Bacterial metabolism of 3-chloroacrylic acid

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Two bacterial strains were isolated with 3-chloroacrylic acid (CAA) as sole source of carbon and energy. Strain CAA1, a *Pseudomonas cepacia* sp., was capable of growth with only the *cis*-isomer of CAA. Strain CAA2, a coryneform bacterium, utilized both isomers of CAA as sole source of carbon and energy. Strain CAA1 contained *cis*-CAA hydratase and strain CAA2 contained two hydratases, one with *cis*-CAA hydratase activity and one with *trans*-CAA hydratase activity. The product of the hydratase activities with CAA was malonate semialdehyde. In both strains malonate semialdehyde was subsequently decarboxylated by a cofactor-independent decarboxylase yielding acetaldehyde and CO_2.

Introduction

The biodegradability of halogenated hydrocarbons receives considerable attention in view of the persistence of some of these compounds in the environment. Degradation of a number of chlorinated hydrocarbons by pure cultures has, however, been demonstrated. In several cases the dechlorination step has also been characterized. Specific dechlorination reactions of chlorinated hydrocarbons that have been studied in detail are the hydrolytic dehalogenases acting on chlorinated alkanes (Kohler-Staub & Leisinger, 1985; Keuning *et al.*, 1985; Scholtz *et al.*, 1987) or 2-chloroalkanoic acids (Motosugi *et al.*, 1982; Smith *et al.*, 1990). The oxidative dechlorination of chlorinated alkanes has also been reported (Yokota *et al.*, 1986). The dechlorination reactions of unsaturated chlorinated hydrocarbons have been less well studied. Within this category of compounds most attention has focussed on the chlorinated ethenes due to their broad use and widespread occurrence in the environment. Transformation of all chlorinated ethenes has been observed under anaerobic conditions (Freedman & Gossett, 1989; Bagley & Gossett, 1990; Fathepure & Boyd, 1988). Aerobic transformation has been observed only for mono-, di- and trichloroethenes. Transformation (co-oxidation) of these compounds has been demonstrated with various types of micro-organisms (e.g. toluene-, methane-, alkane- and ammonium-utilizers) (Wackett *et al.*, 1989).

Micro-organisms utilizing chlorinated ethenes as sole carbon and energy source have been isolated only with monochloroethene (vinyl chloride) (Hartmans *et al.*, 1985). The initial step in the aerobic transformation of chlorinated ethenes is generally assumed to be an epoxidation of the carbon–carbon double bond (Hartmans *et al.*, 1989). The further metabolism of the reactive and unstable chloroepoxides is not known, but extensive dechlorination is often observed.

Aerobic degradation of 3-chloroallyl alcohol, which also contains a chloroethenyl group, has been studied by Belser & Castro (1971). It is formed from the soil disinfectant 1,3-dichloropropene as a result of chemical hydrolysis. The 3-chloroallyl-alcohol-degrading *Pseudomonas* sp. isolated by Belser & Castro (1971) degrades 3-chloroallyl alcohol via 3-chloroacrylic acid. Incubation of whole cells with 3-chloroacrylic acid resulted in the formation of 3-oxopropanoic acid (malonate semialdehyde). The dechlorination of 3-chloroacrylic acid was, however, not investigated in any further detail.

We now report on the dechlorination of 3-chloroacrylic acid in more detail. The 3-chloroacrylic acid degradative pathway and the dechlorination reaction in particular were studied in two bacterial strains isolated with 3-chloroacrylic acid (Hartmans *et al.*, 1988).

Methods

Chemicals. cis- and *trans-*3-chloroacrylic acid (CAA) were from Ventron (Karlsruhe, FRG); 3-chloropropionic acid, *cis-* and *trans-*1,2-dichloroethene, 1,3-dichloropropene, fumaric acid, acrylic acid, crotonic acid, cinnamic acid, methacrylic acid and MOPS were from

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Janssen (Beerse, Belgium). *trans*-2-Pentenoic acid and maleic acid were from Aldrich, 1-chloropropene was obtained from ICN Biomedicals (New York, USA) and vinyl chloride was from Hoek Loos (Schiedam, The Netherlands). 2-Chloroacrylic acid was from Heraeus (Karlsruhe, FRG). 3-Chloroallyl alcohol was a gift from the Institute for Pesticide Research (Wageningen, The Netherlands). L- and *meso*-diamino-pimelic acid (DAP) were from L. Light & Co. (Colnbrook, UK), DL-isocitrate and Coenzyme A were from Sigma and NAD⁺ and NADP⁺ were from Boehringer. Other chemicals were from Merck.

Isolation and cultivation. Enrichment cultures were set up using a mixture of soil and water samples as inoculum in mineral salts medium with one of the following compounds as sole carbon source. 1,3-Dichloropropene and chloroallyl alcohol were added at a concentration of 1 mM in Erlenmeyer flasks sealed with Mininert valves to prevent evaporation. *cis*- or *trans*-CAA were added at a concentration of 2·5 mM. After incubation for 2 weeks at 30 °C 1 ml from the enrichment cultures was transferred to fresh medium with the same carbon source. After growth was observed in these cultures, cells were streaked to purity on glucose/yeast extract agar plates. Non-sterile controls without inoculum were also made and incubated under identical conditions to monitor any chemical hydrolysis of the chlorinated substrates. 1,3-Dichloropropene was assayed by taking headspace samples which were analysed on a gas chromatograph. Chemical hydrolysis or biodegradation of the two other compounds was monitored by determining the rates of formation of Cl⁻ in both the enrichment cultures and the non-sterile controls. From the depletion curve observed for 1,3-dichloropropene in the control flasks a half-life of 92 h was calculated.

