Haloalkane degradation and assimilation by *Rhodococcus rhodochrous* NCIMB 13064

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The bacterium *Rhodococcus rhodochrous* NCIMB 13064, isolated from an industrial site, could use a wide range of 1-haloalkanes as sole carbon source but apparently utilized several different mechanisms simultaneously for assimilation of substrate. Catabolism of 1-chlorobutane occurred mainly by attack at the C-1 atom by a hydrolytic dehalogenase with the formation of butanol which was metabolized via butyric acid. The detection of small amounts of γ -butyrolactone in the medium suggested that some oxygenase attack at C-4 also occurred, leading to the formation of 4-chlorobutyric acid which subsequently lactonized chemically to γ -butyrolactone. Although 1-chlorobutane-grown cells exhibited little dehalogenase activity on 1-chloroalkanes with chain lengths above C₁₀, the organism utilized such compounds as growth substrates with the release of chloride. Concomitantly, γ -butyrolactone accumulated to 1 mM in the culture medium with 1-chlorohexadecane as substrate. Traces of 4-hydroxybutyric acid were also detected. It is suggested that attack on the long-chain chloroalkane is initiated by an oxygenase at the non-halogenated end of the molecule leading to the formation of an ω -chlorofatty acid. This is degraded by β -oxidation to 4-chlorobutyric acid which is chemically lactonized to γ -butyrolactone which is only slowly further catabolized via 4-hydroxybutyric acid and succinic acid. However, release of chloride into the medium during growth on long-chain chloroalkanes was insufficient to account for all the halogen present in the substrate. Analysis of the fatty acid composition of 1-chlorohexadecane-grown cells indicated that chlorofatty acids comprised 75% of the total fatty acid content with C_{14:0}, C_{16:0}, C_{16:1} and C_{18:1} acids predominating. Thus the incorporation of 16-chlorohexadecanoic acid, the product of oxygenase attack directly into cellular lipid represents a third route of chloroalkane assimilation. This pathway accounts at least in part for the incomplete mineralization of long-chain chloroalkane substrates. This is the first report of the coexistence of a dehalogenase and the ability to incorporate long-chain haloalkanes into the lipid fraction within a single organism and raises important questions regarding the biological treatment of haloalkane containing effluents.

Keywords: *Rhodococcus rhodochrous*, haloalkanes, degradation, chlorofatty acids, lipid composition

INTRODUCTION

Many haloalkanes are resistant to microbial attack and persist in the environment to become major pollutants of the biosphere and ground water. The rate of biodegradation of these compounds is largely determined by the rate of cleavage of the carbon–halogen bond. Three principal

mechanisms of microbial dehalogenation have been identified, namely oxidative, reductive and hydrolytic. Oxidative dehalogenation is usually mediated by relatively non-specific monooxygenases which catalyse the formation of *gem*-halohydrins or halogenated epoxides which spontaneously decompose to aldehydes releasing halide ion. In general, such enzymes attack short-chain halo-

carbons (C_1 to C_4). Thus ammonia monooxygenase of the nitrifying bacterium *Nitrosomonas europaea* dehalogenated all monohaloethanes and C_1 to C_4 monochlorinated n-alkanes (Rasche *et al.*, 1990; Vannelli *et al.*, 1990). Similarly the broad substrate specificity of methane oxygenase in methanotrophic organisms allows it to dehalogenate a variety of C_1 and C_2 compounds. Resting cells of *Methanococcus capsulatus* can convert chloromethane to formaldehyde (Stirling & Dalton, 1979) whilst the soluble methane oxygenase of *Methylomonas* sp. and *Methylosinus* sp. is able to dehalogenate, in addition to C_1 compounds, C_2 compounds such as *trans*-1,2-dichloroethylene via chloroxirane type intermediates (Oldenhuis *et al.*, 1989; Janssen *et al.*, 1988b).

Reductive dehalogenation of a range of chlorinated alkanes and alkenes by methanogenic, denitrifying and sulphate reducing organisms has been observed in reducing environments in nature (Vogel & McCarty, 1985; Bouwer & McCarty, 1983; Bagley & Gossett, 1990). The precise enzymic mechanism has not been defined but there is evidence that reduced porphyrin and corrin complexes are able to dehalogenate halogenated compounds efficiently (Marks *et al.*, 1989).

Hydrolytic dehalogenation of haloalkanes involving release of halide ion and formation of the corresponding alcohol has been reported in a number of organisms. D. B. Janssen and co-workers (Janssen *et al.*, 1985; Keuning *et al.*, 1985) have isolated two halidohydrolases with different substrate specificities from a nitrogen fixing hydrogen bacterium *Xanthobacter* sp. able to use 1,2-dichloroethane and C_1 to C_4 terminally halogenated alkanes as sole carbon and energy source. Similar enzymes are produced by several strains of *Ancylobacter aquaticus*, a facultative methylotroph also able to use dichloroethane as sole carbon and energy source (Van den Wijngaard *et al.*, 1992). This group has also purified a broad spectrum halidohydrolase from an *Acinetobacter* sp. capable of degrading C_2 to C_9 terminally halogenated alkanes (Janssen *et al.*, 1987, 1988a).

T. Leisinger and co-workers (Scholtz *et al.*, 1987a, b, 1988) have shown that at least three different haloalkane dehalogenases can be induced in an *Arthrobacter* sp. with a broad range of growth substrates. They described the purification of one of these dehalogenases capable of attacking a wide variety of halogenated compounds. Similar enzymes have been isolated by Yokata *et al.* (1987) from a *Corynebacterium* sp. and by Sallis *et al.* (1990) from *Rhodococcus erythropolis*. An interesting variant of the hydrolytic dehalogenation mechanism in which glutathione acts as a co-factor has been described in a dichloromethane-utilizing methanotrophic *Hyphomicrobium* sp. In this organism an inducible glutathione-S-transferase catalyses the nucleophilic displacement of chloride by reduced glutathione which decomposes chemically to formaldehyde and glutathione (Stucki *et al.*, 1981; Kohler-Staub & Leisinger, 1985).

