Isolation and characterization of a haloalkane halidohydrolase from *Rhodococcus erythropolis* Y2

PAUL J. SALLIS,¹ SUSAN J. ARMFIELD,² ALAN T. BULL¹ and DAVID J. HARDMAN^{1*}

¹ Biological Laboratory, University of Kent at Canterbury, Canterbury, Kent CT2 7NJ, UK

² The Laboratory of the Government Chemist, Queens Road, Teddington, Middlesex TW11 0LY, UK

(Received 23 March 1989; revised 24 August 1989; accepted 4 October 1989)

Rhodococcus erythropolis strain Y2, isolated from soil by enrichment culture using 1-chlorobutane, was able to utilize a range of halogenated aliphatic compounds as sole sources of carbon and energy. The ability to utilize 1-chlorobutane was conferred by a single halidohydrolase-type haloalkane dehalogenase. The presence of the single enzyme in cell-free extracts was demonstrated by activity stain polyacrylamide gel electrophoresis. The purified enzyme was a monomeric protein with a relative molecular mass of 34 kDa and demonstrated activity against a broad range of haloalkanes, haloalcohols and haloethers. The highest activity was found towards α , ω disubstituted chloro- and bromo- C_2 – C_6 alkanes and 4-chlorobutanol. The K_m value of the enzyme for 1-chlorobutane was 0.26 mM. A comparison of the *R. erythropolis* Y2 haloalkane halidohydrolase with other haloalkane dehalogenases is discussed on the basis of biochemical properties and N-terminal amino acid sequence data.

Introduction

The large scale use of halogenated aliphatic compounds in industry and in agriculture has led to widespread contamination of the environment. Many such compounds have been designated as priority pollutants by the United States Environmental Protection Agency (Keith & Teilliard, 1979) because of their recalcitrance, toxicity, carcinogenicity and potential teratogenicity.

The presence of halogen substituents often renders simple chemical structures recalcitrant (Alexander, 1965). Organisms possessing oxygenases, reductases or dehalogenases, which are capable of catalysing the cleavage of the carbon–halogen bonds, can mineralize haloalkanes and thereby utilize them as sources of carbon and energy (Hardman, 1990; unpublished data). Consequently, there has been considerable interest in the possibility of using such organisms and their enzymes for the detoxification of xenobiotic chemicals. In our experience, the halidohydrolase-type dehalogenases are of particular interest as they do not require cofactors and are relatively stable catalysts.

The halidohydrolases which have been extensively studied to date are the 2-haloacid dehalogenases (EC 3.8.1.2; Goldman *et al.*, 1968) and the haloacetate dehalogenases (EC 3.8.1.3; Goldman, 1965). Micro-organisms possessing these enzymes are readily isolated from most soils and many have been shown to produce

more than one halidohydrolase with activity towards mono- and disubstituted 2-haloacids (Hardman & Slater, 1981; Allison *et al.*, 1983).

In contrast, there have been relatively few reports of the hydrolytic dehalogenation of haloalkanes and organisms containing haloalkane halidohydrolases are less readily isolated from the environment. Those haloalkane-degrading organisms which have been characterized usually possess a single enzyme active towards a broad range of haloalkanes and related compounds (Scholtz *et al.*, 1987; Yokota *et al.*, 1987; Janssen *et al.*, 1988). However, Scholtz *et al.* (1988) provided evidence for the presence of three haloalkane dehalogenases in *Arthrobacter* sp. strain HA1.

We report here the isolation of a strain of *Rhodococcus erythropolis*, designated Y2, capable of growth on 1-chlorobutane and a range of other α -substituted chloroalkanes, and the characterization of the single haloalkane halidohydrolase produced by this organism.

Methods

Materials. Chemicals and reference proteins were purchased from Sigma except for 1-chlorododecane (Pfaltz Bauer) and all other halogenated substrates (Aldrich). DEAE-Sephacel and Sephadex G-150 were purchased from Pharmacia-LKB; Biogel HT was from Bio-Rad.

