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Degradation of 2-Chloroallylalcohol by a *Pseudomonas* sp.

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Three *Pseudomonas* strains capable of utilizing 2-chloroallylalcohol (2-chloropropenol) as the sole carbon source for growth were isolated from soil. The fastest growth was observed with strain JD2, with a generation time of 3.6 h. Degradation of 2-chloroallylalcohol was accompanied by complete dehalogenation. Chloroallylalcohols that did not support growth were dechlorinated by resting cells; the dechlorination level was highest if an α-chlorine substituent was present. Crude extracts of strain JD2 contained inducible alcohol dehydrogenase activity that oxidized mono- and dichloroallylalcohols but not trichloroallylalcohol. The enzyme used phenazine methosulfate as an artificial electron acceptor. Further oxidation yielded 2-chloroacrylic acid. The organism also produced hydrolytic dehalogenases converting 2-chloroacetic acid and 2-chloropropionic acid.

Halogenated aliphatic hydrocarbons have become an important class of environmental pollutants due to accidents, improper disposal of wastes, or agricultural application. Biodegradation can be a useful tool for the cleanup of soil and water contaminated with such compounds and for the treatment of waste streams, provided that microorganisms can efficiently degrade these potentially recalcitrant structures. Therefore, information about the biochemical potential that is present in the environment and the distribution and physiology of the organisms carrying out dehalogenation reactions is desirable.

During the last decade, aerobic dehalogenation of aliphatic compounds has become the subject of extensive study. Several bacteria capable of utilizing such compounds for growth have been isolated, and their dehalogenating enzymes have been characterized (8, 15). Most attention has been paid to organisms that convert haloalkanes or halocarboxylic acids by hydrolytic dehalogenases. Biodegradation of chlorinated alcohols, which are intermediates in various chemical processes, has been studied in less detail, although dechlorination was demonstrated in a few cases (1, 14, 28, 30). Secondary alcohols can be dechlorinated by halohydrin dehalogenases via intramolecular substitution yielding epoxides (31). Brominated primary alcohols can be dechlorinated hydrolytically by organisms isolated on chloroalkanes, but the chlorinated analogs are poor substrates for the enzymes (11). In 2-chloroethanol-degrading bacteria, dechlorination follows oxidation of the chlorinated alcohol to 2-chloroacetate (28). The latter compound is a substrate of the hydrolytic 2-haloalkanoic acid dehalogenases, which are relatively common enzymes that have been divided into several classes (9, 20, 34). β-Substituted haloalkanoic acids are not hydrolyzed by these enzymes, however, and only a few cases of dechlorination of these compounds in vitro have been reported (1, 3, 10, 32).

This study concerns the degradation of 2-chloroallylalcohol, which together with other chloroallylalcohols is an intermediate or byproduct in industrial herbicide synthesis (25). The related 2-chloroacrolein (2-chloropropenal) is formed during chlorination of humic material or bleaching of softwood kraft and is mutagenic (20, 29). To our knowledge, no information on the microbial degradation of 2-chloroallylalcohol is available. The 3-chloroallylalcohols are degraded in soil (23) and may serve as growth substrates for pure bacterial cultures under aerobic conditions (1). Degradation proceeds via oxidation of the alcohol to β-chloroacrylate (3-chloropropenoic acid), and dechlorination is thought to be the result of hydration of the double bond, leading to elimination of the halogen from the chemically unstable product to form malonic acid semialdehyde (1, 10, 32). A different route was found for 3-chlorocrotonate (3-chloro-2-butenoic acid), in which dechlorination is probably the result of hydration after formation of the acyl coenzyme A thioester by enzymes involved in the β-oxidation pathway (16).

In this paper, we describe microbial growth on 2-chloroallylalcohol and propose a degradation pathway. A possible structure-activity relationship with respect to chlorine substitution and recalcitrance to biotransformation is presented.

MATERIALS AND METHODS

Isolation and characterization of strains. Bacterial strains able to grow on 2-chloroallylalcohol were isolated from soil samples collected at a chemical production plant and provided by A. Aarts of Monsanto Europe S.A., Louvain la Neuve, Belgium. Batch enrichments (50 ml each) were started in a mineral medium containing 1 mM 2-chloroallylalcohol as the carbon source and inoculated with 5% (vol/vol) soil. After three transfers to fresh medium, cells were streaked on agar plates with 2-chloroallylalcohol supplied via the gas phase. Pure cultures were isolated by repeated streaking, checked for purity on rich media, and maintained on plates with 2-chloroallylalcohol as the carbon source. Three isolates, which closely resembled each other but showed different patterns of chloroallylalcohol utilization, were chosen for further study.

Identifications were carried out according to the Biolog system (Biolog Inc., Hayward, Calif.), with 24-h incubations. Strain JD2 was further characterized by using the API 20NE test (Analytab Products, Plainview, N.Y.) and the MIDI fatty acid analysis (Microbial Identification System Inc., Newark, Del.) by D. Janssens (University of Ghent, Ghent, Belgium). Biolog tests, API tests, and electron mi-

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croscopy with negative staining were independently carried out by J. van den Toorn (Technical University of Delft, Delft, The Netherlands). Other routine tests were performed according to standard procedures (26).