The assimilation of longer-chain chloroalkanes by organisms does not necessarily involve dehalogenation. Thus Murphy & Perry (1983, 1984) have shown that *Mycobacterium* spp. and certain fungi, when growing on 1-chlorohexadecane or 1-chlorooctadecane as sole carbon and energy source, can incorporate these compounds as the corresponding chlorinated fatty acids into the cellular lipid fraction of the organism in high yield. Transformation into chlorinated fatty acids of both longer and shorter chain-length also occurred so that chlorinated fatty acids could account for 85% of the total fatty acid content. It was not clear from these reports whether these organisms possessed a dehalogenase allowing complete metabolism of the haloalkane or whether β -oxidation of the substrate proceeded until proximity of the halogen prevented further degradation.

In this paper we describe an investigation of the growth on a variety of haloalkanes of *Rhodococcus rhodochrous* NCIMB 13064, an organism which appears to use several different mechanisms simultaneously for haloalkane assimilation.

METHODS

Isolation and culture of organism. *Rhodococcus rhodochrous* NCIMB 13064 was isolated by elective culture from a soil sample obtained from an industrial site which had previously been exposed to chlorinated alkanes. The minimal salts medium used contained 1.5 mM 1-chlorobutane.

The organism was grown routinely in 500 ml conical flasks sealed with Teflon lined screw caps fitted with Mini-inert sampling ports (Precision Sampling Co.) containing 200 ml of medium of the following composition ($g\ l^{-1}$): $NaH_2PO_4 \cdot 2H_2O$ (1.6); Na_2HPO_4 (1.4); $(NH_4)_2SO_4$ (0.5); $MgSO_4 \cdot 7H_2O$ (0.2); K_2SO_4 (0.5). The pH of the solution was adjusted to 7.0 and the medium supplemented with a trace element solution ($10\ ml\ l^{-1}$) containing ($g\ l^{-1}$): Na_3EDTA ($2H_2O$) (12.0); $FeSO_4 \cdot 7H_2O$ (2.0); $CaCl_2$ (1.0); $ZnSO_4 \cdot 7H_2O$ (0.4); $MnSO_4 \cdot 4H_2O$ (0.4); $CuSO_4 \cdot 5H_2O$ (0.1). 1-Chlorobutane (1.1 mmol, 115 μ l) was injected through the sampling port of the flask giving an aqueous phase concentration of 2.8 mM after equilibration of aqueous and gaseous phases in the flask. Cultures were incubated at 25 °C on an orbital shaker (200 r.p.m.). When cultured on less volatile long-chain alkanes such as hexadecane and 1-chlorohexadecane flasks were stoppered with cotton wool and contained 200 ml of culture medium plus 1 mmol of substrate.

Growth of the organism was monitored by measuring the optical density at 690 nm. During growth on long-chain alkanes, cells tended to clump together and it was necessary to homogenize the culture prior to measurement of optical density.

Chemicals. 1-Chlorobutane (HPLC grade, purity > 99.5%) and 1-chlorohexadecane (purity > 95%) were obtained from Aldrich. Analysis by gas chromatography/mass spectrometry (GC/MS) showed that concentrations of 4-chlorobutanol and 1,4-dichlorobutane did not exceed 5 p.p.m. in 1-chlorobutane. Similarly, no individual homologue present as an impurity in 1-chlorohexadecane exceeded a concentration of 0.5%. All other chemicals used as substrates were purchased from Aldrich except 1-bromopentadecane which was obtained from Fluka.

Halide analysis. Chloride and bromide ions in centrifuged culture medium were determined by potentiometric titration using a Corning Chloride Analyser 926. Fluoride ion was measured using an Orion model 94-09 combination fluoride electrode.

1-Chlorobutane determination. 1-Chlorobutane in culture medium was determined by gas chromatography using a head-

space assay. Samples of head-space (1 ml) were withdrawn from culture flasks through the sampling port and injected into a Pye Unicam 104 (model 74) gas chromatograph fitted with a flame ionization detector and equipped with a glass column (1.5 m $\times$ 2 mm) packed with Tenax GC (60–80 mesh) and operated at a nitrogen gas flow of 20 ml min⁻¹. The oven temperature was programmed at a rate of 24 Centigrade degrees min⁻¹ from 60 to 200 °C.

Calibration was against samples of head-space from above 1-chlorobutane solutions (2 ml) of known concentration equilibrated at 25 °C in 25 ml screw capped septum vials sealed with Teflon lined silicone discs. The partition coefficient water/air (w/v per w/v) for 1-chlorobutane at 25 °C was found to be 1.51 at 25 °C.

1-Chlorohexadecane determination. Cells, which tended to grow in clumps, were removed using either filtration (Whatman no. 50 paper) or a separating funnel (500 ml). The resulting culture medium (200 ml) was extracted with light petroleum (b.p. 40–60 °C, 3 $\times$ 25 ml) and the extracts bulked. The cells harvested from the medium were washed with light petroleum (b.p. 40–60 °C, 3 $\times$ 10 ml) to remove residual 1-chlorohexadecane. The resulting extracts were combined with those from the culture medium and evaporated to small volume.

1-Chlorohexadecane was determined in the extract by gas chromatography using a Hewlett Packard 5890 fitted with a flame ionization detector and equipped with an Ultra 1 fused silica WCOT column (25 m $\times$ 0.32 mm) with 100% dimethylpolysiloxane as bonded phase. Helium was used as the carrier gas at a flow rate of 1.5 ml min⁻¹. After sample injection using a split ratio of 200:1 the oven temperature was held at 100 °C for 1 min and then programmed at 10 Centigrade degrees min⁻¹ to 250 °C. 1-Chlorohexadecane was quantified by comparison of the peak area at the retention time of the compound with that of a standard solution of the compound.

