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DEHALOGENASES IN SOIL BACTERIA

by David J. Hardman BSc.

A thesis submitted in fulfillment of the requirements
for the degree of Doctor of Philosophy.

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Finally, I gratefully acknowledge the financial support of the Natural Environment Research Council.

Declaration

I declare that this thesis is a report of the research undertaken by myself during the years 1978-1981 in the Department of Environmental Sciences under the supervision of Dr J.H. Slater. It is my own original, unaided work and has, to the best of my knowledge, not been previously described by any other person.

Summary

A number of bacterial strains were isolated from soil, by direct plating and enrichment culture, which were able to grow on chlorinated alkanolic acids as the sole carbon and energy source. Sixteen of these isolates were divided into five groups on the basis of differences in their dehalogenase activities towards monochloroacetic acid (MCA), dichloroacetic acid (DCA), 2-monochloropropionic acid (2MCPA) and 2,2-dichloropropionic acid (22DCPA).

Disc gel electrophoresis of crude extracts identified four distinct dehalogenases exhibiting different electrophoretic mobilities. The groups were characterized by the possession of one or more of these enzymes although one of the groups was split into two on the basis of the possession of two different dehalogenases. In some cases, dehalogenases with the same electrophoretic mobilities, from different isolates, appeared to be identical enzymes, while in others, enzymes from different isolates, with the same electrophoretic mobilities, had different substrate activity profiles.

Representatives of each group were grown in continuous-flow culture with either MCA or 2MCPA as the growth-limiting substrate. In all but one instance the dehalogenase profiles were different from those of the same organism grown in closed culture and were seen to be dependent on both growth rate and the growth-limiting substrate.

The same strains were seen to possess different plasmids with molecular weights ranging from 109 to 190 Md. Five plasmids were characterized on the basis of their restriction endonuclease digest patterns. Curing experiments indicated that these plasmids encoded for the dehalogenase activities and resistance towards tellurium or mercury, selenium and tellurium.

The significance of these results in terms of the evolution of dehalogenase activity is discussed and a model presented which relates the required period of enrichment and the common dehalogenases expressed by each bacterial strain to the presence of the five plasmids.

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INTRODUCTION

1.1 MICROBIAL DEGRADATION OF HALOGENATED ORGANIC COMPOUNDS

1.1.1 Naturally-occurring halogenated compounds

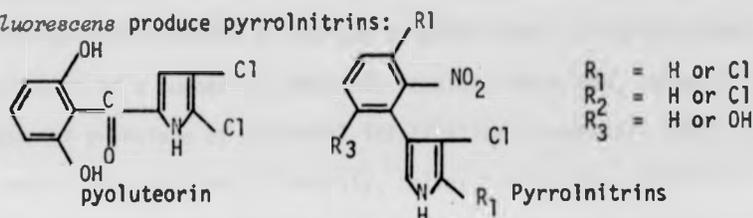
The existence of a number of microorganisms capable of degrading halogenated compounds has been amply demonstrated, largely as a result of the use of chlorine-substituted pesticides and the research into their persistence in natural environments. The biodegradation of these compounds suggests that naturally-occurring, halogen-substituted molecules existed before man polluted the environment with economically important halogenated compounds. A number of surveys have been made of these natural organic molecules (Bracken, 1954; Petty, 1961; Fowden, 1968; Suida & DeBernardis, 1973).

Among the first chlorine-containing organic compounds to be discovered were those produced by fungi and lichens. Clutterbuck *et al.* (1940) studied 27 species of *Aspergillus* and 96 species of *Penicillium* as well as 13 other fungal genera, although only a minority were capable of converting inorganic chlorine-containing compounds into organic compounds. A number of similar compounds are described by Bracken (1954) and by the year 1973 Suida and DeBernardis (1973) were able to list more than 200 halometabolites, of which chlorinated compounds exceeded 150 and brominated exceeded 50.

Halometabolites have been identified in a number of higher organisms. Higher plants have been widely studied for the metabolism of chlorine-containing compounds (Petty, 1961) presumably because of the development and use of chlorinated herbicides (Section 1.1.3). Fluorocitrate and iodinated substances, such as thryoxins, are important in human physiology.

The chlorinated metabolites of fungi show antimicrobial action and several have been used clinically, for example, chlortetracycline, chloramphenicol and griseofulvin as antibiotics. The compound pyoluteorin is a chlorine-containing antibacterial agent produced by *Pseudomonas aeruginosa* (Birch *et al.*, 1964) and *P. pyrocinia* and

P. fluorescens produce pyrrolnitriins:



The marine environment is particularly rich in halogenated compounds with a number of algae producing bromometabolites. Hodgkin *et al.* (1966) isolated, at a high yield, a compound from *Polysiphonia lanosa* which contained two bromine atoms. Craigie and Gruenig (1967) described a number of brominated aromatic compounds isolated from red algae, Suzuki and Kurosawa (1978) isolated two halogenated sesquiterpenes and Beechan and Sims (1979) isolated a novel class of halogenated lactones. Naturally-occurring halocarbons in the air and sea have been described by Lovelock (1973, 1975). Halogen-containing cyclic organic molecules are more common than substituted alkanolic molecules (Petty, 1961). Fowden (1968) noted that only one natural haloalkanoic acid had been identified at that time: this was δ -chlorolaevulinic acid ($\text{Cl-CH}_2\text{-CO-CH}_2\text{-CH}_2\text{-COOH}$) which was believed to be an intermediate in the synthesis of caldariomycin by *Caldariomyces fumago*. However, alkanolic compounds are more difficult to detect than the cyclic structures, as most of the latter compounds have been identified after observing their toxic or chromatic properties. Thus halometabolites lacking the structural peculiarities conferring these properties have

tended to remain unnoticed. A number of naturally-occurring chlorinated alkanolic acids were described by Murray and Riley (1973).

1.1.2 Halogenated xenobiotic compounds

Numerous man-made halogenated compounds eventually contaminate the environment, whether indirectly through the dumping of waste material or more directly by the use of pesticides. Originally the toxic nature of a number of compounds remained unnoticed, largely because the principle of microbial infallibility (Alexander, 1965) gave man a false feeling of security, believing that the extensive catabolic potential of microbial communities and the ubiquity of microorganisms would ensure that any compound released into the biosphere would eventually be degraded. Although metabolic recalcitrance of novel synthetic compounds in the environment became apparent, with increasing levels of undegraded pesticides being found in food chains, it was observed, as in the case of dichlorodiphenyltrichloroethane (DDT), that most of these compounds were eventually degraded (Faber, 1979), if they were susceptible to attack by the catabolic mechanisms already present in the microbial population (Hill, 1978).

With the increasing awareness of the dangers associated with recalcitrance, toxicity and accumulation, investigations into the effects of these compounds on the environment were undertaken.

1.1.3 Halogenated pesticides in the soil environment

A number of factors are responsible for determining the fate of a chemical in the soil (Edwards, 1964; Kunze, 1966): the soil type; type of chemical (Kaufman, 1966; Hammond & Alexander, 1972; Dorn & Knackmuss, 1978; Hance, 1979); method of application; and

the biological population. Kaufman (1964) found that Dalapon was degraded mainly by bacteria in silty and sandy loams, but in loam and silty clay loams numerous fungi were involved.

The initial studies into the persistence of pesticides consisted mainly of experimental systems, such as soil microcosms and perfusion columns, to which samples of the pesticide were added and the rate of disappearance followed by biochemical or biological assay procedures (Holstun & Loomis, 1956; Upchurch & Mason, 1962). The actual microbial populations involved were not studied and often their involvement was assumed after control experimental systems, containing sterile soil, failed to show any breakdown of the compound in question.

One of the major problems of working with these experimental systems is separating biological from physical effects. The physical parameters are beyond the scope of this introduction although the factors involved include adsorption and leaching which are important in determining retention time of a pesticide in the environment (Rao & Davidson, 1979), and in the degradation process itself (Holstun & Loomis, 1956; Kunze, 1966). Bahadir *et al.* (1978) showed that up to 70% oxidative mineralization of the chloro-fluoro-compound CCl_2F_2 occurred upon adsorption to Mecca sand. Photochemical reactions are also thought to play a major role in breakdown through oxidation, although it is difficult to demonstrate this in the environment (Plimmer, 1970, 1972; Crosby, 1972, 1976; Brown, *et al.*, 1978a).

One of the major problems with all experimental systems, such as soil columns, for determining the biological activity is that in taking the soil sample, the environmental conditions are immediately changed, especially if air dried soils are used (Holstun & Loomis, 1956).

Extrapolation to the natural environment of any results should be approached with great care (Wingfield *et al.* 1977).

The effect of pesticides on soil microorganisms has been studied both *in situ* and in the laboratory. They have been seen to select bacterial populations capable of degrading them. When first applied a lag period ensues, before degradation takes place, but with subsequent additions, degradation commences almost immediately. The compounds can be used as carbon, nitrogen or other mineral sources (Alexander, 1977) and can give rise to increased bacterial and fungal populations (Percich & Lockwood, 1978). MacGregor (1963) followed the disappearance of Dalapon from soils and concluded that enrichment caused some members of the microbial population to proliferate. Fletcher (1960) studied the effect of Dalapon (25 ppm) on nitrifying bacteria in soils and found that after an initial inhibition of nitrification of about 1 month the normal rates of nitrification returned. The same conclusions were reached by Hale *et al.* (1957) who examined the effects of herbicides on nitrification under laboratory conditions, and Wainwright (1978) concluded that field rates of application of a number of pesticides had little effect on any of the major biogeochemical cycles in the soil.

Martin (1966) stressed the importance of the differences between the soil environment and pure culture conditions, the pathways of decomposition may be very different. A comparison of the biodegradation of Dalapon by mixed and pure cultures of *Arthrobacter* sp. was undertaken by Beall *et al.* (1964) and Senior *et al.* (1976a & b) used a mixed population of soil microorganisms in chemostat culture with Dalapon as the sole carbon and energy source and selected a seven-membered

community consisting of four primary and three secondary utilizers.

Apart from acting as a nutrient source, pesticides may also be degraded as a result of cometabolism (Horvath & Alexander, 1970), during which the compound is metabolized while the organism utilizes another source of carbon and energy for growth. The inability to grow on the compound that it can metabolize may result from the fact that although the initial enzymes in the pathway can act on a substituted substrate, subsequent enzymes are only active towards unsubstituted forms. The cometabolism of monofluorobenzoates by a mixed soil population was described by Horvath and Flathman (1976) and Rosenberg and Alexander (1980) studied the cometabolism *in vitro* of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) by bacteria. *Pseudomonas aurantiaca* strains can cometabolize 3,4-dichloroalanine while utilizing glucose and propanide for growth (Surovts_eva & Funtikova, 1978).

Halogenated alkanes from pesticides and refrigerants, or produced 'naturally' when water is chlorinated in municipal water treatment plants, are dehalogenated by sewage samples. However, Omori and Alexander (1978a) were unable to isolate an organism able to utilize 1,9-dichlorononane as its sole carbon source. It was suggested that these compounds were also acted upon by cometabolism.

1.1.4 Dehalogenation of halogenated aromatic compounds

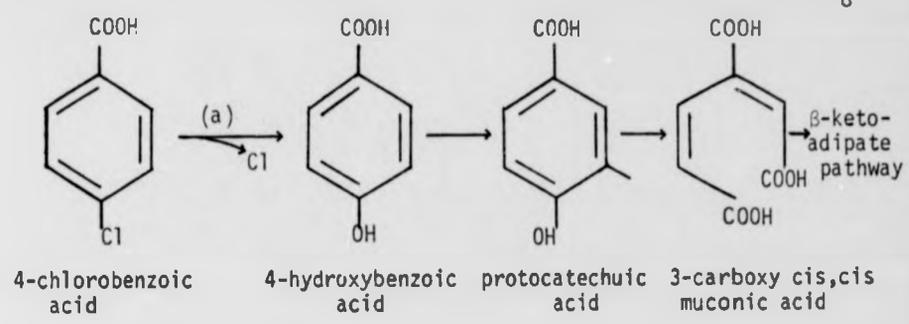
The dehalogenation step in the metabolism of halogenated aromatic compounds apparently occurs in one of three ways. Firstly as a spontaneous reaction during the oxygenase cleavage of the ring structure, secondly as a spontaneous enzymatic reaction after the ring has been cleaved, or thirdly as an initial enzyme reaction before cleavage.

An example of the second pathway is the metabolism of 3-chloro-4-chloro- and 3,5-dichlorobenzoates by a *Pseudomonas* (Hartmann *et al.* 1979). Strains were selected which exhibited various isoenzymes (Section 1.4) with different stereospecificities towards chlorocatechols enabling them to overcome the high degree of stereospecificity demonstrated by the enzymes of the unsubstituted benzoate pathway. The halogen was eventually eliminated from the nonaromatic metabolites generated by ring cleavage.

Bacteria often cannot use fluoro-aromatic compounds as their sole carbon source but can cometabolize them. Knackmuss and Hellwig (1978) suggested that this was due to the accumulation of halocatechols and Harper and Blakley (1971a & b) believed the inability of a bacterium to utilize fluorocompounds was related to its inability to avoid the accumulation of fluoroacetate. Such an accumulation was seen with a *Nocardia* sp. induced to metabolize fluorobenzoate but unable to utilize it as a carbon source because fluoroacetate readily converts to fluorocitrate, which is a potent inhibitor of the TCA cycle.

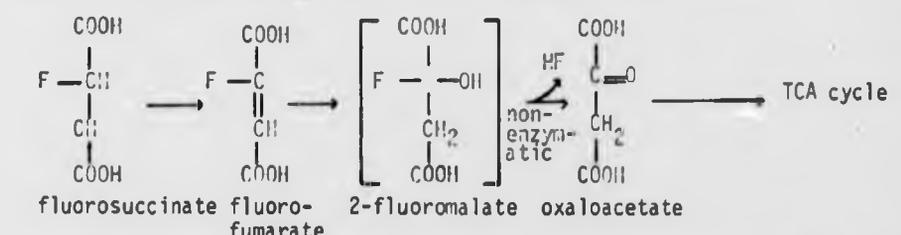
Klages and Lingens (1979, 1980) and Klages *et al.* (1981) provided two examples of the third mechanism. A *Pseudomonas* sp. CBS3 degraded 3-chlorobenzoic acid by cleaving the halogen substituent before ring cleavage, although no halogenated catechols were identified as degradation products, as in other work.

The enzyme catalysing the dehalogenation of 4-chlorobenzoate was found to be induced by its substrate only. The specific induction was observed for 4-chlorobenzoate 4-hydroxylase(a) which appeared to differ from other hydroxylating enzymes in phenylalanine, phenylacetate and benzoate metabolism.

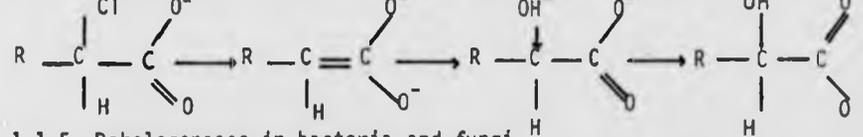


Dehalogenation prior to ring cleavage of 4-chlorophenylacetic acid was observed by Klages *et al.* (1981) which suggested that *Pseudomonas* sp. CBS 3 possessed two different dehalogenating systems which operated before ring cleavage.

Non-enzymatic cleavage of a fluorine atom from 3-fluoro-3-hexenedioic acid via fluorosuccinate, a straight chain molecule, was demonstrated by Harper and Blakley (1971b). The pathway suggested was:



Omori and Alexander (1978b) suggested the following mechanism for the non-enzymatic cleavage of halogens from α -halogenated fatty acids:



1.1.5 Dehalogenases in bacteria and fungi

In order to utilize halogenated substrates, microorganisms must remove the halogen substituent to allow complete mineralization. Spontaneous dehalogenation does occur (Section 1.4.4) but often the partial degradation of the halogenated substrate represents a lethal synthesis by producing toxic metabolites (Peters, 1952).

Bacteria and fungi possess enzymes capable of catalysing the

dehalogenation reaction. The release of halogens in ionic form when organisms utilize halogenated substrates has been demonstrated (Magee & Colmer, 1959; Hirsch & Alexander, 1960; MacGregor, 1963). It is possible that some enzymes catalysing unrelated reactions are capable of dehalogenating the substrate. Garrett *et al.* (1979) demonstrated the dehalogenation of 5-bromo- and 5-iodo-2'-deoxyuridilate by thymidylate synthetase producing the enzyme's normal substrate, 2'-deoxyuridilate. Other adventitious dehalogenations are listed in Table 1.1.

In a number of cases, however, specific dehalogenating enzymes have been identified. These enzymes are known as dehalogenases (Jensen, 1960; 1963) or halohydrolyses (Goldman *et al.* 1968; Little & Williams, 1971). Bollag (1974) segregated these enzymes into three groups: Hydrolytic dehalogenation, $-RCH_2X \rightarrow RCH_2OH$, e.g., fluoroacetate \rightarrow glycollate (Goldman, 1965); reductive dehalogenation, $RCX_3 \rightarrow RCX_2$, e.g., dichlorodiphenyltrichloroethane (DDT) \rightarrow dichlorodiphenyldichloroethane (DDD); and dehydrodehalogenation, $RCH_2CX_3 \rightarrow RCH = CX_2$, e.g., DDT \rightarrow dichlorodiphenylethane (DDE) or Dalapon \rightarrow pyruvate.

The first example of a dehalogenating enzyme was found in DDT-resistant flies and was studied in a purified state. DDT-dehydrochlorinase inactivates DDT by removing a hydrochloric acid group from the trichloroethane moiety. Jensen (1957, 1959) demonstrated the existence of enzymes in bacteria and fungi capable of catalysing dehalogenation reactions. Two types of chloride-liberating enzyme are seen in a strain of *Trichoderma viride* (Jensen 1959), one induced by monochloroacetic acid (MCA) and active towards this compound only, while the other, induced by dichloroacetic acid (DCA), was active towards MCA and DCA.

TABLE 1.1: Fortuitous enzyme dehalogenations (after Goldman, 1972)

Substrate	Enzyme	Product
p-Fluorophenylalanine	Phenylalanine hydroxylase	Tyrosine, F ⁻
p-Iodophenylalanine	Phenylalanine hydroxylase	Tyrosine, I ⁻
β-Chloroalanine	Homeserine dehydratase	Pyruvate, NH ₄ ⁺ , Cl ⁻
Fluorooxaloacetate	Aspartate aminotransferase	Oxaloacetate, F ⁻ , NH ₄ ⁺
Fluorofumarate	Fumarate hydratase	Oxaloacetate, F ⁻
2,2-Difluorosuccinate	Succinate dehydrogenase	Fluorofumarate, F ⁻
L-Monofluorosuccinate	Succinate dehydrogenase	Fumarate, F ⁻
1-Fluoro2,4-dinitrobenzene	Carbonic anhydrase	Dinitrophenol, F ⁻
α-D-Glucosylfluoride	α-D-glucosidase	Glucose, F ⁻

TABLE 1.2.: Dehalogenase induction in bacteria and fungi (after Jensen, 1960) (Bracketed symbols not tested, but were not attacked by growing cultures)

Organism	Inducer	Adaptation caused to substrate			
		MCA	DCA	TCA	22DCPA
<i>Trichoderma viride</i>	MCA	+	-	(-)	(-)
	DCA	+	+	(-)	(-)
<i>Pseudomonas dehalogenans</i> Groups I - II	MCA	+	+	-	-
<i>Pseudomonas dehalogenans</i> Group III	MCA	+	+	-	+
	DCA	+	+	-	+
	TCA	+	+	+	+
	2MCPA	+	+	(+)	+
	22DCPA	+	+	+	+

An alternative hypothesis suggested that DCA induced both enzymes, each specific for MCA in the former case and DCA in the latter.

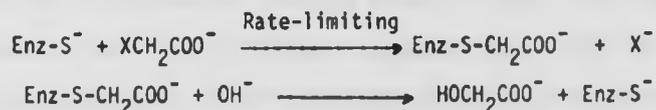
The dehalogenases of bacteria and fungi appear to differ with respect to pH profiles and also substrate specificity (Jensen, 1960). (Table 1.2). Jensen noted that the MCA induced dehalogenase of *Pseudomonas dehalogenans* in groups I and II attacked MCA and DCA, but in group III the specificity was increased to include 22DCPA.