Growth conditions. Bacteria were grown aerobically at 30°C under rotary shaking. To prevent evaporation of substrates, cultivation was carried out in closed flasks filled to one-third of their volume with medium. The mineral medium that was used in all experiments contained (per liter) 5.3 g of $Na_2HPO_4 \cdot 12H_2O$, 1.4 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 30 mg of yeast extract (Difco Laboratories, Detroit, Mich.) and 5 ml of a salts solution (14).

Resting cell assays. Cells were grown on different carbon sources (5 mM) and harvested by centrifugation (10 min, 16,000 × *g*), washed once, and resuspended in 10 mM phosphate buffer (pH 7.1) to a concentration of about 1.8 mg (dry weight) of cells per ml.

Dehalogenation by resting cell suspensions was measured at 30°C in 10 mM phosphate buffer (pH 7.1) containing 5 mM substrate in a final volume of 15 ml. The concentration of cells in the incubation mixtures was 1 mg (dry weight) per ml. At different time points, 0.5-ml samples were analyzed for halide levels. Other samples were analyzed (directly or after esterification) for product formation by high-pressure liquid chromatography or by gas chromatography. When necessary, values were corrected for spontaneous dehalogenation of the substrates in the absence of cells.

Oxygen consumption of suspensions of resting cells was measured with a biological oxygen monitor equipped with a Clark-type oxygen electrode. Cells were resuspended in 10 mM phosphate buffer (pH 7.1) at 30°C in a final volume of 5 ml to a concentration of 1 mg (dry weight) of cells per ml. The reaction was started by the addition of substrate (5 mM final concentration). Oxygen consumption rates were corrected for oxygen uptake in the absence of substrate.

Preparation of crude extracts. Cells were harvested in the late exponential growth phase, centrifuged (10 min, 16,000 × *g*), washed once with 10 mM Tris sulfate buffer (pH 7.5) containing 1 mM EDTA and 1 mM β-mercaptoethanol, centrifuged again (10 min, 27,000 × *g*) and suspended in this buffer at 0°C. After ultrasonic disruption of the ice-cold cells, a crude extract with a protein content of about 40 mg/ml was obtained by centrifugation (10 min, 12,000 × *g*).

Enzyme assays. Alcohol and aldehyde dehydrogenase activities were determined with a biological oxygen monitor. Incubation mixtures contained, in a final volume of 5 ml, 60 mM sodium pyrophosphate buffer (pH 9.0), 23 mM NH_4Cl, 5 mM substrate, and a suitable amount of enzyme. The reaction was started by the addition of 0.2 ml of 30 mM phenazine methosulfate, and oxygen consumption was monitored at 30°C. One unit of activity was defined as the amount of enzyme that catalyzes an oxygen consumption rate of 1 μmol/min. Values were corrected for oxygen uptake in the absence of substrate. Aldehyde dehydrogenase coupled to NAD or NADP reduction was measured as described previously (12).

Dehalogenase activities were assayed at 30°C in 50 mM Tris sulfate buffer (pH 7.5) containing 5 mM substrate in a final volume of 3 ml. The reaction was started by the addition of a suitable amount of crude extract. Dechlorination via the formation of a coenzyme A derivative was determined after addition of 2 mM coenzyme A, 2 mM ATP, and 1 mM Mg^{2+} to reaction mixtures containing 5 mM chloroacrylic acid. Dechlorination of 2-chloroacrylyl coenzyme A was tested with a freshly prepared sample of the thioester (10 mM final concentration) as the substrate. From all of these incubations, 0.5-ml samples were withdrawn at different time points and analyzed for halide levels (2, 13). Nonenzymatic dehalogenation was determined for each substrate and was used for correction of enzymatic dehalogenation rates. It was found negligible for all substrates except 2-chloroacrylyl coenzyme A. One unit of activity is defined as the amount of enzyme catalyzing the production of 1 μmol of halide per min. Specific activities are expressed as units per milligram of protein.

Analytical methods. Chlorinated allylalcohols were quantitatively determined by capillary gas chromatography by using a flame ionization detector. Samples (4.5 ml each) were extracted with 1.5 ml of diethyl ether containing 0.05 mM 1-bromohexane as an internal standard. Extracts of samples were analyzed as described previously (30).

Carboxylic acids were esterified with methanol. Samples (0.9 ml each) were centrifuged (5 min, 12,000 × *g*) and added to 2 ml of methanol and 0.2 ml of 25% H_2SO_4. 3-Chloropropionic acid (10 mM) was added as an internal standard. The mixture was incubated at 100°C for 90 min, and after cooling, extraction with 2 ml of hexane was performed. The hexane layer was analyzed by gas chromatography on a CPWax-52CB (Chrompack, Middelburg, The Netherlands) column as described above.