Identification and quantitation of metabolic intermediates.

Culture medium (400 ml) after centrifugation to remove cells was divided into two equal portions. For extraction of organic acids (i.e. n-butyric, 4-chlorobutyric, 4-hydroxybutyric and succinic acids) a portion was adjusted to pH 2.0 and continuously extracted with diethyl ether for 72 h. The ether extract was dried with anhydrous MgSO₄ and evaporated to small volume. Acids were converted to methyl esters by addition of an equal volume of a solution of diazomethane in ether and this derivatized extract was utilized for GC/MS. For isolation of γ -butyrolactone, culture medium was continuously extracted with diethyl ether for 72 h without prior pH adjustment. The ether extract was dried with anhydrous MgSO₄ and evaporated to small volume prior to analysis by GC/MS.

Both γ -butyrolactone and methyl esters of organic acids were identified and quantified on a Hewlett Packard 5890 gas chromatograph linked to a HP5970 mass selective detector. The GC was fitted with a CP-SIL-19 fused silica WCOT column (25 m $\times$ 0.25 mm) with a bonded phase of 7% cyano, 7% phenyl, 85% methyl, 1% vinylpolysiloxane. Helium was used as the carrier gas at a flow rate of 0.75 ml min⁻¹. After splitless injection of sample the oven temperature was held at 30 °C for 2 min and programmed at 10 Centigrade degrees min⁻¹ to 275 °C. Ion currents between m/e 30 and m/e 350 were monitored. Compounds were identified from their mass spectra and retention time. To quantify each compound the total ion current at the retention time of the compound was compared with that of an authentic sample of the compound of known concentration.

n-Butanol was determined by gas chromatography of the culture medium by the method of Elfaki *et al.* (1991).

Measurement of oxygen uptake by cell suspensions. Cultures (200 ml) were grown as described above. Oxygen consumption by whole cell suspensions was recorded at 25 °C with a 4 ml capacity Clark-type oxygen electrode (Rank Bros.). Incubation mixtures contained 50 mM phosphate buffer pH 7.0, cell suspension (12.6 mg wet wt) and 1 mM substrate in a total volume of 3 ml. Butane was added as a stock solution (10 mM) prepared by weighing an aqueous butane gas solution stored in screw capped septum vials sealed with Teflon lined silicone discs.

Measurement of dehalogenating activity in resting cells.

Cultures (200 ml) were grown aerobically on 1-chlorobutane, as described previously. Cells were harvested in the exponential phase by centrifugation, washed with Tris/H₂SO₄ buffer (50 mM, pH 8.0) and resuspended in the buffer (50 ml) in 250 ml medical flat bottles sealed with Teflon lined screw caps. Dehalogenation of a given substrate by these resting cell suspensions was measured by addition of 0.375 mmol substrate (7.5 mM final concentration) and incubation of the suspension at 25 °C with shaking for 24 h. Cells were then removed by centrifugation and the supernatant was analysed for halide ion. Controls containing buffer and substrate alone were used to measure any abiotic dehalogenation and additional controls containing cell suspensions in the absence of substrate were employed to assess release of endogenous halide ion by cells. Net biological dehalogenation of each substrate was calculated by subtracting the sum of abiotic halide release from the substrate and halide release from cells in the absence of substrate from halide release by the cells in the presence of substrate. All experiments were performed in duplicate. Cellular dehalogenation activity for each substrate is recorded as the release of halide ion (in mmols) expressed as a percentage of the total chloride ion released (in mmols) from 1-chlorobutane under the same conditions.

Determination of fatty acid composition of lipids of *R. rhodochrous*.

Cells (5 g wet wt) were harvested by centrifugation, washed with light petroleum (b.p. 40–60 °C, 3 $\times$ 20 ml) to remove residual substrate. Cellular fatty acids were converted directly to the corresponding methyl esters by the transesterification method of Dunlap & Perry (1967). Wet cells (1 g wet wt) were suspended in 14% BF₃ in methanol/benzene (9:1, v/v; 4 ml) and heated for 12 h at 60 °C in a water bath in a glass stoppered tube. After cooling, water (8 ml) was added and the mixture extracted with light petroleum (b.p. 40–60 °C, 2 $\times$ 10 ml). The extracts were combined, washed with water (10 ml) and concentrated to 1 ml.

To extract free fatty acids from cells of *R. rhodochrous*, cells (5 g wet wt) after washing with light petroleum as described above were adjusted to pH 2.0 and extracted with diethyl ether (3 $\times$ 30 ml). The combined ether extract was shaken with saturated NaHCO₃ (2 $\times$ 20 ml). The aqueous extract was washed with diethyl ether (20 ml), acidified to pH 2.0 and re-extracted with diethyl ether (3 $\times$ 30 ml). The extract was reduced to a small volume and the acids were methylated as described above.

Fatty acid methyl esters (FAME) were identified and quantified by GC/MS on a Hewlett Packard 5890 gas chromatograph linked to an HP 5970 mass selective detector controlled by an HP 300 series computer. The gas chromatograph was fitted with a CP-SIL-88 fused silica WCOT column (50 m $\times$ 0.25 mm) with 100% cyanopropylpolysiloxane as bonded phase. Helium was used as the carrier gas at a flow rate of 0.75 ml min⁻¹. After splitless injection of the sample the oven temperature was held at 130 °C for 5 min, then programmed at 5 Centigrade degrees min⁻¹ up to 230 °C and held at the temperature for 15 min. Ion currents between m/e 30 and 500 were monitored. Individual FAME were identified from their mass spectra and retention times (Murphy & Perry, 1983). To quantify each

Fig. 1

Fig. 2

Fig. 1. Growth of *R. rhodochrous* NCIMB 13064 on 1-chlorobutane. The organism was grown at 25 °C in 500 ml sealed flasks containing 200 ml of minimal salts medium to which 1.1 mmol 1-chlorobutane was added. Growth and metabolite concentrations were measured as described in Methods. ●, Growth; ▽, chloride concn; ▼, 1-chlorobutane concn; □, butyric acid concn.