These enzymes were believed to cleave the halogen from the carbon chain by hydrolysis of the carbon-halogen bond, resulting in the formation of hydroxyalkanoic acids from monosubstituted haloalkanoic acids and oxoalkanoic acids from disubstituted compounds (Foy, 1975). The molecules of HCl then accumulate while the alkanolic acids enter the normal metabolic pathways of the organism.

Davies and Evans (1962) examined sonicated cells of *P. dehalogenans* and found that the extracts catalysed the reaction:



The enzymatic cleavage of the carbon-fluorine bond in fluoroacetate by haloacetate halohydrolyase (Goldman 1965) was seen during the utilization of fluoroacetate by a soil Pseudomonad as its sole carbon source. The following reaction, analogous to the chloroacetate \rightarrow glycollate reaction involving a thioether was suggested:



The halogen (X) determined the rate of the reaction, which was fastest

for F, then Cl and slowly for I. Goldman and Milne (1966) proved that the hydroxyl ion originated from water by using (^{18}O) oxygen. The enzyme also exhibited specificity towards fluoroacetates and would not cleave fluoropropionates.

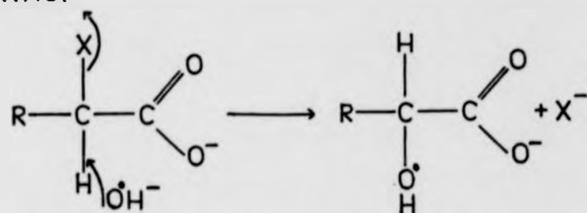
The isolation of two more dehalogenases from a soil pseudomonad, (Goldman *et al.* 1968) again showed different induction mechanisms for each enzyme. One was induced by MCA, the other by DCA. These enzymes were able to dehalogenate mono- and dichloroacetates, iodoacetate, 2-monochloropropionate and 2-chlorobutyrate (2MCBA). A major difference between these two enzymes and haloacetate halohydrase was their lack of sensitivity towards sulphhydryl-blocking agents.

Two mechanisms were put forward to explain the accompanying inversion of configuration during the dechlorination of L-2MCPA and L-2MCBA (Figure 1.1). A further two suggested mechanisms were disregarded as they failed to produce the inversion observed.

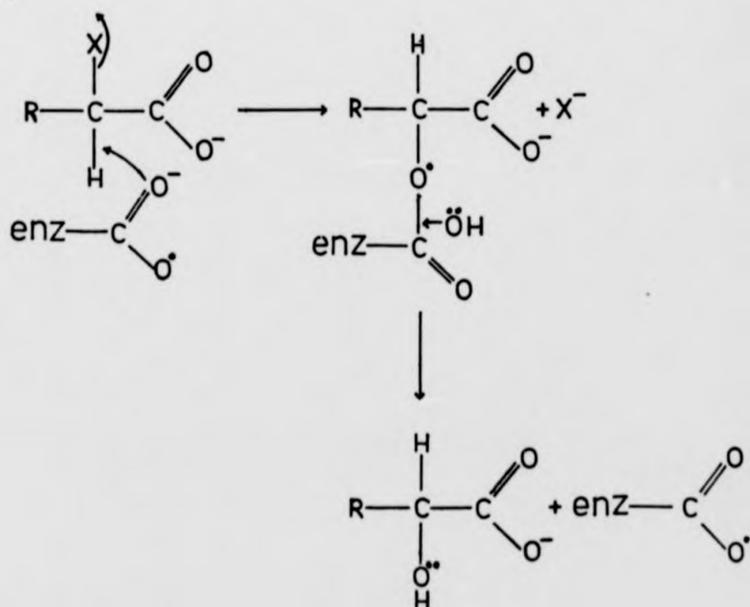
Tonomura *et al.* (1965) isolated a pseudomonad which could use fluoroacetate as a carbon source by converting it to glycollate. The enzyme properties of this organism were similar to those isolated by Goldman *et al.* (1968), as was also the case with the Gram-negative organism isolated by Kelly (1965) which could utilize fluoroacetate or fluoroacetamide as its carbon source. Other bacteria capable of defluorinating sodium fluoroacetate (compound 1080, used in New Zealand for the control of "noxious animals" (Lien *et al.* 1979)) were isolated by Lien *et al.* (1979) who suggested that a rich and diverse indigenous population of defluorinating microorganisms existed in the soil of New Zealand.

Figure 1.1 Two mechanisms suggested by Goldman et al. (1968) to explain the accompanying inversion of configuration during the dechlorination of L-2MCPA and L-2MCBA. O was ¹⁸O used to confirm possible mechanisms.

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TABLE 1.3.: Comparison of bacterial halohydrolyases (after Little & Williams, 1971)

Enzyme sources	Reference	Relative rates of hydrolysis ^b (and Michaelis constants) of substrates								Inhibition by third reagents					
		Monochloroacetate	Monofluoroacetate	Monobromoacetate	Moniodoacetate	Dichloroacetate	L-2-chloropropionate	Dichloropropionate	L-2-chlorobutyrate	pH optima	Stereo specificity	p-chloromercuribenzoate	N-ethylmaleimide	Iodoacetamide	sulphonate p-chloromercuribenzenes
<i>Pseudomonas deltalocumosa</i> NCIB 9062	Davies and Evans 1962	100	0	100	100	17	0	0	-	>9.5	-	50 (10 μ M)	-	-	-
<i>Pseudomonas</i> sp.	Goldman 1965	100 ^a (20)	700 ^a (2.4)	-	0.5 ^a	-	-	-	-	9.3	-	-	80 (40 min)	-15	100 (rapid) (10 μ M)
<i>Pseudomonas</i> sp.	Halidohydrolyase I, Goldman <i>et al.</i> , 1968	100 ^a (1.3)	0 ^a	-	20 ^a	10 ^a (0.08)	50 ^a	10 ^a	10 ^a	9.3-9.4	L-2-halo	-	0	0	0
<i>Pseudomonas</i> sp.	Halidohydrolyase II, Goldman <i>et al.</i> , 1968	100 ^a (1.3)	0 ^a	-	80 ^a	200 ^a (0.08)	120 ^a	80 ^a	80 ^a	9.3-9.4	0-2-hydroxy	-	0	0	0
<i>Pseudomonas deltalocumosa</i> NCIB 9061	Little & Williams 1971	100 (6.24)	0	140 (0.18)	100 (0.10)	18 (0.088)	35 (0.30)	9 (0.26)	9	9.3	vs low	0	0	0	49 (instantaneous)

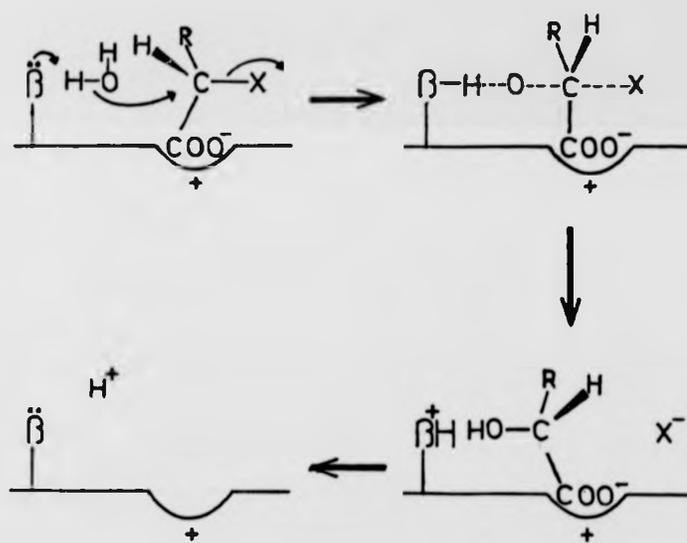
^a Figures taken from Goldman (1969)^b In % of the rate of hydrolysis of Monochloroacetate

Little and Williams (1969, 1971) further studied the haloacetate halohydrolyase of *Pseudomonas dehalogenans* NCIB 9061. They believed that two distinct forms of these enzymes could be distinguished by the effect of thiol reagents on enzyme activity. Those isolated by Davies and Evans (1962) and Goldman (1965) being completely inhibited by these reagents and those of Goldman *et al.* (1968) being virtually unaffected. The *P. dehalogenans* NCIB 9061 enzyme was only slightly affected by the thiol reagents, which was contrary to the results in the 1969 communication. A comparison of the bacterial halohydrolyases isolated in those early studies is shown in Table 1.3.

The inhibition studies suggested that there may be two distinct mechanisms for halide hydrolysis. The mechanism suggested by Goldman (1965) involving the formation of a δ -carboxymethyl intermediate could not be involved, Little and Williams (1971) believed in the enzyme system they isolated. The mechanism suggested for this enzyme is shown in Figure 1.2. It involved base catalysis by a group which the results suggested, was a histidine residue. If the base group was a thiol, then the differences between the two enzyme systems may be due to the degree of protection the thiol group obtains from the rest of the molecule.

Slater *et al.* (1976, 1979) described the differing capabilities of two strains of *Pseudomonas putida*, strains S3 and P3 (Senior *et al.* 1976b) to utilize a range of chlorinated acetic, propionic and butyric acids. Strain P3 possessed enzyme levels 10 to 40 times greater than strain S3 and although S3, a secondary utilizer in the Dalapon degrading community (Senior *et al.* 1976b) could not grow on any of the 9 chlorinated compounds tested, strain P3 could grow on 2MCPA and 22DCPA.

Figure 1.2 Dehalogenation mechanism suggested by Little and Williams (1971) involving a base catalysis by a histidine residue (β).



It was suggested that P3 originated from S3 in the chemostat culture. Chemostat selection studies confirmed that P3-like strains could be selected from pure cultures of S3 (Senior *et al.* 1976b; A.J. Weightman & J.H. Slater, unpublished observations).

This work also indicated that strain P3 contained at least 2 dehalogenases, one mainly active towards chlorinated acetic acids and the other towards chlorinated propionic acids. This work represents the second example of an organism containing two dehalogenases, the first was that of Goldman *et al.* (1968), but in that case the enzymes were separately induced.

The work of Slater's group showed that the dehalogenase systems of *P. putida* exhibited different substrate specificities. One enzyme, the fraction II dehalogenase, was more active towards the halogenated acetates and the other, the fraction I dehalogenase was more active towards the chlorinated propionates and brominated compounds.

Berry *et al.* (1979) found the dehalogenase activity of a *Rhizobium* sp. was greater towards 2MCPA than 22DCPA, in contrast to Kearney *et al.* (1964) who showed that the enzyme from an *Arthrobacter* sp. liberated chloride ions from 22DCPA more readily than from 2MCPA. These differences may represent minor variations in enzyme structure, when more than one enzyme is present in a given organism, or differences in the control mechanisms, or differences in the actual mechanism of reactions as described by Little and Williams (1971).

Thus far, the enzymes considered have all acted upon the substitution of the carbon-2 of each compound. However, Castro and Bartnicki (1965) isolated a pseudomonad capable of converting 3-bromo-

-propanol to 3-hydroxypropionate which was then completely mineralized. Such compounds are used as nematocides in soils, so their biodegradation may be mediated by such organisms. A strain of *Micrococcus denitrificans* (Bollag & Alexander, 1971) could utilize 3-chloropropionic acid (3MCPA) as its sole carbon source. Its dehalogenase was specific for 3-carbon-substituted compounds and, as no 3-hydroxypropionate as an intermediate was observed, it was tentatively suggested that the mechanism involved dehydrodehalogenation.

The release of halogen substituents from a number of xenobiotic compounds has been shown to be mediated by a number of different bacterial and fungal genera. The ability may have evolved as a result of contact with halogenated compounds produced by other microorganisms and could represent a detoxification mechanism, as a number of these compounds are bactericidal. For example, the possession of such enzymes may prevent the lethal synthesis of fluorocitrate from fluoroacetate by dehalogenating the latter. This would incidentally enable the microorganism to use the halogenated compound as a source of energy, carbon, nitrogen or other minerals.

1.2 CONTINUOUS-FLOW CULTURE

Traditionally batch culture systems have been used for the study of microbial growth. These cultures are characterized by initially high concentrations of all nutrients and no through-put of materials once the medium has been inoculated. Thus it is a discontinuous process in which the environment is continually changing due to nutrient depletion and increasing levels of metabolites, producing only transient states (Bull, 1974). Organisms cultured under these conditions have a history.

The behaviour of organisms, at a given time, is influenced by the growth and metabolism of their ancestors which developed under different environmental conditions.

The continuous-flow culture of microorganisms divorces them from their history at any given time, by maintaining a constant environment. There are two types of continuous-flow system:

(1) the turbidostat where the organisms grow at, or very close to, their maximum specific growth rate (u_{max}). The actual rate being determined by the genetic properties of the organism; that is, growth under internal control.

(2) the second type, known as chemostats, subject cultures to external control such that the growth rate is determined by the rate of supply of a limiting growth substrate. Continuous-flow cultures balance the input of nutrients with the output of organisms, metabolic products and unused nutrients, enabling the attainment of steady state conditions, during which the average values of all culture and organism properties remain theoretically constant over an indefinite time. Since the initial development by Monod (1950) and Novick and Szilard (1950a) the continuous culturing of organisms, both prokaryotic and eukaryotic, has become increasingly important in research and industry (Bull, 1974; Parkes, 1982).

1.2.1 Theoretical considerations of continuous culture

The derivation of the mathematical equations used to describe the continuous culture of microorganisms will not be described, as numerous accounts have been published (Herbert *et al.*, 1956; Tempest, 1970, 1978; Bull, 1974; Pirt, 1975; Harder *et al.* 1977; Slater, 1979), although specific details pertinent to this work are necessary.

The basis from which the mathematical models have been derived is an equation, analogous to the Michaelis-Menten enzyme kinetics equation, suggested by Monod (1942), which describes the relationship between growth-limiting substrate concentration and growth rate. It reflects the similarity between the bacterial cell and an enzyme molecule reacting with the substrate (S) (Schulze & Lipe, 1964):

$$\mu = \mu_{\max} \frac{S}{K_S + S} \quad (1)$$

where μ is the specific growth rate at a given growth-limiting substrate concentration, S; μ_{\max} is the maximum specific growth rate obtained when S is large and not limiting; K_S is the substrate saturation constant corresponding to the concentration of S at which μ is $0.5 \mu_{\max}$, μ is specific for each organism and the physicochemical environment in which the organism is growing.

In chemostat cultures, the growth rate is determined by the rate of supply of the limiting substrate and is controlled by the fractional rate of medium displacement, that is the dilution rate, D (units: h^{-1}) which is defined as:

$$D = \frac{F}{V} \quad (2)$$

Where F is the medium flow through the fermenter (units: $l h^{-1}$) and V is the volume of the culture (units: l). Thus D is a measure of the number of culture volume changes achieved in unit time. The reciprocal of D represents the mean residence time, θ (units: h) which is the average time an organism remains in the vessel. θ is related to the organism's doubling time, t_d , (units: h) by:

$$t_d = \frac{\theta \ln 2}{\mu} \quad (3)$$

from which the chance of an organism undergoing cell division before being washed out of the culture vessel is:

$$e^{-Dt_d} \quad (4)$$

Hence the greater the culture doubling time the higher the probability that an organism will be removed from the system before it divides.

An analysis of chemostat theory in terms of organism biomass, x (units: g dry wt ℓ^{-1}), and substrate concentration in the culture vessel, S (units: g substrate ℓ^{-1}) when $\mu = D$ and $dx/dt = 0$ and $ds/dt = 0$ (that is, when the system is in steady state) leads to the derivation of the steady state organism biomass and growth-limiting substrate (3) equations:

$$\bar{s} = K_s \frac{D}{\mu_{\max} - D} \quad (5)$$

and

$$\bar{x} = Y \left[S_R - K_s \frac{D}{\mu_{\max} - D} \right] \quad (6)$$

where Y is the observed growth yield, the ratio of weight of cells produced to weight of substrate utilized; and S_R is the initial concentration of the growth-limiting substrate.

Equations (5) and (6) indicate the self-regulating capacity of chemostat cultures to produce steady-states at different values of D because the dilution rate determines S which in turn determines μ (Fenc1, 1966). Thus, changing the dilution rate results in a change in μ , producing new and unique steady-state values. So an infinite number of steady states can be obtained by varying D and S_R . Minor changes in biomass concentration or limiting-substrate concentration at a fixed dilution rate results in opposing changes in the specific

Figure 1.3 *The self-regulating capacity of chemostat cultures*
(after Veldkamp, 1976).

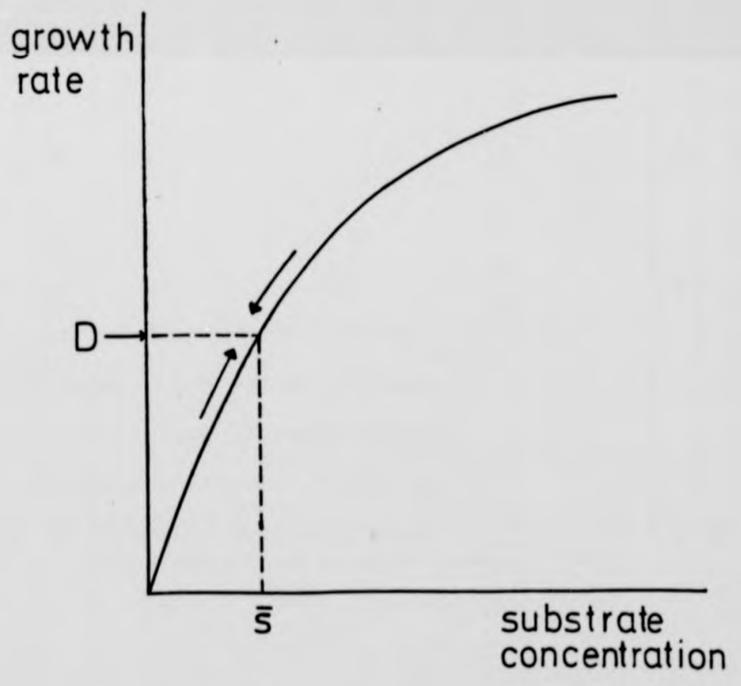
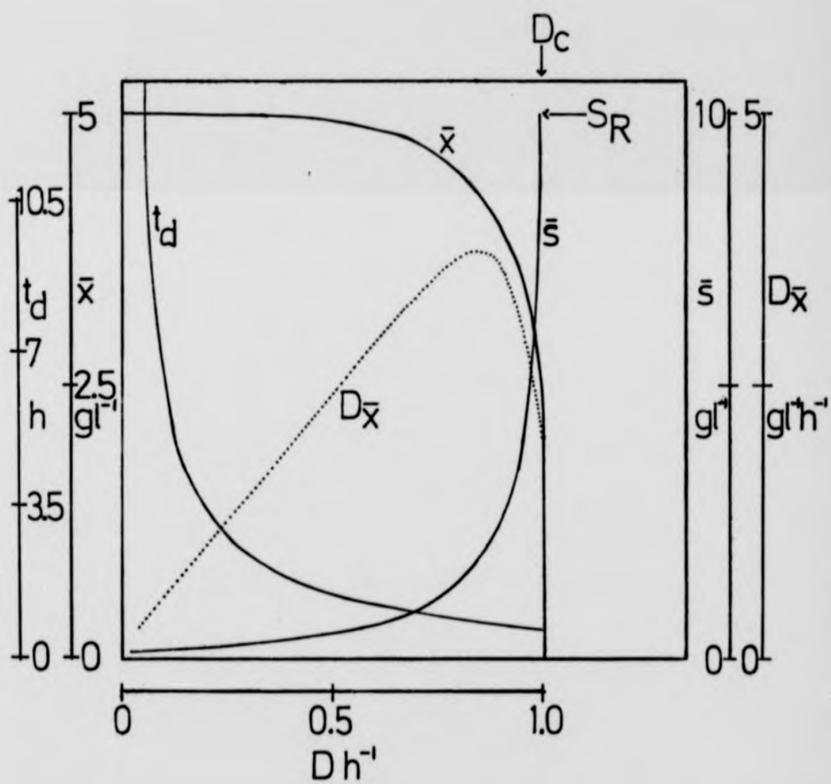


Figure 1.4 Theoretical steady-state conditions derived from equations (5) and (6) \bar{x} , steady-state biomass concentration; \bar{s} , steady-state substrate concentration; S_R , initial substrate concentration; t_d , culture doubling time; $D_{\bar{x}}$, productivity. (after Bull, 1974).



ration;

1974).

growth rate of the culture, such that the original steady state is restored (Figure 1.3).