Chloroacrylic acids were analyzed by high-pressure liquid chromatography. Samples were prepared by filtration through a 0.2-μm-pore-size filter with a diameter of 13 mm. The chromatography system consisted of a Waters chromatography pump and an injector (Rheodyne model 7125) with a 20-μl sample loop. A cation-exchange column for organic acid analysis (4.6 by 300 mm) (Chrompack) equipped with a guard column was used with 0.01 N H_2SO_4 as the solvent and a flow rate of 0.8 ml/min at 50°C. Elution was monitored with a Chrompack UV-VIS variable wavelength spectrophotometer set at 210 nm, and peaks were recorded and integrated with an integration package (Kontron instruments) on an IBM PC-compatible computer.

Protein was determined by the method of Bradford with the Bio-Rad assay, with bovine serum albumin as the standard.

Halide levels were determined by the colorimetric method of Bergmann and Sanik (2).

Chemicals. 2-Chloroacrylic acid was prepared as described elsewhere (4). 2-Chloroacrylyl coenzyme A was prepared as described by Stadtman (27). Coenzyme A and ATP were obtained from Boehringer, and phenazine methosulfate was obtained from ICN. Chloroallylalcohols were a kind gift of A. Aarts (Monsanto Europe S.A.). Where necessary, the compounds were purified by vacuum distillation until a purity of at least 95% was reached. 2,3-Dichloroallylalcohol was only available as a mixture of 60% *cis* and 40% *trans* isomer. All other organic compounds were obtained from commercial sources and were checked for purity by gas chromatography.

RESULTS

Isolation and characterization of strains. Bacterial cultures able to grow on 2-chloroallylalcohol were isolated from soil after repeated transfers of batch enrichments (30°C) containing 1 mM 2-chloroallylalcohol as the sole carbon source. Plate purification on nutrient broth agar yielded three different pure cultures, designated strains JD1, JD2, and JD3.

All strains were gram-negative, nonmotile rods which were catalase and oxidase positive. Glucose fermentation

was negative, and reduction of sulfate was not observed. The three strains behaved very similarly on agar plates with a range of different carbon sources. The organisms had identical physiological profiles in the Biolog test, which resulted in them being classified in the group *Pseudomonas fluorescens*, with similarity scores of 0.786, 0.707, and 0.657 for strains JD1, JD2, and JD3, respectively. Testing of strain JD2 with the API 20NE system did not result in an identification at the species level, but the highest similarity was found with *P. fluorescens* (identity score, 63.1 to 70.4%). A comparison of the fatty acid profile of strain JD2 with those in the MIDI data base resulted in them being identified as *Pseudomonas putida* biotype B (score, 0.307 to 0.703) or *Pseudomonas chlororaphis* or *aureofaciens* (score, 0.293 to 0.577). These identification tests suggest that strain JD2 should be placed in the *fluorescens* complex of the rRNA group 1 of the genus *Pseudomonas*, which contains *P. fluorescens*, *P. putida*, and *P. aureofaciens* (21). Strain JD2 was nonmotile, however, and production of fluorescent pigments was weak on King A and King B media. Electron microscopy revealed the absence of flagella. On the basis of these results, a formal classification within the *fluorescens* complex was not possible, which can be due to the heterogeneity within the biotype varieties placed in this group. Alternatively, strain JD2 could represent a new species.

Growth of strains JD1 and JD2 on 2-chloroallylalcohol was accompanied by complete substrate utilization and production of equimolar amounts of chloride. The specific growth rates on 2-chloroallylalcohol were 0.17 and 0.19 h^{-1} for strains JD1 and JD2, respectively. Strain JD3 showed linear growth on 2-chloroallylalcohol, with incomplete substrate consumption. A specific growth rate could therefore not be determined, but growth was slow at all stages compared with that of strains JD1 and JD2. The ability of strains JD1, JD2, and JD3 to utilize 2-chloroallylalcohol was a stable property, because no 2-chloroallylalcohol-negative variants were detected after eight serial transfers of the organisms on nutrient broth plates.

Because strain JD2 showed the best growth on 2-chloroallylalcohol, this organism was chosen for further study. The organism was able to grow on ethanol, *n*-propanol, *n*-butanol, acetic acid, propionic acid, acrylic acid, crotonic acid, *trans*-2-hexenic acid, lactic acid, pyruvic acid, and glucose but not on methanol, 2-propanol, 2-butanol, acrolein, acetone, hydroxyacetone, or toluene. In batch cultures incubated with 2-propanol and 2-butanol, the production of acetone and 2-butanone, respectively, was detected by gas chromatography-mass spectrometry. Furthermore, no growth was observed with 2-chloroethanol, 2,3-dichloropropanol, 3-chloro-1,2-propanediol, trichloroacetic acid, 3-chloropropionic acid, 2,3-dichloropropionic acid, 2-chloroacrylic acid, *cis*-3-chloroacrylic acid, *trans*-3-chloroacrylic acid, *trans*-3-chlorocrotonic acid, allylchloride, 1,2-dichloroethane, 1-chloropropane, 2-chloropropane, 1,2-dichloropropane, 1,3-dichloropropane, 1,2-dibromopropane, *cis*-1,3-dichloropropene, *trans*-1,3-dichloropropene, and 2,3-dichloropropene.

