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Dehalogenation of haloalkanes by *Rhodococcus erythropolis* Y2

The presence of an oxygenase-type dehalogenase activity complements that of an halidohydrolase activity

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Abstract

Rhodococcus erythropolis Y2 produced two types of dehalogenase: a hydrolytic enzyme, that is an halidohydrolase, which was induced by C_3 to C_6 1-haloalkane substrates, and at least one oxygenase-type dehalogenase induced by C_7 to C_{16} 1-haloalkanes and *n*-alkanes. The oxygenase-type activity dehalogenated C_4 to C_{18} 1-chloroalkanes with an optimum activity towards 1-chlorotetradecane. The halidohydrolase catalysed the dehalogenation of a wide range of 1- and α,ω-disubstituted haloalkanes and α,ω-substituted haloalcohols. In resting cell suspensions of hexadecane-grown *R. erythropolis* Y2 the oxygenase-type dehalogenase had a specific activity of 12.9 mU (mg protein)$^{-1}$ towards 1-chlorotetradecane (3.67 mU mg^{-1} towards 1-chlorobutane) whereas the halidohydrolase in 1-chlorobutane-grown batch cultures had a specific activity of 44 mU (mg protein)$^{-1}$ towards 1-chlorobutane.

The significance of the two dehalogenase systems in a single bacterial strain is discussed in terms of their contribution to the overall catabolic potential of the organism.

Introduction

Microorganisms have evolved a number of different enzyme-catalysed mechanisms to dehalogenate haloorganic compounds and thereby utilize them as substrates for growth. These enzymes are collectively termed 'dehalogenases' (Goldman 1965). Four types of dehalogenases have been described in aerobic microorganisms: oxygenases catalysing the dehalogenation of haloaromatics, haloalkanes, and haloalkanoic acids; glutathione-dependent dehalogenases active towards haloalkanes; epoxidases which dehalogenate haloalcohols; and hydrolytic dehalogenases (halidohydrolases) which cleave halogen substituents from haloaromatic and haloaliphatic compounds (for reviews see Hardman 1991; Leisinger & Bader 1993; Slater 1994).

The choice of enrichment substrate used to isolate haloalkane-utilising strains is known to influence the type of dehalogenation system selected. The use of 1,9 dichlorononane (Omori & Alexander 1978) or 1-chlorohexadecane (Armfield SJ, unpublished data) led to the isolation of strains possessing oxygenase-type dehalogenases, whereas the use of dichloromethane (DCM), 1,2-dichloroethane (1,2DCE) or 1-chlorobutane has resulted in the isolation of halidohydrolases (Janssen et al. 1989; Scholtz et al. 1987; Yokota et al. 1987; Sallis et al. 1990). Tsang et al. (1983) also showed that a hydrolase was involved in the dehalogenation of 1-chlorobutane and an oxygenase dehalogenated C_8 and C_9 compounds. Both types of enzyme could dehalogenate 1-chlorohexane; however, in this instance the two dehalogenation systems did not coexist in individual bacterial isolates.

We have reported previously that *Rhodococcus erythropolis* Y2, isolated by enrichment culture on 1-chlorobutane, could utilize a wide range of halogenated compounds as growth substrates, an ability conferred by the presence of a haloalkane halidohydrolase (Sallis et al. 1990). In this paper we report further studies of the dehalogenation capacities of *R. erythropolis* Y2 and provide evidence that this organism contains two dehalogenase activities. In addition to the previously described halidohydrolase, an oxygenase-type dehalogenase activity is induced by haloalkanes of carbon chain length 7 and higher while there is an indication of the presence of a second oxygenase-type dehalogenase which is active towards 3-monochloropropionate (3MCPA).

Methods

Culture conditions

Rhodococcus erythropolis Y2 (Sallis et al. 1990) was grown in 1.0 l screw-top bottles containing 250 ml defined growth medium (Slater et al. 1979) at pH 7.0 modified to contain (l^{-1}); $Na_2HPO_42H_2O$, 2.67 g; KH_2PO_4, 1.36 g; yeast extract 20 mg; tryptone 50 mg; and sodium succinate 25 mg. Carbon substrates were added to a final concentration of 5.0 mM. Haloalkane substrates were added directly to the growth medium in an undiluted form. When dichloroacetate (DCA), monochloroacetate (MCA), 2-monochloropropionate (2MCPA), or 3MCPA were used as growth substrates they were added from filter sterilised stock solutions (1 M, pH 7.0). Cultures were incubated on a reciprocating shaker (120 rpm) at 30° C for 3 days. Growth was monitored as OD_{600} and by the release of chloride ions, the concentrations of which were determined electrocoulometrically (Marius Chlor-o-Counter, FT Scientific Instruments, Tewkesbury, UK) (Sallis et al. 1990).