Theoretical steady-state conditions derived from equations (5) and (6) have been frequently represented as seen in Figure 1.4 (Herbert *et al.*, 1956; Fenc1, 1966; Bull, 1974; Veldkamp, 1976; Slater, 1979). This indicates that the dilution rate cannot be made greater than μ_{\max} if steady state conditions are to be maintained. The maximum value of the dilution rate is known as the critical dilution rate (D_{crit}) and is nearly equal to μ_{\max} . If $D > D_{\text{crit}}$ then exponential wash-out of the organisms occurs, at which time $S = S_R$. D_{crit} is not constant but depends on the organism and the initial concentration of the growth-limiting substrate (S_R):

$$D_{\text{crit}} = \mu_{\max} \frac{S_R}{K_S + S_R} \quad (7)$$

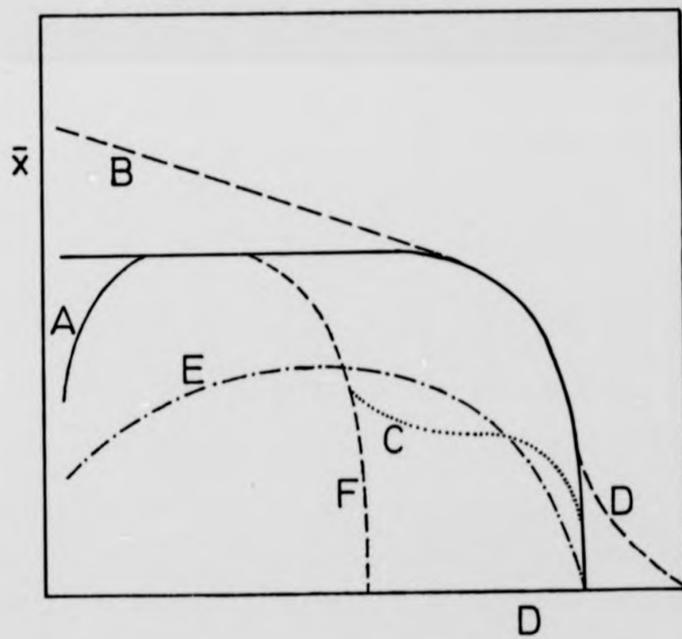
As well as a maximum specific growth rate and the D_{crit} value, evidence is also accumulating to support the theory of a minimum growth rate. Bull (1974) suggested a value of 6% of μ_{\max} for the minimum growth rate (μ_{\min}). Below μ_{\min} cultures apparently fail to grow correctly. Practice indicates that the chemostat can support steady state populations over the range μ_{\min} to D_{crit} or approximately 5 to 95% μ_{\max} .

1.2.2 Departures from theory

The kinetic model described above is based on ideal parameters and as a result is oversimplified. The theoretical predictions are often found to be inexact when compared with practical results.

Experimental departures from the predicted steady state organism concentrations as shown in Figure 1.5 are caused by the following phenomena. A decrease in organism concentration at low dilution rates (A)

*Figure 1.5 Departures from the theory of continuous culture
(after Bull, 1974). See text for details.*



can occur in carbon limited chemostats due to maintenance energy requirements which could also explain the existence of a μ_{\min} value. The opposite effect, at low dilution rates (B), occurs in cultures under nitrogen or phosphorus limitation. Phosphorus limitation restricts nucleic acid synthesis and low nitrogen concentrations restrict protein synthesis. However, the polysaccharide and lipid content of the cells continue to rise as intracellular reserves, so producing larger biomass concentrations than would be expected.

A change in dilution rate may also lead to shifts in metabolism (C) which result in a decrease in substrate utilization efficiency, so resulting in a decrease in biomass concentration. The model assumes perfect and instantaneous mixing of fresh medium with the growing culture, which in reality does not occur, and so some regions of the culture may effectively exist in a pocket of higher or lower dilution rate (Herbert *et al.*, 1956; Pirt, 1975). When in a pocket of lower dilution rate an apparent increase in D_{crit} can be observed (D), a phenomenon also caused by wall growth.

Monod's equation (equation (1)) describes substrate concentration as being asymptotic to μ_{\max} . However, some substrates are toxic even at low concentrations. This causes a decrease in the expected biomass concentration (E). Similar results are also seen if metabolic products are toxic and so become inhibitory.

The requirement for a growth factor, for example a vitamin or amino acid, can produce an apparently low D_{crit} (F). Initially the growth factor is supplied in the original inoculum, but with time it is gradually diluted. Jannasch (1963, 1965) discussed the growth

of bacteria at low population densities and suggested that below certain substrate concentrations organisms are unable to grow whatever the dilution rate. It was also suggested that these results supported the concept of growth-stimulating relationships between organisms in a population, enabling growth on a medium which does not meet all the growth requirements of a single organism. Thus, the internal growth factor becomes limiting when the population density falls below certain values.

Reference should be made to the discussion by Powell (1967) of formulae which he suggested represented quite closely the transition of batch cultures from the exponential to stationary phases. This provides a variation from the model of Monod based on the dependence of growth rate on substrate concentration. A further model system (Yamané *et al.*, 1979) described the start-up conditions in batch and fed-batch cultures to initiate chemostat growth, and was offered to enable more efficient start-up strategies for continuous culture.

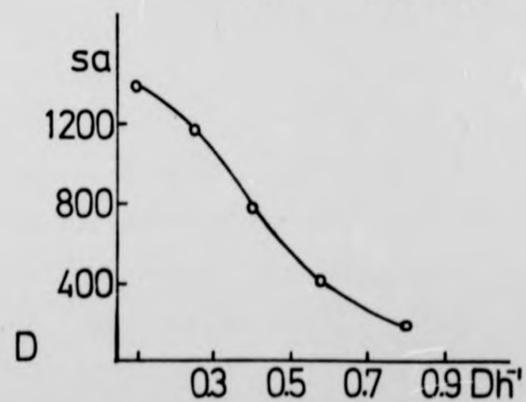
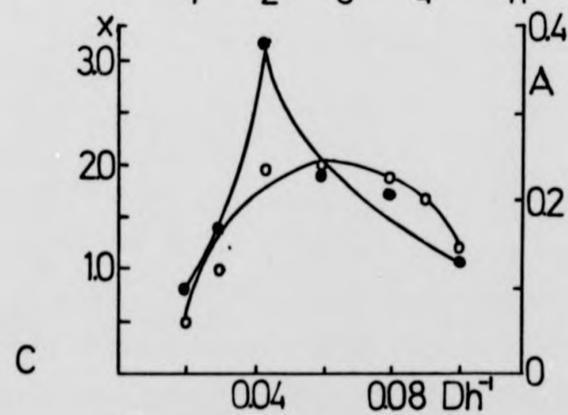
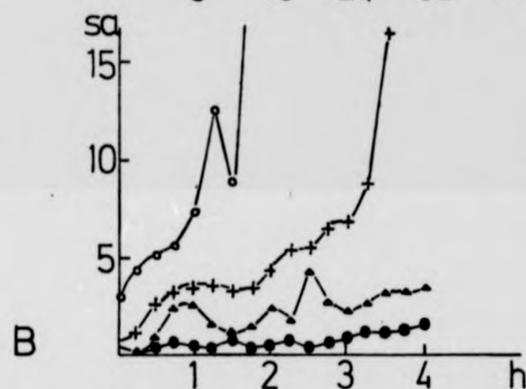
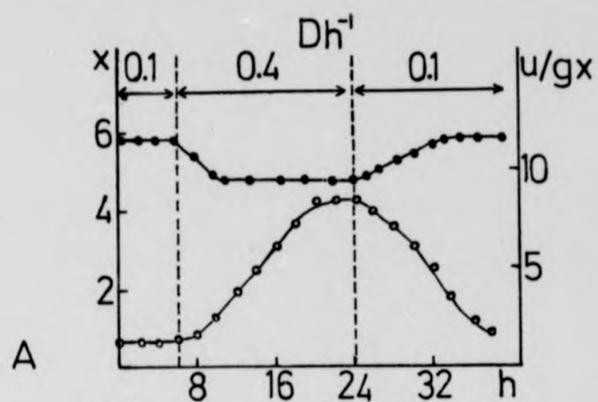
1.2.3 Enzyme and metabolic regulation in chemostat cultures

The steady state, obtainable in chemostat cultures, enables quantitative changes in enzyme levels, in response to environmental parameters, to be observed. The chemostat is also an ideal system in which to study the control mechanisms involved. Furthermore it allows the selection of constitutive strains and hyperproducing organisms exemplified by the *Escherichia coli* β -galactosidase experiments (Horiuchi *et al.* 1962a; Vojtíšek *et al.*, 1969; Pavlasová *et al.*, 1980a & b, 1981).

Pardee (1961) suggested that bacteria have evolved so as to

Figure 1.6 Effect of dilution rate on enzyme activity.

- A: Changes in the level of isocitrate dehydrogenase in Candida utilis after changes in the dilution rate. (O), Isocitrate dehydrogenase; (●), biomass. (after Fencl, et. al. 1972)
- B: Effect of D (h^{-1}) on amidase synthesis by P. aeruginosa growing with succinate + acetamide. At time zero the incoming medium was changed from 10mM succinate to 10mM succinate + 20mM acetamide. Results recorded as amidase specific activity ($\mu\text{m}(\text{mg dry wt})^{-1}\text{min}^{-1}$) against D (h^{-1}) (after Boddy et al., 1967) (O) D 1 0.22; (+) D 1 0.38; (▲), D 1 0.54; (●) D 1 0.76.
- C: Effect of D on the synthesis of pectin lyase in a pectin-limited chemostat (●) steady state specific activity ($\Delta A_{500\text{nm}} \text{min}^{-1} \text{mg protein}^{-1}$) (O) biomass (gl^{-1}) (after Almengor-Hecht & Bull, 1978).
- D: Effect of D on the β -galactosidase activity ($\text{nmol ONPG hydrolysed}(\text{mg dry weight})^{-1} \text{min}^{-1}$) of Klebsiella aerogenes 418 (after Dean, 1972).



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multiply as rapidly as possible. However, because few rich environments, outside the laboratory, exist which permit the maximum growth rate being obtained, it was suggested that bacteria have developed control mechanisms which help to bring the intracellular environment into conditions suitable for rapid growth. For example, permeases can create high intracellular substrate concentrations, even when extracellular levels are extremely low (Pardee, 1961).

Enzyme production can be linked with growth rate and hence, in chemostat cultures to the dilution rate. Dean (1972) identified five types of dependency of enzyme activity on dilution rate, namely: the enzyme activity remains constant irrespective of dilution rate; activity passes through a maximum value at a given dilution rate; activity passes through a minimum value at a given dilution rate or activity increases or decreases as the dilution rate increases (Figure 1.6).

When in a steady state, growth rates and yields remain constant. However, this does not necessarily apply to individual enzymes in the organisms (Sikyta & Fenc1, 1976). In a number of experiments levels of certain enzymes have been shown to oscillate about an average value, a phenomenon more pronounced in the production of extracellular enzymes. Sikyta and Fenc1 (1976) suggested two explanations for this oscillatory behaviour. Firstly, that the changes in enzyme levels are the consequence of the loss of culture history (Section 1.2), or secondly that these changes are caused by the accumulation of low or non-producing mutants within the chemostat population, which would cause the observed oscillations by apparently changing the overall specific activity of the culture for the enzyme concerned. Macleod *et al.* (1975) working with *Escherichia coli* ML308 grown in a chemostat, observed regular

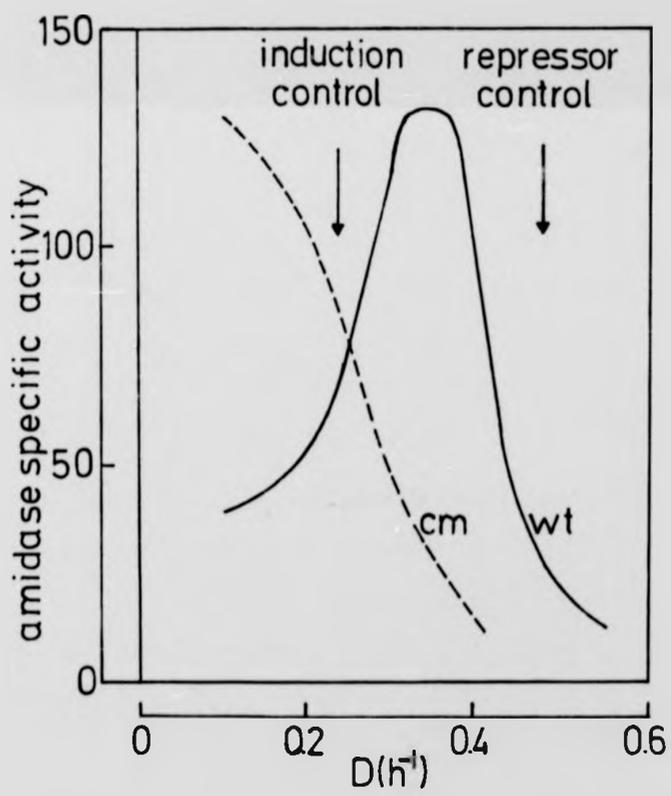
oscillations in enzyme activity even after steady state conditions had been reached. The amplitude of the oscillations was 20% of the mean value and had a periodicity of one hour.

Theoretical considerations (Goodwin, 1966;) predicted the periodic oscillation of enzyme synthesis, and that the oscillations depend to some extent on growth rate. Dean (1969) reported the damped oscillatory nature of *Aerobacter (Klebsiella) aerogenes* during the switch from batch to continuous culture. Such damped oscillations have also been observed after increasing the dilution rate from one value to another.

Boddy *et al.* (1966) observed fluctuations in the bacterial biomass concentration and amidase levels in chemostat cultures of *Pseudomonas aeruginosa* strain 8602 and Boddy *et al.* (1967) suggested that the oscillatory nature of the biomass was a reflection of the oscillating enzyme level, and hence, only reflected changes in concentration of the inducer or catabolite repressor. Although biomass fluctuation may result from the oscillation of an enzyme the periodicity of each need not be in phase with each other.

In batch culture the dual control of enzyme synthesis, induction and repression, manifests itself as diauxic growth, but in continuous culture the balance between the opposing controls is far more obvious. Boddy *et al.* (1966, 1967) and Clarke *et al.* (1968) provided a good example with the aliphatic amidase synthesis in *Pseudomonas aeruginosa* strain 8602. The effect of increasing the dilution rate on enzyme activity (Figure 1.7) was interpreted in the following way by Clarke *et al.* (1968). At low dilution rates catabolite repression is minimal so the
rate

Figure 1.7 The effect of increasing dilution rate on the amidase activity of Pseudomonas aeruginosa strain 8602 (after Clarke et al. 1968), cm - constitutive mutant
wt - wild type.



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of amidase synthesis is dependent on the rate at which acetamide becomes available. Under these conditions an increase in dilution rate results in an increase in the specific activity of the amidase. As the dilution rate increased above 0.3h^{-1} , the growth rate increased to a point where metabolic intermediates form at a rate sufficiently high to cause catabolite repression. At a dilution rate of 0.6h^{-1} the enzyme concentration had decreased considerably, with the specific activity remaining similar to overnight batch cultures.

A constitutive mutant of *P. aeruginosa* demonstrated a gradual decrease in specific activity with increasing dilution rate (Figure 1.7) which is consistent with increasing catabolite repression, resulting from an increased rate of metabolite formation of the enzyme which requires no induction. Similar dual control was cited as the explanation for the specific activity of pectin lyase passing through a maximum value at a dilution rate of approximately 0.04h^{-1} in a pectin-limited chemostat (Almengor-Hecht & Bull, 1978).

Clarke *et al.* (1968) concluded that the effect of an inducer depended on the metabolic state of the organism with respect to the production of catabolite repressor molecules. That is, when the concentration of metabolites is high, enzyme repression is severe, so changes in inducer levels will have little effect on enzyme synthesis. When the carbon pool is low, catabolite repression will be negligible and enzyme activity will depend on the inducer concentration.

Other environmental parameters also have an effect on enzyme activity. Marriott *et al.* (1981) found that in *Azotobacter beijerinckii* the specific activity of adenylate kinase was relatively unaffected by

dilution rate under nitrogen or oxygen limitation, but inversely related to it under carbon-limited conditions. While in the same organism adenosine monophosphate nucleosidase activity was inversely related to the dilution rate under all three limiting conditions. Bolton and Dean (1972) demonstrated that acid phosphatase synthesis in *Klebsiella aerogenes* varied with growth rate and the composition of the growth medium, the highest activity occurring in glucose-limited cultures.

The response of *Escherichia coli* enzyme regulation to removal of glucose-carbon limitation in a chemostat was investigated by Harvey (1970). During limited growth the enzyme levels were precisely regulated to give balanced exponential growth. The rates of reaction were determined by zero order kinetics, that is solely by the enzyme concentrations. However, on removal of carbon limitation, the enzyme levels became the rate-limiting factor. Thus the specific growth rate before and immediately after removal of glucose limitation remained the same, until the enzyme levels were increased. The response to the presence of unlimited substrate was studied by Harvey (1970) by looking at the levels of amino acid synthesizing enzymes. These effectively control growth rate after the onset of unlimited conditions, because of the inability to immediately increase amino acid production required for ribosome synthesis and other vital functions which enables an increase in growth rate.

Jensen and Neidhardt (1969) followed the response of histidase specific activity in *Aerobacter (Klebsiella) aerogenes*. Upon the imposition of a biosynthetic restriction, by limiting either sulphate or a required amino acid, L-arginine, the specific rate of production

of histidase gradually reduced. This was caused by the dilution of the organism's complement of histidase by growth, in turn due to catabolite repression of enzyme synthesis. However, Jensen and Neidhardt (1969) believed that three other processes were involved in the limitation of histidase activity. Firstly the operation of the histidase pathway was somehow restricted; secondly the activity of the enzyme decreased and thirdly adjustment in the catabolite metabolism whereby repressing catabolites were converted to inert materials (CO_2 or lactate) occurred. This latter event may also prevent catabolite repression of branching pathways which have multiple end products (Section 1.3.4). Once the biosynthetic limitation was lifted the processes were reversed. Almost instantaneously the rate of histidase formation increased, as would be predicted by the release of catabolite repression. However, before histidase levels had increased significantly the growth rate had trebled. This suggested that the restriction on enzyme activity and the diversion of carbon to excreted metabolites could be quickly reversed.

These mechanisms may represent specific examples of the concept of total integration of the cell which implies that the synthesis of enzymes in cells are integrated into a network of closed interdependent cycles. Thus, as conditions change, so the levels of all enzymes re-adjust to levels best suited to the new environment.

1.2.4 Bacterial evolution in chemostat cultures

The low concentration of the limiting nutrient in a chemostat culture creates a highly competitive environment in which the fitness of an organism is dependent upon the efficiency with which it can

utilize the limiting nutrient. In mixed cultures this results in one organism becoming dominant in the population (assuming the substrate can be utilized by a single species rather than by a community) with the slower-growing, less competitive organism being washed out.

A single species present in a number of mutated forms may also be regarded as a mixed culture. If one of these mutations affects the organism's growth rate then its survival in the chemostat will depend on how the mutation affects the metabolism of the growth-limiting nutrient. A mutation which increases the rate of utilization of the substrate will enhance the growth rate which, assuming the mutant remains in the chemostat long enough to divide (Section 1.2.1, equations (3) and (4)), would outcompete the parental strain. This would result in an increased population density and rate of biomass production reflecting the greater adaptation to the environment.

The theoretical and experimental basis of microbial evolution in the chemostat was discussed by Novick and Szilard (1950b). Since then the chemostat has been used to study the evolution of catabolic systems in which the activity of "substrate capturing enzymes" are rate limiting (Bull & Brown, 1979).