Isolation and maintenance of *Rhodococcus erythropolis*. The bacterium, subsequently identified and designated *R. erythropolis* Y2, was isolated

by batch-enrichment culture. Soil samples were obtained from a discharge point at an industrial site where haloalkanes had been used for several decades. Soil samples (5 g) were incubated in 250 ml screw-top bottles containing 20 ml defined growth medium (Slater *et al.*, 1979) at pH 7.0 containing modified quantities of (l^{-1}): $Na_2HPO_4 \cdot 12 H_2O$, 4 g; KH_2PO_4 , 1 g; $NaHCO_3$, 1 g; yeast extract, 20 mg; $CoCl_2 \cdot 6H_2O$, 100 μ g; $NiCl_2 \cdot 6 H_2O$, 20 μ g; and H_3BO_3 , 100 μ g. 1-Chlorobutane was added as the sole carbon and energy source at 0.9 g l^{-1} . Enrichment cultures were incubated on a reciprocating shaker (120 r.p.m.) at 30 °C for 3 d. Samples (1 ml) from enrichment cultures were subcultured into the above medium (100 ml) in 300 ml screw-top bottles and incubated as above. After two further subcultures samples of the culture were serially diluted in order to produce separated colonies on nutrient agar. Inocula from all individual colonies were tested for their ability to grow in the above liquid medium with 1-chlorobutane as growth substrate. One isolate (Y2) was obtained which grew as an axenic culture.

Isolate Y2 was grown routinely in screw-top bottles containing less than 30% volume of defined growth medium and 1-chlorobutane at a final concentration of 0.7 g l^{-1} . Growth was monitored as OD_{600} and by Cl^{-} release (Marius Chlor-O-Counter, FT Scientific Instruments, Tewkesbury, UK). Several terminally substituted chloroalkanes were tested for their ability to act as growth substrates under similar culture conditions.

Preparation of bacterial extracts. Bacteria were harvested by centrifugation at 9000 g for 10 min, washed and resuspended in 100 mM-glycine/NaOH buffer, pH 9.1, then disrupted by five passages through a French pressure cell (SLM Instruments, Urbana, USA) at 138 MPa. Intact bacteria and debris were removed by centrifugation at 48000 g for 40 min to yield a cell-free extract which could be stored without further treatment for at least 3 months at $-20^{\circ}C$ without detectable loss of dehalogenase activity.

Dehalogenase purification. The dehalogenase was purified from the cell-free extract using the three chromatographic steps described by Tsang *et al.* (1988) with the following modifications. (i) DEAE-Sephacel. The column was equilibrated with 20 mM-Tris/ H_2SO_4 buffer, pH 8.5, and eluted with an 1800 ml linear gradient of sodium sulphate (0–400 mM) in the same buffer. (ii) Hydroxyapatite. Active fractions from the previous step were diluted 1.5-fold with 30 mM-glycine/NaOH buffer, pH 9.1, before being applied to the column. The column was then eluted with a 600 ml linear gradient (0–100 mM) of sodium phosphate in 30 mM-glycine/NaOH buffer, pH 9.1. (iii) Gel-filtration. Active fractions from the previous step were concentrated by ultrafiltration (Amicon PM-10 membrane) to 5 ml, applied to a Sephadex G-150 column equilibrated with 100 mM-glycine/NaOH buffer, pH 9.1, and eluted with the same buffer. Active fractions were pooled, concentrated and stored at $-20^{\circ}C$ after addition of 1,4-dithiothreitol (100 μ M).

Enzyme assay. Dehalogenase activity was measured in 10 ml glass-vials closed with Suba-seals in the following reaction mixture: 5 ml 100 mM-glycine/NaOH buffer, pH 9.1, 4 μ l 1-chlorobutane (equivalent to 7.6 mM). After equilibration of the reaction mixture by shaking (120 r.p.m.) at 30 °C for 15 min, the assay was initiated by addition of the enzyme preparation (5–200 μ l). Samples (1 ml) were withdrawn periodically and Cl^{-} content determined by the method of Bergmann & Sanik (1957).

During kinetic studies, enzyme-substrate reaction mixtures were shaken within a plugged 10 ml syringe containing a 6 mm diameter glass-bead. By advancing the syringe piston during each sample withdrawal, the headspace volume was reduced to zero thus eliminating substrate losses through partition equilibrium.

The pH dependence of the haloalkane dehalogenase was determined in a reaction mixture containing 50 mM-Tris/50 mM-glycine/50 mM-

maleate adjusted to the required pH value by addition of NaOH/ H_2SO_4 before addition of substrate and enzyme.

An anaerobic assay was done by sparging O_2 -free nitrogen through a sample of 100 mM-glycine/NaOH buffer, pH 9.1, contained in a Suba-sealed glass-vial at 0 °C for 2 h prior to the addition of substrate and enzyme. Incubation was at 30 °C.

