentration of the substrate in the gas out flow using the Oswald solubility $c(H_2O)/c(air)$ of 14.8 (Frische et al. 1981).

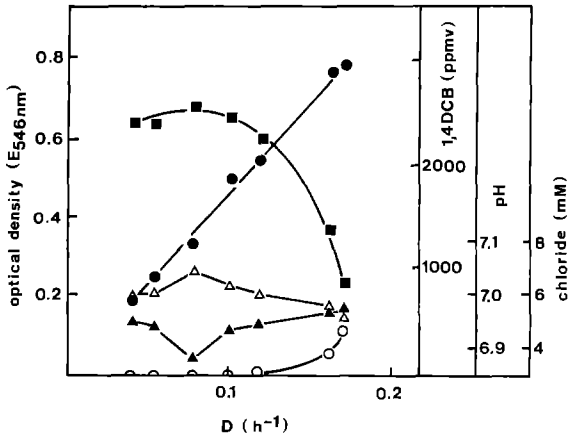

Fig. 3. Steady state relationship in a continuous culture of strain R3 with 1,4DCB as the substrate. The steady state values of turbidity (■), substrate in the incoming (●) and outgoing air (○) (10 liters per h), chloride (△) and pH (▲) at different dilution rates were obtained after six exchanges of the total volume after each change of the dilution rate. The substrate in the incoming air was changed in proportion to the dilution rate. The flow rate of the gas was adjusted by flowmeters. The concentration of the 1,4DCB in the incoming and outgoing air was monitored spectrophotometrically at 227.5 nm. UV cuvettes with a 5-cm light path were used

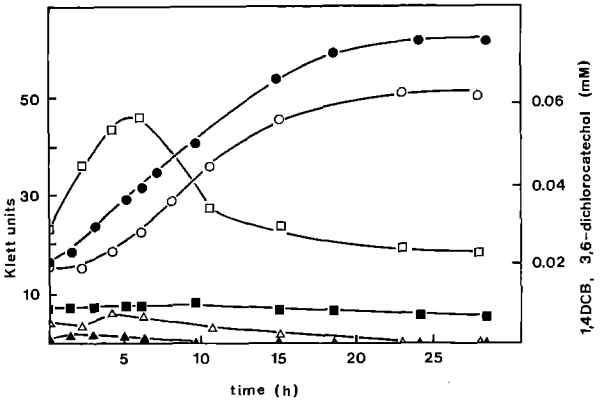

Fig. 2. Utilization of 1,4DCB by strain RHO1 (● ■ ▲) and strain WR1323 (○ □ △). Solid 1,4DCB at a concentration of 2.0 mM (the concentration was calculated as if it was being totally absorbed to the medium) was directly added to the medium. The concentration of substrate (▲ △) and 3,6-dichlorocatechol (■ □) were followed by high-pressure liquid chromatography. Growth (● ○) was observed turbidimetrically at 520—580 nm with a Klett-Summerson photometer

Oxidation of aromatic hydrocarbons by whole cells

Table 2 summarizes the data on the oxygen-consumption in the presence of various benzene derivatives. Strain RHO1 consumed oxygen in the presence of all the substrates at a much slower rate than the three other strains.

Cells grown in the presence of succinate did not show any activity with the substrates tested. Oxygen-consumption occurred at rates which correspond to endogenous respiration.

The consumption of p-xylene occurred at very low rates. Strain R3 failed to use this compound as a substrate for oxidation. When cell suspensions were incubated with 2,5-dichlorophenol, which has been postulated to be an intermediate of 1,4-dichlorobenzene metabolism (Ballschmiter et al. 1977), no oxygen uptake at the expense of this compound was measured with either strain.

Table 2. Rates of oxygen uptake by washed cell suspensions of strains RHO1, WR1323, R3, and B1

Assay substrate	Rate of oxygen uptake (µl oxygen per minute) Strain RHO1	Strain WR1323	Strain R3	Strain B1
benzene	(100) 0.87	(100) 3.14	(100) 2.51	(100) 2.88
chlorobenzene	(375)	(105)	(160)	(105)
1,4-dichlorobenzene	(300)	(30)	(60)	(55)
p-xylene	(50)	(10)	(<2)	(20)
2,5-dichlorophenol	(<2)	(<2)	(<2)	(10)

1,4DCB-grown cells were harvested during exponential growth. Oxygen uptake rates were determined by Warburg respirometry as previously described (Reineke and Knackmuss 1984) and are expressed as specific activities (µl oxygen per minute of a cell suspension of absorbance 5 at 546 nm, corrected for endogenous uptake). Relative activities of oxidation (given in parenthesis) are referred to benzene as 100%