Horiuchi *et al.* (1962b) used the chemostat to isolate *Escherichia coli* mutants which constitutively produced elevated levels of β -galactosidase. By using lactose as the growth-limiting substrate, they isolated hyperproducing mutants which produced levels of β -galactosidase representing 25% or more of the total cell protein concentration, and a specific activity increased four-fold over the parental strain.

Constitutive, hyperproducing strains of *E. coli* have also been studied by Pavlasová *et al.* (1980a) who investigated the effect of cyclic adenosine monophosphate on the production of β -galactosidase and tryptophanase and Pavlasová *et al.* (1980b) selected hyperproducing D-serine deaminase (D-sdase) mutants of *E. coli* by chemostat enrichment using D-serine as the nitrogen source. Strains were isolated which exhibited a 25-fold increase in enzyme specific activity with respect to parental strains. 90% of these mutants produced the enzyme constitutively, although it was noted that of the two possible mutations which could lead to constitutivity of this enzyme, an activator or an operator mutation, the chemostat only selected activator mutants which were very sensitive to catabolite repression. Pavlasová *et al.* (1981b) also selected for mutants of *Escherichia coli* which simultaneously hyperproduced both D-sdase and β -galactosidase.

Pavlasová *et al.* (1980a & b) and Horiuchi *et al.* (1962b) found that, when growing on media not containing the hyper-enzyme's substrate, the growth rates of these strains were 20% less than that of the parental strains and Horiuchi *et al.* (1962b) found that the hyperproduction ability was unstable, possibly as a consequence of their lower growth rates. The observed 4-fold increase in specific activity in the hyper-strains when freshly isolated from the chemostat was lost on continued subculturing in non-lactose-containing medium (Section 1.4.2).

Hegeman and Rosenberg (1970) used the work of Zamenhof and Eichdorn (1967) to describe competition experiments in chemostat cultures between prototrophic and auxotrophic strains of *Bacillus subtilis*, and demonstrated that in all cases, when supplied with the

necessary metabolite(s), the auxotrophic strains always outcompeted the prototrophs. The effect was most noticeable if the biosynthetic block was earlier rather than later in the pathway. The reasons put forward were that auxotrophs were having to synthesise fewer metabolic intermediates than the competitors. Studies such as these enable further understanding of the mechanisms of evolution of metabolic pathways and the results paralleled with observations of the differences in the regulatory mechanisms in coliform and soil microorganisms (Section 1.4.5).

Mixed culture studies on the degradation of the herbicide Dalapon (Senior *et al.* 1976b) demonstrated the evolution of an additional primary utilizer. The mutated organism was identified as *Pseudomonas putida* strain PP3 and was similar in all other respects to one of the secondary utilizers in the original population. Pure culture work later suggested that the ability to degrade Dalapon was probably the result of a spontaneous mutation in a pre-existing gene which produced an enzyme activity towards 2-monochloropropionic acid and dichloroacetic acid.

Further studies on the dehalogenase system (Weightman & Slater, 1980) involved exposing *P. putida* strain PP3, growing on 2-monochloropropionic acid, to 2-monochlorobutanoic acid (2MCBA) in order to select strains capable of growth on 2MCBA. Although this was not achieved, two strains were isolated which differed from the parental organism in respect of their dehalogenating system. The results were explained as the production of elevated levels of one of the two dehalogenating enzymes possessed by the parental strain. However, unlike the

β -galactosidase elevated levels, the new strains continued to demonstrate the novel dehalogenation pattern after subculturing in batch culture; suggesting that the mutation was stable.

The selective pressure exerted by the chemostat on growth rate of the organism is also shown in the work of Godwin and Slater (1979). The effect of the possession of a plasmid on the growth rate of an organism has been shown to be competitively disadvantageous when the genes on the plasmid are of no immediate value. It was demonstrated that with time mutant varieties of the plasmid TP120 were selected in *Escherichia coli* K-12 grown in a chemostat under carbon-limited and phosphorus-limited conditions. The mutations, in the form of deletions, resulted in the loss of resistance to one or more of the antibiotics normally coded for by TP120. The strains of *E. coli* which possessed these reduced resistances were capable of higher maximum specific growth rates than the parent strain. This may represent an example of a deletion of redundant genetic material to decrease the genetic load (Section 1.3.5) to enable the organism to compete more effectively in the environment.

Batch liquid or plate methods are of more value if the traditional mutagens are to be used as if they are added to chemostat cultures they would lead to culture washout (Bull & Brown, 1979). However, the selectional pressure of increased growth rate enhanced by the chemostat system provides, possibly, a more natural selection pressure for the evolution of new or improved systems to utilize a given substrate. As the chemostat focuses selection on given enzymes in a large population it effectively telescopes evolutionary time with regard to cell generations which, although described for *Saccharomyces cerevisiae* by Francis and

Hanschke (1972) is even more significant for bacterial populations.

Bull and Brown (1979) noted that the chemostat has been used greatly to study mutation, but that to their knowledge there had been no report of its use in gene transfer experiments, and suggested that the rapid mixing required for aeration and homogeneity may prevent such events occurring. The problems of lack of cell to cell contact in the chemostat may be overcome by use of a system such as described by Gowland and Slater (1982). The culture vessel is packed with ballontini beads which provide an inert surface to which the microorganisms attach. This attachment allows cell to cell contact over time periods concomitant with transfer of genetic material.

The chemostat enables environmental selection pressures to be focused on a target site and its inherent stability enables conditions to be maintained over periods of time sufficient for adaptation to occur.

1.2.5 Continuous culture in microbiological research

The choice of batch or continuous culture as laboratory growth systems has led to a great deal of debate as to their relative merits. Proponents of each system often underestimate the problems of modelling the natural environment in the laboratory; both systems can be used to great effect. However, with either system care should be taken when extrapolating results back to the natural environment.

Those advocating the use of the chemostat often exaggerate its effectiveness as a model system. As Jannasch (1974) noted the chemostat "is neither meant to reproduce nor is it capable of reproducing the natural habitat. Its sole purpose is to make physiological,

biochemical and genetic responses of whole populations amenable to arithmetic analysis. There is no equivalent for the 'dilution rate' in a natural population." In natural conditions growth conditions change continually so there are no steady state conditions. Thus selection in nature would favour organisms capable of coping with growth rate changes which does not happen in chemostats.

One major difference in the use of open and closed culture systems becomes apparent in enrichment studies. Batch cultures, starting with high concentrations of all substrates, result in competition and selection based solely on their μ_{max} values. These organisms are capable of high growth rates and normally have low substrate specificities that is, they are zymogenous, and will outcompete autochthonous organisms (Jannasch, 1967a; Parkes, 1982; Slater & Hardman, 1982). The autochthonous organisms grow at slow rates but are able to utilize low substrate concentrations. Thus batch enrichment selects for organisms which are of less significance in the natural oligotrophic environment. However, the presence of zymogenous organisms indicates some functional role for them in the environment and their presence and activity must not be ignored.

The same effect can be seen in chemostat enrichments if the dilution rate is too fast. The slow-growing organisms will be diluted more rapidly than the faster growing ones, so enriching for the latter group (Jannasch, 1967a; However, by enriching at low substrate concentration levels and low dilution rates, enrichment of the autochthonous organisms is possible (Jannasch, 1967b).

The selection of microorganisms by continuous culture has been reviewed by Harder *et al.* (1977) and Parkes (1982) but one observation

worth noting is the usual practice of inoculating a fermenter and allowing the organisms to grow in batch before turning on the medium flow. Subsequent enrichment only selects from those organisms which have been enriched or survived the batch phase, so changing the composition of the original inoculum and opening this enrichment system to the same problems exhibited by batch techniques (Brown *et al.*, 1978).

There are advantages in the use of continuous culturing in the laboratory; some of these are described below.

The growth of a microbial population in a chemostat is limited by one nutrient in the medium, which is normally at a level which provides a high biomass concentration in order to facilitate analysis. Under these conditions growth is obtained while the organism is not saturated with substrate, as is the case with closed systems. This enables studies to be made into the influence of growth rate on a number of physiological characteristics. In most natural environments the populations are likely to be growing at submaximal rates for most of the time so the chemostat system enables investigations under more natural conditions, in this respect (Veldkamp, 1976). Basic macromolecular composition (Herbert, 1961); enzyme specific activities (Dean, 1972; Clarke & Lilly, 1969) enzyme complement (Almengor-Hecht & Bull, 1978; Bushell & Bull, 1981) and intracellular pool size and constituents (Brown & Stanley, 1972) have all been investigated in continuous culture.

Growth for long periods under constant conditions enables long term studies on the biochemistry and physiology of the population. External effects on these parameters can be studied in isolation from

previous events in the history of the culture (see Section 1.2).

It is also possible to use low substrate concentrations, including those not present at limiting values to obtain low population densities (Jannasch, 1963, 1965, 1967b).

Continuous culture provides the best system for investigating mixed cultures or microbial communities (Veldkamp & Jannasch, 1972; Jannasch & Mateles, 1974). in the study of interactions between components of the community, such as competition (Godwin & Slater, 1979) predation, (Lampert, 1976; Curds & Bazin, 1977), also the breakdown of compounds by symbiotic relationships (Daughton & Hsieh, 1977; Slater, 1978) and evolutionary changes within a population (Senior *et al.*, 1976). Larsen *et al.* (1978) reviewed the possibilities for mixed culture in industrial processes.

Continuous culture apparatus has been developed from the basic systems as described by Marcus and Halpern (1966) and Baker (1968) to much more sophisticated, though not necessarily more reliable, systems for the cultivation of many different cell types: Wardley-Smith and White (1975) used bacterial luminescence and optical density to control the bacterial culture in their 'luminostat'. Fay and Kulasooriya (1973) developed a system for the culture of photosynthetic microorganisms and Carpenter (1968) and Palmer *et al.* (1975) continuously cultivated algal cells. Fungi have been cultured in different ways by Blain *et al.* (1979) and Amon and Arthur (1979). Numerous studies of *Trichoderma* species in continuous culture apparatus have been undertaken in the study of cellulase production, for example Ryu *et al.* (1979) and Ghose and Sahai (1979). Studies on planktonic species (Conway *et al.*,

1976; Feuillade, 1979) and on Euglenid populations (Chisholm *et al.*, 1975) show further possibilities for continuous culture.

It has also been used for the cultivation of higher plant cells (Kurz, 1971; Wilson *et al.*, 1971; Bertola & Klis, 1979) and also for mammalian cells (Pirt & Callow, 1964) in the study of RNA tumor viruses (Manly, 1975) and for the production of interferon (Tovey *et al.*, 1973).

Thus it can be seen that the chemostat continuous flow technique is being widely used in many areas of microbiological research. One aspect not discussed is the use of chemostats in research into bacterial films, the importance of which in the natural environment was investigated by Jannasch and Pritchard (1972). However, further information can be found in a review on microbial films in fermenters by Atkinson and Fowler (1974).

1.3 ISOENZYMES

1.3.1 Definition

The terms isozymes (Markert & Møller, 1959) or isoenzymes (Wroblewski & Gregory, 1961) are used to describe a family of enzymes which have similar functions and are normally used to differentiate between enzymes expressed by the same organism, the earliest examples were described by Umbarger and Brown (1958). Brewer (1970) suggested that isoenzymes are multiple molecular forms of an enzyme derived from the same organism having at least one substrate in common. However, a number of different definitions have been proposed. Some favour a more restricted description, describing isoenzymes as those multi-enzymic forms which can be shown to be genetically independent proteins.

TABLE 1.4: Multiple forms of enzymes (from IUPAC-IUB, 1971)

Group	Reason for multiplicity	Example
1	Genetically independent proteins	Malate dehydrogenase in mitochondria + cytosol.
2	Heteropolymers (Hybrids) of two or more polypeptide chains, non-covalently bound	Lactate dehydrogenase
3	Genetic variants (allelic)	Glucose 6-phosphate dehydrogenase in man
4	Proteins conjugated with other groups	Phosphorylase a and b
5	Proteins derived from one polypeptide chain	The family of chymotrypsins arising from chymotrypsinogen
6	Polymers of a single subunit	Glutamate dehydrogenase of m.w. 1,000,000 and 250,000
7	Conformationally different forms	All allosteric modifications of enzymes

However, the frequent occurrence of slight modifications in the primary structure of proteins can lead to difficulties in nomenclature. Dixon *et al.* (1979) queried the number of amino acids which can be substituted before a protein, classed as an isoenzyme, has to be described as a different protein.

In 1971 the International Union of Biochemistry Commission in Biochemical Nomenclature published their recommendations on the nomenclature of multiple enzymic forms (IUPAC-IUB, 1971) and recommended that the term isoenzyme (or isozyme) should only apply to those multiple forms of enzymes arising from genetically determined differences in primary structure, and not to those derived by the modification of the primary sequence. By this definition those enzymes in groups 1, 2 and 3 of Table 1.4 are true isoenzymes and the others, derived by post-translational modification, are secondary isoenzymes (Dixon *et al.*, 1979).

1.3.2 The causes of enzyme multiplicity

As indicated in Section 1.3.1, the causes of enzyme multiplicity can be divided into two categories. The primary-genetic causes, in a diploid genome, can result from expression of multiple alleles or multiple loci. The former produces either one type of subunit, if the individual is homozygous for the allele, or two, if the individual is heterozygous. Such enzyme multiplicity produces only two isoenzymes in a given individual. However, from one individual to another a considerable range of subunits can be produced from a variety of alleles in the gene pool (Rider & Taylor, 1980).

Multiple genetic loci can be present in diploid and haploid genomes and this is the usual reason for genetically based isoenzymes in

prokaryotes. Each locus produces a different enzyme, or subunit of an enzyme, and expression of each can be controlled independently. In a multicellular organism, this can lead to given subunits being produced at different rates in individual cells enabling the organism to produce an enzyme with specific characteristics tailored to suit the requirements or conditions in a cell or type of tissue (Ferdinand, 1976). The best example, and one of the most studied set of isoenzymes, that of lactate dehydrogenase, a tetrameric enzyme, composed of two or three subunit types (Markert & Ursprung, 1962; Dietz & Lubrano, 1967; Pappas & Rodrick, 1971; Markert, 1975; Agatsuma & Takeuchi, 1976; Agatsuma & Tsukii, 1980; Rider & Taylor, 1980). The first family of catalytic enzymes in bacteria, produced by two distinct genes, found in *E. coli* K-12, was ornithine carbamoyl transferase which was shown to be under dual genetic control by Legrain *et al.* (1972). However, in haploid organisms multiple loci cannot account for different isoenzymes from one member of a species to the next, as each would possess the same loci. However, such differences could arise by mutation of the loci by amino acid substitution (Section 1.4). Secondary isoenzymes (Dixon *et al.*, 1979) are derived by post-translational modification of a single protein. The modification may or may not have biological significance, but can lead to identification as separate isoenzymes by various separation techniques.

Modification is achieved in many ways including the addition of carbohydrates or other compounds. Alkaline phosphatase isoenzymes, identified by electrophoretic mobilities, have been shown to contain different amounts of neuraminic (sialic) acid, which produce different

net charges on the proteins (Wilkinson, 1970). Several enzymes of the glycogen pathway are modified by phosphorylation and dephosphorylation as part of the metabolic control mechanisms (Dixon *et al.*, 1979). Proteolysis from the N-terminal end of the polypeptide chain causing the loss of a length of the chain or the loss of one or two amino acids can produce isoenzymic patterns. Further modification can also be achieved by alterations in amino acid side chains. Aldolase isoenzymes are encoded by three genetic loci. However, the product of the A locus is found as two subunit types, A_{α} and A_{β} , in vertebrate skeletal muscle. The product of the locus is represented by A_{α} which is slowly converted to A_{β} by deamination of an asparagine residue near the carboxyl terminus. (Midelfort & Mehler, 1972; Horecker, 1975).

Chlorinesterase appears to consist of separate aggregates of polypeptide chains (Wilkinson, 1970). Such polymerization has been shown to produce isoenzyme forms of amylase and glutamate dehydrogenase. Although Sundaram and Fincham (1964) suggested that isoenzymes of glutamate dehydrogenase in *Neurospora crassa* resulted from conformational differences in the structure of the enzyme, conformationally different forms, that is, conformers (Dixon *et al.*, 1979) are difficult to identify due to the restricted nature of the differences and the ease of interconversion. These changes are probably essential for enzymic activity, but have gained little support as far as being a cause of multiple enzymic forms.

The term isozyme has been suggested (Markert, 1975) as a general description of multiple enzymic forms however they are produced.

Once the reason for the production has been determined specific terms such as; allelic, non allelic, homopolymeric, heteropolymeric,

epigenetic, conjugated, should be used to describe the isoenzymes further.

1.3.3 Apparent enzyme multiplicity

Isoenzymes are frequently distinguished by electrophoretic studies and the use of the zymogram technique, developed by Hunter and Markert (1957) provides a simple method of identification. However, such techniques can lead to the production of artefacts. The unnatural ion concentrations may lead to non-specific binding of ligands. Proteolysis, resulting from the liberation of proteolytic enzymes on disrupting the cells, during storage or other forms of degradation can lead to apparent detection of multiple enzymes. If the term isoenzyme is restricted to just genetically encoded proteins then electrophoretic identification becomes more difficult. For example, alkaline phosphatase from *Escherichia coli* produces three bands on electrophoresis. This enzyme is a dimer, the subunits being products of the same gene. It is the post-translational conjugation with carbohydrates to differing degrees which leads to the multiple forms (Scrimgeour, 1977).

Apparent isoenzymes of rabbit muscle phosphoglucose isomerase were shown to result from the oxidation of available sulphhydryl groups and were classed as pseudo-isoenzymes (Blackburn *et al.*, 1972). The interconversion between three forms by sulphhydryl group oxidation or reduction, using dithiothreitol, indicated the presence of a single protein in different states. Hopkinson (1975) observed several isoenzyme systems and found a progressive change in electrophoretic mobility when extracts were stored at 4°C. On storage the overall anodal mobilities increased although the relative staining intensities

remained constant. It was found that the mobilities returned to the original pattern exhibited by fresh extracts if sulphydryl reducing agents, mercaptoethanol or dithiothreitol were present. Hopkinson (1975) suggested that the use of such thiol reagents would prove useful in the study of isoenzymic systems.

A different class of isoenzymes was suggested by Noltman (1975) to explain the results obtained with three isoenzymes of yeast phosphoglucose isomerase. These isoenzymes have been shown to be genetically determined, unlike the rabbit muscle enzyme (Blackburn *et al.*, 1972), yet all their properties, except for electrophoretic mobility, appear largely the same. Noltman (1975) suggested the term 'charge isoenzymes' to indicate the differences. The effects described so far result in an overestimate of isoenzyme involvement. However, the zymogram method can also result in an underestimate. Only a third of the possible base substitutions result in amino acid changes which cause alterations in the net charge of the protein (Lyman-Allen & Meremiuk, 1971).

Markert and Møller (1959) highlighted the problem of determining the existence of true isoenzymes when studying enzymes with broad substrate specificities, as for example with the esterases, in which each is probably a member of a different family of enzymes which have distinct, but overlapping, substrate specificities. Such enzymes cannot be classed as isoenzymes. They represent an example of the grey area in the definition of isoenzymes (Section 1.3.1).

Other procedures used to study isoenzymes involve the selective inactivation of each isoenzyme. These methods include inactivation by heat, urea and related compounds and EDTA, and have been discussed by

Moss (1979). Urea and guanidine hydrochloride were used by Withycombe *et al.* (1965) while investigating organ specificity of lactate dehydrogenase. Urea was shown to inhibit differentially lactate dehydrogenase dependent upon its source, either ox liver, heart or human heart. Sanford *et al.* (1981) used anionic and cationic surfactants to selectively inactivate lactate dehydrogenase isoenzymes, and found that one homopolymer was inactivated by the anionic, the other by the cationic surfactant.

Experimental procedure can also give rise to misleading results due to the unphysiological nature of the assay methods. Often substrate concentrations greatly in excess of those found *in vivo* are used to obtain optimal activities *in vitro*. Such high concentrations may lead to the substrate being altered by an enzyme for which it has a low affinity, which under normal physiological conditions would not act on the substrate. So under the experimental conditions the low affinity enzyme(s) could appear as part of a multienzymic system (Rider & Taylor, 1980). Suppressor mutations have been shown to be overcome by the presence of high substrate concentrations (Jensen, 1976). These conditions may also lead to misinterpretation of studies on mutated enzymes which *in vivo* may have deleterious effects yet *in vitro* work equally as well as the original enzyme.