The mineral salts medium contained, per litre of deionized water: 3·88 g K₂HPO₄, 2·13 g NaH₂PO₄.2H₂O, 2·0 g (NH₄)₂SO₄, 0·1 g MgCl₂.6H₂O, 10 mg EDTA, 2 mg ZnSO₄.7H₂O, 1 mg CaCl₂.2H₂O, 5 mg FeSO₄.7H₂O, 0·2 mg Na₂MoO₄.2H₂O, 0·2 mg CuSO₄.5H₂O, 0·4 mg CoCl₂.6H₂O and 1 mg MnCl₂.2H₂O.

Cultures were grown in the mineral salts medium with 0·1% (w/v) carbon source, unless indicated otherwise. When CAA was used as a carbon source it was sterilized separately by filtration and added at a concentration of 5 mM. Culture conditions, maintenance of strains, culture harvesting and storage of harvested cells were as previously described (Hartmans & de Bont, 1986).

Preparation of cell extracts. Crude cell extracts and dialysed extracts were prepared as described previously (Hartmans & de Bont, 1986).

Separation of hydratases. Cell extract (150 mg protein) from glucose-grown (0·25%, w/v) strain CAA2 cells, which had been induced with 2 mM-*cis*-CAA in the late-exponential growth phase, was fractionated on a DEAE-Sepharose CL-6B column (25 × 2·5 cm). Elution was with 50 mM-potassium phosphate buffer, pH 7·0, with a linear gradient of 100–500 mM-NaCl at a flow rate of 50 ml h⁻¹. Fractions of vol. 8·3 ml were collected.

Fractions containing hydratase activity were concentrated and washed with phosphate buffer (50 mM, pH 7·0), in order to reduce the Cl⁻ concentration, by ultrafiltration across an Amicon YM 10 membrane in an Amicon 8050 concentrator. These hydratase preparations could be stored at −20 °C without any apparent loss of activity and were used for K_m, pH optimum and substrate specificity determinations.

Isolation of 2,4-dinitrophenylhydrazones. The partially purified hydratase preparations were incubated with the appropriate CAA isomer until reaction was complete. After filtration over an Amicon YM 10 filter (to remove protein) the filtrate was reacted with 2,4-dinitrophenylhydrazine and analysed by mass spectrometry as described by van den Tweel & de Bont (1985).

Enzyme assays. All assays were done at 30 °C. Spectrophotometric assays were done on a Perkin-Elmer 550A spectrophotometer. Activities are expressed as nmol product formed min⁻¹ (mg protein)⁻¹. One unit (U) is the amount of enzyme which catalyses the transformation of 1 μmol substrate min⁻¹.

(*i*) *3-Chloroacrylic acid hydratase.* The reaction mixture (total vol. 2 ml) contained 100 μmol potassium phosphate, pH 7·0, 10 μmol phenylhydrazine hydrochloride and cell extract. The reaction was initiated by the addition of 10 μmol *cis*- or *trans*-CAA and phenyl-hydrazone formation was recorded at 315 nm. By comparing phenyl-hydrazone formation rates with Cl⁻ liberation rates (amperometrically) under the same conditions, a molar absorption coefficient for the phenylhydrazone of 2·2 × 10³ l mol⁻¹ cm⁻¹ was calculated for both isomers of CAA. At 300 nm an absorption coefficient of 4·7 × 10³ l mol⁻¹ cm⁻¹ was calculated. Van den Tweel & de Bont (1985) reported an absorption coefficient for the phenylhydrazone of malonate semialdehyde of 4 × 10³ l mol⁻¹ cm⁻¹ at 300 nm.

(*ii*) *Fumarase (EC 4.2.1.2).* Activity was assayed at 240 nm (fumarate formation) in a reaction mixture (total vol. 2 ml) containing 100 μmol potassium phosphate and 5 μmol L-malate and enzyme. An absorption coefficient of 2·3 × 10³ l mol⁻¹ cm⁻¹ was used in calculating fumarase activities.

(*iii*) *Malonate semialdehyde decarboxylase (EC 4.1.1.–).* Activity was determined by assaying CO_2 or acetaldehyde formation in 30 ml serum bottles containing 50 μmol potassium phosphate, pH 7·0, 5 μmol *cis*-CAA, 0·2 U partially purified *cis*-CAA hydratase devoid of malonate semialdehyde decarboxylase activity and cell extract in a total volume of 1 ml.

(*iv*) *Malonate decarboxylase (EC 4.1.1.–).* Activity was determined by assaying CO_2 formation in 30 ml serum bottles containing 50 μmol potassium phosphate, pH 7·0, 5 μmol malonate and cell extract in a total volume of 1 ml.

(*v*) *Acetaldehyde dehydrogenase (EC 1.2.1.3).* The reaction mixture (total vol. 2 ml) contained 100 μmol Tris/HCl, pH 8·0, 2 μmol NAD⁺ and cell extract. The reaction was started by the addition of 10 μmol acetaldehyde and NADH formation was recorded at 340 nm.

(*vi*) *Isocitrate lyase (EC 4.1.3.1).* The reaction mixture (total vol. 2 ml) contained cell extract, 100 μmol Tris/HCl, pH 8·0, 100 μmol MgCl₂ and 10 μmol phenylhydrazine hydrochloride. The reaction was started by the addition of 20 μmol isocitrate and hydrazone formation was recorded at 324 nm. Activities were calculated using an absorption coefficient for the phenylhydrazone of 1·7 × 10⁴ l mol⁻¹ cm⁻¹.