Growth characteristics. Growth of strain JD2 on 2-chloroallylalcohol resulted in the disappearance of substrate and the simultaneous formation of biomass and equimolar amounts of chloride, with no indication of the accumulation of chlorinated intermediates (Fig. 1). Concentrations of 2-chloroallylalcohol above 15 mM were toxic, inhibiting growth.

Whether strain JD2 could utilize halogenated compounds that are structurally related to 2-chloroallylalcohol and its possible degradation products was determined (Table 1).

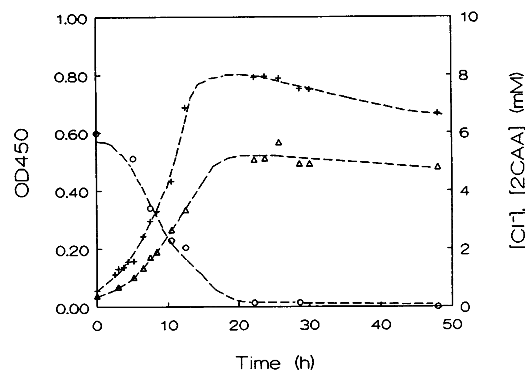

FIG. 1. Growth of strain JD2 on 2-chloroallylalcohol (2CAA) in batch culture. +, optical density at 450 nm (OD450); △, chloride concentration (millimolar); ○, 2-chloroallylalcohol concentration (millimolar).

The results show that besides 2-chloroallylalcohol and the nonchlorinated analog allylalcohol, *cis*-3-chloroallylalcohol is the only chloroallylalcohol that supported some growth of strain JD2 in batch culture. Growth was slow with incomplete substrate utilization, however. Other halogenated compounds that supported growth were 2-chloropropanol, 3-chloropropanol, chloroacetic acid, dichloroacetic acid, and 2-chloropropionic acid. It is remarkable that 2-chloroallylalcohol was a much better substrate for the organism than 2-chloropropanol and the nonchlorinated analog allylalcohol. With all chlorinated substrates, growth was accompanied by chloride release that strongly exceeded that of uninoculated controls.

Induction of 2-chloroallylalcohol metabolism. The induction of the 2-chloroallylalcohol degradation pathway in strain JD2 was investigated by measuring oxygen consumption and enzyme activities in cells grown on different substrates. Chloroallylalcohol stimulated oxygen consumption with alcohol-grown cells but not with citrate-grown cells, indicating

TABLE 1. Utilization of chlorinated compounds by strain JD2

Substrate[a]	Growth[b]	Generation time (h)[c]	Chloride produced (mM) in[d]: Inoculated culture	Chloride produced (mM) in[d]: Sterile control
Allylalcohol	++	5.0	ND[e]	ND
2-Chloroallylalcohol	++	3.6	5.46	0.06
trans-3-Chloroallylalcohol	−		0.32	0.07
cis-3-Chloroallylalcohol	+	>10[f]	2.97	<0.02
2,3-Dichloroallylalcohol	−		0.46	0.02
3,3-Dichloroallylalcohol	−		0.18	0.03
Trichloroallylalcohol	−		0.25	0.08
2-Chloropropanol	+	13.1	2.06	0.17
3-Chloropropanol	+	14.4	2.28	<0.02
Chloroacetic acid	++	3.8	4.29	0.07
Dichloroacetic acid	+	5.7	6.12	0.02
2-Chloropropionic acid	++	2.5	4.95	0.08

[a] Carbon sources were added at 5 mM final concentration.
[b] Growth was scored by an increase in turbidity (A_{450}) to above 0.2 within 1 day (++) or 5 days (+). −, no growth within 5 days.
[c] Generation times were determined after inoculation of fresh medium (1% inoculum) with cells from a culture pregrown on 2-chloroallylalcohol.
[d] Chloride levels were determined after 5 days of incubation at 30°C.
[e] ND, not determined.
[f] Slight growth visible but nonexponential.

TABLE 2. Oxygen consumption rates of resting cells of strain JD2 after growth on various carbon sources

Growth substrate[b]	Oxygen consumption (nmol/min per mg [dry weight] of cells) with substrate added[a]						
	Allylalcohol	2-Chloroallylalcohol	2-Chloropropionic acid	Citrate	Acrolein (0.1 mM)	Acrolein (2.5 mM)	Acrylic acid
Allylalcohol	79	19	80	<5	43	7	80
2-Chloroallylalcohol	42	81	97	<5	21	27	—[c]
n-Propanol	52	19	—	—	—	—	63
2-Chloropropionic acid	<5	<5	149	—	33	18	—
Citrate	17	<5	14	135	18	20	—

[a] Substrates were added at 5 mM final concentration unless stated otherwise.
[b] Late-exponentialphase cultures, pregrown on various substrates, were added at 0.9 mg (dry weight) of cells per ml.
[c] —, not tested.

the involvement of an inducible alcohol oxidation pathway (Table 2). 2-Chloroallylalcohol gave slower oxygen uptake in allylalcohol-grown cells than in 2-chloroallylalcohol-grown cells and vice versa, which suggests that different enzymes are involved in the metabolism of the two compounds. This is also indicated by the observation that acrolein (2-propenal) inhibited oxygen consumption by allylalcohol-grown cells but not by 2-chloroallylalcohol-grown cells. Acrylic acid stimulated oxygen consumption in both allylalcohol- and *n*-propanol-grown cells. Therefore, in the first step, allylalcohol metabolism in strain JD2 probably does not involve a hydratase induced by unsaturated aliphatic compounds.