1.3.4 The biological significance of isoenzymes

Enzyme polymorphisms provide cells with a further ability to adapt to their changing environment, over short time intervals (Gillespie & Kojima, 1968). As substrate concentrations are usually low under physiological conditions, the enzyme with the greatest affinity for the

substrate will determine the rate of reaction. Thus, as the substrate affinity, K_m , for each enzyme depends upon the physiological environment, a change in conditions, may result in a significant rise in the K_m of the rate determining form, but a decrease in the K_m of an alternative form, so the alternate form becomes the rate limiting enzyme. The role in adaptation to the changing environment is exemplified by the isoenzymic patterns in the rainbow trout. When acclimated to different temperature conditions, rainbow trout exhibit distinct warm and cold isoenzymes. (Somero & Hochachka, 1971). The warm isoenzymes exhibit their lowest K_m values (that is their highest affinity for the substrate) at temperatures greater than 10-12°C, whereas the cold isoenzymes function optimally at 2-5°C. Such isoenzymes of lactate dehydrogenase have also been demonstrated in the fish *Fundulus heteroclitus* (Powers & Powers, 1975). These isoenzymes allow the poikilotherms to adapt to seasonal changes in temperature.

Rhizobial nitrogen metabolism includes two isoenzymes of glutamine synthetase (Darrow & Knotts, 1977), although, the mechanisms of control differ markedly for each isoenzyme (Fuchs & Keister, 1980). One was controlled by adenylation in response to the level of ammonia, whereas the other was repressed by ammonia. This may represent a response to environmental levels of ammonia.

Lactate dehydrogenase isoenzymes exhibit different sensitivities to substrate inhibition. In regions where anaerobic glycolysis occurs, for example, a skeletal muscle, one homopolymer predominates, but where no lactate accumulates in the heart and the brain, the other homopolymer is present. As the various heteropolymers exhibit intermediary functions these may be found in different tissues.

Wilkinson (1970) suggested that the lactate dehydrogenase isoenzymes act as regulators in the oxidative and glycolytic pathways.

Isoenzymic control of metabolic functions is of great importance in branched chain (converging or diverging) metabolic pathways.

End product inhibition of an initial enzyme in a given pathway as a mechanism of feed back control (Umbarger, 1961), ensures that as levels of the product increase so the activity of the enzyme decreases. However, when one enzyme controlling a reaction common to more than one pathway is subject to endproduct inhibition by one of the products, the other pathway is also regulated and could lead to the organisms being starved of the second metabolite. To overcome this problem the common reaction can be controlled by isoenzymes, each under individual control by the individual end products of each pathway.

The first example of this was identified by Umbarger and Brown (1958) and other examples of such mechanisms are seen in *Escherichia coli* in the aspartate pathway which leads to the production of lysine, isoleucine and methionine. Each end product specifically inhibits one of three isoenzymes of aspartokinase. In this case these enzymes are not at the branching point in the pathway, but represent three separate branches running in parallel (Datta, 1969). Homoserine dehydrogenase, involved in the production of isoleucine and methionine demonstrates two isoenzymes each controlled by one of the end products. Acetohydroxy acid synthetase III, one of three isoenzymes in the *E. coli* K-12 pathway for biosynthesis of valine, leucine and isoleucine, was shown

to be inhibited by leucine (DeFelice, 1977; McCandliss *et al.*, 1978), whereas isoenzyme I was multivalently controlled by leucine and valine. Isoenzymes controlling the biosynthesis of aromatic compounds in *E. coli* have also been shown to be regulated by individual end products - tyrosine, phenylalanine and tryptophan (Doy & Brown, 1965; McCray & Herman, 1976; Jensen, 1976).

Multiple enzymes are also seen when their function can be either catabolic or biosynthetic, each enzymic form is controlled by different end products. However, the question arises as to whether functionally different forms should be classed as isoenzymes (Brown & Kline, 1975). *Neurospora crassa* possesses two dehydroquinases and two shikimate oxidoreductases. Normally one of each pair functions in a biosynthetic pathway the other pair in a catabolic one, but Giles and Case (1975) showed that under certain circumstances the two dehydroquinases could substitute for each other, suggesting functional similarities and so possible evolutionary relatedness. These may represent isoenzymes which have evolved into almost individual enzymes, as may be the case for the isoenzymes of a ketoglutamic semialdehyde dehydrogenase in *Pseudomonas putida* (Koo & Adams, 1974). Two aliphatic amidases in *Aloaligenes eutrophus* and their different regulatory controls lead Friedrich and Mitrenga (1981) to suggest that one, the formidase, was involved with nitrogen metabolism, whilst the other, the valeramidase, was concerned with carbon metabolism.

From the foregoing it is obvious that a large number of isoenzyme systems have been investigated in organisms across the whole evolutionary spectrum and there is an increasing awareness that enzyme multiplicity is the rule rather than the exception. The frequent

occurrence of small modifications in the primary structure of proteins often gives rise to problems in nomenclature, for it has not been determined just how many amino acid substitutions are required to change one protein to another protein which cannot be considered an isoenzyme.

Characterization of a particular multienzyme system involves both genetic and biochemical studies which can class enzymes as primary or secondary isoenzymes. The active centre of the isoenzymes necessarily must remain immutable so that its activity can be maintained. Dixon *et al.* (1979) believe that as the number of known forms of an enzyme increases it may be necessary to name the enzymes according to their particular active centres.

The question as to whether multienzymic systems allow populations to adapt to their environment has stimulated a great deal of discussion. Many advocate a form of selection to balance the polymorphism, while others argue that the observed polymorphisms are selectively neutral. The use of isoenzymes as genetic markers has proved useful in the study of the evolution of metabolic functions and provides a model for the investigation of control mechanisms.

1.3.5 The neutralist-selectionist controversy

There are a number of theories as to how metabolic and catabolic pathways developed and all have been used to explain the occurrence of enzyme polymorphism. Two contrasting views exist as to the reason for the existence of polymorphic enzymes.

Firstly, there is the concept of neutral or near-neutral mutation and random genetic drift which involves no selection pressures and has been called non-Darwinian evolution (Scandalios, 1975). This represents

the neutralist point of view.

The second theory attributes polymorphism to balancing selection, such that enzyme polymorphism is only maintained if it confers a selective advantage by making the organism more competitive.

The maintenance of polymorphism should, in theory, entail evolutionary cost, in terms of the genetic load. Thus it would be expected that the number of polymorphisms in a natural population would not be great, as an excessive genetic load would lead to extinction (Johnson 1974). However, as described above (Section 1.3.2) a great number of polymorphisms have been demonstrated and one explanation is that the electrophoretic variants contain only minor differences in their tertiary structure, which affect mobility, but not function and are hence selectively neutral. The existence of selectively neutral polymorphisms in a population would depend entirely on random genetic drift, some would be eliminated, others maintained at low frequencies. Occasionally some would be spread fortuitously and achieve higher frequencies in the population producing polymorphic forms of the protein within a population (Harris *et al.*, 1977).

The neutralist hypothesis does not exclude deleterious mutations, which would be eliminated or kept at a low frequency, or advantageous mutations, which are regarded as uncommon compared to neutral mutations but which would be maintained.

Evolutionary pressures act on an organism by affecting the products of many interacting genes. The genotype is the target but environmental pressures act to select the phenotype most fitted to the environment (Lyman-Allen & Heremiuk, 1971). The selectionist's hypothesis of

polymorphism is that most mutations are deleterious to enzymic function and hence to the fitness of the organism and so they are eliminated, or kept at a low frequency, by natural selection. Occasionally a mutation may occur which confers some selective advantage to the organism in the given environment and will consequently be spread through the population as the organism's chance of survival in the competitive environment is increased due to its increased ability to utilize the resources (Krebs, 1981).

Johnson (1971) suggested that if selection acts to maintain polymorphism then enzymes controlling rates of pathways would be most sensitive as sites of action for selective forces and if such action maintained polymorphism, then it would be enhanced at such control points.

As described (Section 1.3.4), enzyme polymorphism associated with rate-limiting reactions indicate a selective advantage for organisms possessing such polymorphisms. Such observations cannot indicate selectively equivalent forms of a gene (Johnson, 1974).

The balance theory (Powell, 1975) represents a third hypothesis to explain the existence of polymorphic proteins. It allows for greater variability by various forms of balancing selection. It suggests evolution is not controlled by stochastic or deterministic processes but by a combination of both.

1.3.6 Isoenzymes and phylogeny

A study of isoenzymes exhibited by different taxonomic groups has enabled a study of the evolutionary relationships of different genera and species. Lactate dehydrogenase is a commonly used system as it has

been widely studied. Agatsuma and Tsukii (1980) used electrophoretic studies of these isoenzymes to examine different syngens of *Paramecium caudatum* and Borden *et al.* (1977) used a number of isoenzyme forms of eight enzymes in their studies on *Tetrahymena* species. The esterases and acid phosphatases of ten syngens of *T. pyriformis* were used in phylogenetic studies by Corbett (1970) and Lyman-Allen and Weremiuk (1971).

Bacterial phylogeny has been based more on differences in regulatory control of isoenzymic systems. Durham *et al.* (1980) and Durham and Ornston (1980) studied the enzyme protocatechuate 3,4-dioxygenase in *Azotobacter*, *Pseudomonas* and *Acinetobacter* species and concluded that they were all derived from the same ancestral gene. The regulatory control of the β -oxoadipate pathway show differences in evolution, reflected in different regulatory control mechanisms, *P. aeruginosa* and *P. putida* are closely related and show similar regulatory mechanisms (Ornston & Parke, 1976). The catabolism of aromatic compounds has also been used by Dagley (1971) in studying relationships in *Pseudomonas* strains, and *Moraxella calcoacetica* and demonstrated entirely different control mechanisms in the different genera. The regulatory controls in the three *Pseudomonas* spp. studied were very similar.

1.4 THE EVOLUTION OF BACTERIAL ENZYME SYSTEMS

A great deal of experimental data on evolution of enzyme systems was presented by Hegeman and Rosenberg (1970), and Markert *et al.* (1975) used the lactate dehydrogenase isoenzymic system as a model of the evolution of gene structure, function and regulation.

1.4.1 Retroevolution

The earliest theory proposed to account for the evolution of biosynthetic pathways was that of Horowitz (1945). The hypothesis of retrograde evolution suggested the step-wise and sequential recruitment of enzymes in reverse order and depended on the assumption that each intermediate of the backwardly evolving pathway was readily available in the prebiotic environment. If the pathway evolved from the beginning it would not be selectively advantageous until the whole pathway was complete. However if evolution of the pathway occurred in reverse, one step at a time towards the initial reaction, then each mutation would confer a selective advantage since a new, useable end product would be available for growth (Horowitz, 1965). Major problems with this theory concern the extreme lability of many of the intermediates, as well as the barrier to their transport in the absence of specialised transport systems. The evolution of catabolic pathways, cannot^b be accounted for by this hypothesis (Jensen, 1976).

1.4.2 Gene duplication

Horowitz (1965) evoked the theory of E.B. Lewis, that the origin of new genetic material involved tandem duplication followed by functional differentiation. Many aspects of gene duplication, including the mechanisms involved have been discussed by Ohno (1970). New genetic material is created by gene duplication by a variety of mechanisms, for example transposition to give a second copy of the gene, tandem duplications due to unequal crossing over or mini-insertion-like duplications (Cullum & Saedler, 1981) or frame shift mutations (Ohno, 1970). One of the resulting genes is redundant and is thus free of selectional pressures which prevented the parental gene from changing.

A mutation resulting in the change of a residue directly involved in catalysis would cause loss of function, which in most cases would always be lethal (Watts & Watts, 1968a; Kimura & Ohta, 1974). Whilst one of the daughter genes provides a viable phenotype, the other provides genetic material which can undergo mutation to produce new enzymes, with different capacities, although they are initially related to the original gene. Under different environmental conditions the mutated gene product may again become active and provide the organism with a new gene product.

The existence of silent or cryptic genes has been demonstrated in a number of cases. Campbell *et al.* (1973) deleted the *lac Z* for β -galactosidase in *Escherichia coli* strain K-12 and selected for reacquisition of activity and found a new enzyme which possessed different kinetic properties to the *lac Z* product. The new gene, *ebg*, demonstrated no close ancestry to the *lac Z* gene and no evidence has been found to show β -galactosidase polymorphism in *E. coli* under normal circumstances. The *ebg* locus may represent a silent gene or a multistep mutation of a gene to produce the new enzyme to hydrolyse lactose. Syvanen and Roth (1972) demonstrated the redundancy of a second ornithine transcarbamylase gene in *E. coli* K-12 separated from the active gene by 14 min on the *E. coli* genome map. Schaeffler and Malány (1969) investigated the expressed and cryptic phospho- β -glucosidases in *Enterobacteriaceae* and suggested their use in studying evolutionary relationships. The position of duplicate genes relative to each other would vary according to the method of duplication (Watts & Watts, 1968b). Duplication can occur either by small changes in segments of the chromosome or by duplication of the whole chromosome, polyploidization in higher plants

represents a form of duplication. Zipkas and Riley (1975) proposed that the genome of *E. coli* underwent two sequential duplications; 90° and 180° relationships between functionally related genes, they believed, were too great to be due to chance alone. Gene pairs giving rise to isoenzymes of malate synthetase, glycerol phosphate dehydrogenase and phosphotransferase system enzymes are located at approximately 90° from each other. Those isoenzymes not possessing this relationship were believed to represent examples of convergent evolution. Sequence analysis of a number of proteins indicated that gene duplications have occurred fairly frequently in evolution.

The ability to perform a new function is unlikely to be associated with a great change in structure as this is more likely to lead to complete loss of function, due to incompatibility with the protein's tertiary structure. Rigby *et al.* (1974) and Watts and Watts (1968a) cite Annelid phosphagen kinases as such an example. They all have one type of catalytic site but their functions are altered by mutations of amino acids in other parts of the molecule associated with the following: substrate binding, specificity towards the substrate, conformational changes associated with catalytic activities and those amino acids associated with maintaining the tertiary and quaternary structure of the enzyme.

Mutants of *Pseudomonas aeruginosa* have been isolated which can utilize novel amides which could not support growth of parental strains. Betz *et al.* (1974) showed that this was the result of a number of point mutations in the amidase gene of the bacterial strains.

Freese and Yoshida (1965) believed that enzymes with very different

specificities, but possessing a number of features in common, including cofactor requirements, may also indicate genes of common ancestry.

The importance of gene duplication in evolution has been questioned by a number of experimentors. Horiuchi *et al.* (1962a & b) produced hyper constitutive mutants of *Klebsiella aerogenes* with duplicated genes but the system was unstable and the duplication was readily lost when cultures were removed from the chemostat. Other evidence, reviewed by Omston and Parke (1976), indicated that duplicated structural genes were unstable within bacteria and were lost with a frequency as high as 1.0×10^{-2} per cell plated.

It is possible that if the duplicated genes could be maintained long enough for point mutations to occur then the gene's stability would be increased. However, Wu *et al.* (1968) believed that although gene duplication followed by divergence could account for the appearance of multicistronic operons encoding for proteins involved in homologous functions, such a mechanism could not account for metabolic sequences involving dissimilar reactions.

1.4.3 Derepression as a mode of evolution

In many cases the selection of altered enzymic activity in laboratory experiments involved an initial mutation in the regulatory genes (Clarke, 1978). Almost always this involves the derepression of an enzyme. It has been shown that under such conditions these enzymes are capable of acting upon new substrates, which are chemically related to the normal substrate but could not induce the enzyme. Such substrate ambiguity in itself provides a mode of evolution.

Mutations that lead to constitutivity may result from the

inactivation of a repressor protein and could occur more often than mutations which change the structure of regulatory proteins so that novel substrates could induce enzyme synthesis. Wu *et al.* (1968) believed that derepression as a first step in the adaptation to a new substrate was more likely than mutation of the enzyme directly. Once derepressed, a further modification may occur which increases the efficiency of the new function and then, as the cistrons' utility has been increased, subsequent gene duplication could increase the amount of protein. In this way adaptation to a new demand would be achieved without necessarily having to sacrifice old functions. Unlike primary duplication of genes, constitutive mutations appear to be stable and Ornston and Parke (1976) suggested that two steps are required for the evolution of new regulatory genes. Firstly a mutation inactivates the original regulatory gene which results in a constitutive product, then further mutation(s) reassert the inductive control by the new substrate.

Mutants of *Klebsiella aerogenes* which had acquired the ability to utilize the novel pentitol xylitol, by oxidation to D-xylulose, were found to produce ribitol dehydrogenase and D-ribulokinase constitutively (Wilson & Mortlock, 1973). The former catalysed the oxidation of xylitol to D-xylulose and provides an example of substrate ambiguity which was not possible until derepression had occurred. Izumori *et al.* (1980) demonstrated the derepression of a non-specific pentose reductase which enabled *Mycobacterium smegmatis* mutants to utilize unnatural pentose sugars. Brown and Clarke (1970) isolated mutants of *Pseudomonas aeruginosa* strain 8602 which were able to utilize butyramide as a growth substrate and demonstrated that one

group of mutants produced the amidase constitutively allowing them to produce large amounts of amidase in the presence of the non-inducing butyramide. It should be noted that a second class of mutants were thought to represent a class of mutants altered in the amidase structural gene, as the substrate specificities had altered, resulting in increased activity towards butyramide.

Another example of mutation causing constitutive enzyme production enabled *Escherichia coli* mutants to utilize putrescine as the sole carbon and nitrogen source, the enzyme involved was putrescine α -ketoglutarate transaminase (Kim, 1963).

The subsequent formation of a new control mechanism (Ornston & Parke, 1976) was cited as the reason for the presence of two genes for lysozyme in the Embden goose, a 'Chick type' and a 'Goose type'. Yet each showed tissue specificity even though all tissues had both genes. Arnheim (1975) suggested that the expression patterns may have evolved during the evolution of the two genes from a common ancestor.

1.4.4 Substrate ambiguity - the fortuitous error concept

Substrate ambiguity can be further involved in the evolution of a new metabolic pathway in that an enzyme already present, active towards the novel substrate, however slowly, may be recruited into a preexisting multistep sequence enabling the new substrate to be metabolized. In some cases the evolution of a single enzyme may initiate a new multistep pathway, which is important as the breakdown of a substrate requires a whole pathway and not just one enzyme (Clarke, 1974). The fortuitous error concept of enzyme recruitment (Jensen, 1976) dispenses with the problems associated with the retrograde evolution theory. It permits

individual alterations in enzyme capabilities to be of selective value by allowing such mutants to be of immediate use to the organism by bridging existing pathways to produce erroneous new pathways.

If enzyme recruitment, to produce a new pathway, were involved the corresponding genes are likely to be dispersed around the genome. Translocation could collect them into a single operon if selective pressures were present, which could explain how the genes for the metabolism of aromatic acids in *Pseudomonas aeruginosa* (Rosenberg & Hegeman, 1969) and *P. putida* (Leidigh & Wheelis, 1973) are clustered more than would be expected solely on the basis of regulatory requirements.

Thus new enzyme functions may be established most readily by recruitment of proteins already catalysing analogous reactions. An hydrolase may mutate to a protein with sufficient hydratase or aldolase activity to allow a new pathway to function (Jeffcoat & Dagley, 1973). The *E. coli* *ebg* gene function after deletion of the *lao Z* gene (Campbell *et al.* 1973) may represent an example of this. Wu *et al.* (1968) cite the example of *Aerobacter aerogenes* strains PRL R3 and 1033 in which a single mutation is sufficient to permit growth on xylitol. The mutation derepresses ribitol dehydrogenase which can metabolize xylitol (Wilson & Mortlock, 1973) to xylulose which is an intermediate in the D-arabitol pathway. Thus the utilization of a new carbon source required the participation of enzymes in two pre-existing pathways.