(*vii*) *Isocitrate dehydrogenase (EC 1.1.1.42).* The reaction mixture (total vol. 2 ml) contained cell extract, 100 μmol Tris/HCl, pH 8·0, 2 μmol NADP⁺ and 2 μmol MgSO₄. The reaction was started by adding 8 μmol isocitrate and NADPH formation was recorded at 340 nm.

Analytical methods. Protein was determined by the Lowry method using bovine serum albumin as standard. CO_2 concentrations were determined by analysing headspace samples on a gas chromatograph (Hartmans *et al.*, 1985). Acetaldehyde and vinyl chloride (at 180 °C) and 1,3-dichloropropene (at 210 °C) were assayed on a Packard 430 gas chromatograph fitted with a Porapak R column (Hartmans *et al.*, 1985). Cl⁻ were determined colorimetrically (Bergmann & Sanik, 1957) or amperometrically (Hartmans *et al.*, 1985). The substrate specificity of the hydratases with the chlorinated substrates was determined by incubating enzyme and 5 mM-substrate in 100 mM-MOPS/KOH buffer and assaying Cl⁻ formation by the colorimetric method. The substrate specificity of the hydratases with the nonchlorinated substrates was determined spectrophotometrically at 220 nm by incubating 0·5 mM-substrate in 50 mM-potassium phosphate buffer, pH 7, with 0·05 Units of partially purified hydratase ml⁻¹. Chromatography of whole-cell hydrolysates was done as described by Becker *et al.* (1964). *Arthrobacter globiformis* (ATCC 8010), *Arthrobacter simplex* (NCIB 8929) and

Brevibacterium linens (ATCC 9175) were used as reference strains containing lysine, L-DAP and meso-DAP respectively. Phenyl-hydrazones were extracted with ethyl acetate, which was subsequently removed by evaporation, and assayed by reverse phase HPLC using a C-18 column (200 × 3 mm; Chrompack) and detected at 280 nm by means of a Perkin-Elmer variable wavelength detector. The eluent was acrylonitrile in Milli-Q water (60 : 40, v/v) at a flow rate of 0·6 ml min^{-1}.

Results and Discussion

Isolation and characterization of new isolates

To isolate micro-organisms containing CAA-dechlorinating activity, enrichment cultures were set up with 1,3-dichloropropene, chloroallyl alcohol or one of the CAA isomers as the sole carbon source. With 1,3-dichloropropene and chloroallyl alcohol no differences were observed either in substrate depletion or Cl$^-$ formation between enrichment cultures and the controls without inoculum. Although biodegradation of 1,3-dichloropropene has been observed (van Dijk, 1980), to our knowledge, no pure cultures have been isolated. In contrast, enrichment cultures with either isomer of CAA as sole carbon source resulted in rapid microbial growth. Growth was also observed in the non-sterile control containing *trans*-CAA. Strains CAA1 and CAA2 were isolated from the enrichment cultures with *cis*-CAA and *trans*-CAA respectively.

Strain CAA1 was a Gram-negative, straight and motile rod. It was catalase- and oxidase positive and possessed β-galactosidase activity. Denitrification, indole formation, urease activity and gelatin hydrolysis were negative. Glucose, arabinose, mannose, mannitol, *N*-acetylglucoside, gluconic acid, capric acid, adipic acid, malic acid and phenylacetic acid were all utilized as growth substrates. Maltose was not utilized. Based on these observations strain CAA1 was identified as a strain of *Pseudomonas cepacia*. The organism also utilized acetate, propionate, fumarate and malonate as growth substrates. *trans*-CAA, acrylic acid and·methanol were not utilized. The doubling time of strain CAA1 with *cis*-CAA was 2·6 h and with malonate, a possible intermediate of *cis*-CAA metabolism, 2·2 h.

Strain CAA2 was creamy to light-orange in colour. The isolate was Gram-positive and not acid fast. Catalase and oxidase tests were positive. The morphology of strain CAA2 depended on the age of the culture. In exponentially growing cultures strain CAA2 occurred as irregular rods (Fig. 1a). In the stationary growth phase strain CAA2 formed coccoid cells, often in the V-arrangement typical of coryneform bacteria (Fig. 1b). Thin-layer chromatography of strain CAA2 hydrolysates showed *meso*-DAP to be present. Based on the

presence of *meso*-DAP and the microscopic morphology, strain CAA2 was tentatively designated as a *Brevibacterium* sp. (Jones & Keddie, 1986). The isolate grew with both CAA isomers, as well as with acetate, propionate and fumarate. Malonate, acrylic acid and methanol were not utilized as sole carbon source. The doubling time of strain CAA2 with *cis*-CAA was 3 h. The doubling time with *trans*-CAA was difficult to determine as concentrations higher than 2–4 mM-*trans*-CAA clearly inhibited growth.

Neither strain grew with 1,3-dichloropropene or chloroallyl alcohol at a concentration of 1 mM.