Preparation of bacterial extracts

Bacteria were harvested and disrupted by passage through a French pressure cell as described previously (Sallis et al. 1990). When *R. erythropolis* Y2 was grown on 1-chlorohexadecane or hexadecane, cells were sonicated on ice, 4 × 1 min exposure to a sonic probe (MSE Soniprep 150; amplitude 22 μ), prior to passage through the French press. This procedure produced an emulsion of the substrate which promoted dispersal of the bacterial flocs which formed when growth was on these substrates.

Enzyme assay for dechlorination

Dehalogenase activities in cell extracts (halidohydrolase-type dehalogenase) or resting cells (oxygenase-type dehalogenase) were measured colorimetrically (Bergmann & Sanik 1957) as the rate of release of chloride ions from 1-chlorobutane or 1,2-dibromomethane (10 mM) (Sallis et al. 1990). The unit of dehalogenase activity (U) was defined as the amount of enzyme catalysing the release of 1 μmol Cl^- min^{-1} (mg soluble protein)$^{-1}$.

Determination of fluoride ion concentration

Fluoride ion concentration was determined using an ion specific electrode (Orion Solid State model 94-09, Orion Instruments, UK). The sample (50 ml) was placed in a 100 ml plastic beaker and 25 ml of CDTA buffer added (cyclohexane-1,2-diaminetetracetic acid, 4 g in 800 ml distilled water, dissolved by the addition of 40% wv^{-1} NaOH; 57 ml glacial acetic acid; 58 g sodium chloride). The pH was adjusted to 5.8 with 40% (wv^{-1}) NaOH and the solution made up to 1 L with distilled water). The solution was mixed and the electrodes allowed to stabilise for 5 min before the readings were taken. The fluoride electrode was calibrated using 500 and 5000 μgl^{-1} sodium fluoride in distilled water.

Polyacrylamide gel electrophoresis (PAGE)

Halidohydrolase-type dehalogenase activity was visualised after electrophoresis on nondenaturing polyacrylamide gels (PAGE) by an activity reaction in the gel (Hardman & Slater 1981). Following electrophoresis the gels were incubated in 1,4-dichlorobutane for 30 to 120 min, washed in distilled water to remove excess substrate and then immersed in $AgNO_3$ to visualize the dehalogenase proteins as bands of precipitated AgCl (Hardman & Slater 1981). Haloalkane substrates were also incorporated into gels as an emulsion by sonication prior to casting (Sallis et al. 1990). Halidohydrolase-type activity was visualised after electrophoresis (4 h, 30 mA) as regions of clearance in the emulsion gel during incubation at 37° C for 30 to 120 min in a humidified atmosphere.

Protein determination

Protein concentrations in cell extracts were measured by the method of Bradford (1976) using bovine serum albumin as the standard. Whole cell protein concentrations were determined, following solubilisation in alkali (Scholtz et al. 1987), using the Folin reagent (Lowry et al. 1951).

Dehalogenase induction

The dehalogenation systems in *R. erythropolis* Y2 were induced by a modification of the procedure described by Yokota et al. (1987). Bacteria were grown on sodium succinate (10 mM) for 40 h (final culture absorbance A_{600nm} 1.2). The cultures were harvested by centrifugation at 9000 g for 10 min, washed twice and resuspended in 50 mM phosphate buffer, pH 7.2, to a concentration of 60 g wet weight cell L^{-1}. Aliquots of this suspension (50 ml) were incubated in 250 ml baffled flasks covered with Subaseals with 0.2% halogenated substrate in an orbital shaker (20 h, 30° C, 200 rpm). Induction was followed by determination of Cl^- release with time, in the presence and absence of chloramphenicol (0.5 mg ml^{-1}).

Induced bacteria were harvested by centrifugation at 9000 g for 10 min, washed and resuspended in 100 mM-glycine/NaOH buffer, pH 9.1 containing 0.5 mM dithiothreitol (DTT), then disrupted as described previously (Sallis et al. 1990).

Dehalogenase activities in resting cells

Bacterial cultures were grown on 1-chlorobutane until the A_{600nm} was greater than 0.8, (or for 2 days when cultured on hexadecane); after harvesting by centrifugation, the bacteria were washed and resuspended in 100 mM Tris-SO_4 buffer, pH 8.0, to a final concentration of 50–100 mg wet weight cell ml^{-1}. Resuspended cells (5 ml) were incubated with halogenated substrate (10 mM) at 30° C, 200 rpm in 100 ml baffled flasks covered with Subaseals. Oxygenase-type dehalogenase activities were then determined by measuring Cl^- release with time and relative activities towards the different substrates defined by reference to the dehalogenation rate of 1-chlorobutane.