The utilization of L-1,2-propanediol by *Escherichia coli* has been demonstrated to be made possible by the recruitment of enzymes

from the L-fructose and L-lactate metabolic pathways (Cocks *et al.*, 1974). The enzyme lactaldehyde reductase which normally functioned during anaerobic growth on L-fucose mutated to an enzyme catalysing the reverse reaction, acting as a dehydrogenase during aerobic growth on L-1;2propandiol. The genetic loss of a second enzyme of a normal pathway in *Pseudomonas putida* enabled the mutant strain to utilize phenol by another inducible pathway normally used for the metabolism of benzoic acids (Feist & Hegeman, 1969).

Translocation as a method of collecting together genes into a single operon has been discussed, but other genome rearrangements could in themselves lead to new metabolic capabilities. Fusion of new promoters to structural genes would modify their expression. Cullum and Saedler (1981) described the *E. coli* K-12 *gal* operon fusing with a new promoter, initiated by IS1, resulting in constitutive expression of the operon. They suggested insertion sequences could mobilize genes as new transposons, and move them to different parts of the genome, so collecting together genes of a pathway, or creating new systems. Cornelis *et al.* (1978) put forward the idea that the *E. coli* *lac* operon arose as a result of such fusion events. Genome rearrangements of this type could allow rapid evolution of new functions and control mechanisms by the rearrangement of genetic material and the association with the new combination of genes and a promoter so modifying their expression.

1.4.5 Summary of the adaptative mechanisms in evolution

Possibly all of the foregoing mechanisms are, or have been, involved in the evolution of the metabolic pathways seen in the microorganisms which currently exist.

It is assumed (Jensen, 1976) that primitive enzymes exhibited broad specificities, so maximizing the catalytic versatility of an ancestral organism which functioned with limited enzyme resources. Such substrate ambiguity would have produced a number of minor products which although fortuitously formed, may have been useful to the organism, and would have conferred a selective advantage on organisms capable of increased production of those compounds.

Gene duplication provides an immediate way of increasing the amount of any protein, so compensating for low activity of an enzyme towards a new substrate (Cullum & Saedler, 1981). No matter how rare the event, such multiplication would be selected for if the protein could produce more of the advantageous product. This has been observed in *Klebsiella* (Rigby *et al.*, 1974) for xylitol utilization and in cells for the production of ribosomal RNA which, while short in variety, has to be made in large quantities. With time, specialization by mutation of one of the duplicated genes, could further increase product formation. Lerner *et al.* (1964) suggested that with time the ribitol dehydrogenase (Wilson & Mortlock, 1973), which already possessed xylitol dehydrogenating capability, if in an environment rich in xylitol, may become increasingly efficient as a xylitol dehydrogenase, at the expense of its activity towards ribitol. Gene duplication would allow both functions to be maintained and the evolution of a xylitol-inducing mechanism would save synthetic function so being selectively advantageous.

Hence, substrate ambiguity and gene duplication followed by mutation could have lead to increased genetic material of the organism. However, gene duplication may result in the organism entering an evolutionary cul-de-sac (Koch, 1972) as mutation in a single gene would eventually no

longer afford a high selective advantage. Also potentially lethal mutations may render the duplicate copies inactive, thus although the original copy can continue to function the inactive genes would represent a genetic load of no value. The greater the number of duplications, the greater the chance of an excessive build-up of useless genetic material (Ohno, 1970).

At a later time the substrate which was limiting may become no longer so, thus the genes will serve a passive role and may be subjected to non-selective mutation, which could produce potentially superior enzymes or new ones. If the substrate again became limiting the organism possessing the potentially superior enzyme would out-compete the antecedent forms. The original enzyme would be vestigial so could undergo mutation or deletion decreasing the genetic load (Hegeman & Rosenberg, 1970; Johnson, 1974). The effect of loss of biosynthetic function on microbial evolution has been discussed by Zamenhof and Eichdorn (1967).

As the number of enzymes in an organism increased, by gene duplication, so the organism would be able to evolve enzymes with increased specificity and hence increased metabolic efficiency. Regulation, to avoid unnecessary metabolic activity would be selected for. The evolution of regulatory control under different conditions may be considered by comparing the regulatory mechanisms of coliform bacteria and soil pseudomonads. In general pseudomonads apparently have less complex and less effective regulatory control of biosynthetic mechanisms than coliforms. This possibly reflects the nutritionally rich environments in which coliforms exist. The coliforms inhibit production of metabolites

when they are available in the environment (Harder *et al.*, 1977). However, the soil bacteria possess complex mechanisms to govern their extensive catabolic pathways which again may reflect the organism's growth environment.

Regulatory enzymes catalyse the rate limiting reactions in a given pathway, hence even minor alterations in their properties will have an effect on the metabolic flux of the pathway concerned. The development of separate regulatory controls for each isoenzyme form would allow the organism to produce the most effective isoenzyme for the prevailing environmental conditions. In multicellular organisms isoenzymes allow tissue specificity and differential expression during ontogenetic development.

Once such a position has been achieved differential use would mean each gene would be exposed to different selection pressures. Thus the isoenzymes would gradually diverge by the accumulation of different mutations. So finally, although acting on the same substrate, the isoenzymes would acquire individual properties.

The evolution of metabolic regulation has recently been reviewed by Baumberg (1981) and the duplication of regulatory genes by Ohno (1970).

Increased availability of novel substrates would select for organisms capable of utilizing a greater range of substrates, thus development of enzyme polymorphism by gene duplication would be selectively advantageous. However, the role of duplication in evolution of metabolic pathways today, according to experimental data, is not as great as it possibly was in ancestral organisms (Hegeman, 1972).

With the great range of enzymes available to an organism the role of substrate ambiguity, enabling recruitment of enzymes from existing pathways may be to allow utilization of novel substrates. With gene duplication now regulating the specific activity of existing genes by the gene **dosage** effect.

When considering evolution of a new metabolic pathway it is also necessary to remember that before a new pathway can act on a substrate it must be transported into the cell. Some apparent gains in ability to utilize a substrate result from mutations which change cellular permeability. (Hegeman & Rosenberg, 1970).

One mechanism for the acquisition of a new genetic material not so far discussed is that of transfer of genetic material from one organism to another. The role of plasmids in evolution and the increased metabolic diversity of an organism will be discussed in Section 1.5.

1.5 PLASMIDS

1.5.1 Plasmids: An introduction to their concept

Thirty years ago Lederberg (1952), proposed the term plasmid as a generic term for any extrachromosomal hereditary determinant. Plasmids are closed circular double-stranded DNA molecules existing in bacteria, which replicate independently of the chromosome and are inherited by both daughter cells on cell division. Although plasmids have mostly been studied in bacteria, analogous entities do exist in eukaryotes.

The first transmissible plasmid to be identified was the F factor of *E. coli* K-12, which was recognised by its ability to transfer parts of the host chromosome. However, the majority of plasmids were initially

TABLE 1.5: Examples of bacterial plasmids (after Broda, 1979 and Clarke 1981)

Plasmid	Size (Megadaltons)	Host	Phenotype Determined
Col E1	4.2	<i>E. coli</i>	colicinogeny
λ dv	8.6	<i>E. coli</i>	lambda immunity
p1258	19.0	<i>S. aureus</i>	resistance to antibiotics and heavy metals
OCT	27.0	<i>P. putida</i>	octane utilization
RP.4	36.0	<i>P. aeruginosa</i>	Cb Km Nu Tc resistance
SAL	51.0	<i>P. putida</i>	salicylate utilization
Ent	53.0	<i>E. coli</i>	enterotoxin
R1	62.0	<i>S. typhimurium</i>	ApCmKmSmSu resistance
F	63.0	<i>E. coli</i>	sex factor
TOL	78.0	<i>P. putida</i>	toluene utilization
Ti	95-156	<i>Agrobacterium tumefaciens</i>	plant tumours
R46) Col 1b)		<i>E. coli</i>	UV. resistance

recognised by a change in phenotype, conferred by their presence, followed by the discovery that the genes responsible were located on a plasmid. Some of the properties encoded by plasmids are shown in Table 1.5:

In the rest of this section it is proposed to discuss the phenotypes conferred on bacteria by the possession of catabolic or degradative plasmids. Further information on the genetics, structure, epidemiology and biochemistry can be found in: Clowes (1972); Willetts (1972); Chakrabarty (1976); Cohen (1976); Bennett and Richmond (1978); Broda (1979) and Holloway *et al.* (1979).

1.5.2 Degradative plasmids

Genes of all essential biosynthetic activities are located on the chromosome. However, the range of compounds microorganisms can catabolize, especially members of the *Pseudomonas* genus, would require more genetic information than is present in the bacterial genome. Around 1970 Gunsalus and colleagues discovered that some of the more esoteric catabolic functions in pseudomonads were unstable. They could be irreversibly lost and the function conjugationally transferred to different strains. The SAL plasmid (Chakrabarty, 1972) coded for the degradation of salicylate, CAM plasmid (Rheinwald *et al.*, 1973) carried the genes for camphor utilization and others for the degradation of naphthalene (Dunn & Gunsalus, 1973), octane (Chakrabarty *et al.*, 1973), toluene, m-xylene and p-xylene (Williams & Murry, 1974) have been identified.

Catabolic plasmids, although particularly abundant in the *Pseudomonas* group, do occur in other organisms. Sucrose and lactose fermentation by *Salmonella* and *Klebsiella* strains may be dependent on

plasmid presence (Reeve & Braithwaite, 1974; Wohlhieter *et al.*, 1975) and a number of plasmids conferring the ability of lactose utilization upon various Enterobacteriaceae have been identified and their expression and regulation studied (Guiso & Ullmann, 1976). Hydrogen metabolism by *Alcaligenes eutrophus* requires the presence of a large (200 Md) plasmid which was shown to confer the autotrophic ability on hydrogenase-minus strains (Andersen *et al.*, 1981).

Agrobacterium tumefaciens the causative agent of crown-gall in plants carries a plasmid responsible for its pathogenicity and also for a catabolic enzyme which converts octopine (or nopaline) to products which serve as carbon and nitrogen sources for the bacterium (Montoya *et al.*, 1977). Several plasmids found in virulent strains of *Agrobacterium rhizogenes* the causative agent of hairy-root in dicotyledenous plants, were compared by Constantino *et al.* (1980) Kawasaki *et al.* (1981a) isolated a plasmid bearing strain of a *Moraxella* species which was capable of dehalogenating fluoroacetate. The enzyme involved, haloacetate halohydrolyase, was shown to be coded for on the pU01 plasmid (Kawasaki *et al.* 1981b). Kiel *et al.* (1981) identified a plasmid associated dehalogenation enzyme which enabled *Pseudomonas* sp CBS3 to utilize 4-chlorobenzoate.

The possible importance of degradative plasmids in the environment, to enable the biodegradation of xenobiotic compounds was discussed by Chakrabarty (1978), and put into practice by Reineke & Knackmuss (1979) when they constructed a novel strain of *Pseudomonas* sp B13, by transferring the TOL plasmid from *P. putida* mt-2 (WR101) which could utilize various chlorosubstituted benzoates as its sole source of carbon and energy. However, the evolution of such a system may already be

represented in the natural environment by a plasmid bearing strain of *Aloaligenes paradoxus* (Pemberton & Fisher, 1977; Fisher *et al.*, 1978). Evolution of the degradative ability of this plasmid, following apparent gene duplication and mutation (Section 1.4.2) was observed by Pemberton *et al.* (1979). This resulted in plasmid bearing strains being able to utilize both phenoxyacetic acid and 2,4-dichlorophenoxyacetic acid. Further work by Don and Pemberton (1981) resulted in the isolation of six pesticide degrading plasmids from *A. paradoxus* and *A. eutrophus*.

Degradative plasmids may code for complete pathways or partial degradation. An example of the former is the degradation of toluene and xylene (Worsey & Williams, 1975; Friello *et al.*, 1976). A plasmid termed pAC25, specifying the complete biodegradation of 3-chlorobenzoate by a strain of *P. putida*, isolated from sewage, was characterized by Chatterjee *et al.* (1981) which suggested that the laboratory evolution studies of Reineke & Knackmuss (1979) have taken place in the natural environment, producing new enzymes capable of degrading this compound, presumably in response to the widespread release of 3-chlorobenzoate and other haloaromatic compounds into the environment.

An example of an incomplete pathway encoded for by a plasmid is the naphthalene to salicylate pathway. Dunn and Gunsalus (1973) demonstrated the presence of a plasmid (NAH) in *P. putida*, Pp67 which coded for the early enzymes in naphthalene oxidation, however the *P. putida* strain PpG63, although degrading the substrate did not appear to possess a plasmid, in this case it was suggested the enzyme activities were encoded on the chromosome. Other partial degradative pathways include

2,4-D to 2,4-dichlorophenol (Don & Pemberton, 1981) and pCB to p-chlorobenzoate.

The NAH plasmid codes for the degradation of naphthalene to salicylate. Thus organisms possessing the SAL plasmid as well, can then convert salicylate to pyruvate and eventually completely mineralize it (Chakrabarty, 1972). Other plasmids such as CAM and OCT code for the breakdown of these substrates to isobutyric acid and aliphatic aldehydes, respectively, which can then be used by most pseudomonads using chromosomally coded enzymes (Farrell & Chakrabarty, 1979).

It is not proposed to discuss the detailed work that has been undertaken on the TOL plasmid, but recent work covering most of the details can be found in Williams and Worsey, 1976; Duggleby *et al.*, 1977; Broda *et al.*, 1979 and Williams, 1981.

A number of degradative plasmids have been shown to be plasmid aggregates. The OCT plasmid is an aggregate of a non-infectious OCT plasmid and an infectious plasmid, designated as K (Chakrabarty, 1974). The OCT and CAM plasmid cannot coexist in the same organism, they are incompatible, however Chou *et al.* (1974) described a situation where they can. They believed there are regions of homology between the two plasmids which allow recombination producing a cotransducing CAM-OCT plasmid which has become a single replicative system. Chakrabarty *et al.* (1978) provide a further example with the hydrocarbon degradative plasmids of *Pseudomonas putida*. By transferring the TOL plasmid (76 Md) from *P. putida* to *Pseudomonas aeruginosa* PAO the plasmid dissociates into a non-infectious toluene degradative plasmid TOL* (28 Md) and a transfer plasmid TOL Δ (48 Md). Chakrabarty and Friello (1974) demonstrated dissociation on transfer from one host to another.

The octane utilizing factor first recognised in *Pseudomonas oleovorans* dissociates into three independent plasmids, OCT, MER and K when the composite plasmid is transferred into *P. putida* (MER codes for mercury resistance). Such composite plasmids allow great versatility in bacterial biodegradation as many recombinations could occur *in vivo*.

1.5.3 Evolution of plasmid-associated catabolic pathways

The role of plasmids in evolution of microorganisms has been described by Bennett and Richmond (1978); Farrell and Chakrabarty (1979); Sakoguchi and Okanishi (1980); and Clarke (1981).

The evolution of metabolic pathways in bacterial populations (Section 1.4) could be greatly enhanced by exchange of genetic material between similar organisms. It provides a means of reducing the need for the whole population to possess genetic information which under given environmental conditions is of no value but may become selective after the conditions change. This is a way of reducing the genetic load (Section 1.4.5). Plasmid mediated transfer of catabolic enzymes' genes occurs freely within the *Pseudomonas* genus and between this and other genera (Chakrabarty *et al.*, 1973; Benson & Shapiro, 1978; Don & Pemberton, 1981).

Certainly plasmids coding for degradative or other functions appear to be ubiquitous in nature, of 13 strains of *Pseudomonas* isolated from soils by Williams and Worsey (1976) all appeared to carry TOL plasmids superficially the same as the *P. putida mt-2* plasmid (pWWO). Soil bacteria isolated from enrichment-cultures selecting for amidase activities apparently possessed a number of different plasmids (J.H. Slater, P.C. Gowland and D.J. Hardman, unpublished observations).

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Many of the hydrocarbons for which degradative plasmids have been identified are toxic to microorganisms, thus such plasmids may have evolved as a rapid means of detoxification under locally high toxic levels. The advantage of only a few organisms carrying the genes being the lack of an unnecessary genetic load when these compounds are at sub-inhibitory levels.

Dunn and Gunsalus (1973) discovered two strains of naphthalene degrading *P. putida* one which coded for the necessary enzymes by plasmid, the other by chromosomal genes. From an evolutionary standpoint, the question arises as to whether the ability to degrade the compound was of plasmid origin in both strains, but became integrated into the *P. putida* G63 strain or that NAH plasmid, or part of it, was once an integral part of the *P. putida* chromosome but was later excised.

Guiso and Ullmann (1976) found that the plasmid-coded lactose systems had many features in common with the *Escherichia coli lac* operon, and suggested that the plasmids could have acquired the lactose genes from an *E. coli* chromosome.

It is also possible for plasmid and chromosomal genes to function together. In this way isoenzymic forms of an enzyme could be produced. Hall and Reeve (1977) discovered a third β -galactosidase in a *Klebsiella* strain RE1544. This strain contains two *lac* operons, one on the chromosome and one on a *lac* plasmid. They also found a system similar to the *E. coli ebg* gene when both the known *lac* operons produced no active enzyme. Schmid and Schmitt (1976) demonstrated two α -galactosidases in *E. coli* K-12, one of which was plasmid encoded.

Degradative plasmids provide a metabolically cheap method for storing information, which, in the laboratory, is non-essential but can confer a selective advantage under certain conditions. They increase the metabolic potential of strains possessing them and provide a method of transfer of genetic material and by integrating and excising with bacterial chromosomes may transfer new combinations of genes so providing new pathways or possibly providing an additional enzyme to complete the formation of a new metabolic route by joining two pre-existing pathways.

CHAPTER 2

MATERIALS AND METHODS2.1 CONDITIONS FOR CULTURING THE BACTERIAL ISOLATES2.1.1 Batch Culture

The bacterial isolates were grown in batch culture using a defined growth medium (Slater *et al.* 1979), pH 7.0, which contained in g l^{-1} in double-glass-distilled water: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $(\text{NH}_4)_2\text{SO}_4$, 0.5; KH_2PO_4 , 0.5 and K_2HPO_4 , 1.5 plus 10 ml (l of defined growth medium) $^{-1}$ trace element solution containing in g l^{-1} double-glass-distilled water: NaEDTA, 12.0; NaOH, 2.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.4; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1; conc. H_2SO_4 , 0.5 ml; Na_2SO_4 10.0; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 and $\text{Fe}_3\text{SO}_4 \cdot 7\text{H}_2\text{O}$, 2.0.

This basal salts solution was placed in conical flasks in the following volumes: 50 ml in 100 ml flasks, 100 ml in 250 ml flasks and 500 ml in 2000 ml flasks and autoclaved at 15 lb sq.in $^{-2}$ for 15 min.

Stock solutions of the halogenated alkanolic acids (10% wv^{-1} , pH 7.0) filter sterilised (Gallenkamp filter holder) with 0.45 μ filters (Oxoid, Basingstoke, Hampshire), were added to the sterile basal salts solution to give a final concentration of 0.5g carbon l^{-1} .

After inoculation the flasks were incubated at 30°C on an orbital shaker (Gallenkamp, London) at 200 rev min^{-1} . Maintenance of cultures in batch was achieved by sub-culturing (1 to 5 ml) into fresh medium every 24 to 48 h. For enzyme studies cultures were grown in 500 ml volumes and inoculated with 25 to 50 ml of an overnight culture of the given organism.

2.1.2 Culturing on solidified media

Solid media were prepared using the defined medium, described

above, solidified with lab 'M' agar ($1.5\% \text{ wv}^{-1}$). The medium containing the agar was autoclaved, 15 lb sq.in^{-2} for 15 min, and one of the chlorinated substrates: monochloroacetic acid (MCA), dichloroacetic acid (DCA), 2-monochloropropionic acid (2MCPA) or 2,2-dichloropropionic acid (2,2DCPA), added to give a final concentration of $0.5 \text{ g carbon l}^{-1}$, immediately before pouring the medium into the Petri dishes.

Stock cultures were maintained in universal bottles with solidified defined medium, as described, supplemented with yeast extract (10 mg l^{-1} ; Hirsch & Alexander, 1960). The cultures were stored at 4°C and remained viable for more than a year. Sub-culturing was carried out infrequently in order to minimise changes in the isolates morphology and physiology by repeated sub-culturing and hence selection of fitter strains.