CAA dechlorinating activities

The initial step in CAA metabolism was studied using cell extracts. Cell extracts of *cis*-CAA-grown cells of both strains exhibited rapid Cl$^-$ formation upon addition of *cis*-CAA. Dialysis of crude extracts did not affect the specific rate at which Cl$^-$ was liberated from *cis*-CAA. Cell extracts of *cis*-CAA-grown strain CAA1 did not dechlorinate *trans*-CAA, whereas cell extracts of strain CAA2 grown with *cis*-CAA dechlorinated both isomers of CAA. Extracts of *trans*-CAA-grown strain CAA2 also dechlorinated both isomers of CAA. The cofactor-independent Cl$^-$ formation observed in cell-free extracts after *cis*-CAA addition could be explained by assuming an enzymic hydration of the double bond of *cis*-CAA. Hydration of the double bond would yield 3-chloro-2-hydroxypropanoic acid or the unstable 3-chloro-3-hydroxypropanoic acid, which rapidly chemically decomposes to malonate semialdehyde (3-oxopropanoic acid) and HCl. This would be in accordance with the previously reported formation of malonate semialdehyde by whole cells incubated with *cis*-CAA (Belser & Castro, 1971). A similar type of reaction has been reported for the hydration by fumarase of monofluorofumarate yielding α-fluorohydrin (α-fluoromalate) which subsequently decomposed to oxaloacetate. Hydration of monochlorofumarate with fumarase, however, yielded the chemically stable β-chloro-*threo*-L-malate (Marletta et al., 1982).

Larger amounts of cells were required to study the dechlorinating activities in more detail and to identify the product of the reaction. As CAA is relatively expensive, strain CAA2 was grown on glucose with 2 mM-*cis*-CAA added in the late-exponential growth phase (2 h before harvesting) to produce biomass with dechlorinating activities. Cells cultivated in this manner contained specific dechlorinating activities similar to the levels present in cells grown with *cis*-CAA as sole carbon and energy source. With extracts from cells cultivated in
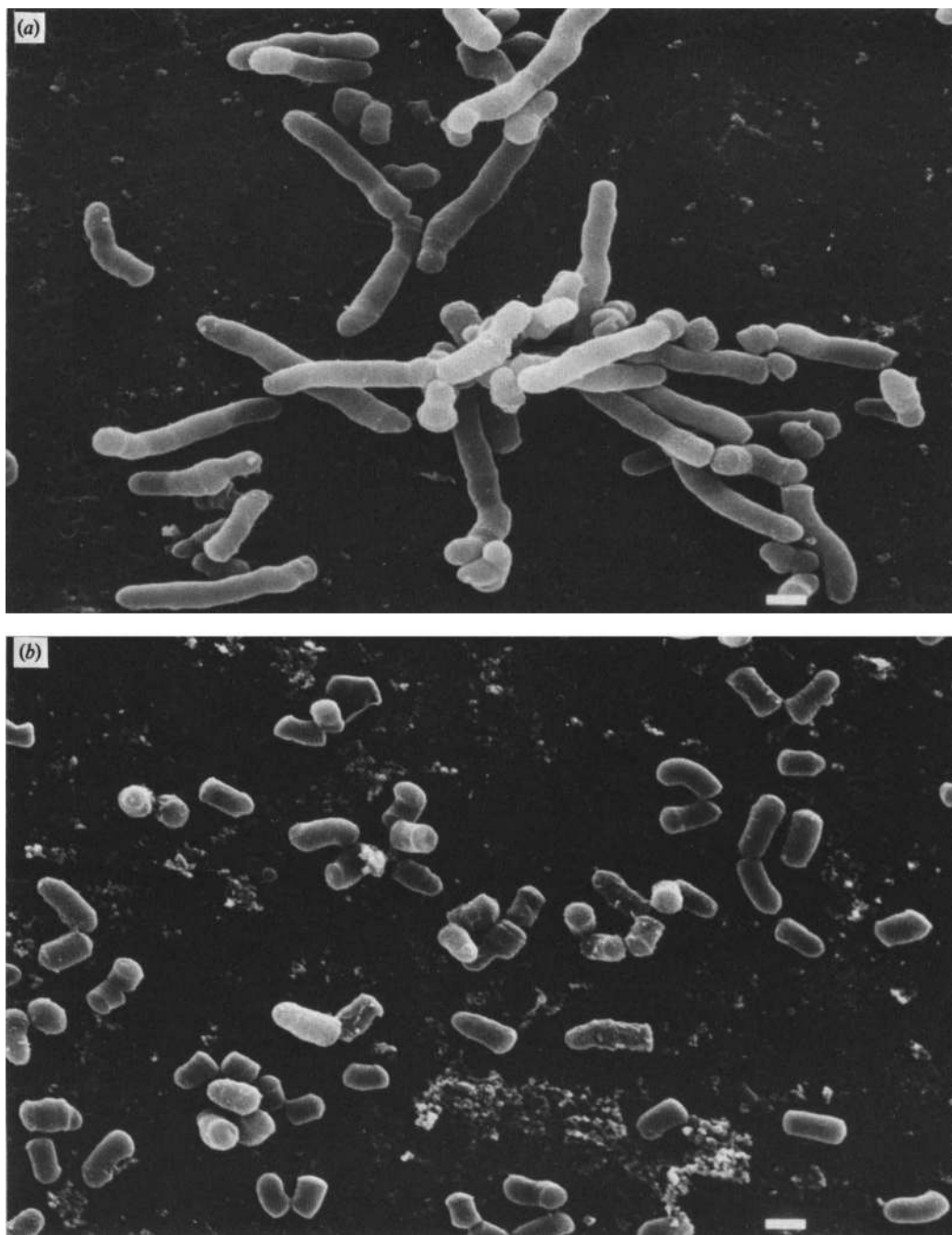
Fig. 1. Photomicrographs of strain CAA2 after growth for 8 h (*a*) and 96 h (*b*) on mineral salts medium with 0·1 % (w/v) glucose. Bars, 1 μm.

this manner the dechlorinating activities of strain CAA2 were investigated in more detail.