Fig. 2. Growth of *R. rhodochrous* NCIMB 13064 on 1-chlorohexadecane. The organism was grown at 25 °C in 500 ml flasks stoppered with cotton wool on 200 ml of minimal salts medium containing 5 mM 1-chlorohexadecane. Growth and metabolite concentrations were measured as described in Methods. ●, Growth; ▼, chloride concn; □, 1-chlorohexadecane concn; ▽, γ -butyrolactone concn.

FAME the total ion current at the retention time of the compound was expressed as a percentage of the sum of the ion currents associated with all the FAME in the extract.

RESULTS

1-Chlorobutane degradation by *R. rhodochrous*

The time course of growth of *R. rhodochrous* on 1-chlorobutane measured as optical density at 690 nm is shown in Fig. 1. The accumulation of chloride ion in the medium closely parallels growth and is consistent with the disappearance of 1-chlorobutane from the medium. n-Butyric acid also accumulates transiently in the medium during growth attaining a peak concentration of 0.12 mmol per flask during the exponential phase of growth. Additionally, culture medium sampled at 21 and 31 h contained n-butanol at concentrations of 0.024 mmol per flask and 0.016 mmol per flask respectively. The presence of these intermediates suggested that degradation of substrate was initiated by attack on the carbon atom attached to the halogen atom. Further evidence of catabolism by this route was provided by respiration studies on 1-chlorobutane-grown cells which showed high rates of oxygen uptake with 1-chlorobutane, n-butanol and n-butyric acid but negligible oxygen uptake on n-butane and n-pentane (Table 1). Acetate but not succinate was also readily oxidized implying that n-butyric acid was probably metabolized by β -oxidation. When washed resting cells harvested from 1-chlorobutane-grown cultures were incubated with 1-chlorobutane under anaerobic conditions equimolar n-butanol (confirmed by GC/MS) and chloride ion accumu-

lated in the medium suggesting the presence of a hydrolytic dehalogenase.

To ascertain whether any degradation of 1-chlorobutane from the non-halogenated end of the molecule occurred, culture medium (200 ml) from 1-chlorobutane-grown cultures was adjusted to pH 6.5 or pH 2.0 and continuously ether-extracted in an attempt to isolate any trace metabolic intermediates. GC/MS analysis of these extracts revealed the presence of only one unknown compound in significant quantities. The electron impact mass spectrum of the compound exhibited fragments at m/z 86 (molecular ion, 100%), m/z 42 ($M-\text{CO}_2$, 83%), m/z 56 ($M-\text{CH}_2\text{O}$, 43%), m/z 41 ($M-\text{HCO}_2$, 38%) and m/z 85 ($M-\text{H}$, 25%). The retention time and mass spectrum of the compound were identical to that of γ -butyrolactone. The compound was present in both pH 6.5 and pH 2.0 extracts in similar amounts and was detected, albeit in trace quantities, in samples from all stages of growth from 24 h onwards. A maximum concentration of 5 μM was attained as growth peaked at 36 h but the compound could still be detected in the medium at 3 μM when measurements were terminated at 70 h. It seems probable that this compound arises by chemical lactonization of 4-chlorobutyric acid which could be produced by oxygenase attack at the non-chlorinated end of the molecule. Measurement of chloride release from 4-chlorobutyric acid solutions at pH 8.0 indicated a half-life for the compound of about 20 min rising to 2 h at pH 6.5 although reduction of the pH to 3.0 extended the half-life to 22 h. GC/MS examination of diethyl ether extracts of such solutions at both pH 6.5 and 3.0 indicated that γ -

Table 1. Oxidation of various substrates by cell suspensions of *R. rhodochrous* grown on 1-chlorobutane

Substrate	O ₂ uptake* (relative to that with n-butyric acid as substrate)
n-Butyric acid	100†
n-Butanol	189
4-Chlorobutanol	113
Butane-1,4-diol	21
4-Hydroxybutyric acid	3
Butane	0
Pentane	0
1-Chlorobutane	68
γ-Butyrolactone	0
Acetic acid	22
Succinic acid	0

* Corrected for endogenous O₂ uptake.

† Represents an O₂ uptake of 668 nM O₂ min⁻¹ (mg protein)⁻¹ after correction for an endogenous rate of 40 nM O₂ min⁻¹ (mg protein)⁻¹.

butyrolactone was the only detectable organic product formed from 4-chlorobutyric acid under these conditions. Under normal cultural conditions this rapid lactonization would ensure that accumulation of significant quantities of 4-chlorobutyric acid in the medium did not occur unless there was substantial production of the compound during bacterial catabolism.

Dehalogenating activity of resting cells of *R. rhodochrous* with various substrates

1-Butanol-grown cells do not possess dehalogenase activity but growth on 1-chlorobutane induces an enzyme which permits dehalogenation of a number of substrates. The relative activities of 1-chlorobutane-grown resting cells of *R. rhodochrous* in dehalogenating a range of halogenated substrates is shown in Table 2, which also indicates which compounds act as growth substrates. Dehalogenation of 1-halosubstituted alkanes with chain-lengths between C₃ and C₉ was observed. The corresponding bromo- and iodo-compounds also appeared to be cleaved at rates similar to those of the chloro-compounds but only a limited release of fluoride from the fluoroalkanes tested was found. The low or insignificant rate of dehalogenation of chloro-, iodo- and bromo-compounds with chain-lengths exceeding 10 carbon atoms by resting cells did not appear to affect the ability of these compounds to act as growth substrates implying that enzymic dehalogenation is not the only mechanism available to the organism to initiate degradation of these molecules. Conversely, C₅ to C₈ bromo-alkanes, whilst readily dehalogenated by resting cells did not act as growth substrates, suggesting that perhaps these compounds were not capable of inducing expression of the dehalogenase enzyme.