2.1.3 Chemostat continuous-flow culture

2.1.3.1 Continuous-flow culture system

The culture system utilised was a simple glass system which consisted of a Quickfit Pyrex glass culture vessel, 1.0l (Gallenkamp, London) fitted with a glass lid possessing five ports through which the necessary culture additions and removals were made. The joint between the lid and growth vessel was sealed with silicon vacuum grease and held in place with a metal spring.

Fresh medium was continuously supplied to the vessel using a flow inducer (MHRE 7, Watson-Marlow, Falmouth, Cornwall) from a Pyrex glass vessel (20l) through silicon rubber tubing. A burette placed in the medium input line allowed determination of the flow-rate of medium to the culture vessel, from which the dilution rate ($D \text{ h}^{-1}$) was

calculated. Aseptic replacement of the medium vessel was aided by the presence of a metal hood which covered the joint in the medium line to which the new vessel was to be connected. To prevent growth back from the culture vessel to the medium pot, a glass medium break was inserted in the medium line which provided a break in the column of fresh medium, so hindering grow-back.

Culture homogeneity was ensured by continuous stirring with a 5 cm magnetic follower operated by a magnetic stirrer (Gallenkamp, London).

The culture was supplied with humidified air at a rate of 1000 ml air min^{-1} through a glass sparge placed 1 to 2 cm above the magnetic follower to ensure effective gas dispersion.

The temperature (30°C) was controlled by the use of a warm-finger connected to a Churchill heating unit (Churchill Instruments, Uxbridge, Middlesex) and measured with a thermometer situated in a water-filled Pyrex glass pocket in the central part of the lid.

Culture volume was maintained at 1.0 λ by use of an overflow positioned so that when the medium volume exceeded 1.0 λ waste gases could not escape, causing a pressure build-up, so forcing the excess medium out through the waste line into a Pyrex glass vessel (20 λ). This was aseptically replaced at intervals with an empty sterile vessel.

Sampling the culture was achieved by opening a Hoffman clip on the sample outlet and collecting the effluent in a sterile glass bottle (25 ml) or in a large vessel if more sample as required. The rate of sampling could be increased by closing the waste medium line, so increasing the pressure in the culture vessel.

Once the apparatus had been assembled, with the exception of the

medium vessel it was autoclaved at 15 lb sq.in⁻² for 20 min, after which the medium and inoculum were added.

2.1.3.2 Growth medium

The basal medium used was described in Section 2.1.1 and was supplemented with yeast extract (15 mg l⁻¹), with either MCA or 2MCPA as the sole carbon and energy sources at 0.5 g carbon l⁻¹, a final concentration which ensured the culture was carbon limited. The basal salts solution was autoclaved at 15 lb sq.in⁻² for 45 min before sterile trace element solution, containing the yeast extract, and the filter sterilised carbon source were added.

2.1.3.3 Inoculation

The chemostat growth vessel was inoculated aseptically through one of the ports with an overnight batch culture (50 ml) of a given isolate (Section 2.1.1). For an initial period of 24 to 48 h the cultures were grown under closed conditions after which the required medium flow rate was started.

On a number of occasions a second or third period of batch growth was required, as the initial culture washed out. After the further periods of batch growth the organisms became adapted to the conditions in the chemostat and even the high dilution rates, equivalent to 86% ν_{\max} (Section 2.2.2), failed to wash the cultures out.

2.1.3.4 Determination of steady state

A culture was considered to be in a steady state once the dilution rate had been constant for at least three times the culture doubling time. Culture absorbance at 600 nm measured using a Unicam SP1700 Ultraviolet spectrophotometer (Pye-Unicam) in 3.0 ml glass cuvettes with

a 10 cm light path, and chloride release (measured as described in Section 2.3.1) was followed for evidence that steady state conditions had been achieved.

2.2 ISOLATION AND GROWTH OF BACTERIA

2.2.1 Methods of Isolation

Two approaches were used to isolate bacteria capable of utilising the chlorinated alkanic acids MCA, DCA, 2MCPA and 22DCPA as sole carbon and energy sources. Firstly a sample (20g) of soil was shaken in sterile 50 ml Tris-sulphate buffer (0.02M), pH 7.0, for 20 min. After allowing solid material time to settle, 5 min, the liquid was decanted and serially diluted (1.0 ml) in volumes (9 ml) of sterile Tris-sulphate buffer (0.02M) pH 7.0 and aliquots (0.1 ml) spread onto solidified defined medium (Section 2.1.2) containing the appropriate chlorinated substrate.

Each dilution series of petri-dishes was prepared in triplicate; to the first set yeast extract (10 mg l^{-1}) was added (Hirsch & Alexander, 1960) to another soil extract was added. To the third no supplements were added.

The soil extract was prepared using soil (400g, wet weight), added to tap water (960 ml) and autoclaved at $15 \text{ lb sq. in}^{-2}$ for 1 h and left in an unopened autoclave overnight, then autoclaved again and the extract decanted and filtered through paper, then through a 0.45μ membrane filter (Oxoid, Basingstoke, Hampshire). Aliquots (300 ml) of the filtrate were finally autoclaved at $15 \text{ lb sq. in}^{-2}$ for 20 min and stored until required at 4°C . This procedure was based on that of Gordon (1968).

Once inoculated the plates were incubated at 30°C for up to 7 d after which isolates capable of growth on the halogenated substrates were purified by repeated dilution streaking onto Petri-dishes containing the substrate on which they were isolated as the carbon and energy source.

The second procedure involved an initial batch enrichment. Samples (10g) of soil were added to 100 ml defined growth medium with MCA, DCA, 2MCPA or 22DCPA as the carbon source and incubated for 7 d (Section 2.1.1). After the enrichment phase samples (1.0 ml) were serially diluted into volumes (9 ml) of $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (0.1M), pH 7.0, (Gomori, 1955) and aliquots (0.1 ml) spread on solidified defined medium (Section 2.1.2) containing the appropriate carbon source. Single colonies were selected after incubation and purified by repeated dilution streaking, as previously described. All isolates were maintained on Petri-dishes and on agar slopes as described in Section 2.1.2.

2.2.2 Determination of maximum specific growth rate

The maximum specific growth rates (μ_{max}) of the isolates were determined by aseptically transferring an aliquot (1 to 2 ml) of an overnight culture, grown on defined medium (Section 2.1.1), into sterile defined medium containing the same substrate as in the overnight culture. The inocula added gave an initial absorbance of 0.01 to 0.02 at a wavelength of 600 nm in a Corning colorimeter (model 252). Culture absorbance was recorded at 30 min intervals until the maximum population was reached.

The results were calculated from the gradient of a \log_{10} absorbance against time of incubation plot and recorded as values for μ_{max} with units of reciprocal hours.

2.3 MEASURE OF DEHALOGENASE ACTIVITY

2.3.1 Estimation of free chloride ions

An estimation of free chloride ions was used to indicate dehalogenase activity in liquid cultures (Section 2.1.1) and enzyme assay studies (Section 2.3.2).

Free chloride ions were assayed using a Marius Chlor-0-Counter (F.T. Scientific, U.K.). A base counting solution (25 ml), containing in ml \times^{-1} double-glass-distilled water: glacial acetic acid, 100; Conc. Nitric Acid, 8.0; NaCl (0.5M), 1.0 and gelatin-thymol blue indicator solution, 1.0 was placed into a small beaker. The indicator solution consisted of, in mg 100 ml⁻¹ double-glass-distilled water: white powder gelatin, 600; thymol, 10.0 and thymol blue pH indicator, 10.0, made up as a 10-fold concentration and stored at 4°C until required then diluted using double-glass-distilled water.

The background chloride, due to the NaCl solution, was titrated and samples (1.0 ml) were added and free chloride was titrated against silver ions, generated coulometrically, resulting in the precipitation of insoluble silver chloride. The titration end-point was detected amperometrically by silver electrodes measuring the appearance of free silver ions. The time of the titration was directly proportional to the chloride ion concentration and was represented by a time based numerical read-out.

For enzyme studies the base counting solution was changed after each set of 4 readings in order to account for changes in counts due to the state of the counting solution. However, for free chloride ion counts in batch cultures, 12 to 15 samples could be counted before the solution required changing.

2.3.2 Assay procedure for dehalogenase activity

Samples (400 or 800 ml) of late exponential cultures were harvested by centrifugation at 5000g for 15 min at 4°C, and the pellet washed and resuspended in refrigerated (4°C) Tris-sulphate buffer (0.02M), pH 7.9. Cell-free extracts were prepared by disrupting the whole organisms by two passages through a French pressure cell (American Inst. Co. Ltd., Maryland) at 83 MPa (ca 11,500 lbs sq.in⁻²), and the remaining whole organisms and cell debris removed by centrifugation at 30,000g for 45 min at 4°C. The supernatant was decanted and dithiothreitol added to give a final concentration of 1.0mM. Dithiothreitol cleaves disulphide bonds and protects sulphydryl groups, so stabilising enzymes especially if sulphydryl groups are found in the enzymes' active site.

If cell-free extracts were not to be assayed immediately for dehalogenase activity they were stored at -20°C, at which temperature they remained active for several months. Immediately before assaying the extracts were thawed and stored on ice (4°C).

The assay mixture of 5.5 ml contained, in double-glass-distilled water: Tris-sulphate buffer (100 mM), pH 7.9; NaCl (0.005 mM); chlorinated alkanolic acid (0.1 to 0.2mmol) and 0.1 to 1.0 ml cell free extract. The chlorinated substrates were added as one of 10% wv⁻¹ stock solutions, pH 7.0: MCA (156 µl); DCA (215 µl); 2MCPA (179 µl) and 22DCPA (236 µl). The buffer was added to overcome the acidic products of the enzyme reaction which in the buffers absence would inhibit further enzymic activity.

The assay mixture, including the cell-free extract, but minus the chlorinated substrate, was equilibrated at 30°C in a water bath for 10 min. The reaction was initiated by adding the substrate. Chloride release

was measured in samples (1 ml; Section 2.3.1) taken at intervals during the first 20 min of reaction.

Once extracts were known to possess dehalogenase activity the NaCl was not included in the assay mixture, the volume being made up with double-glass-distilled water, as the additional counts resulting from the presence of the NaCl only added to the time taken to count the free chloride ions resulting from the dehalogenase activity.

The results were expressed as relative dehalogenase activities which were the ratios of rates of chloride release from DCA, 2NCPA and 22DCPA standardised to the rate of chloride release from MCA, the rates being determined from plots of Chlor-O-Counter counts (1 ml assay mixture)⁻¹ against assay time. A second expression of dehalogenase activity was as enzyme specific activity. This was determined for each substrate by converting the chloride ion counts to μmolCl^- released, in accordance with the calibration of the counter - 100 counts on range 10 represents $1\mu\text{molCl}^-$. The final expression of specific activity also involved determining the protein concentration of the cell free extract and had units of $\mu\text{mol substrate converted (mg protein)}^{-1} \text{ min}^{-1}$.

2.3.3 Modifications to the enzyme assay procedure for further characterisation of the enzyme systems

To investigate the effect of the pH of the assay mixture on dehalogenase activity the assay mixture buffer and strength were changed. The buffer was TTA buffer which was chosen because it was effective over the complete range required, pH 6.0 to 11.0. TTA buffer (0.2M) contained in g l^{-1} double-glass-distilled water: Trizma base, 24.24; N tris (Hydroxymethyl)methyl-2-aminæthane sulphonic acid (Tes), 45.84 and glacial

acetic acid, 19.8. This produced a pH of approximately 4.5, which was adjusted to the required pH by the addition of concentrated NaOH.

The assay procedure was as described in Section 2.3.2. However no water was added to the assay mixture, the additional volume being made up with additional buffer. The buffer was adjusted to values of pH 6.0 to 11.0 in whole pH units and cell-free extracts were assayed at each pH value against MCA, DCA, 2MCPA and 22DCPA. The stability of each substrate was investigated at the highest and lowest pH values over a time period equivalent to the assay period.

The substrate specificity of the dehalogenation reactions were also studied using the assay procedure previously described (Section 2.3.2). For this the following substrates were prepared as 10% (wv^{-1}) stock solutions and used as described: trichloroacetic acid (TCA); 3monochloropropionic acid (3MCPA); 2monochlorobutyric acid (2MCBA); 3monochlorobutyric acid (3MCBA); 4monochlorobutyric acid (4MCBA); 2monobromopropionic acid (2MBPA); monobromoacetic acid (MBA) and 2monobromobutyric acid (2MBBA).

2.3.4 Determination of protein concentration

The protein concentration of the cell-free extracts was determined using the Biuret Method of Gornall *et al.* (1948), with bovine serum albumin (BSA) as the reference protein. The Biuret assay solution contained, in 500 ml double-glass-distilled water: $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.5g and COOK. $(\text{CHOH})_2\text{COONa} \cdot 4\text{H}_2\text{O}$, 6.0g, to which 300 ml of autoclaved NaOH (10% wv^{-1}) was added. The NaOH was prepared as a 60% (wv^{-1}) stock solution and autoclaved at 10 lb sq.in^{-2} for 10 min to remove the carbonates. The removal of the carbonates was necessary because they

interfere with the colorimetric response. The 800 ml solution was made up to 1.0% with double-glass-distilled water and stored in a screw-cap plastic bottle. A plastic bottle was used because the solution is unstable when stored in glass.

A standard curve was prepared using 0.5 to 8.0 mg BSA by adding the necessary volume of a 10 mg ml^{-1} stock solution to 1 ml of double-glass-distilled water.

The Biuret solution (4 ml) was added and mixed, then left to stand at room temperature for exactly 30 min, after which the absorbance was measured at 550 nm using a spectrophotometer (SP1700 - Pye-Unicam), in 3.0 ml glass cuvettes with a 1.0 cm light-path. A reagent blank was used as a zero BSA concentration.

The protein concentration of the cell-free extracts was determined after dilution to 0.5 or 0.25 full strength in 1.0 ml double-glass-distilled water and the Biuret solution added and absorbance measured as before. The protein concentrations were then determined by reference to the standard curve.

2.4 ELECTROPHORETIC STUDIES OF THE DEHALOGENASE SYSTEMS

2.4.1 Preparation of disc polyacrylamide gels

The electrophoretic techniques used were based on those of Laemmli (1970) and Fairbanks *et al.* (1971) and were described in Weightman & Slater (1980).

Polyacrylamide gels were prepared from the following stock solutions, which were stored at 4°C : Tris-sulphate buffer (0.75M), pH 8.8 and 6.8; acrylamide solution containing in g 100 ml^{-1} double-glass-distilled water: acrylamide, 40 and NN'-methylene-bis-acrylamide,

1.5; $(\text{NH}_4)_2\text{S}_2\text{O}_8$, 2% (wv^{-1}) - freshly prepared on day of use; NNN'N'-tetramethylethylenediamine (TEMED), 5% (wv^{-1}) and dithiothreitol, 100 mM. The acrylamide solution being light sensitive was stored in a foil covered bottle.

The stock solutions were used to give final concentrations in the gels as follows. In the running gel: Tris-sulphate buffer (0.325M), pH 8.8, acrylamide, 80.0 g l^{-1} ; NN'-methylene-bis-acrylamide, 3.0 g l^{-1} ; dithiothreitol, 1.0 mM; $(\text{NH}_4)_2\text{S}_2\text{O}_8$, 1.0 g l^{-1} and TEMED, 0.5 g l^{-1} . In the stacking gel: Tris-sulphate buffer, (0.125M), pH 6.8, acrylamide, 30.0 g l^{-1} ; NN'-methylene-bis-acrylamide, 1.08 g l^{-1} ; $(\text{NH}_4)_2\text{S}_2\text{O}_8$, 1.0 g l^{-1} and TEMED, 0.5 g l^{-1} .

The gels were formed by pipetting the running gel solution between two glass plates, shaped to fit the electrophoresis tank, sealed with a piece of thin walled silicon-rubber tubing and held together with flip-back clips. The solution was added until the gap between the plates was filled to within ca 2.5 cm of the top of the front plate. The gel was left standing for approximately 10 min until the gel was seen to be solidifying, approximately 2mm below the surface, at which time double-glass-distilled water was carefully layered over the surface to produce an horizontal surface to the top of the running gel (Davis, 1964).

The stacking gel was prepared, and the water removed from the gel surface. The running gel surface was washed with ca 5 ml of stacking gel solution which was discarded (Cooksey, 1971), the stacking gel added and a well-former put into place. After ca 10 min the gel was ready for use.

Before samples were added the gels were pre-run overnight at 4°C in a vertical electrophoresis tank (Raven Scientific Ltd, Suffolk) at 25 mA, supplied as a constant current by a power pack (Shandon Southern Instruments, Runcorn, Cheshire). The electrode buffer contained in double-glass-distilled water: Trizma base, 0.025M; glycine, 0.192M and dithiothreitol, 1.0mM. The pH was adjusted to 8.3 by addition of additional Trizma base. Once pre-run, the buffer was exchanged for fresh buffer and samples were loaded.

2.4.2 Sample preparation and electrophoresis

Cell-free extract (0.5 ml), prepared as described in Section 2.3.2, was added to (0.5 ml) glycerol solution (20% wv^{-1}) in Tris-sulphate buffer (0.02M), pH 7.9 and 50 μl bromophenol blue (0.5% wv^{-1}).

Samples (50 μl) were loaded into the preformed wells of the pre-run gel and electrophoresed at 4°C for 2 to 3 h at 30 mA, until the marker dye was 1 to 2 cm from the bottom of the gel.

2.4.3 Visualisation of dehalogenase activity

The electrophoretically separated dehalogenase activities were visualised by the method developed by Weightman & Slater (1980). After removal of the stacking gel the running gel was incubated in Tris-sulphate buffer (0.2 M), pH 7.9 at 30°C, in the presence of halogenated substrates (50 mM) for 30 min. For the initial gels a mixture of MCA and DCA (50 mM) was used. In later studies on substrate specificities, four wells were loaded with samples of the same extract and after electrophoresis, the gels were cut into sections in line with the wells and marker-dye, and each section was incubated with either MCA, DCA, 2MCPA or 22DCPA as the sole halogenated substrate.

After the incubation period the gels, or sections of gels, were washed twice in double-glass-distilled water, to remove surplus substrates, and placed in a solution of AgNO_3 (0.1M). Free chloride ions, present in the regions of dehalogenase activity, precipitated with the silver, producing light sensitive white bands of AgCl , thus visualising the positions of dehalogenase activity on the gel.

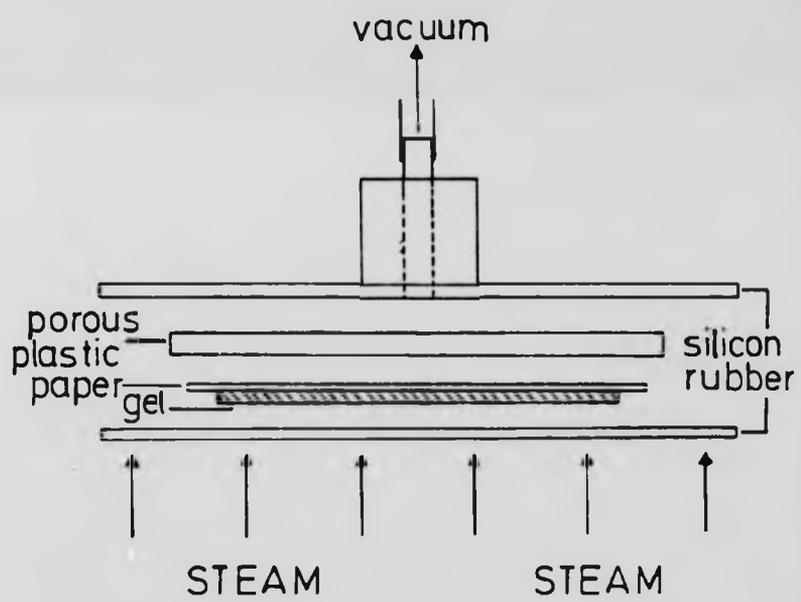
In order to prevent background fogging of the gel, which would mask enzymic activity, the gels after treatment with AgNO_3 , were washed and placed in glacial acetic acid (5% wv^{-1}) for 10 min, then soaked for 12 h in double-glass-distilled water. The degree of fogging could be minimised by ensuring that the substrates were fresh and had a low free chloride ion concentration.