Catabolic enzyme activities in cell free extracts

We have suggested that in the chlorobenzene-degrading strain WR1306 the degradation proceeds via 3-chlorocatechol and then further via the maleylacetate pathway with the elimination of chloride between 2-chloromuconate and 4-carboxymethylenebut-2-en-4-olide (Reineke and Knackmuss 1984). An analogous route could be expected for the metabolism of 1,4DCB, since the same typical enzyme activities for the breakdown of chlorocatechols were induced in 1,4DCB-grown cells (Table 3). During growth on 1,4DCB the following enzyme activities were induced: Catechol 1,2-dioxygenase with high activity for chlorosubstituted catechols, muconate cycloisomerase activity for 2-chloromuconate, hydrolase activity for *trans*-4-carboxymethylenebut-2-en-4-olide and reductase activity for maleylacetate. In contrast, in cells grown on succinate, these enzyme activities were not induced. In the strains B1, R3 and WR1323, catechol 2,3-dioxygenase activity was observed in 1,4DCB-grown cells. This was expected since these strains also grow on benzene and this enzyme is used to degrade benzene via the *meta*-pathway. The catechol 2,3-dioxygenase activity was absent in strain RHO1 under any condition of growth, which might explain its failure to use benzene as the growth substrate.

Discussion

Here we propose a complete degradative pathway for 1,4DCB based upon the enzyme activities that we found and the identification of 3,6-dichloroca-

Table 3. Specific activities of catabolic enzymes in cell-free extracts of 1,4DCB- and succinate succinate-grown cells

Enzyme	Substrate	Specific activities: 1,4DCB/succinate-cells Strain RHO1	Strain WR1323	Strain R3	Strain B1
Catechol 1,2-dioxygenase	catechol	(100) 1.62/0.02	(100) 0.41/0.01	(100) 0.38/0.01	(100) 0.79/0.02
	3-chlorocatechol	(130)	(180)	(175)	(105)
	4-chlorocatechol	(110)	(160)	(160)	(105)
	3,5-dichlorocatechol	(70)	(120)	(90)	(85)
	3,6-dichlorocatechol*	(50)	(80)	(95)	(50)
Catechol 2,3-dioxygenase	catechol	<0.01/ND	0.34/ND	0.54/ND	0.06/ND
Muconate cycloisomerase	muconate	(100) 0.09/ND	(100) 0.11/ND	(100) 0.08/ND	(100) 0.1/ND
	2-chloromuconate	(270)	(300)	(340)	(270)
4-Carboxymethylenebut-2-en-4-olide hydrolase	*trans*-4-carboxymethylenebut-2-en-4-olide	2.84/<0.01	4.05/<0.01	3.2/<0.01	4.6/<0.01
Maleylacetate reductase	maleylacetate	0.76/<0.01	0.4/<0.1	0.68/0.02	0.8/0.03

For preparation of cell-free extracts cells were harvested during exponential growth. Enzyme activities were determined as described (Dorn and Knackmuss 1978; Gaal and Neujahr 1980; Nozaki et al. 1970; Schmidt and Knackmuss 1980) and are expressed as absolute specific activities (µmol per minute per mg of protein). Relative activities of catechol 1,2-dioxygenase and muconate cycloisomerase (given in parenthesis) are referred to catechol or muconate as 100%, respectively, measured with extracts from 1,4DCB-grown cells

ND, not determined

*, 2,5-dichloromuconate: $\varepsilon_{260\,nm} = 14{,}250\ l \cdot mol^{-1} \cdot cm^{-1}$

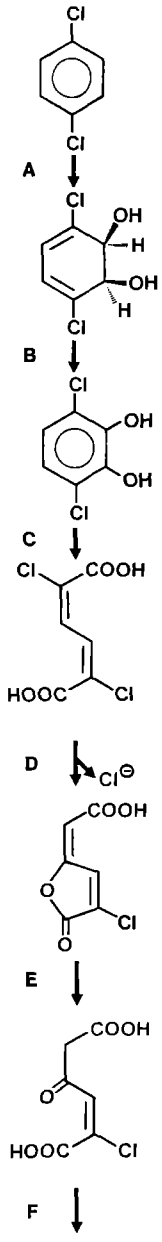

Fig. 4. Proposed catabolic pathway of 1,4-dichlorobenzene. The enzymes involved are as follows: A, benzene dioxygenase; B, dihydroxycyclohexadiene oxidoreductase; C, catechol 1,2-dioxygenase; D, chloromuconate cycloisomerase; E, 4-carboxymethylenebut-2-en-4-olide hydrolase; F, maleylacetate reductase