The activities were partially purified using anion-exchange column chromatography. Fractionation of extracts on a DEAE-Sepharose CL-6B column resulted in separation of the *cis*- and *trans*-CAA dechlorinating activities (Fig. 2), indicating that the two isomers are

dechlorinated by two distinct enzymes. Fumarase activity was also located. No fumarase activity coincided with either of the two CAA-dechlorinating activities. The fractions containing the respective dechlorinating activities were pooled and dialysed to remove excess Cl⁻. These partially purified preparations were used to identify the product of the dechlorinating reaction and to
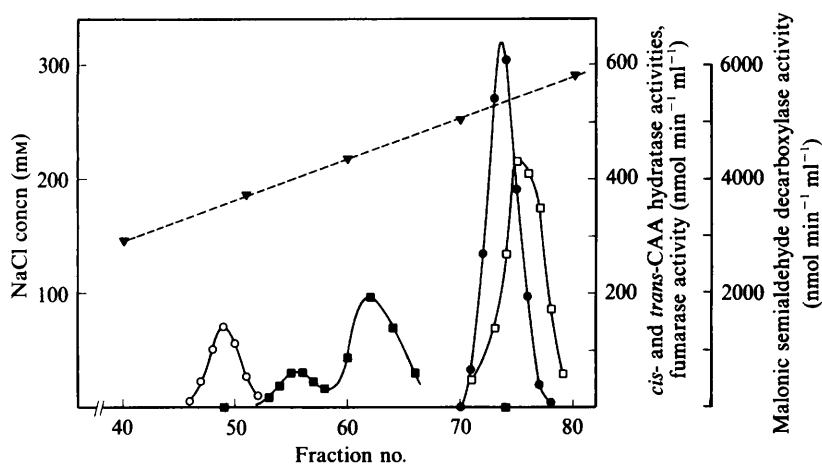
Fig. 2. Separation of *cis*-CAA hydratase (O), fumarase (■), *trans*-CAA hydratase (●) and malonic semialdehyde decarboxylase (□) activities with a DEAE-Sepharose CL-6B column (25 × 2·5 cm). Elution was with 50 mM-potassium phosphate buffer, pH 7·0, with a linear gradient of 100–500 mM-NaCl (▼) at a flow rate of 50 ml min⁻¹. Fraction vol. was 8·3 ml.

determine the pH optimum, the K_m for CAA and the substrate specificities. The specific activities of the partially purified preparations of the *cis*-CAA and *trans*-CAA dechlorinating activities were, respectively, 1300 and 2600 nmol min⁻¹ mg protein⁻¹.

Product identification

The product of the CAA dechlorinating activity was assumed to be malonic semialdehyde. To confirm this hypothesis 2,4-dinitrophenylhydrazones were prepared after incubating the partially purified *trans*-CAA dechlorinating activity with its substrate. Van den Tweel & de Bont (1985) reported that the mass spectra of the malonate semialdehyde and acetaldehyde 2,4-dinitrophenylhydrazones are identical due to the decarboxylation of malonate semialdehyde during 2,4-dinitrophenylhydrazone preparation. The mass spectrum of the 2,4-dinitrophenylhydrazone of the product formed by the *trans*-CAA dechlorinating activity was indeed identical to that of the 2,4-dinitrophenylhydrazone of authentic acetaldehyde and to the spectrum of the 2,4-dinitrophenylhydrazone of acetaldehyde published by Kanner & Bartha (1982). Further identification of the product was undertaken using HPLC, as the identification of acetaldehyde only gave indirect evidence for the formation of malonate semialdehyde from CAA.

Analysis of the unsubstituted phenylhydrazones formed during the standard CAA hydratase assay using phenylhydrazine, rather than 2,4-dinitrophenylhydrazine, was possible with reverse-phase HPLC. Both dechlorinating activities gave phenylhydrazones with the same retention time (4·1 min) upon HPLC analysis

and the same UV spectrum. Incubation of cell extracts of 3-butyn-1-ol-grown *Pseudomonas* BB1 (van den Tweel & de Bont, 1985) with propynoic acid and phenylhydrazine yields the phenylhydrazone of malonate semialdehyde. This phenylhydrazone was, based on HPLC and UV analysis, identical to the phenylhydrazones that were formed from CAA. The phenylhydrazone of acetaldehyde also had the same UV spectrum (dissolved in ethyl acetate) but was, probably due to the very low solubility in the HPLC eluent, not detectable with HPLC. The phenylhydrazone of pyruvate had a markedly different UV spectrum and also a different retention time (2·7 min) upon HPLC analysis.

Based on these results it is concluded that strain CAA2 contained two distinct enzymes that dechlorinate the respective isomers of CAA, both yielding malonate semialdehyde as product. Belser & Castro (1971) had already demonstrated that whole cells could transform CAA to malonate semialdehyde, but they proposed a difficult-to-envisage 'hydroxylation' of CAA as the dechlorinating step. Although the dechlorinating enzymic activities could perhaps be characterized as dehalogenases (halidohydrolases), characteristics such as the pH optimum and substrate specificity indicate that these enzymes share more similarity with hydratases like fumarase. Therefore, it is assumed that the CAA hydratases should indeed be classified as hydratases (EC 4.2.1.–), rather than as dehalogenases.