Table 2. Growth of *R. rhodochrous* on various substrates compared with dehalogenating activity of 1-chlorobutane-grown cells for these substrates

Dehalogenation was measured as μmol halide ion released when 0.375 mmols substrate was incubated with 50 ml Tris/H₂SO₄ buffer (50 mM, pH 8.0) containing 5 × 10⁶ resting cells ml⁻¹ for 24 h at 25 °C in a 250 ml bottle. Corrections were made for abiotic halide release and dehalogenation is expressed as a percentage of halide ion released when 1-chlorobutane was substrate. Abiotic dehalogenation of all of the substrates was less than 5% of the total added over 24 h with the exception of 5-chlorovaleric acid (30%). ND, Not determined.

Compound	Utilization as growth substrate	Dehalogenation
Chloromethane	—	0
1-Chloroethane	—	0
1-Chloropropane	+	121
1-Chlorobutane	+	100
1-Chloropentane	+	112
1-Chlorohexane	+	125
1-Chloroheptane	+	121
1-Chlorooctane	+	43
1-Chlorononane	—	35
1-Chlorodecane	+	32
1-Chlorododecane	+	19
1-Chlorotetradecane	+	12
1-Chlorohexadecane	+	5
1-Chlorooctadecane	+	0
1-Bromopropane	+	98
1-Bromobutane	+	102
1-Bromopentane	—	112
1-Bromohexane	—	109
1-Bromoheptane	—	110
1-Bromooctane	—	135
1-Bromodecane	+	48
1-Bromododecane	+	20
1-Bromopentadecane	+	0
1-Bromohexadecane	+	15
1-Bromooctadecane	+	8
1-Iodopropane	+	92
1-Iodobutane	+	30
1-Iododecane	+	ND
1-Iodododecane	+	21
1-Iodoheptadecane	+	0
1-Iodooctadecane	+	0
1-Fluoropentane	—	1
1-Fluorohexane	—	0.6
1-Fluorotetradecane	+	0
2-Chlorobutane	—	0
2-Bromobutane	—	31
1,4-Dichlorobutane	+	218
1,6-Dichlorohexane	+	121
3-Chloropropanol	—	102
6-Chlorohexanol	+	79
1-Chloroacetic acid	—	0
3-Chloropropionic acid	—	12
5-Chlorovaleric acid	—	30
R-2-Chloropropionic acid	—	0
L-2-Chloropropionic acid	—	0

Dehalogenation of certain diterminally substituted substrates such as 1,4-dichlorobutane also occurred readily but substrates with mid-chain substituents were not in general attacked. ω -Halo-alcohols were rapidly dehalogenated and the corresponding acids were similarly degraded although chemical lactonization of some of these compounds was also very rapid under the assay conditions used. This made it difficult to determine with accuracy the rate of enzymic dehalogenation of 4-chlorobutyric acid in particular and this data is not included. However, both of the homologous acids 3-chloropropionic acid and 5-chlorovaleric acid underwent biological dehalogenation at significant rates rendering it not unreasonable to assume that 4-chlorobutyric acid can act as a substrate for enzymic dehalogenation. The overall range of substances dehalogenated by whole cells of *R. rhodochrous* bears a close resemblance in a number of respects to the substrate specificity of the haloalkane halidohydrolase isolated by Sallis *et al.* (1990) from *Rhodococcus erythropolis*, although it will not be possible to assess the precise degree of similarity until the substrate specificity of the purified *R. rhodochrous* enzyme is determined.

1-Chlorohexadecane degradation by *R. rhodochrous*

In view of the ready growth of *R. rhodochrous* on higher chlorinated alkanes despite the failure of 1-chlorobutane-grown resting cells of the organism to dehalogenate these substrates, growth of the organism on 1-chlorohexadecane was examined in some detail. The time course of growth on the substrate is shown in Fig. 2. Although measurement of optical density provided only an approximate indication of the extent of growth because cells of the organism tended to clump together with this substrate, growth had apparently attained a maximum after between 30 and 70 h incubation. Disappearance of 1-chlorohexadecane from the culture medium was complete after approximately 70 h but only about 40% of the organic chlorine originally present in the substrate had been released as chloride ion by this time, suggesting that chlorinated degradation products were accumulating in the culture medium. Chloride continued to be released into the medium over an extended period of time but even after 20 d incubation only 80% mineralization of organic chloride had been achieved. A GC/MS examination of ether extracts of the culture medium at pH 6.5 and 2.0 was conducted in order to identify possible metabolites. Extracts at both pH 6.5 and 2.0 revealed the presence of γ -butyrolactone. Concentrations of the compound were significantly higher than in 1-chlorobutane-grown cultures and are recorded for the various stages of growth in Fig. 2. γ -Butyrolactone levels increased during the exponential phase reaching 1 mM as maximum growth was achieved. It would appear that little further metabolism of the compound occurred as concentrations had declined only slightly when measurements were terminated at 120 h.