Dechlorination of 2-chloroallylalcohol was found with resting cells grown on allylalcohol, 2-chloroallylalcohol, or *n*-propanol and therefore is not exclusively induced by chlorinated compounds (Table 3). 2-Chloroallylalcohol-grown cells, however, possessed a higher level of dechlorinating activity towards 2-chloropropionic acid than cells grown on other substrates. Cells grown on 2-chloropropionic acid did not dechlorinate 2-chloroallylalcohol because they lacked the initial alcohol-oxidizing enzyme (Table 2).

Dechlorination of other chloroallylalcohols by resting cells. Strain JD2 is capable of growth on only two out of seven chloroallylalcohols. The presence of enzymatic activity towards these compounds in cells cultivated on 2-chloroallylalcohol was investigated. Incubation of the substrates with resting cells resulted in a conversion of more than 99% within 24 h for six out of seven chloroallylalcohols tested. Only trichloroallylalcohol was still present at 70% of the original concentration after 24 h of incubation (Table 4).

For some substrates, intermediates were identified on the basis of retention time comparison after high-pressure liquid chromatography or by gas chromatography-mass spectrometry. The β-chlorinated compounds *trans*-3-chloroallylalcohol and 3,3-dichloroallylalcohol were oxidized to their corresponding acrylic acids without dechlorination taking place. α-Chlorinated allylalcohols (2-chloroallylalcohol, *cis*-2,3-dichloroallylalcohol, and *trans*-2,3-dichloroallylalcohol) were almost completely degraded and dechlorinated, with the formation of minor amounts of 2-chloroacrylic acid from 2-chloroallylalcohol and two unidentified products from the dichloro compounds, possibly the two isomers of 2,3-dichloroacrylic acid. Trichloroallylalcohol was only partially converted, but its degradation was accompanied by 50% dechlorination. The detection of 2-chloroacrylic acid from 2-chloroallylalcohol indicates that biodegradation of chloroallylalcohols proceeded via oxidation of the alcohol and formation and subsequent dechlorination of the corresponding acrylic acid.

TABLE 3. Dechlorination activities of resting cells of strain JD2 after growth on various carbon sources

Growth substrate	Chloride specific release rate (nmol/min per mg [dry weight] of cells)[a]	
	2-Chloroallylalcohol	2-Chloropropionic acid
Allylalcohol	8	20
2-Chloroallylalcohol	12	71
n-Propanol	11	16
2-Chloropropionic acid	<1	39
Citrate	<1	6

[a] Chloride production rates were measured with a 5 mM concentration of the substrate indicated.

Although 2-chloroethanol was not a growth substrate for strain JD2, washed cells of this organism dechlorinated this compound. Growth is apparently hindered by the lack of some other enzyme than a functional dehalogenase. With 2-chloroallylalcohol-grown cells, some dechlorination of 2-chloroacrylic acid could be observed, but conversion was slow and far from complete (Table 4).

Alcohol oxidation in crude extracts. Activities of enzymes that are possibly involved in chloroallylalcohol metabolism were tested with crude extracts prepared from cells grown on different substrates. 2-Chloroallylalcohol-grown cells of strain JD2 contained a phenazine methosulfate-linked alcohol dehydrogenase activity (Table 5), with activity for 2-chloroallylalcohol, indicating alcohol oxidation as the first metabolic step. The alcohol dehydrogenase was active at pH 9 in the presence of ammonium. No activity was found with NAD or NADP. Similar activities were found with other chloroallylalcohols. Substrates with both an α- and a β-chlorine substituent (2,3-dichloroallylalcohol, trichloroallylalcohol, and 2,3-dichloropropanol) were poor substrates for the enzyme. Other alcohols that were converted are *n*-propanol and 3-chloropropanol. Methanol was not oxidized.

In incubations with allylalcohol and 2-chloroallylalcohol, no aldehydes could be detected by gas chromatography. However, methanol-esterified samples of these incubations contained a compound with a retention time on gas chromatography identical to that of methylacrylate and 2-chloromethylacrylate, respectively. The latter compound was also identified by gas chromatography-mass spectrometry, giving the expected parent molecular ion at m/z = 120 and a fragmentation pattern consistent with the structure of monochloroacrylmethyl ester. The mass spectrum was compared with those in the CAOS/CAMM spectral data base (University of Nijmegen, Nijmegen, The Netherlands), confirming the identification as 2-chloroacrylmethyl ester. This implies

TABLE 4. Degradation of chloroallylalcohols by resting cells of strain JD2 grown on 2-chloroallylalcohol[a]