One unit of dehalogenase activity (U) was defined as the amount of enzyme catalysing the release of 1 μ mol Cl^{-} min^{-1} .

1-Chlorobutane dehalogenation product determination. The dehalogenation product of 1-chlorobutane was determined by GLC. A megabore column (25 m $\times$ 0.530 mm, J & W Scientific), with DB-Wax-bonded liquid phase, was used with helium as carrier gas. The column temperature was 50 °C and detection was by flame ionization. The product identity was confirmed by reference to chromatograms of a number of C_4 standards.

Protein determination. Protein was measured by the method of Bradford (1976) using BSA as the standard.

PAGE. Dehalogenase activity was visualized after native PAGE by an activity reaction in the gel (cf. Hardman & Slater, 1981). Haloalkane substrates were incorporated into the gel as an emulsion by sonication of 10–200 μ l haloalkane with 40 ml acrylamide solution immediately before casting the gel. Dehalogenase activity bands were visualized after electrophoresis (4 °C) as regions of clearing in the emulsion after incubation at 37 °C for 20–90 min, depending on the number of enzyme units loaded onto the gel.

Isoelectric point determination. The isoelectric point (pI) of the purified dehalogenase was determined using Ampholine PAG plates (LKB), by reference to protein standards according to the manufacturer's recommendations.

Determination of molecular mass. The molecular mass of the purified denatured dehalogenase was determined by SDS-PAGE using 7–20% polyacrylamide gradient gels (Tsang *et al.*, 1988). Molecular mass standards were MW-SDS-70L molecular mass markers (Sigma): BSA, 66 kDa; chick egg albumin, 45 kDa; rabbit glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; bovine carbonic anhydrase, 29 kDa; bovine trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa; bovine milk α -lactalbumin, 14.2 kDa. Gels were stained with Coomassie blue G-250.

The native molecular mass was determined by gel-filtration chromatography using a Sephadex G-150 column (2.6 $\times$ 100 cm) equilibrated with 200 mM-Tris/ H_2SO_4 buffer, pH 8.0. The dehalogenase preparation (5 ml) was applied to the column and eluted with the same buffer (flow rate 0.2 ml min^{-1}). The column was calibrated with blue dextran, 2000 kDa; BSA, 66 kDa; chicken egg albumin, 45 kDa; bovine carbonic anhydrase, 29 kDa; and horse heart cytochrome c , 12 kDa.

N-Terminal amino acid sequence. The purified enzyme was dialysed against 20 mM- KH_2PO_4 /NaOH buffer, pH 7.5, and concentrated to 1 mg ml^{-1} by ultrafiltration (Amicon PM-10 membrane) before lyophilization. The N-terminal amino acid sequence was determined using automated Edman degradation/HPLC analysis with an automatic protein sequencer (model 8710; JayTee Biosciences, Herne Bay, Kent, UK).

Results

Characterization of the bacterial isolate

Bacterial isolate Y2, capable of utilizing 1-chlorobutane as its sole source of carbon and energy, was isolated by

batch-enrichment from a soil sample which had previously been exposed to haloalkanes. This isolate was identified by the National Collection of Industrial and Marine Bacteria (Torry Research Station, Aberdeen, UK) as *Rhodococcus erythropolis*. The genus was determined as a result of chemotaxonomic analysis based on the fatty acid profile and the presence of mycolic acids (Goodfellow, 1986). However, the strain was atypical in utilizing benzoate and tyrosine as growth substrates. In addition to growth on 1-chlorobutane, *R. erythropolis* Y2 could utilize the following α -substituted chloroalkanes: 1-chloropropane, 1-chloropentane, 1-chlorohexane, 1-chlorododecane, 1-chlorotetradecane, 1-chlorohexadecane and 1-chlorooctadecane.

The dehalogenase of *R. erythropolis* Y2 was inducible and active towards a considerably greater range of halogenated aliphatic compounds than would support growth of the organism. The enzyme catalysed the cleavage of a number of mono- and disubstituted chloro- and bromoalkanes with carbon chain lengths from C_1 to C_{16} (Table 1). The enzyme had greater activity towards terminally (α and ω) substituted substrates than towards those with mid-chain substituents.