Analysis of the pH 2 extract after derivatization with diazomethane indicated that the culture medium contained two non-halogenated organic acids which were

identified from the mass spectra and retention times of their methyl esters as succinic and 4-hydroxybutyric acids. Both were observed in trace quantities at all stages of growth from 24 h onwards, each reaching maximum levels as growth peaked at 48 h when concentrations of succinic and 4-hydroxybutyric acid were respectively 3 μ M and 5 μ M. A chlorinated organic acid was also detected by GC/MS in the pH 2.0 extract. The electron impact mass spectrum of the methylated acid exhibited fragments at m/z 74 [$\text{CH}_2 = \text{C}(\text{OH})\text{OCH}_3$, 100%], m/z 105 ($M - \text{OCH}_3$, 37%), m/z 59 (COOCH_3 , 18%), m/z 41 (17%), m/z 107 (12%) and m/z 77 (11%). The ratio of ions at m/z 105 and m/z 107 suggested that the compound contained a chlorine atom. The mass spectrum and retention time of the methyl ester were found to be identical to those of methyl 4-chlorobutyrate. The compound was present at all stages of growth from 24 h onward attaining a maximum concentration of 6 μ M at 48 h. Amounts observed were several orders of magnitude too low to explain the incomplete mineralization of organic chlorine noted during growth on 1-chlorohexadecane. Experiments involving the incubation of 4-chlorobutyric acid in sterile culture medium indicated that the traces of 4-hydroxybutyric acid found did not arise chemically from 4-chlorobutyric acid or γ -butyrolactone.

Fatty acid composition of lipids of *R. rhodochrous*

A report by Murphy & Perry (1983) that *Mycobacterium* spp. could incorporate 1-chlorohexadecane into the cellular lipid fraction when growing on the compound as sole carbon and energy source suggested a possible explanation of the low chloride yield during growth of *R. rhodochrous* on this substrate. Fatty acid compositions of cells harvested after growth on 1-chlorohexadecane, hexadecane and 1-chlorobutane were therefore determined as described in Methods. Although no chlorofatty acids were found in 1-chlorobutane- or hexadecane-grown cells, chlorofatty acids comprised 75% of the total fatty acid content in 1-chlorohexadecane-grown cells with $\text{C}_{14:0}$, $\text{C}_{16:0}$, $\text{C}_{16:1}$ and $\text{C}_{18:1}$ acids being the major chlorinated compounds (Table 3). Nearly all fatty acids were present in bound form, free fatty acids constituting less than 1% of the total fatty acids present in 1-chlorohexadecane-grown cells. Interestingly the chain-length and degree of unsaturation of the chlorofatty acids were markedly different in a number of respects from those of the unsubstituted fatty acids present in cells grown on hexadecane. Thus although the $\text{C}_{16:0}$ acids were the major components in both hexadecane- and 1-chlorohexadecane-grown cells, $\text{C}_{14:0}$ and $\text{C}_{18:1}$ acids were present in much enhanced concentrations mainly at the expense of the $\text{C}_{16:1}$ acid in cells growing on the chlorinated substrate. Indeed, surprisingly the fatty acid composition of 1-chlorohexadecane-grown cells with respect to chain-length and degree of unsaturation corresponded more closely to that of 1-chlorobutane-grown cells than hexadecane-grown cells. The reason for this change in the character of the lipid components is not immediately apparent. Nevertheless, quite clearly 1-

Table 3. Fatty acid composition of cells of *R. rhodochrous* grown on 1-chlorobutane, 1-chlorohexadecane or hexadecane

Non-Cl, Non-chlorinated fatty acid; Cl, chlorinated fatty acid.

Fatty acid	Percentage of total fatty acids						
	1-Chlorobutane-grown cells		1-Chlorohexadecane-grown cells			Hexadecane-grown cells	
	Non-Cl	Cl	Non-Cl	Cl	Total	Non-Cl	Cl
C _{6:0}	—	—	—	0.8	0.8	—	—
C _{8:0}	—	—	—	4.6	4.6	1.2	—
C _{10:0}	—	—	—	3.8	3.8	3.0	—
C _{12:0}	4.1	—	—	—	—	1.0	—
C _{14:0}	14.9	—	1.1	16.4	17.5	7.0	—
C _{14:1}	—	—	—	—	—	0.7	—
C _{15:0}	1.9	—	2.1	1.6	3.7	0.7	—
C _{16:0}	34.6	—	15.1	27.4	42.5	45.3	—
C _{16:1}	8.5	—	3.0	10.2	13.2	38.1	—
C _{17:0}	—	—	—	—	—	0.9	—
C _{18:0}	2.6	—	—	—	—	0.1	—
C _{18:1}	15.6	—	3.7	9.9	13.6	1.5	—
C _{19:0}	9.3	—	0.3	—	0.3	0.5	—
C _{20:0}	8.5	—	—	—	—	—	—
Total	100	0	25.3	74.7	100	100	0

chlorohexadecane is degraded via the methyl end of the molecule and the chlorinated C_{16:0} acid formed is not only assimilated into the cellular lipids *per se* and β -oxidized to the C_{14:0} acid but is readily desaturated to the C_{16:1} acid and chain-lengthened to the C_{18:1} compound. Conversion of the C_{16:0} chloroacid to the C_{16:1} and C_{18:1} chloroacids occurred to a much greater extent in *R. rhodochrous* than was reported for *Mycobacterium* spp. by Murphy & Perry (1983). In the latter investigation the main chloroacids after growth on 1-chlorohexadecane were C_{16:0} and its β -oxidation product C_{14:0} which together comprised 88% of the total chlorofatty acids content. In *R. rhodochrous* by comparison C_{16:0} and C_{14:0} chloroacids amounted to only 59% of the total chlorofatty acids present.