Effect of aeration on dehalogenase activities

Measurement of dehalogenase activities under anaerobic conditions was made by sparging 0_2-free nitrogen gas through cell-free extracts at room temperature for 1 h prior to the addition of the halogenated substrate.

Effect of cell permeabilisation on dehalogenase activities

Bacterial cells were permeabilised either by treatment with Triton X-100 as described by Miozzari et al. (1978) or with toluene (DeSmet et al. 1978). Viable cell counts were used to establish the extent of permeabilisation.

Effect of inhibitors on dehalogenase activities

Resting cell activity against 1-chlorobutane or 1-chlorotetradecane (10 mM) was assayed in the presence of 1 mM dinitrophenylhydrazine (DNP), phenazine methyl sulphate (PMS), or sodium azide (0.02%).

Effect of pH on dehalogenase activities

Resting cell dehalogenation activity of hexadecane- and 1-chlorobutane-grown cells was assayed at various pH values using 200 mM TES-glycine-maleate buffer. The pH did not vary by more than 0.1 unit during the assay.

Electron microscopy

Cells of *Rhodococcus erythropolis* Y2 were fixed according to the procedure of Kellenberger et al. (1958), washed twice in distilled water and stained in 2% (wv^{-1}) uranyl acetate for 2 h at room temperature. Samples were washed in distilled water, dehydrated sequentially in a graded ethanol/water series (30%, 60%, 90%, 100%) for 15 min each followed by three, one-hour incubations in absolute ethanol then embedded in Spurr resin (Spurr 1969). The resin was polymerised overnight (16 h) at 60° C. Thin sections were cut with a diamond knife (LKB Ultratone 111) and mounted on copper grids, post-stained in lead citrate (Reynolds 1963) and examined in a Philips EM410 transmission electron microscope. As a positive control, PHB production was quantified in *Bacillus megaterium* NCIMB 12470, an organism known to produce PHB under conditions of excess exogenous carbon and energy (Macrae & Wilkinson 1958).

Determination of intracellular hexadecane concentration

Hexadecane was extracted from 40 h cultures by the method of Scott & Finnerty (1976). The hexadecane content was quantified by gas-liquid chromatography with a Perkin Elmer LC10-100 gas chromatograph, using a DB wax column (J&W Scientific). Operating conditions were: column temperature 100° C, injector temperature 120° C, detector temperature 240° C, helium flow rate 20 ml min^{-1}. Hexadecane concentrations were determined by reference to a standard curve of hexadecane concentration against integrated peak area.

Determination of intracellular poly-β-hydroxybutryric acid (PHB) concentration

1-Chlorobutane and hexadecane-grown cultures of *R. erythropolis* Y2 were extracted for PHB according to the procedure of Williamson & Wilkinson (1958) and PHB assayed by the method of Law and Slepecky (1961). Concentrated H_2SO_4 (10 ml) was added to the bacterial culture which was then heated for 10 min at 100° C. After cooling and thorough mixing the absorbance of the cell digest at A_{235nm} was measured against a sulphuric acid blank. The amount of crotonic acid was calculated from the molar extinction coefficient of 1.55×10^4. *Bacillus megaterium* strain NCIMB 12470, grown in the medium of Macrae and Wilkinson (1958), was used as a reference source of PHB.

Results

Growth on haloalkane and alkane substrates and evidence for more than one dehalogenase activity

In addition to 1-chlorobutane, *Rhodococcus erythropolis* Y2 could utilise the following α-substituted chloroalkanes for growth: 1-chloropropane, 1-chlorobutane, 1-chloropentane, 1-chlorohexane, 1-chlorododecane, 1-chlorotetradecane, 1-chlorohexadecane and 1-chlorooctadecane. The isolate could grow on *n*-alkanes of carbon chain lengths 7 to 18 but could not utilize *n*-pentane or *n*-hexane. The biomass concentration produced from growth on the *n*-alkanes was 3- to 6-fold greater than that on the 1-chlorosubstituted alkanes for chain lengths 7 to 18.

Table 1. Dehalogenase activities of *Rhodococcus erythropolis* Y2.

Treatment	Oxygenase activity[1]	Halidohydrolase activity[2]
Permeabilisation		
Triton-X 100	0	100
Toluene	0	40
Inhibitors		
Phenazine methyl sulphate	7	81
2,4-Dinitrophenol	55	92
Sodium azide	72	95

Activities are expressed as percentages of non-permeabilised cell controls.
[1] Growth substrate: hexadecane; assay substrate: 1-chlorotetradecane.
[2] Growth substrate: 1-chlorobutane; assay substrate: 1-chlorobutane.