Table 1. *Relative activity of the crude haloalkane halidohydrolase of R. erythropolis Y2 towards halogenated aliphatic substrates*

Enzyme assays contained 5 ml 100 mM-glycine/NaOH buffer, pH 9.1, 50 μ l of cell-free extract, and 4 μ l of one of the substrates (or 40 μ l of a 10%, w/v, neutral solution of the substrate for the haloacids). Relative activities were determined by comparison with the activity towards 1-chlorobutane [$0.012 \text{ U (mg protein)}^{-1}$ standardized to 100].

Substrate	Relative activity	Substrate	Relative activity
1-Chloropropane	8	2-Chlorobutane	3
1-Chlorobutane	100	2-Bromobutane	67
1-Bromobutane	100	1,2-Dichlorobutane	4
1-Chloropentane	132	1,2-Dibromopropane	132
1-Chlorohexane	107	Chloroacetate	0
1-Bromohexane	66	2-Chloropropionate	0
1-Chloroheptane	104	2-Chloroethanol	15
1-Chlorooctane	72	3-Chloropropan-1-ol	18
1-Chlorononane	85	4-Chlorobutan-1-ol	121
1-Chlorododecane	53	bis(2-Chloroethyl)ether	60
1-Chlorotetradecane	10		
1-Chlorohexadecane	8		
1-Chlorooctadecane	0		
Dichloromethane	0		
Dibromomethane	3		
1,2-Dichloroethane	6		
1,2-Dibromoethane	802		
1,3-Dichloropropane	202		
1,4-Dichlorobutane	232		
1,6-Dichlorohexane	168		
1,9-Dichlorononane	61		
1,10-Dichlorodecane	61		

Although there was no difference between the dehalogenation rates of 1-chloro- and 1-bromobutane, there was a marked difference in the activity of the enzyme against 2-chloro- and 2-bromobutane. Similarly, 1,2-dibromoethane was degraded more rapidly (more than 100-fold) than 1,2-dichloroethane.

Several other haloalkanes were dehalogenated by the *R. erythropolis* Y2 dehalogenase, including alcohols and an ether with terminal chloro-substituents (Table 1). However, 2-chloropropionic acid and chloroacetic acid were not substrates for the enzyme.

Polyacrylamide gel electrophoretic studies of the dehalogenase

Native PAGE run with gels containing emulsions of different haloalkanes showed the same single clearance band with an R_F of 0.76 in each substrate emulsion (Fig. 1). Slight darkening of the gel at R_F 1.00 was due to marker dye colouration and not to substrate clearing. Streaking of the substrate gel above the activity band was the result of enzyme activity occurring at 4 °C during electrophoresis. Variation in the solubility of the different haloalkane substrates meant that the degree of emulsion clearance in the gels was not proportional to the relative activities of the enzyme towards each substrate (Table 1).

Enzyme purification

The enzyme purification protocol produced a single dehalogenase activity peak at each chromatographic step, thus confirming the native PAGE analysis. Specific enzyme activity was increased 21-fold during purification (Table 2), which gave a final preparation containing one major protein band on SDS-PAGE (Fig. 2). Although the protein was not completely homogeneous in the Sephadex G-150 effluent, indicated by minor bands at high sample loading (Fig. 2), the major band stained by Coomassie blue R250 (Fig. 2) was identified as the dehalogenase by SDS-PAGE of gel slices taken from the clearing zones in native gels.

Table 2. *Purification of the haloalkane halidohydrolase of R. erythropolis Y2*

Step	Vol. (ml)	Protein (mg ml ⁻¹)	Specific activity (U mg ⁻¹)	Recovery (%)	Purification (-fold)
Cell-free-extract	47.0	9.7	0.016	100	—
DEAE-Sephacel	270.0	0.2	0.11	81	6.9
Biogel HT	3.2	3.5	0.14	21	8.8
Sephadex G-150	1.5	2.1	0.33	14	21.0

Fig. 1. Native PAGE of *R. erythropolis* Y2 cell-free extract. Substrate emulsions in tracks 1 to 6 were; 1,2-dibromopropane, 1,2-dibromoethane, 2-bromobutane, 1,9-dichlorononane, 1,6-dichlorohexane and 1-chlorobutane, respectively. Each track was loaded with 250 µg protein.