Substrate	Substrate left (mM)	Chloride produced (mM)	Product
2-Chloroallylalcohol	<0.01	6.36	2-Chloroacrylic acid[b]
trans-3-Chloroallylalcohol	<0.01	0.61	*trans*-3-Chloroacrylic acid[b]
cis-3-Chloroallylalcohol	<0.01	2.04	*cis*-3-Chloroacrylic acid[b]
trans-2,3-Dichloroallylalcohol	0.02	11.80	NI[c]
cis-2,3-Dichloroallylalcohol	<0.01		
3,3-Dichloroallylalcohol	<0.01	0.66	3,3-Dichloroacrylic acid[d]
Trichloroallylalcohol	3.57	1.96	NI
2-Chloroethanol	<0.01	6.00	
2-Chloroacrylic acid	4.09	0.99	

[a] Substrates (5 mM each) were added as indicated, and the remaining substrate, chloride, and products were determined after 24 h of incubation at 30°C.
[b] Identified by high-pressure liquid chromatography.
[c] NI, product not identified.
[d] Identified with gas chromatography-mass spectrometry.

that 2-chloroallylalcohol is rapidly oxidized via 2-chloroacrolein to 2-chloroacrylate.

The presence of an aldehyde dehydrogenase in cells of strain JD2 grown on different substrates was demonstrated (Table 6). Dehydrogenase activity with acrolein and propionaldehyde was present in all crude extracts tested, and levels of activity with these substrates were higher than with 2-chloroallylalcohol. Crude extracts of cells grown on 2-chloroallylalcohol contained low levels of activity towards formaldehyde and acetaldehyde (data not shown).

Aldehyde dehydrogenase activity was detected with phenazine methosulfate but not with NAD or NADP as the electron acceptor. Levels of aldehyde dehydrogenase activity were elevated during growth on alcohols or 2-chloropropionic acid compared with levels during growth on citrate. The alcohol dehydrogenase was induced only during growth on alcohols, indicating that the aldehyde and alcohol dehydrogenases are different enzymes.

Dechlorination of halogenated compounds in crude extracts. The dehalogenation of chlorinated compounds was further studied in crude extracts of cells grown on chlorinated and nonchlorinated compounds. A crude extract prepared from 2-chloroallylalcohol-grown cells did not show chloride release upon incubation with 2-chloroallylalcohol. This suggests that dechlorination is not the first step in the degradation pathway of 2-chloroallylalcohol, which is in agreement with the observation that 2-chloroallylalcohol is rapidly oxidized to 2-chloroacrylic acid in crude extracts. Halide production, however, was observed with chloroacetate, dichloroacetate, trichloroacetate, and 2-chloropropionate (Table 7).

Relative dechlorination activities were dependent on the growth substrate. During growth on allylalcohol and 2-chloroallylalcohol, a dehalogenase with a relatively high level of activity for 2-chloropropionic acid was induced. A dehalogenase with a higher level of activity for dichloroacetic acid was induced during growth on 2-chloropropionic acid and propanol. During growth on citrate, no dechlorinating activity was present in the cells. This indicates that strain JD2 contains two inducible dehalogenases with activity towards α-substituted haloalkanoic acids. These enzymes did not dechlorinate 2-chloroacrylic acid, the central metabolite in 2-chloroallylalcohol degradation, or other chloroacrylic acids (data not shown).

A likely pathway of chloroacrylic acid degradation is hydration of the double bond, resulting in an unstable intermediate. This could occur directly with 2-chloroacrylic acid, as with the 3-chloroacrylic acids (10, 33), or after formation of an activated intermediate, e.g., in a coenzyme A-dependent reaction, as was found with β-substituted crotonic acids (16). The first possibility was tested by incubating crude extracts with 2-chloroacrylic acid. No chloride release could be detected, even if the compound was incubated with high concentrations of crude extract protein.

However, the addition of cofactors involved in coenzyme A thioester formation also did not result in 2-chloroacrylic acid consumption or chloride production by the crude extract. Possibly, activation of 2-chloroacrylic acid required more than coenzyme A, ATP, and Mg^{2+}, or not all of the enzymes involved in this conversion were active in the crude extract. Therefore, synthetically produced 2-chloroacrylyl coenzyme A was added to the crude extract and chloride

TABLE 5. Alcohol dehydrogenase activities in crude extract from 2-chloroallylalcohol-grown cells

Substrate	Sp act (mU/mg of protein)
Allylalcohol	23.5
2-Chloroallylalcohol	20.1
trans-3-Chloroallylalcohol	21.0
cis-3-Chloroallylalcohol	24.1
2,3-Dichloroallylalcohol	9.2
3,3-Dichloroallylalcohol	23.2
Trichloroallylalcohol	<1
Methanol	<1
n-Propanol	49.1
2-Chloroethanol	2.4
3-Chloropropanol	32.6
2,3-Dichloropropanol	<1
1-Chloro-2,3-propanediol	<1

TABLE 6. Alcohol dehydrogenase and aldehyde dehydrogenase activities in crude extracts of cells grown on various carbon sources