The pH optimum of *cis*-CAA hydratase was rather broad and lay between pH 7·3 and 8·0, whereas the pH optimum of *trans*-CAA hydratase was more pronounced at pH 7·3. The K_m for *cis*-CAA was 2·5 mM and the K_m for *trans*-CAA was 0·2 mM. In both cases slight substrate inhibition was observed at substrate concentrations

above 5 mM. The relatively broad pH optimum observed for *cis*-CAA hydratase is possibly a reflection of the substrate concentration of 2·5 mM which was used in the activity assay. A similar observation has been made for fumarase activity where the pH optimum (pH 6·9) was much less pronounced when the fumarate concentration in the activity assay approached the K_m value (Alberty *et al.*, 1954). The pH optima of the two hydratases are therefore very similar to the pH optimum of fumarase. In contrast, the pH optima of dehalogenases are much higher. For haloalkane dehalogenases pH optima of 8·2 and 9·5 have been determined (Keuning *et al.*, 1985; Scholtz *et al.*, 1987) and for 2-haloacid dehalogenases pH optima of 9·5 are common (Smith *et al.*, 1990).

The substrate specificity of the two hydratases was very high. Several chlorinated compounds were tested as potential substrates for both hydratases. No enzymic liberation of Cl⁻ (detection level <3% of the activity measured with the CAA isomer) was, however, detected with 1,3-dichloropropene, 3-chloroallyl alcohol, 2-chloroacrylic acid, 1-chloropropene, vinyl chloride, *cis*-1,2-dichloroethene, *trans*-1,2-dichloroethene or 3-chloropropionic acid.

Special attention was given to vinyl chloride as we are interested in methods to remove this carcinogenic compound from industrial wastes (Hartmans *et al.*, 1985). Hypothetically, hydration of vinyl chloride would result in the formation of either 1-chloroethanol or 2-chloroethanol. 1-Chloroethanol would decompose to acetaldehyde and HCl, but 2-chloroethanol is stable under the assay conditions. By adding 1,2-dichloroethane-grown cells of *Xanthobacter autotrophicus* GJ10 (Janssen *et al.*, 1985) to the incubation mixture of CAA hydratase with vinyl chloride, any 2-chloroethanol that would be formed would be oxidized and dehalogenated resulting in Cl⁻ formation. However, in the presence of cells of *X. autotrophicus* GJ10 no Cl⁻ formation was detected, indicating that vinyl chloride is not hydrated by the CAA hydratases of strain CAA2. Apparently, the presence of a carboxyl group adjacent to the chloroethenyl group is a prerequisite for enzymic hydration of the carbon–carbon double bond by the two CAA hydratases of strain CAA2.

Besides chlorinated compounds several non-chlorinated unsaturated carboxylic acids were tested as substrates for the partially purified hydratases. Hydration of acrylic acid, propynoic acid, crotonic acid, *trans*-2-pentenoic acid, cinnamic acid, methacrylic acid and maleic acid was, however, not observed with either of the two hydratase preparations (detection level lower than 1% of the activity observed with the appropriate CAA isomer as substrate).

The high substrate specificity of the CAA hydratases also indicates that these enzymes apparently share more

similarity with a hydratase like fumarase, which has a relatively high substrate specificity (Teipel *et al.*, 1968), than with the dehalogenases, which generally transform a wide range of substrates (Smith *et al.*, 1990). The observed high substrate specificity gives rise to speculations concerning the physiological function of these enzymes. If CAA is indeed the only substrate transformed, this could indicate that these CAA hydratases have evolved as a result of the large-scale application in agriculture of the nematocide 1,3-dichloropropene. In that case, however, the CAA utilizers would also be expected to grow on chloroallyl alcohol, the product formed upon chemical hydrolysis of 1,3-dichloropropene.

A better understanding of the evolutionary origin of the CAA hydratases will, however, require characterization of the nucleotide sequences of the corresponding genes. In this respect the recent data showing extensive sequence homology between fumarase and L-aspartate ammonia-lyase (Takagi *et al.*, 1986) is very interesting. Possibly the CAA hydratases share a common evolutionary origin with these enzymes.

CAA degradation pathway

Having established that the product of CAA dechlorination was malonic semialdehyde, the degradative pathway of this not-very-common metabolic intermediate was studied. Two enzymic activities oxidizing malonic semialdehyde have been described previously. A NAD⁺-dependent dehydrogenase (EC 1.2.1.15) which oxidizes malonic semialdehyde to malonate (Nakamura & Bernheim, 1961) and a NAD⁺- and CoA-dependent malonate semialdehyde oxidative decarboxylase (EC 1.2.1.18) forming acetyl-CoA (Yamada & Jakoby, 1960). Interestingly, *cis*-CAA-dependent formation of CO_2 was detected in dialysed extracts without adding NAD⁺ or CoA. Incubation of *trans*-CAA with the partially purified preparation of *trans*-CAA hydratase also resulted in CO_2 formation. No CO_2 formation was observed when *cis*-CAA was incubated with the partially purified *cis*-CAA hydratase.

This indicated that the partially purified *trans*-CAA hydratase preparation contained a cofactor-independent malonate semialdehyde decarboxylase, presumably yielding acetaldehyde and CO_2, which was not present in the partially purified *cis*-CAA hydratase. Subsequent experiments with the partially purified *trans*-CAA hydratase did indeed reveal simultaneous formation of acetaldehyde and CO_2 from *trans*-CAA. Combining of the *trans*-CAA and *cis*-CAA hydratase preparations also resulted in acetaldehyde and CO_2 formation from *cis*-CAA. When phenylhydrazine was added to the

Table 1. *Enzyme activities in strain CAA1*

Activities are expressed in nmol min^{-1} (mg protein)$^{-1}$.