Fig. 2. Gradient SDS-PAGE of *R. erythropolis* Y2 halohydrase at different stages of enzyme purification. Tracks 1 and 8, SDS-7 marker proteins (7×10 µg protein); 2, cell-free extract (200 µg protein); 3, DEAE-Sephacel effluent (30 µg protein); 4, Biogel HT effluent (30 µg protein); 5, Biogel HT effluent concentrate (200 µg protein); 6, Sephadex G-150 effluent (10 µg protein); 7, Sephadex G-150 effluent concentrate (80 µg protein).

Characterization of the purified enzyme

The haloalkane dehalogenating activity of *R. erythropolis* Y2 was the result of a single broad-specificity enzyme which was equally active under aerobic and anaerobic assay conditions. The product of its activity towards 1-chlorobutane was identified as n-butanol by GLC, indicating that the enzyme catalysed a hydrolytic dehalogenation.

The dehalogenase had optimum activity at pH 9.2 but

retained more than 50% of its maximum activity over the range pH 7.5–11.0. Activity was very low at pH 5–6. The isoelectric point of the enzyme was estimated to be 4.7.

Dehalogenase activity increased with assay temperature up to 37 °C. Above this temperature enzyme activity declined as thermal inactivation became significant. From an Arrhenius plot of the temperature/activity data an enzyme activation energy of 43 kJ mol⁻¹ was estimated.

The effect of 1-chlorobutane concentration on the

Table 3. Effect of inhibitors on haloalkane halidohydrolase activity from *R. erythropolis* Y2

Enzyme assays contained 5 ml 100 mM-glycine/NaOH buffer, pH 9.1, which had been made 1 mM with respect to each of the inhibitors, 4 μ l 1-chlorobutane and 50 μ l of a solution of purified enzyme. The amount of inhibition was determined by reference to a control assay containing no inhibitor. The specific activity of the control assay was 0.30 U (mg protein)⁻¹.

Inhibitor	Inhibition (%)	Inhibitor	Inhibition (%)
CaCl ₂	46	<i>N</i> -Ethylmaleimide	69
CuSO ₄	100	Dibromomethane	48
FeSO ₄	83	Dichloromethane	23
NiSO ₄	93	Dichloroacetate	0
ZnSO ₄	89	EDTA	0
HgCl ₂	100		
K ₂ SO ₄	0		
KCl	0		

dehalogenase activity was determined over the range 0.2–1.0 mM. The Eadie–Hofstee transformation of the data produced an apparent K_m of 0.26 mM and a value for V of 0.38 μ mol Cl⁻ min⁻¹ (mg protein)⁻¹.

The effect of inhibitors on the hydrolysis of 1-chlorobutane is shown in Table 3. Thiol reagents (Hg²⁺, *N*-ethylmaleimide) were effective inhibitors as were a number of other divalent metal ions (e.g. Cu²⁺, Zn²⁺) in comparison with the monovalent metal ion K⁺. Halocompounds which were poor substrates for the enzyme (i.e. dichloro- and dibromomethane; Table 2) were effective inhibitors of 1-chlorobutane dehalogenation although the type of inhibition was not investigated. However, the non-substrate halocompound dichloroacetate did not act as an inhibitor.

The molecular mass of the enzyme was estimated to be 36 kDa by gel-filtration and 34 kDa by SDS-PAGE (Fig. 2); it was therefore considered to be a monomeric protein.

The N-terminal amino acid sequence for the first 20 residues of the purified enzyme was: Ser-Glu-Ile-Gly-Thr-Gly-Phe-Pro-Phe-Asp-Pro-His-Tyr-Val-Glu-Val-Leu-Gly-Glu-Arg.

Discussion

The range of haloalkane growth substrates utilized by *R. erythropolis* Y2 (C₃–C₁₆) is considerably greater than that reported for most other haloalkane-utilizing bacteria, with the exception of *Arthrobacter* sp. strain HAl which is able to utilize halosubstituted C₂–C₁₆ alkanes (Scholtz *et al.*, 1988). Janssen *et al.* (1987) described a bacterial isolate, GJ70, capable of growth on terminally substituted C₂–C₉ alkanes whilst Yokota *et al.* (1986)

reported growth of two bacterial isolates on agar containing 1,10-dichlorodecane. *Xanthobacter autotrophicus* GJ10 (Janssen *et al.*, 1985) had a considerably more restricted substrate profile, being capable of growth only on mono- and disubstituted C₂ and C₃ compounds.