Growth substrate	Sp act (mU/mg of protein)			
	Allylalcohol	2-Chloroallylalcohol	Acrolein	Propionaldehyde
Allylalcohol	9.1	5.0	21.1	58.6
2-Chloroallylalcohol	20.4	14.4	48.9	74.6
n-Propanol	5.8	4.9	12.4	15.1
2-Chloropropionic acid	2.0	<1	49.6	67.8
Citrate	1.8	<1	7.1	9.6

TABLE 7. Dehalogenase activities in crude extracts of strain JD2 grown on various carbon sources

Growth substrate	Dehalogenase sp act (mU/mg of protein)[a]			
	Chloro-acetate	Dichloro-acetate	Trichloro-acetate	2-Chloropro-pionate
Allylalcohol	9	23	ND[b]	24
2-Chloroallylalcohol	117	178	26	201
n-Propanol	79	388	ND	19
2-Chloropropionate	146	560	ND	59
Citrate	<5	ND	ND	<5

[a] No activity was found with 3-chloropropionic acid, 2-chloroacrylic acid, *cis*-3-chloroacrylic acid, and *trans*-3-chloroacrylic acid.
[b] ND, not determined.

release was monitored. Under the assay conditions used, however, the thioester appeared to be very unstable, and nonenzymatic liberation of chloride was observed in controls not containing the extract. It was therefore not possible to detect enzymatic hydration of 2-chloroacrylyl coenzyme A in crude extracts of strain JD2.

DISCUSSION

The results presented in this paper show that 2-chloroallylalcohol can serve as a substrate for bacterial growth. The newly isolated bacterial strains JD1 and JD2 both completely degraded 2-chloroallylalcohol and grew with a generation time of about 4 h. Degradation was accompanied by dechlorination, indicating detoxication. The strains were very similar. On basis of the Biolog and API tests, fatty acid analysis, and several other classification tests, strain JD2 was identified as a *Pseudomonas* sp., although the lack of motility seems to contradict this. The classification of the organism is under further investigation (15a).

The first step in 2-chloroallylalcohol metabolism in strain JD2 was oxidation of the alcohol to the aldehyde (Fig. 2). The alcohol dehydrogenase involved was not active with NAD or NADP but could use phenazine methosulfate as an artificial electron acceptor. This alcohol dehydrogenase was induced during growth on alcohols and could convert primary alcohols but not methanol. Activity with phenazine methosulfate but not with nicotinamide cofactors is a characteristic of the quinoprotein alcohol dehydrogenases that have been identified in several gram-negative bacteria, in which they are located in the periplasmic space (6). In methylotrophic bacteria, quinoprotein alcohol dehydrogenases that are active with primary alcohols but not with aldehydes have been described. In some nonmethylotrophic bacteria, on the other hand, an alcohol dehydrogenase that is active with primary and secondary alcohols and aldehydes but not with methanol is present (6). Possibly, this type of alcohol dehydrogenase is present in strain JD2. Secondary alcohols did not support growth of JD2, because the ketones produced were not metabolized by the organism.

The substrate range of alcohol oxidation was determined by the position of both the hydroxyl and chlorine substituents. The presence of both α- and β-chlorine substituents had a negative effect on the oxidation of both propanols and propenols. This effect was not due to the number of chlorine substituents, because 3,3-dichloroallylalcohol was readily oxidized. Trichloroallylalcohol, however, was not oxidized by crude extracts or suspensions of resting cells, indicating the importance of the degree of chlorination. It is not known whether the inhibition is dependent on the position of the β-chlorine substituent, because *trans*- and *cis*-2,3-dichloroallylalcohol were only available as a mixture.

Further oxidation was carried out by an aldehyde dehydrogenase that could deliver electrons to phenazine methosulfate. This activity appeared to be constitutively expressed and oxidized acrolein and propionaldehyde. The aldehyde-oxidizing activity appeared not to be caused by an alcohol dehydrogenase since cells grown on 2-chloropropionic acid showed high levels of aldehyde activity but low levels of alcohol oxidation activity. The rapid conversion of chloroacroleins to chloroacrylic acids is considered an important step, preventing the toxic effects of these reactive intermediates.

The results suggest that the catabolic pathway of chloroallylalcohol and its dechlorination are specific for this chlorinated compound. Oxygen uptake experiments indicate that there is no simultaneous induction of the enzymes involved in allylalcohol and chloroallylalcohol oxidation. Furthermore, organisms that can grow on allylalcohol do not usually degrade 2-chloroallylalcohol. These findings indicate the presence of specific enzymes for chloroallylalcohol catabolism.

In mammals, allylalcohol can be oxidized to glycidol through oxygenation or to acrylate by NAD-dependent dehydrogenases (22). With the latter conversion, acrolein is an intermediate, and this compound is responsible for the toxic effects of allylalcohol. The herbicides sulfallate, diallate, and triallate are converted by rats, yielding the corresponding chloroacrylates (18). The toxic effects of these agrochemicals are attributed to the formation of the carcinogenic 2-haloacroleins (19, 25, 29). Chloroacrolein was found, by the mouse skin assay, to initiate tumors (24). Oxidation of *cis*- and *trans*-3-chloroallylalcohol by cell suspensions of an unidentified *Pseudomonas* strain also led to the production of the corresponding chloroacrylic acids (1). In strain JD2, probably all chloroallylalcohols are degraded via oxidation of the alcohol to the corresponding chloroacrylic acid.