DISCUSSION

The postulated routes by which 1-chlorobutane and 1-chlorohexadecane are degraded by *R. rhodochrous* are summarized in Fig. 3. Catabolism of 1-chlorobutane is initiated principally by attack at the C-1 atom by a hydrolytic dehalogenase resulting in the formation of n-butanol which is oxidized to n-butyric acid and metabolized by β -oxidation via acetate. The dehalogenase has recently been purified and characterized by Stafford (1993). However, the presence of trace quantities of γ -butyrolactone in the culture medium indicates that a small proportion of 1-chlorobutane is metabolized via attack at the non-chlorinated end of the molecule to form 4-chlorobutyric acid which cyclizes with elimination of chloride to γ -butyrolactone which appears to be resistant

to further degradation. Although γ -butyrolactone and 4-hydroxybutyric acid can both act as substrates for growth of *R. rhodochrous*, induction of enzymes for the catabolism of each occurs only after 9 to 10 d incubation in the presence of the compound (at 1.5 mM).

The wide haloalkane substrate range of *R. rhodochrous* is similar to that reported for *R. erythropolis* Y2 by Sallis *et al.* (1990) who also noted that this bacterium could grow on terminally halogenated C₁₄, C₁₆ and C₁₈ alkanes without showing any short-chain haloalkane dehalogenase activity. They suggested that this might be due to the presence in the organism of another dehalogenase specific for long-chain haloalkanes. This explanation was supported by evidence from Scholtz *et al.* (1988) that *Arthrobacter* HA1 possessed several different haloalkane halohydrolases. However, although HA1 could grow on 1-bromohexadecane, no dehalogenase activity against this compound was detected in cell-free extracts. As is the case for *R. erythropolis* Y2 (Sallis *et al.*, 1990), the dehalogenase of *R. rhodochrous* is inducible. However, we have found that C₅ to C₈ bromoalkanes cannot act as growth substrates even though they are attacked by the dehalogenase induced by growth on 1-chlorobutane. Unlike their chloro-analogues, these compounds do not appear to act as inducers. This indicates an intriguing specificity for the repressor system functioning in this bacterium and may provide a means to further study the regulation of dehalogenase expression through the selection of repressor mutants.

In contrast, 1-chlorohexadecane appears to be metabol-

Fig. 3. Possible routes of degradation of 1-chlorobutane and 1-chlorohexadecane in *R. rhodochrous* NCIMB 13064. * Metabolites detected in medium during growth on 1-chlorobutane; † metabolites detected in medium during growth on 1-chlorohexadecane.

ized by *R. rhodochrous* predominantly via initial attack on the non-halogenated end of the molecule with degradation proceeding by β -oxidation until 4-chlorobutyric acid is formed. Two potential fates are then conceivable for the compound: spontaneous cyclization to γ -butyrolactone or dehalogenase attack with the formation of 4-hydroxybutyric acid which is subsequently metabolized via succinic acid and the TCA cycle. The accumulation of 1 mM γ -butyrolactone in the culture medium during the growth phase when total chloride release into the medium is about 1.6 mM suggests that most of the 4-chlorobutyric acid formed is subject to chemical lactonization. The

resistance of γ -butyrolactone to further degradation can be ascribed to relatively slow induction of enzymes for its catabolism. However, the traces of 4-hydroxybutyric acid and succinic acid found in the culture medium suggest that further metabolism of γ -butyrolactone can occur. Both compounds have been implicated in the degradation of tetrahydrofuran via γ -butyrolactone by a *Rhodococcus* sp. studied by Bernhardt & Diekmann (1991). If the dehalogenase induced during growth on 1-chlorobutane is also induced during growth on 1-chlorohexadecane a proportion of the 4-chlorobutyric acid formed could be subject to enzymic dehalogenation as the enzyme is

capable of utilizing ω -chlorofatty acids as substrates (Table 2). Thus although it was not possible to determine the rate of enzymic dehalogenation of 4-chlorobutyric acid itself, both of the homologues 3-chloropropionic acid and 5-chlorovaleric acid were subject to enzyme attack. However, evidence obtained by Stafford (1993) using enzyme-specific antibody probes suggests that the dehalogenase is not induced to any significant extent during growth on 1-chlorohexadecane. Thus it seems unlikely that appreciable enzymic dehalogenation of 4-chlorobutyric acid occurs.

The incorporation of the product of oxygenase attack on 1-chlorohexadecane, 16-chlorohexadecanoic acid, directly into cellular lipids represents a third route of substrate assimilation and accounts at least in part for the incomplete mineralization of the chlorinated substrate. It is possible that chlorinated alkanes are also incorporated into the mycolic acids which can constitute up to 60 % of the dry weight of the cell envelope of nocardioform bacteria of this type (Fox *et al.*, 1990). The slow release of chloride over an extended period after maximum growth was achieved on 1-chlorohexadecane may reflect a slow metabolic turnover of this large cellular fraction during the stationary phase involving various catabolic enzymes including the dehalogenase.

Chain-length would seem to be the primary factor determining whether degradation of 1-haloalkanes is initiated mainly by attack on the halogenated or the non-halogenated end of the alkane molecule with dehalogenase attack predominating with chain-lengths of C_{10} and less whilst oxygenase attack dominates at chain-lengths of C_{12} and above. The co-existence of a dehalogenase enzyme and a propensity to assimilate long-chain haloalkanes directly into lipids in a single organism raises important questions as regards biological treatment of haloalkane-containing effluent. Consideration may need to be given to the harvesting and safe disposal of biomass after such treatment.