The observation that cell-free extracts of *R. erythropolis* Y2 catalysed an oxygen-independent dehalogenation of 1-chlorobutane and that the product of the reaction was n-butanol suggests that the enzyme catalyses a hydrolytic dehalogenation, and so can be classified as a halidohydrolase.

Activity stain PAGE, utilizing Ag⁺ to precipitate free halide ions in regions of dehalogenase activities, has been employed to identify electrophoretically distinguishable 2-haloacid halidohydrolases (Slater *et al.*, 1979; Hardman & Slater, 1981; Allison *et al.*, 1983). The same technique was used by Scholtz *et al.* (1988) to visualize one of the dehalogenase activities in *Arthrobacter* sp. HAl. In this paper we describe a more direct visualization procedure which utilizes the greater solubility of the reaction product of enzymic dehalogenation so that regions of dehalogenase activity are identified as zones of clearance in the substrate emulsion contained in the gel (see Fig. 1). Using this technique, a single halidohydrolase was identified in cell-free extracts of *R. erythropolis* Y2, and was subsequently purified.

The halidohydrolase of *R. erythropolis* Y2 possesses one of the broadest substrate ranges reported to date (cf. Scholtz *et al.*, 1987; Yokota *et al.*, 1987; Janssen *et al.*, 1988). This halidohydrolase showed little or no activity towards terminally halogenated C₁₄, C₁₆ and C₁₈ alkanes, but the organism could utilize these haloalkanes as substrates for growth. This observation suggests that a second enzyme may be present, which is either active in the dehalogenation of these longer-chain haloalkanes or in their modification to short-chain halocompounds.

The biochemical characteristics of the *R. erythropolis* Y2 halidohydrolase closely resembled those of enzymes from *Corynebacterium* sp. m-15-3 (data from Yokota *et al.*, 1987) and *Arthrobacter* sp. HAl (data from Scholtz *et al.*, 1987). The three enzymes have similar pH optima, ranging from pH 9.2–9.5, and are monomeric proteins with a molecular mass of 34–36 kDa. The isoelectric point for the *R. erythropolis* Y2 enzyme (4.7) is similar to that for the *Corynebacterium* sp. m-15-3 enzyme (4.5). Additionally, the activation energy of the *Arthrobacter* sp. HAl dehalogenase (40 kJ mol⁻¹) is very similar to that of the *R. erythropolis* Y2 halidohydrolase (42.9 kJ mol⁻¹). The other haloalkane dehalogenases reported to date – in *Xanthobacter autotrophicus* GJ10 (Keuning *et al.*, 1985) and strain GJ70 (Janssen *et al.*, 1988) – have fewer apparent similarities, with more acidic pH optima and higher activation energies (55–

59 kJ mol⁻¹), while the dehalogenase of GJ70 had a notably lower molecular mass (28 kDa).

The K_m values of the dehalogenases from *X. autotrophicus* strain GJ70, *Corynebacterium* sp. m-15-3, *Arthrobacter* sp. HA1 and *R. erythropolis* Y2 for 1-chlorobutane all fall in the range 0.06–0.40 mM. However, the V value for the *R. erythropolis* Y2 halidohydrolase is considerably lower [0.382 µmol min⁻¹ (mg protein)⁻¹] than those for the other enzymes [approximately 1.5 µmol min⁻¹ (mg protein)⁻¹].

The most notable similarity between the *R. erythropolis* enzyme and that of *Arthrobacter* sp. HA1 is the 100% homology between their N-terminal amino acid sequences; at least 17 of the first 18 amino acid residues are identical. (*Arthrobacter* sequence data from Scholtz *et al.*, 1987.) This is in contrast to major differences between the N-terminal sequences of these two enzymes and that of the *X. autotrophicus* GJ10 enzyme (Keuning *et al.*, 1985), and the *Hyphomicrobium* sp. DM2 enzyme (Kohler-Staub *et al.*, 1986). The degree of N-terminal homology between the *R. erythropolis* Y2 and *Arthrobacter* sp. HA1 halidohydrolases raises interesting questions about the evolution of these enzymes. These actinomycetes were isolated from geographically separate industrial sites. The presence of biogenic haloalkanes in the environment might suggest that haloalkane dehalogenases have not evolved recently, thus the possibility of parallel evolution from a common ancestral protein or the spread of dehalogenase gene(s) or plasmids is a plausible explanation of this homology. *R. erythropolis* Y2 has been shown to harbour at least one plasmid (C. Fletcher, V. Vienravi, A. T. Bull & D. J. Hardman unpublished data). The evolution of dehalogenase activities from ancestral hydrolases has been proposed as a mechanism by which the multiple forms of the 2-haloacid dehalogenases arose (Tsang *et al.*, 1988) and this hypothesis is being tested further in the system reported in this paper.