The haloalkane halidohydrolase of *R. erythropolis* Y2 is known to have little or no activity toward terminally halogenated C_{14}, C_{16} and C_{18} alkanes (Sallis et al. 1990). However, this strain could utilise these compounds as growth substrates, a result which suggested the presence of a second dehalogenase. Resting cell suspensions of *R. erythropolis* Y2 induced by growth of the culture in the presence of 1-chlorobutane, catalysed dehalogenation under both aerobic and anaerobic conditions and were previously shown to contain a single halidohydrolase (Sallis et al. 1990). In contrast, resting cell suspensions induced by hexadecane, 1-chlorotetradecane, 1-chlorohexadecane or 1-chlorooctadecane possessed a dehalogenase activity that was strictly aerobic. Permeabilisation of hexadecane-grown cells resulted in complete loss of the aerobic dehalogenase activity (Table 1). The dehalogenase activity of hexadecane-grown cells was also sensitive to all inhibitors tested, whilst these compounds had little effect on the halidohydrolase activity of cultures grown on 1-chlorobutane. Further evidence for the presence of more than one dehalogenase was provided by the inhibitor studies (Table 1).

Dehalogenase induction studies

Both types of dehalogenase activity were inducible and induction did not occur in the presence of chloramphenicol (0.5 g l^{-1}). The halidohydrolase-type activity was induced by C_3 to C_6 1-chloroalkanes, 1,4-dichlorobutane and 4-chlorobutan-1-ol. Attempts to

Table 2. Halidohydrolase induction in *Rhodococcus erythropolis* Y2

Inducer	Specific activity (mU mg^{-1} protein)	Relative induction[1]
1-Chloropropane	0.92	12
1-Chlorobutane	8.24	100
1-Chloropentane	6.55	79
1-Chlorohexane	6.59	80
1-Chloroheptane	0.72	9
1-Chlorooctane	0.34	4
1-Chlorononane	0.34	4
1-Chlorodecane	0.54	7
1-Chlorododecane	0.21	3
1,4-Dichlorobutane	7.68	93
4-Chlorobutan-1-ol	3.30	40
1,2-Dibromobutane	0.32	4
Succinate	0.32	4

[1] Compared to induction by 1-chlorobutane.

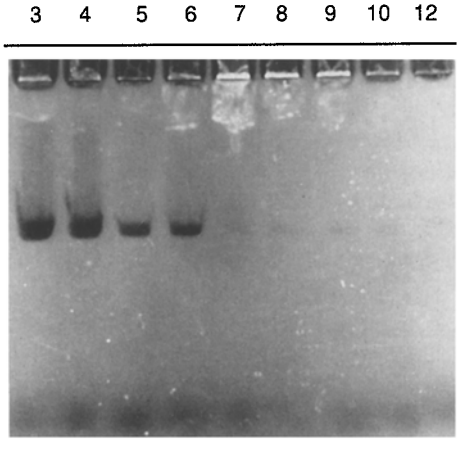

Fig. 1. Native PAGE of *Rhodococcus erythropolis* Y2 cell free extracts. Cultures were induced on 1-chloro-alkanes (C_3 to C_{12} indicated as lanes 3 to 12). The enzyme-detection substrate was 1,4-dichlorobutane.

induce cultures with 1-chloroalkanes of chain length 7 to 12 resulted in a low level constitutive halidohydrolase activity which was comparable to the activity of succinate-grown cultures (Table 2).

Following induction by C_3 to C_6 substrates cell free extracts of *R. erythropolis* expressed a single halidohydrolase-type dehalogenase activity as defined on polyacrylamide emulsion gels (Fig. 1). The dehalogenase band had an R_f of 0.51 which was the same as the enzyme induced by growth of *R. erythropolis* Y2 on 1-chlorobutane. The constitutive activity towards 1-chlorobutane, observed as faint banding on the gel (lanes C_7–C_{12}), represented 4% of the maximum activity obtained after induction with 1-chlorobutane (ie. 0.3 mU mg^{-1}protein; cf. succinate-grown cultures, Table 2). 1,4-Dichlorobutane did not serve as a growth substrate but it was a good inducer of, and substrate for, the halidohydrolase activity (Table 2). Cell free extracts of 1-chlorobutane-grown cells exhibited a halidohydrolase-type dehalogenase activity towards 1,2-dibromoethane which was 6- to 8-fold higher than that for 1-chlorobutane (Sallis et al. 1990), but 1,2-dibromoethane neither induced the halidohydrolase activity nor served as a growth substrate for the bacterium.