REFERENCES

- Bagley, D. M. & Gossett, J. M. (1990). Tetrachloroethane transformation to trichloroethene and *cis*-1,2-dichloroethene by sulphate-reducing enrichment cultures. *Appl Environ Microbiol* **56**, 2511–2516.
- Bernhardt, D. & Diekmann, H. (1991). Degradation of dioxane, tetrahydrofuran and other cyclic ethers by an environmental *Rhodococcus* strain. *Appl Microbiol Biotechnol* **36**, 120–123.
- Bouwer, E. J. & McCarty, P. L. (1983). Transformations of halogenated organic compounds under denitrification conditions. *Appl Environ Microbiol* **45**, 1295–1299.
- Dunlap, K. R. & Perry, J. J. (1967). Effect of substrate on the fatty acid composition of hydrocarbon utilizing microorganisms. *J Bacteriol* **94**, 1919–1923.
- Elfaki, A. E., Dirar, H. A., Collins, M. A. & Harper, D. B. (1991). Biochemical and microbiological investigations of sigda – a Sudanese fermented food derived from sesame oilseed cake. *J Sci Food Agric* **57**, 351–365.
- Fox, A., Gilbert, J. & Morgan, S. L. (1990). Analytical microbiology: a perspective. In *Analytical Microbiology Methods. Chromatography and Mass Spectrometry*, pp. 1–17. Edited by A. Fox, S. L. Morgan, L. Larsson & G. Odham. New York: Plenum Press.
- Janssen, D. B., Scheper, A., Dijkhuizen, L. & Witholt, B. (1985). Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. *Appl Environ Microbiol* **49**, 673–677.
- Janssen, D. B., Jager, D. & Witholt, B. (1987). Degradation of n-haloalkanes and α,ω -dichloroalkanes by wild-type and mutants of *Acinetobacter* sp. strain GJ70. *Appl Environ Microbiol* **53**, 561–566.
- Janssen, D. B., Gerritse, J., Brackman, J., Kalk, C., Jager, D. & Witholt, B. (1988a). Purification and characterization of a bacterial dehalogenase with activity toward halogenated alkanes, alcohols and ethers. *Eur J Biochem* **171**, 67–72.
- Janssen, D. B., Grobбен, G., Hoekstra, R., Oldenhuis, R. & Witholt, B. (1988b). Degradation of *trans*-1,2-dichloroethene by mixed and pure cultures of methanotrophic bacteria. *Appl Microbiol Biotechnol* **29**, 392–399.
- Keuning, S., Janssen, D. B. & Witholt, B. (1985). Purification and characterization of a hydrolytic haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. *J Bacteriol* **163**, 635–639.
- Kohler-Staub, D. & Leisinger, T. (1985). Dichloromethane dehalogenase from *Hyphomicrobium* sp. IM2. *J Bacteriol* **162**, 676–681.
- Marks, T. S., Allpress, J. D. & Maule, A. (1989). Dehalogenation of lindane by a variety of porphyrins and corrins. *Appl Environ Microbiol* **55**, 1258–1261.
- Murphy, G. L. & Perry, J. J. (1983). Incorporation of chlorinated alkanes into fatty acids of hydrocarbon-utilizing mycobacteria. *J Bacteriol* **156**, 1158–1164.
- Murphy, G. L. & Perry, J. J. (1984). Assimilation of chlorinated alkanes by hydrocarbon-utilizing fungi. *J Bacteriol* **160**, 1171–1174.
- Oldenhuis, R., Vink, R. L. J. M., Janssen, D. B. & Witholt, B. (1989). Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. *Appl Environ Microbiol* **55**, 2819–2826.
- Rasche, M. E., Hicks, R. E., Hyman, M. R. & Arp, D. J. (1990). Oxidation of monohalogenated ethanes and n-chlorinated alkanes by whole cells of *Nitrosomonas europaea*. *J Bacteriol* **172**, 5368–5373.
- Sallis, P. J., Armfield, S. J., Bull, A. T. & Hardman, D. J. (1990). Isolation and characterization of a haloalkane halohydrolase from *Rhodococcus erythropolis* Y2. *J Gen Microbiol* **136**, 115–120.
- Scholtz, R., Leisinger, T., Suter, F. & Cook, A. M. (1987a). Characterization of 1-chlorohexane halohydrolase, a dehalogenase of wide substrate range from an *Arthrobacter* sp. *J Bacteriol* **169**, 5016–5021.
- Scholtz, R., Schmuckle, A., Cook, A. M. & Leisinger, T. (1987b). Degradation of eighteen 1-monohaloalkanes by *Arthrobacter* sp. HA1. *J Gen Microbiol* **133**, 267–274.
- 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. *Appl Environ Microbiol* **54**, 3034–3038.
- Stafford, T. M. (1993). *The microbial degradation of chloroalkanes*. PhD thesis. The Queens University of Belfast, Belfast, UK.
- Stirling, D. J. & Dalton, H. (1979). The fortuitous oxidation and cometabolism of various carbon compounds by whole-cell suspensions of *Methylococcus capsulatus* (Bath). *FEMS Microbiol Lett* **5**, 315–318.
- Stucki, G., Galli, R., Ebersold, H. R. & Leisinger, T. (1981). Dehalogenation of dichloromethane by cell extracts of *Hyphomicrobium* DM2. *Arch Microbiol* **130**, 366–371.
- Vannelli, T., Logan, M., Arciero, D. M. & Hooper, A. B. (1990). Degradation of halogenated aliphatic compounds by the ammonia-

oxidizing bacterium *Nitrosomonas europaea*. *Appl Environ Microbiol* **56**, 1169–1171.

Van den Wijngaard, A. J., Van der Kamp, K. W. H. J. J., Van der Ploeg, J., Pries, F., Kazemier, B. & Janssen, D. B. (1992). Degradation of 1,2-dichloroethane by *Ancylobacter aquaticus* and other facultative methylotrophs. *Appl Environ Microbiol* **58**, 976–983.

Vogel, T. M. & McCarty, P. L. (1985). Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chlor-

ide and carbon dioxide under methanogenic conditions. *Appl Environ Microbiol* **49**, 1080–1083.

Yokota, T., Omori, T. & Kodama, T. (1987). Purification and properties of haloalkane dehalogenase from *Corynebacterium* sp. strain m15-3. *J Bacteriol* **169**, 4049–4054.

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