techol as a metabolite (Fig. 4). An analogous pathway has recently been described for an *Alcaligenes* sp. and a *Pseudomonas* sp. (Schraa et al. 1986; Spain and Nishino 1987). Ballschmiter et al. (1977) have shown that hydroxylation of chlorobenzenes by what they interprete as monooxygenase attack is carried out by soil microorganisms to give chlorophenols as the initial metabolites. However, such a pathway via 2,5-dichlorophenol was not used by the strains studied in this paper for the degradation of 1,4DCB. 3,6-Dichlorocatechol was subject to *ortho*-cleavage. Cycloisomerization of 2,5-dichloromuconate with elimination of chloride, yielding a chlorosubstituted 4-carboxymethylenebut-2-en-4-olide, was proposed in analogy to the reaction demonstrated for 2,4-dichloromuconate in the degradation of 2,4-D. By use of a hydrolase, a chloromaleylacetate was formed. Chapman (1979) has proposed a NADH-dependent reaction for the degradation of β-chloromaleylacetate. Maleylacetate was thought to be the first product generated by β-elimination of hydrogen chloride, which was further reduced by the same enzyme to give 3-oxoadipate.

Besides the *ortho*-pathway described, the initial enzyme of the *meta*-pathway, the catechol 2,3-dioxygenase, was present during growth with 1,4DCB in all of the strains except strain RHO1. However, the *meta*-pathway was found to be unproductive for haloaromatics degradation and must be prevented to allow breakdown of chlorocatechols via the *ortho*-pathway. Different strategies might be used to avoid misrouting of chlorocatechols into the *meta*-pathway.

i) The basal level of catechol 2,3-dioxygenase is low and neither the chlorosubstituted substrate nor a metabolite is able to induce the enzyme.

ii) A spontaneously occurring mutation in the gene for the catechol 2,3-dioxygenase is selected, as has been shown for 4-chlorocatechol as the ring-fission substrate (Jeenes et al. 1982; Reineke et al. 1982a).

iii) The catechol 2,3-dioxygenase will be inactivated either by the substrate or suicidally by the product of its turnover, a reactive acylchloride, which has been shown for the enzymes from strain F1 and *P. putida* strain mt-2 with the substrate 3-chlorocatechol (Bartels et al. 1984; Klečka and Gibson 1981).

The third mechanism should also function for 3,6-dichlorocatechol. However, the high level of catechol 2,3-dioxygenase in 1,4DCB-grown cells clearly indicated that a suicidal inactivation did not occur with 3,6-dichlorocatechol. Misrouting into the *meta*-pathway was avoided since 3,6- as well as 3,5-dichlorocatechol were not able to function as a substrate for the catechol 2,3-dioxygenase (unpublished results).

However, independent from the presence or absence of the catechol 2,3-dioxygenase the cleavage of the 3,6-dichlorocatechol was found to be a critical step in the degradation of 1,4DCB in all of the strains studied, since it accumulated when the

media were saturated with 1,4DCB. A similar observation was reported for the degradation of chlorobenzene by strain WR1306 (Reineke and Knackmuss 1984). Chlorinated catechols were shown to be cleaved by an *ortho*-pyrocatechase which showed high specific activity for chlorinated substrates, in contrast to an enzyme which degrades nonchlorinated aromatic compounds. The oxygen concentration for half-maximum reaction velocity of such *ortho*-pyrocatechase type II in strain B13 with chlorocatechols was found to lie above the oxygen concentration in air-saturated water (Dorn and Knackmuss 1978). This might explain the great tendency of strain WR 1323 to accumulate 3,6-dichlorocatechol, since this organism harbors the B13 *ortho*-pyrocatechase II. In contrast, cultures of strain RHO1 accumulated far less 3,6-dichlorocatechol than those of the other strains. However, the lower accumulation can not be due to the fact that it is an enriched organism, since strains R3 and B1, which were also enriched accumulate high concentrations of 3,6-dichlorocatechol.

The enrichment conditions used to isolate the strains RHO1, R3 and B1 selected for organisms capable of growth at high 1,4DCB concentrations. Analysis of the kinetic properties of the strain R3 indicated that both the maximum specific growth rate (μ_{max}) and the Monod constant (K_s) are high, with values of 0.175 h^{-1} and 1.2 mg/liter, respectively. Similar kinetic properties have been reported by Stanlake and Finn (1982) for an *Arthrobacter* sp. capable of growth on pentachlorophenol, with a high Monod constant estimated to be approximately 60 mg/liter. Hill and Robinson (1975) published a value of <1.0 mg/liter for phenol with a *Pseudomonas putida*. Since concentrations of 1,4DCB in aquatic environments are in the range of 0.1—0.4 µg/l (Pearson 1982), which is below the K_s value, organisms such as strain R3 will not show efficient removal of 1,4DCB under natural conditions.

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