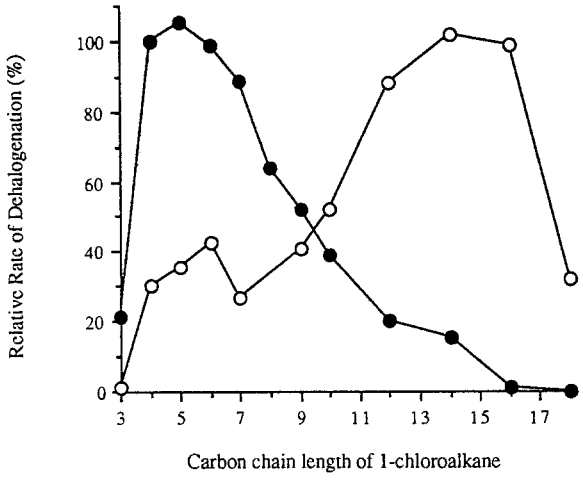

Fig. 2. Relative resting cell dehalogenation rates of *Rhodococcus erythropolis* Y2. Growth was made on hexadecane (o) and 1-chlorobutane (•) and dehalogenation was measured against 1-chloroalkane substrates of carbon chain lengths C_3 to C_{18}.

The oxygenase-type dehalogenase activity of *R. erythropolis* Y2 was induced by growth in the presence of C_7 to C_{18} 1-chloroalkanes and by the corresponding *n*-alkanes. Experiments to determine whether the substrates 1-chloropropane, 1-chlorobutane, 1-chloropentane and 1-chlorohexane were inducers of the oxygenase-type dehalogenase were not performed.

Hexadecane-grown cells were used for resting cell studies of the oxygenase-type dehalogenase because a greater biomass could be obtained than on 1-chlorohexadecane and also any chloride release could be attributed to activity on the assay substrate. Attempts to remove 1-chlorohexadecane from cells

grown on the substrate by shaking in fresh buffer were unsuccessful. The dehalogenation activity of hexadecane-grown cells was variable and unstable and as such contrasted with the halidohydrolase activity of resting cell suspensions of 1-chlorobutane-induced cultures which was very stable. When stored at 4° C, 50% of the halidohydrolase-type activity remained after 200 d and 80% activity retained after 300 d at - 20° C. At 30° C the activity of the resting cell suspensions fell to 50% of the initial value after 9 d.

Substrate range of resting cell suspensions

Resting cell suspensions of *R. erythropolis* Y2, grown on 1-chlorobutane or hexadecane, demonstrated a wide substrate range (Table 3). The halidohydrolase-type activity demonstrated a preference for short chain compounds whereas the oxygenase-type dehalogenase was more active towards long chain substrates (Fig. 2). Both resting cell systems had greater activity towards terminally substituted substrates than towards those with mid-chain substituents. Carbon-iodine bonds were more readily cleaved by the oxygenase-type dehalogenase which also showed weak activity towards carbon-fluoride bonds, the latter were resistant to cleavage by the halidohydrolase.

3MCPA was effectively dehalogenated by resting cells and was a good substrate for oxygenase-type activity present in the cells, but it was a poor substrate for the halidohydrolase.

Dehalogenase pH optimum of resting cells

Resting cell suspensions of cultures grown on 1-chlorobutane appeared to have a similar pH optimum (pH 9.2) to the purified halidohydrolase (Sallis et al. 1990). Cells grown on hexadecane had a broad optimum over the pH range 7–10.

Electron microscopy

The growth physiology of *R. erythropolis* Y2 on *n*-alkanes and 1-chloroalkanes of carbon chain lengths C_{12} to C_{18} was such that the biomass was present in pellet form. The addition of surfactants, co-substrates and propan-2-ol (1% vv^{-1}) as an organic phase to the culture medium did not change this morphology. Electron microscopy of bacteria harvested after 40 h batch culture on hexadecane revealed the presence of large electron-transparent structures. Succinate or 1-chlorobutane-grown cells did not contain these struc-

Table 3. Relative activity of resuspended whole cells of *Rhodococcus erythropolis* Y2 induced by 1-chlorobutane or hexadecane towards halogenated aliphatic substrates.