Enzyme	Growth substrate		
	cis-CAA	Malonate	Succinate
cis-CAA dehalogenase	240	<5	<5
MSA decarboxylase	530	40	30
Malonate decarboxylase	<20	470	<20
Acetaldehyde dehydrogenase	40	35	35
Isocitrate lyase	825	695	5
Isocitrate dehydrogenase	585	680	550

Table 2. *Enzyme activities in strain CAA2*

Activities are expressed in nmol min^{-1} (mg protein)$^{-1}$.

Enzyme	Growth substrate		
	cis-CAA	Ethanol	Succinate
cis-CAA dehalogenase	450	<10	<10
trans-CAA dehalogenase	1200	<10	12
MSA decarboxylase	1160	55	60
Acetaldehyde dehydrogenase	25	200	5
Isocitrate lyase	1020	1010	15
Isocitrate dehydrogenase	560	645	855

incubation mixture, acetaldehyde and CO_2 was no longer observed, but the Cl$^-$ formation rate was unaffected.

As malonate semialdehyde was not available from a commercial source the partially purified *cis*-CAA hydratase preparation, devoid of malonate semialdehyde decarboxylase, was used to produce malonate semialdehyde from *cis*-CAA *in situ* to assay malonate semialdehyde decarboxylase activity. With this assay we were able to locate the malonate semialdehyde decarboxylase activity peak in the fractions from the DEAE-Sepharose column. As anticipated, malonate semialdehyde decarboxylase activity more or less coincided with *trans*-CAA hydratase activity (Fig. 2).

With the observed enzyme activities a degradative pathway for *cis*- and *trans*-CAA in *cis*-CAA-grown strain CAA2 can be envisaged which proceeds via malonate semialdehyde to acetaldehyde. Subsequently, enzyme activities in crude extracts of *cis*-CAA-grown cells of both strains and in extracts of cells grown with control substrates were compared. As can be seen in Tables 1 and 2, CAA hydratase and malonate semialdehyde decarboxylase activities were present only after growth with *cis*-CAA. NAD$^+$-dependent acetaldehyde dehydrogenase activities in *cis*-CAA-grown cells of both strains were rather low. No phenazine methosulphate (PMS)-dependent acetaldehyde dehydrogenase activity could be detected in extracts of ethanol-grown cells of strain CAA2. In spite of the observed low acetaldehyde
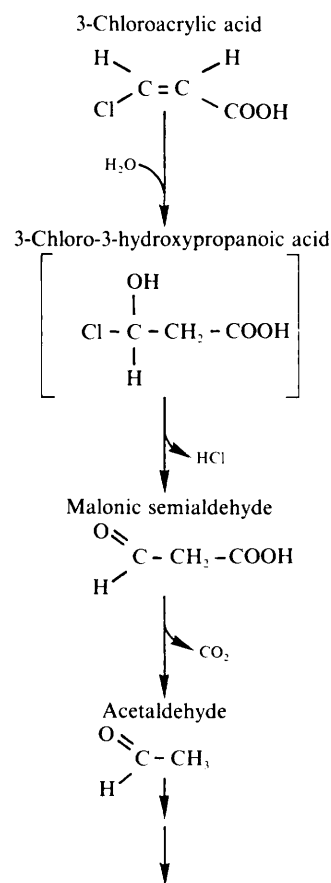
Fig. 3. Proposed pathway for *cis*-CAA degradation in *Pseudomonas cepacia* strain CAA1 and the coryneform strain CAA2.

dehydrogenase activities we propose for both strains the *cis*-CAA degradation pathway summarized in Fig. 3. Induction of isocitrate lyase activity during growth with *cis*-CAA is also in agreement with the above pathway.

The presence of malonate decarboxylase activity in cell extracts of malonate-grown strain CAA1, and the absence of this activity in *cis*-CAA-grown cells, is a further confirmation that, in this isolate, malonate is not an intermediate of *cis*-CAA metabolism.

Having established the pathway for *cis*-CAA degradation an explanation for the inhibition of strain CAA2 by the growth substrate *trans*-CAA was still required. Initial *trans*-CAA concentrations higher than 2–4 mM gave inconsistent growth and often resulted in a transient accumulation of acetaldehyde. This accumulation of the toxic aldehyde possibly explained the poor growth of strain CAA2 with *trans*-CAA. No acetaldehyde formation was observed with *cis*-CAA as carbon source at similar concentrations, probably because *cis*-CAA hydratase activity is lower.

References

ALBERTY, R. A., MASSEY, V., FRIEDEN, C. & FUHLBRIGGE, A. R. (1954). Studies of the enzyme fumarase. III. The dependence of kinetic constants at 25° upon the concentration and pH of phosphate buffers. *Journal of the American Chemical Society* **76**, 2485–2493.

BAGLEY, D. M. & GOSSETT, J. M. (1990). Tetrachloroethene transformation to trichloroethene and *cis*-1,2-dichloroethene by sulfate-reducing enrichment cultures. *Applied and Environmental Microbiology* **56**, 2511–2516.

BECKER, B., LECHEVALIER, M. P., GORDON, R. E. & LECHEVALIER, H. A. (1964). Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. *Applied Microbiology* **12**, 421–423.