The recalcitrance to biodegradation of other chloroallylalcohols in strain JD2 is apparently determined both at the level of the alcohol and at the level of the chloroacrylic acid.

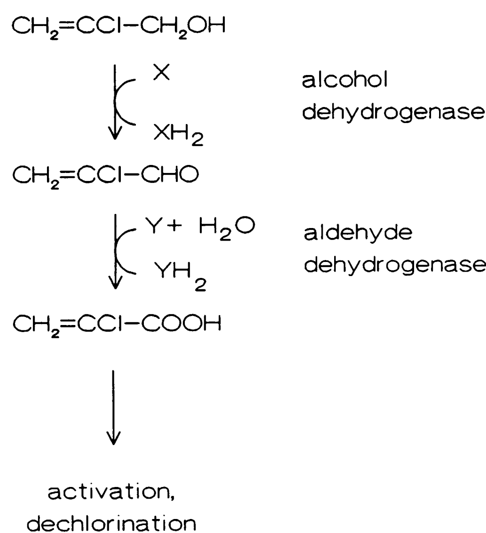

FIG. 2. Proposed route for 2-chloroallylalcohol metabolism in strain JD2.

With regard to dechlorination rates, it is obvious that the presence of an α-chlorine substituent greatly enhances dechlorination. A similar structure-activity relationship was found for chloroallylalcohols and chloroacroleins and their mutagenicity in the Ames test (17, 19, 25). It was proposed that in these cases, the electron-withdrawing effect of a chlorine substituent at the α-carbon of acroleins enhances a nucleophilic addition at the β position (18, 19).

In the case of chloroacrylic acids, the presence of an α-chlorine substituent may also enhance the formation of a thioester by its electron-withdrawing effect. The position of the chlorine substituent appeared more important than the degree of chlorination. Thus, although trichloroallylalcohol was only partly oxidized, the corresponding trichloroacrylic acid was better dechlorinated than 3,3-dichloroacrylic acid. Oxidation of a chloroallylalcohol therefore seems to be facilitated when there are no chlorines in both the α and β positions, whereas dechlorination is increased by the presence of a chlorine substituted on the α position.

The dechlorination reaction, which is proposed to occur at the level of 2-chloroacrylic acid or an activated derivative thereof, is not yet understood. At least two different 2-haloacid dehalogenases are present in strain JD2, but these enzymes do not have any activity for 2-chloroacrylic acid. It is remarkable that α-chlorinated alcohols and carboxylic acids especially are good growth substrates for strain JD2. An exception is 2-chloroethanol, for which the rate-limiting metabolic step may be the slow oxidation by the alcohol dehydrogenase. Although an efficient dechlorinating system for its oxidation product chloroacetic acid was present, there is no indication that this dehalogenase is responsible for dehalogenation of chloroallylalcohols.

Dechlorination of β-halogenated three- and four-carbon carboxylic acids has been demonstrated in a few cases (3, 10, 16, 32). In crude extracts of the coryneform bacterium strain FG41, two dehalogenases are present that act with the two different isomers of 3-chloroacrylic acid and convert these compounds to malonic acid semialdehyde, probably via hydration. The same activity has been found in another coryneform bacterium and in an unidentified *Pseudomonas* sp. (10). This mechanism is not responsible for the dechlorination of 2-chloroacrylic acid in strain JD2, because in crude extracts no direct hydration of this compound occurred. A different mechanism for the dechlorination of β-chlorinated crotonates and 3-chlorobutyrate has been proposed. Dechlorination of these compounds in crude extracts of a bacterial strain isolated on *trans*-3-chlorocrotonic acid depended on the presence of coenzyme A, ATP, and Mg^{2+} (16). Intermediates have not yet been demonstrated, however.

Our results suggest a degradation pathway for 2-chloroallylalcohol in strain JD2 via 2-chloroacrylic acid (Fig. 2). It is most likely that an intermediate is activated, followed by addition of water, nucleophilic attack of a hydroxyl group, or saturation of the double bond. Evidence for the hydration of acrylyl-coenzyme A to produce lactate has been obtained with *Escherichia coli* and *Salmonella typhimurium* (7, 33). This reaction could not be demonstrated in strain JD2, however, because crude extracts failed to catalyze dechlorination under the different conditions tested, and enzymatic dehalogenation of 2-chloroacrylyl coenzyme A could not accurately be measured because of the chemical instability of the compound. Theoretically, another dechlorination mechanism could be the conversion of 2-chloroacrylic acid to chloropropionic acid followed by hydrolytic dehalogenation. The same reductive reaction has been found during the anaerobic conversion of acrylic acid (5). It is clear that there are different mechanisms for the biological dehalogenation of α-chlorinated acrylic acids, β-chlorinated acrylic acids, α-chlorinated saturated carboxylic acids, and vicinal chloroalcohols (15).

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