Substrate	Relative activity of resting cells	
	1-Chlorobutane induced	Hexadecane induced
1-Bromoethane	92	124
1-Chloropropane	21	16
1-Chlorobutane	100	100
1-Bromobutane	108	152
1-Iodobutane	73	186
1-Chloropentane	105	121
1-Iodopentane	65	210
1-Chlorohexane	99	145
1-Bromohexane	78	179
1-Iodohexane	35	107
1-Fluorohexane	0	6
1-Chloroheptane	87	90
1-Chlorooctane	62	141
1-Chlorononane	51	138
1-Fluorononane	0	2
1-Chlorodecane	38	179
1-Chlorododecane	20	303
1-Chlorotetradecane	16	352
1-Bromotetradecane	27	324
1-Chlorohexadecane	0	345
1-Chlorooctadecane	0	107
Dichloromethane	6	0
Dibromomethane	20	24
1,2-Dichloroethane	13	0
1,2-Dibromomethane	87	114
1,2-Dichloropropane	0	0
1,2-Dibromopropane	57	17
1,3-Dichloropropane	152	55
1,4-Dichlorobutane	155	141
1,3-Dibromobutane	44	52
1,6-Dichlorohexane	113	145
1,9-Dichlorononane	66	179
1,10-Dichlorodecane	60	248
2-Chlorobutane	4	0
2-Bromobutane	25	90
2-Iodobutane	66	128
2-Chloropentane	4	24
2-Chlorooctane	9	107
Chloroacetic acid	3	0
Dichloroacetic acid	4	0
2-Chloropropionic acid	3	0
3-Chloropropionic acid	16	293
2-Chloroethanol	10	24
3-Chloropropan-1-ol	21	121
4-Chlorobutan-1-ol	189	472
4-Chlorobenzyl chloride	25	ND
3-Chlorobenzyl chloride	23	ND
Chlorobenzene	0	ND
4-Chlorophenol	0	ND

ND = not determined.

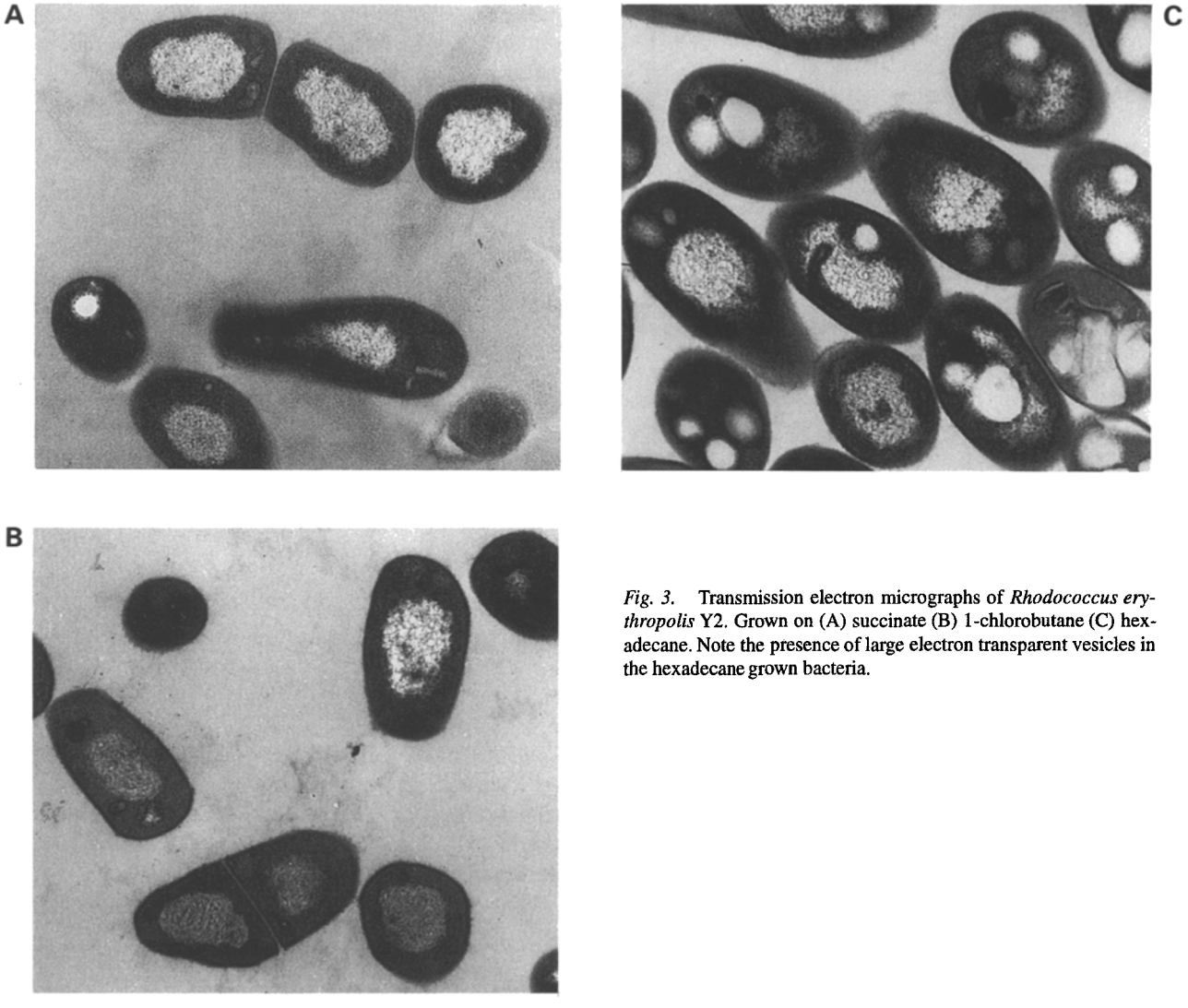

Fig. 3. Transmission electron micrographs of *Rhodococcus erythropolis* Y2. Grown on (A) succinate (B) 1-chlorobutane (C) hexadecane. Note the presence of large electron transparent vesicles in the hexadecane grown bacteria.