BELSER, N. O. & CASTRO, C. E. (1971). Biodehalogenation – the metabolism of the nematocides *cis*- and *trans*-3-chloroallyl alcohol by a bacterium isolated from soil. *Journal of Agricultural and Food Chemistry* **19**, 23–26.

BERGMANN, J. G. & SANIK, J. (1957). Determination of trace amounts of chlorine in naphtha. *Analytical Chemistry* **29**, 241–243.

VAN DIJK, H. (1980). Dissipation rates in soil of 1,2-dichloropropane and 1,3- and 2,3-dichloropropenes. *Pesticide Science* **11**, 625–632.

FATHEPURE, B. Z. & BOYD, S. A. (1988). Dependence of tetrachloroethylene dechlorination on methanogenic substrate consumption by *Methanosarcina* sp. strain DCM. *Applied and Environmental Microbiology* **54**, 2976–2980.

FREEDMAN, D. L. & GOSSETT, J. M. (1989). Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. *Applied and Environmental Microbiology* **55**, 2144–2151.

HARTMANS, S., DE BONT, J. A. M., TRAMPER, J. & LUYBEN, K. CH. A. M. (1985). Bacterial degradation of vinyl chloride. *Biotechnology Letters* **7**, 383–388.

HARTMANS, S. & DE BONT, J. A. M. (1986). Acetol monooxygenase from *Mycobacterium* Py1 cleaves acetol into acetate and formaldehyde. *FEMS Microbiology Letters* **36**, 155–158.

HARTMANS, S., JANSEN, M. W. & DE BONT, J. A. M. (1988). 3-Chloroacrylic acid metabolism in bacteria. In *Microbial Physiology and the Manufacturing Industry*, p. 205. Edited by C. Ratledge, A. Szentirmai, G. Barabás & F. Kevei. Budapest: OMIKK.

HARTMANS, S., DE BONT, J. A. M. & HARDER, W. (1989). Microbial metabolism of short-chain unsaturated hydrocarbons. *FEMS Microbiology Reviews* **63**, 235–264.

JANSSEN, D. B., SCHEPER, A., DIJKHUIZEN, L. & WITHOLT, B. (1985). Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. *Applied and Environmental Microbiology* **49**, 673–677.

JONES, D. & KEDDIE, R. M. (1986). Genus *Brevibacterium*. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1301–1313. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

KANNER, D. & BARTHA, R. (1982). Metabolism of acetylene by *Nocardia rhodochrous*. *Journal of Bacteriology* **150**, 989–992.

KEUNING, S., JANSSEN, D. B. & WITHOLT, B. (1985). Purification and characterization of hydrolytic dehalogenase from *Xanthobacter autotrophicus* GJ10. *Journal of Bacteriology* **163**, 635–639.

KOHLER-STAUB, D. & LEISINGER, TH. (1985). Dichloromethane dehalogenase of *Hyphomicrobium* sp. strain DM2. *Journal of Bacteriology* **162**, 676–681.

MARLETTA, M. A., CHEUNG, Y. F. & WALSH, C. (1982). Stereochemical studies on the hydration of monofluorofumarate and 2,3-difluorofumarate by fumarase. *Biochemistry* **21**, 2637–2644.

MOTOSUGI, K., ESAKI, N. & SODA, K. (1982). Purification and properties of a new enzyme, DL-2-haloacid dehalogenase, from *Pseudomonas* sp. *Journal of Bacteriology* **150**, 522–527.

NAKAMURA, K. & BERNHEIM, F. (1961). Studies on malonic semialdehyde dehydrogenase from *Pseudomonas aeruginosa*. *Biochimica et Biophysica Acta* **50**, 147–152.

SCHOLTZ, R., LEISINGER, TH., SUTER, F. & COOK, A. M. (1987). Characterization of 1-chlorohexane halidohydrolase, a dehalogenase of wide substrate range from an *Arthrobacter* sp. *Journal of Bacteriology* **169**, 5016–5021.

SMITH, J. M., HARRISON, K. & COLBY, J. (1990). Purification and characterization of D-2-haloacid dehalogenase from *Pseudomonas putida* strain AJ1/23. *Journal of General Microbiology* **136**, 881–886.

TAKAGI, J. S., TOKUSHIGE, M., SHIMURA, Y. & KANEHISA, M. (1986). L-Aspartate ammonia-lyase and fumarate hydratase share extensive sequence homology. *Biochemical and Biophysical Research Communications* **138**, 568–572.

TEIPEL, J. W., HASS, G. M. & HILL, R. L. (1968). The substrate specificity of fumarase. *Journal of Biological Chemistry* **243**, 5684–5694.

VAN DEN TWEEL, W. J. J. & DE BONT, J. A. M. (1985). Metabolism of 3-butyn-1-ol by *Pseudomonas* BB1. *Journal of General Microbiology* **131**, 3155–3162.

WACKETT, L. P., BRUSSEAU, G. A., HOUSEHOLDER, S. R. & HANSON, R. S. (1989). Survey of microbial oxygenases: trichloroethylene degradation by propane-oxidizing bacteria. *Applied and Environmental Microbiology* **55**, 2960–2964.

YAMADA, E. W. & JAKOBY, W. B. (1960). Aldehyde oxidation. V. Direct conversion of malonic semialdehyde to acetyl-coenzyme A. *Journal of Biological Chemistry* **235**, 589–594.

YOKOTA, T., FUSE, H., OMORI, T. & MINODA, Y. (1986). Microbial dehalogenation of haloalkanes mediated by oxygenase or halidohydrolase. *Agricultural and Biological Chemistry* **50**, 453–460.