We thank Miss Clare Fletcher for excellent technical assistance, the Department of Trade and Industry for its support through the Laboratory of the Government Chemist/University of Kent BIO-TRANS project and JayTee Biosciences for the use of their model 8710 automatic protein sequencer.

References

- ALEXANDER, M. (1965). Biodegradation: problems of molecular recalcitrance and microbial fallibility. *Advances in Applied Microbiology* **7**, 35–76.
- ALLISON, N., SKINNER, A. J. & COOPER, R. A. (1983). The dehalogenase of a 2,2-dichloropropionate-degrading bacterium. *Journal of General Microbiology* **129**, 1283–1293.
- BERGMANN, J. G. & SANIK, J. (1957). Determination of trace amounts of chlorine in naphtha. *Analytical Biochemistry* **29**, 241–243.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- GOLDMAN, P. (1965). The enzymatic cleavage of C–F bond in fluoroacetate. *Journal of Biological Chemistry* **240**, 3434–3438.
- GOLDMAN, P., MILNE, G. W. A. & KEISTER, D. B. (1968). Carbon-halogen bond cleavage. III. Studies on bacterial halidohydrolases. *Journal of Biological Chemistry* **243**, 428–434.
- GOODFELLOW, M. (1986). Genus *Rhodococcus*. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1472–1481. Edited by P. H. A. Sneath. Baltimore: Williams & Wilkins.
- HARDMAN, D. J. (1990). Biotransformation of halogenated compounds: enzymatic cleavage of carbon halogen bonds. *Critical Reviews in Biotechnology* (in the Press).
- HARDMAN, D. J. & SLATER, J. H. (1981). Dehalogenases in soil bacteria. *Journal of General Microbiology* **123**, 117–128.
- 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.
- JANSSEN, D. B., JAGER, D. & WITHOLT, B. (1987). Degradation of n-haloalkanes and α , ω -dihaloalkanes by wild-type and mutants of *Acinetobacter* sp. strain GJ70. *Applied and Environmental Microbiology* **53**, 561–566.
- JANSSEN, D. B., GERRITSE, J., BRACKMAN, J., KALK, C., JAGER, D. & WITHOLT, B. (1988). Purification and characterization of a bacterial dehalogenase with activity toward halogenated alkanes, alcohols and ethers. *European Journal of Biochemistry* **171**, 67–72.
- KEITH, L. H. & TEILLIARD, W. A. (1979). Priority pollutants I: a perspective view. *Environmental Science and Technology* **13**, 416–423.
- KEUNING, S., JANSSEN, D. B. & WITHOLT, B. (1985). Purification and characterization of hydrolytic haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. *Journal of Bacteriology* **163**, 635–639.
- KOHLER-STAU, D., HARTMANS, S., GALLI, R., SUTER, F. & LEISINGER, T. (1986). Evidence for identical dichloromethane dehalogenases in different methylotrophic bacteria. *Journal of General Microbiology* **132**, 2837–2843.
- SCHOLTZ, R., LEISINGER, T., 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**, 5015–5021.
- SCHOLTZ, R., MESSI, F., LEISINGER, T. & COOK, A. M. (1988). Three dehalogenases and physiological restraints in the biodegradation of haloalkanes by *Arthrobacter* sp. strain HA1. *Applied and Environmental Microbiology* **54**, 3034–3038.
- SLATER, J. H., LOVATT, D., WEIGHTMAN, A. J., SENIOR, E. & BULL, A. T. (1979). The growth of *Pseudomonas putida* on chlorinated aliphatic acids and its dehalogenase activity. *Journal of General Microbiology* **114**, 125–136.
- TSANG, J. S. H., SALLIS, P. J., BULL, A. T. & HARDMAN, D. J. (1988). A monobromoacetate dehalogenase from *Pseudomonas cepacia* MBA4. *Archives of Microbiology* **150**, 441–446.
- 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.
- YOKOTA, T., OMORI, T. & KODAMA, T. (1987). Purification and properties of haloalkane dehalogenase from *Corynebacterium* sp. m-15-3. *Journal of Bacteriology* **169**, 4049–4054.