tures (Figs 3a, b, c). Bacteria that produce PHB have been reported to contain electron transparent inclusions (Dunlop & Robards 1973; Scott & Finnerty 1976). Consequently *R. erythropolis* Y2 was tested for an ability to produce PHB when grown on hexadecane. Analysis of the electron-transparent bodies showed that, when compared with the control strain, *R. erythropolis* Y2 contained little PHB when grown on 1-chlorobutane and hexadecane, but there were significant quantities of hexadecane inclusions when grown on that substrate (Table 4). In this work the PHB values were not corrected for unidentified interfering substances (Law & Slepecky 1961). However, because the succinate and 1-chlorobutane grown cells were carbon-limited and would not be expected to have contained PHB, then when such a correction is used, the hexadecane-grown cells only contained 15–45 nmol PHB mg^{-1} dry weight of cells as compared with 1587 nmol in the cells of *B. megaterium.*

Discussion

The range of haloalkanes (C_3 to C_{18}) utilised by *Rhodococcus erythropolis* Y2 as substrates for growth is similar to that reported for *Arthrobacter* sp. strain

Table 4. Analysis of the cell contents of *Rhodococcus erythropolis* Y2 grown in batch cultures on 1-chlorobutane, hexadecane and succinate.

Organism	Substrate	Hexadecane	PHB
		(nmol mg^{-1} dry weight)	
R. erythropolis Y2	hexadecane	147	240
R. erythropolis Y2	1-chlorobutane	0	221
R. erythropolis Y2	succinate	0	199
B. megaterium	glucose	0	1587

HA1 which can utilise halosubstituted alkanes in the range C_2 to C_{16} (Scholtz et al. 1988). Janssen et al. (1987) described a bacterial isolate, GJ70, that was capable of growth on terminally substituted C_2 to C_9 alkanes and Yokota et al. (1986) reported the growth of *Corynebacterium* sp. m15-3 on terminally substituted C_4 and C_6 alkanes. In contrast *Xanthobacter autotrophicus* GJ10 (Janssen et al. 1985) demonstrated a considerably more restricted substrate profile being capable of growth only on mono- and di-substituted C_2 and C_3 compounds.

Organism GJ70, *Corynebacterium* sp. m15-3 and *X. autotrophicus* GJ10 all metabolised haloalkanes through an initial dehalogenation by a single halidohydrolase-type dehalogenase, and all were unable to grow on the unsubstituted *n*-alkane analogues. *Arthrobacter* sp. HA1 produced three haloalkane halidohydrolases but no oxygenase-type dehalogenase. *R. erythropolis* Y2 is the first isolate reported to contain both halidohydrolase and oxygenase-type dehalogenases.

Microorganisms belonging to a variety of genera which produce oxygenases are capable of utilising haloalkanes as substrates for growth. The broad specificity of methane monooxygenases (MMO) and their ability to catalyse dehalogenation has been known for several years (Higgins et al. 1979). The MMO of *Methylococcus capsulatus* (Bath) catalyses the oxidation of chloromethane, bromomethane, dichloromethane and chloroform (Colby et al. 1977) and a similar activity has been ascribed to the MMO of *Methylosinus trichosporium* (Higgins et al. 1979). These halogenated alkanes do not serve as growth substrates but are co-metabolized by the non-specific MMO in the presence of methane or methanol. Although methane monooxygenases demonstrate wide substrate specificities, the enzymes from different methanogens catalyse different reactions with haloalkanes as substrates (Dalton 1980). Imai et al. (1986) isolated a thermophilic methanotroph strain H-2 that oxidised mono- and dichloroalkanes of carbon lengths C_5 and C_6 to the corresponding acids and haloacids. In this instance the reaction appeared to involve terminal hydroxylation. *Methylococcus capsulatus* apparently converted bromomethane to formaldehyde via the putative intermediate bromoethanol (Stirling & Dalton 1980).

Previous studies with non-growing cell suspensions have shown that alkanes can induce monooxygenases that also have broad substrate specificities and which are capable of catalysing dehalogenation. The *n*-undecane-utilising strain *Pseudomonas* sp. B studied by Omori & Alexander (1978) contained an inducible oxygenase-type dehalogenase that could dehalogenate C_5 to C_9 mono- and di-substituted haloalkanes. However, degradation of these compounds again was fortuitous and they did not serve as carbon sources for growth. Omori & Alexander (1978) suggested that dehalogenation was co-metabolic and linked to the activity of an enzyme catalysing a step in the metabolism of structurally similar alkanes. Yokota et al. (1986) reported the ability of resting cell suspensions of the hydrocarbon-utilising bacterium *Pseudomonas butanovora* to dehalogenate the halogenated analogues of alkane growth substrates under aerobic conditions. From $^{18}O_2$ studies these authors concluded that an oxygenase was involved in the dehalogenation of haloalkanes.

Bacterial alkane monooxygenases are poorly characterised. The activity of a number of these enzymes is dependent on an electron transport chain consisting of two or three proteins, for example, the rubredoxin ω-hydroxylases from octane-grown *Pseudomonas oleovorans* (McKenna & Coon 1970), however, the specificity of this alkane monooxygenase towards halogenated alkanes was not studied. The cytochrome P_{450} activity induced in *Pseudomonas putida* PpG-786 by camphor, mediates the dehalogenation of bromotrichloromethane and it was suggested that the mechanism involved reductive dehalogenation (Lam & Vilker 1986). The oxygenase-type dehalogenase of *R. erythropolis* Y2 has a strict requirement for oxygen and, since this activity was inhibited by detergents and solvents and was significantly reduced by the presence of electron transport inhibitors, it appears to be a multicomponent membrane-bound system which requires electron transport for activity.

The oxygenase-type dehalogenase of *R. erythropolis* Y2 can cleave, albeit weakly, carbon-halogen bonds of substrates containing the most electronegative halide, fluoride; this activity is not possessed by the halidohydrolase. Thus, resting cell suspensions of hexadecane-grown *R. erythropolis* Y2 demonstrated weak activity towards fluorinated alkanes but the purified halidohydrolase did not possess this ability. Consistent with these findings is the fact that *Arthrobacter* strain HA1 (Scholtz et al. 1987), which possesses three halidohydrolases, could not utilise or dehalogenate fluoropentane.

Extensive intracytoplasmic membrane structures have been reported in *Methylosinus trichosporium* 0b3b and *Methylococcus capsulatus* (Bath) when grown under conditions of copper excess or oxygen limitation and producing particulate methane monooxygenases (Stanley et al. 1983; Cornish et al. 1985); no such membranes were found in these isolates when they were producing the soluble MMO. Growth of *R. erythropolis* Y2 on hexadecane induced a particulate oxygenase and it was thought that internal structural differences between these and 1-chlorobutane-induced cells might be detectable by electron microscopy. However, *R. erythropolis* Y2 contained a number of intracytosplasmic membranes even when grown on succinate and the only obvious difference noted was the accumulation of hexadecane inside the cells when grown on the hexadecane. The lack of electron-transparent vesicles when the organism was growing on 1-chlorobutane suggests that the substrate is transported into the bacterial cell and metabolised thereafter.

The relaxed specificity of the *n*-alkane oxygenase in *R. erythropolis* Y2 enables it to catalyse the dehalogenation of the long chain haloalkanes, so increasing the growth substrate range of the organism. By catalysing the initial dehalogenation the enzyme provides metabolites which can feed directly into the β-oxidation cycle. Although the halidohydrolase demonstrates very weak activity against the longer chain substrates (C_7–C_{11}), it does not catalyse the dehalogenation of these compounds as effectively as the oxygenase. The bacterium appears to have evolved regulatory mechanisms which prevent the expression of the halidohydrolase when the organism is presented with the haloalkane substrates of carbon chain lengths of greater than six. Such regulation results in the optimal use of two types of dehalogenase for growth on a wide range of haloalkanes. However, we have yet to determine whether shorter chain substrates (1-chloropropane, 1-chlorobutane, 1-chloropentane and 1-chlorohexane) can act as inducers of the oxygenase-type dehalogenase. These substrates induced the halidohydrolase-type activity, and so experiments necessarily would have required the incubation of resting cells in the presence and absence of oxygen to discriminate between the halidohydrolase and the oxygenase-type dehalogenase.

R. erythropolis Y2 may also express a third dehalogenase system which catalyses the dehalogenation of 3MCPA. The very different chemical nature of 3MCPA compared to the long chain haloalkanes may indicate that the oxygen-dependent dehalogenation of 3MCPA was mediated by a second oxygenase-type dehalogenase. This hypothesis remains to be tested.

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