The light sensitive nature of the AgCl bands meant that on exposure to light the bands became dark brown in colour, and when placed on a white background the bands became more distinct. The resolution could be increased by exposing the gels, after preserving, to UV light for 2 to 3 min.

2.4.4 Gel preservation

The apparatus used is shown in Figure 2.1, a piece of Whatman 3 mm chromatographic paper (Whatman Labsales Ltd, Maidstone, Kent) cut larger than the gel was soaked in double-glass-distilled water. This was placed onto the bottom silicon rubber sheet and the gel carefully laid on top, the wetted paper and addition of further water allowed the gel to be positioned correctly. The paper was then reversed, so that the gel was sandwiched between the paper and the rubber sheet. The rest of the apparatus was assembled as shown and the air removed to form an air-tight seal. The apparatus was then placed over a steam bath and the

Figure 2.1: Apparatus for preserving polyacrylamide gels



temperature of 90-95°C and the vacuum removed the water from the gel so that the gel was dried down to a thin film on the paper, so producing a permanent record. However, it was found that if constantly exposed to light the gels grew progressively darker.

2.4.5 Modifications to incubation conditions of electrophoresed samples

The effect of an increase in the pH of the buffer in which the gel was incubated in the presence of the halogenated substrates was studied. A gel carrying three electrophoresed cell-extract samples was cut into three and incubated in either Tris-sulphate buffer (0.5 M), pH 7.9 or glycine NaOH buffer (0.5 M), pH 9 or 10 (Gamori, 1955). The dehalogenase activity after incubation was visualised as described in Section 2.4.3.

The effect of sulphhydryl blocking agents on dehalogenase activity was investigated using electrophoresed samples. Six samples were electrophoresed and the gel sectioned (Section 2.4.3), two sections were incubated for 30 min as described, the rest were placed in a solution of N-ethylmaleimide (1.0 mM) in Tris-sulphate buffer, (0.02 M), pH 7.9 for 5, 10, 15 or 20 min, washed in double-glass-distilled water and incubated as before (Section 2.4.3). The dehalogenase activity was visualised and the effect of N-ethylmaleimide on the amount of activity qualitatively described.

2.5 ELECTROPHORESIS OF THE PROTEIN COMPONENTS OF THE BACTERIAL ISOLATES

2.5.1 Electrophoretic separation of the protein components

The procedures described were based on those of Weber & Osborn (1969) and Laemmli (1970).

The stock solutions were the same as for the enzyme separation

gels (Section 2.4.1), without the use of dithiothreitol but with a stock solution of sodium dodecyl sulphate (SDS) ($2\% \text{ wv}^{-1}$), prepared on the day of use.

The running gel consisted of: Tris-sulphate buffer, pH 8.8, acrylamide solution; $(\text{NH}_4)_2\text{S}_2\text{O}_8$ and TEMED at the concentrations previously indicated, with SDS, $0.1\% \text{ (wv}^{-1})$. The stacking gel was also as described with the addition of SDS, $0.1\% \text{ (wv}^{-1})$. The electrode buffer contained, in double-glass-distilled water: Trizma base, 0.025M, glycine, 0.192M and SDS, $0.1\% \text{ (wv}^{-1})$, and additional Trizma base added to give a final pH of 8.3.

Samples were prepared from cell-free extracts, prepared as described in Section 2.3.2, 0.5 ml of a stock solution containing: Tris-sulphate buffer (0.2M), pH 6.8; 2mercaptoethanol, $2\% \text{ (wv}^{-1})$ and SDS, $2\% \text{ (wv}^{-1})$, was added to cell-free extract (0.5 ml) in an Eppendorf vial and incubated at 45°C for 60 min. After incubation two drops, from a pasteur pipette, of glycerol were added together with $50 \mu\text{l}$ of bromophenol blue ($0.5\% \text{ wv}^{-1}$) and samples (25 to $50 \mu\text{l}$) loaded onto the gel and electrophoresed at 25 mA for 5 to 6 h at 4°C .

2.5.2 Protein visualisation

The proteins were stained by the method described by Weber & Osborn (1969). The electrophoresed gel was placed in a solution of PAGE blue G90 (1.25g) in methanol ($50\% \text{ vv}^{-1}$), 454 ml and glacial acetic acid, 46 ml, overnight, after which the gel was removed, washed in double-glass-distilled water and placed in a destaining solution of: glacial acetic acid, 75 ml and methanol, 50 ml in double-glass-distilled water, 875 ml, for 24 h. The destaining rate could be increased by using

100 ml methanol and decreasing the volume of water and by changing the destaining solution after 2 - 3 h.

Prolonged soaking of polyacrylamide gels in glacial acetic acid solutions causes them to shrink as they are dehydrated (Sargent, 1969). Because of this, gels were soaked in double-glass-distilled water for 2 h to rehydrate the gels before measuring migration distances.

Finally the gels were photographed, using a 35 mm film with a Praktica MTL3 camera with a red, or a red and yellow filter and preserved as described in Section 2.4.4.

2.6 SCREENING FOR THE PRESENCE OF PLASMID DNA IN THE BACTERIAL ISOLATES

2.6.1 Growth of isolates for plasmid analysis

Screening for the presence of plasmids in the bacterial isolates was achieved using cultures growing on defined medium with the relevant chlorinated substrate (Section 2.1.1) though the strain E22 used 2MCPA as its carbon source. The initial screening used batch cultures of all the organisms except for strain E20 which could only utilise MCA when grown in continuous culture. For this organism samples were taken from cultures growing at 19% its μ_{\max} value.

2.6.2 Procedure for rapid screening of bacteria for plasmid DNA

This technique was modified from the method described by Eckhardt (1978). Two reagents were used: lysozyme mixture which contained: lysozyme, 7,500 U ml^{-1} ; ribonuclease A, 0.3 U ml^{-1} ; bromophenol blue 0.05% (wv^{-1}) and ficoll, 20% (wv^{-1}). This was prepared in Tris-borate buffer, (89 mM), pH 8.2 which contained: Trizma base, 89 mM; EDTA, 2.5 mM and boric acid, 89 mM.

The ribonuclease A (10.0 mg ml^{-1}) was initially dissolved in

sodium acetate buffer (500 mM), pH 4.0, and incubated at 98°C for 2 min, before diluting into the rest of the lysozyme mixture to give the required concentration.

The second reagent was an SDS mixture, which contained in Tris-borate buffer (89 mM), pH 8.2: SDS, 0.2% (wv^{-1}) and ficoll, 10% (wv^{-1}).

Volumes (0.1 to 1.5 ml) of cultures in early to mid exponential phase were measured into Eppendorf vials and pelleted in a Beckman microfuge B by centrifugation for 3 to 5 min. The supernatant was discarded and the pellet dried of any remaining surface liquid by carefully touching with tissue paper, resuspended in lysozyme mixture (15 μ l) and incubated at room temperature for 5 min.

After incubation the samples were loaded into wells on an horizontal 0.7% (wv^{-1}) agarose gel. The gels were 18 x 16 cm and made up in Tris-borate buffer (89 mM), pH 8.2, 100 ml and were loaded in one of three ways dependent upon the degree of cell lysis in the lysozyme mixture. Either the lysozyme treated cells (15 μ l) were loaded into the wells and the SDS mixture (25 to 30 μ l) layered on top and electrophoresed; or if less lysis had occurred, once the SDS mixture had been added the two solutions were gently mixed by 2 or 3 sideways movements of a Schuco clip (Schuco Scientific Ltd. London) before electrophoresing. Alternatively, if little or no lysis had occurred, the SDS mixture (30 μ l) was added directly to the resuspended cells in the Eppendorf vials and mixed briefly using a whirlimixer (Fisons Ltd. Loughborough, Leicestershire) after which the samples (40 to 45 μ l) were loaded onto the gel and electrophoresed.

Electrophoresis at 60 mA, using a Shandon Southern power pack, for

60 or 75 min with Tris-borate buffer (89 mM), pH 8.2 as the electrode buffer, was performed on home-made apparatus consisting of 2 developing trays with platinum electrodes connected to the power pack. The horizontal gel was connected to the buffer tanks with absorbant paper towels.

After electrophoresis the DNA was stained with ethidium bromide ($0.2 \mu\text{gml}^{-1}$) for 20 min, rinsed thoroughly in cold water and photographed while on a short-wave UV transilluminator type C61 (Ultra-Violet Products Inc. U.S.A.) using a Polaroid CU5 Land Camera (Polaroid Corp., U.S.A.) with Polaroid type 665 film. The camera was fitted with two UV and one orange filter, the film exposed for 2 min at an aperture of f4.7 and developed at 20°C for 40s.

2.7 PLASMID ASSOCIATED DEHALOGENASE ACTIVITY

2.7.1.1 Curing Experiments

Samples (1.0 ml) of overnight cultures (50 ml) grown on defined medium (Section 2.1.1) with succinate ($0.5\text{g carbon l}^{-1}$) as the carbon source, and ethidium bromide at concentrations of 10, 20, 40, 60, 80 and $100 \mu\text{gml}^{-1}$ were serially diluted into Tris-sulphate buffer, (0.02M), pH 7.0. Aliquots (0.1 ml) were spread onto Petri dishes with either succinate or 2MCPA as the carbon source (Section 2.1.2) and incubated at 30°C for 24 h and 48 h respectively. After incubation colony counts were determined and the values on succinate and 2MCPA plates for each concentration of ethidium bromide compared.

Colonies (100) from the succinate containing plates were transferred, using sterile tooth-picks onto succinate and 2MCPA plates to examine for the ability or otherwise to grow on 2MCPA, which was further investigated

60 or 75 min with Tris-borate buffer (89 mM), pH 8.2 as the electrode buffer, was performed on home-made apparatus consisting of 2 developing trays with platinum electrodes connected to the power pack. The horizontal gel was connected to the buffer tanks with absorbant paper towels.

After electrophoresis the DNA was stained with ethidium bromide ($0.2 \mu\text{gml}^{-1}$) for 20 min, rinsed thoroughly in cold water and photographed while on a short-wave UV transilluminator type C61 (Ultra-Violet Products Inc. U.S.A.) using a Polaroid CU5 Land Camera (Polaroid Corp., U.S.A.) with Polaroid type 665 film. The camera was fitted with two UV and one orange filter, the film exposed for 2 min at an aperture of f4.7 and developed at 20°C for 40s.

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Colonies (100) from the succinate containing plates were transferred, using sterile tooth-picks onto succinate and 2MCPA plates to examine for the ability or otherwise to grow on 2MCPA, which was further investigated

by inoculating a number of these organisms into succinate-defined medium (Section 2.1.1) incubating overnight, and transferring a sample (25 ml) into fresh medium (25 ml) containing 2MCPA, incubating for 24 h and assaying for free chloride ions as previously described (Section 2.3.1). A control of the parental organism was used to ensure sufficient time had elapsed for chloride release to be measured.

Cultures of the isolates incapable of growth on 2MCPA were screened for the presence of plasmid DNA by the method described (Section 2.6.2) using a plasmid containing strain of *Pseudomonas* sp. strain E4 as a control.

2.7.1.2 Rate of reversion to parental phenotype

The rate of reversion of the ethidium bromide treated cells, characterised by absence of dehalogenase activities back to the parental phenotype, characterised by the presence of dehalogenase activities, was investigated.

Cultures cured of dehalogenase activity were repeatedly cultured on succinate-defined medium (Section 2.1.1) and aliquots (0.1 ml) spread onto 2MCPA-containing plates (Section 2.1.2) at intervals.

After incubation for 48 h the plates were studied for the presence of large colonial growth. This experiment was repeated three months after the original work on cured strains of *Pseudomonas* sp. strain E4.

2.7.2 Mating Experiments

Further evidence for the plasmid association of dehalogenase activity in the soil isolates was sought by attempting to transfer the plasmids from the soil isolates to organisms possessing no dehalogenase activity, and selecting for such transconjugants.

Two approaches were used, firstly attempts were made to transfer the plasmid in *Pseudomonas* sp. strain E4 back into a cured strain of this isolate strain E41, and secondly, mating experiments between the soil isolates and other *Pseudomonas* species were set up.

2.7.2.1 Selection of antibiotic resistant strains used as recipients in mating experiments

Two selection procedures were used, though the second proved to be the more efficient. The first involved culturing the required organism on succinate-defined medium (Section 2.1.1) or on nutrient broth (13g Nutrient broth L^{-1}) overnight, and spreading a sample (0.1 ml) onto succinate plates (Section 2.1.2) or nutrient agar plates (2% wv^{-1}) supplemented with an antibiotic (50 to 100 μgml^{-1}) and incubating at 30°C until colonial growth was observed. The colonies were transferred onto media containing higher concentrations of the antibiotic and onto media with a second antibiotic at the lower concentration. The resistant organisms were finally transferred to media containing two antibiotics at the required concentrations.: streptomycin (Sm), 750 μgml^{-1} ; kanamycin (Km), 100 or 200 μgml^{-1} and chloramphenicol (Cm) 100 μgml^{-1} .

The second approach was used to isolate strains of *Pseudomonas aeruginosa* strain PA01162 and *P. putida* strain KT 2440 (kindly supplied by Prof. K. Timmis, Max-Planck Institute, Berlin) which were resistant either to Km (200 μgml^{-1}) and Cm (100 μgml^{-1}) or Km (200 μgml^{-1}) and ampicillin (Ap) (50 μgml^{-1}) respectively.

Both strains were inoculated into nutrient broth and incubated at 30°C overnight. Samples (1.0 ml) were transferred into fresh nutrient

broth and incubated for 2 to 3 h to initiate growth, after which the antibiotics were added at half the required concentrations. After overnight incubation samples (0.1 ml) were spread onto nutrient agar plates containing both pairs of antibiotics at the required concentrations and incubated for 48 h at 30°C. The resistant organisms were cultured and maintained on fresh antibiotic containing media.

The resistant strains were used as recipients in mating experiments and were selected for by the double resistances each possessed.

2.7.2.2 Method for membrane mating experiments

Overnight cultures (50 ml) (Section 2.1.1) of donor and recipient organisms were grown under selective conditions. For donor cultures the selective pressure was the presence of the halogenated substrated as the sole carbon source for the soil isolates and the presence of Km (100 μgm^{-1}) in a nutrient broth culture medium for *P. aeruginosa* PAOBR which carried the resistance factor R68-45 (supplied by B.W. Holloway, Monash, Australia). The recipient organisms were cultured in nutrient broth with the two relevant antibiotics present at half the final concentration used to maintain the resistant organisms.

The recipient organisms were washed and resuspended in Tris-HCl buffer (20 mM), pH 7.0 by centrifugation at 5,000g for 10 min. Given volumes, to ensure more recipient than donor cells were present, of the donor and recipient cultures were mixed in a Gallenkamp filter and filtered onto 0.45 μ membranes (Sartorius Instruments Ltd., Sutton Surrey). The filters were transferred aseptically onto nutrient agar and incubated overnight at 30°C. Samples (1 ml) of the two parental cultures were serially diluted in Tris-HCl buffer (20 mM), pH 7.0 and

broth and incubated for 2 to 3 h to initiate growth, after which the antibiotics were added at half the required concentrations. After overnight incubation samples (0.1 ml) were spread onto nutrient agar plates containing both pairs of antibiotics at the required concentrations and incubated for 48 h at 30°C. The resistant organisms were cultured and maintained on fresh antibiotic containing media.

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The recipient organisms were washed and resuspended in Tris-HCl buffer (20 mM), pH 7.0 by centrifugation at 5,000g for 10 min. Given volumes, to ensure more recipient than donor cells were present, of the donor and recipient cultures were mixed in a Gallenkamp filter and filtered onto 0.45 μ membranes (Sartorius Instruments Ltd., Sutton Surrey). The filters were transferred aseptically onto nutrient agar and incubated overnight at 30°C. Samples (1 ml) of the two parental cultures were serially diluted in Tris-HCl buffer (20 mM), pH 7.0 and

TABLE 2.1: Outline of mating experiments attempted in order to transfer dehalogenase activity from a donor to a recipient as proof of the plasmid association with dehalogenase activity.

Pseudomonas sp. strain JR81 was a glycolate utilizer isolated by J.R. Beeching and selected for resistance to Rif and Sm. *P. aeruginosa* strain 8602/6 was supplied by G. Willshaw (Collingdale)

DONOR	RECIPIENT	SELECTIVE PRESSURE FOR ISOLATION OF TRANSCONJUGANTS
<i>Pseudomonas</i> sp. strain E4	<i>Pseudomonas</i> sp. strain JR81 (Rif ^R /Sm ^R)	2MCPA + Rif (250 μgml^{-1}) + Sm (750 μgml^{-1})
<i>Pseudomonas</i> sp. strain E4	<i>Pseudomonas aeruginosa</i> strain 8602/6 (Rif ^R)	2MCPA + Rif (250 μgml^{-1})
<i>Pseudomonas</i> sp. strain E4	<i>Pseudomonas aeruginosa</i> strain (Tc ^R /Km ^R /Ap ^R)	2MCPA + Tc (150 μgml^{-1}) Km (150 μgml^{-1}) Ap (50 μgml^{-1})
<i>Pseudomonas</i> sp. strains E3, E4 & E6 <i>Pseudomonas/Alteromonas</i> sp. strain E2 <i>Alcaligenes</i> sp. strains E20 & E22	<i>Pseudomonas</i> sp. strain E41 (Km ^R /Sm ^R)	2MCPA + Km (200 μgml^{-1}) Sm (750 μgml^{-1})
<i>Pseudomonas</i> sp. strains E4 & E6	<i>Pseudomonas aeruginosa</i> PA01162 (Km ^R /Cm ^R)	2MCPA + Km (200 μgml^{-1}) Cm (100 μgml^{-1})
<i>Pseudomonas</i> sp. strain E4R	<i>Pseudomonas aeruginosa</i> strain PA01162 (Km ^R /Cm ^R)	2MCPA + Km (200 μgml^{-1}) Cm (100 μgml^{-1})
<i>Pseudomonas</i> sp. strains E4 & E6	<i>Pseudomonas putida</i> strain KT2440 (Km ^R /Ap ^R)	2MCPA + Km (200 μgml^{-1}) Ap (50 μgml^{-1})

spread onto succinate-defined medium Petri dishes (Section 2.1.2) to ascertain the numbers of organisms used in the mating experiment. Colony counts were made after 24 to 48 h incubation.

After overnight incubation the bacteria on the membranes were suspended in sterile Tris-HCl buffer (20 mM), pH 7.0 (3 ml), by whirlimixing, and samples (0.1 ml) spread onto the selection plates and incubated at 30°C for 7 to 14 d. These plates contained 2MCPA as the carbon source, which selected against the recipients, and the two antibiotics to which the recipients were resistant, to select against the donor organisms. Any bacteria capable of growth on those media could only be transconjugants.

Such organisms were purified by dilution streaking onto fresh selection plates, after which they were transferred onto Kings' A plates (King *et al.*, 1954), if the recipient was a *P. aeruginosa* strain, and screened for plasmids as described in Section 2.6.2.

2.7.2.3. Membrane mating experiments

The membrane mating experiments attempted are summarised in Table 2.1.

2.8 RESISTANCE TO HEAVY METALS

The growth inhibitory effect of various heavy metals was investigated by spreading overnight cultures of the soil isolates on defined medium (Section 2.1.1) and the cured strains resulting from ethidium bromide treatment, on defined medium with succinate (0.5g carbon μl^{-1}) as the carbon source, to produce a lawn of bacteria. A 7mm well was cut into the centre of each petri dish, using a 7mm cork-borer, and 175 μl of the heavy metal solution (1 or 100 mM)

pipetted into it. The heavy metals were: mercury (HgCl_2); selenium (Na_2SeO_4); tellurium ($\text{Na}_2\text{TeO}_4 \cdot 2\text{H}_2\text{O}$); zinc (ZnSO_4); arsenate (AsO_4); cadmium (CdCl_2); cobalt (CoSO_4); copper ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$); nickel ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$) or lead ($\text{Pb}(\text{NO}_3)_2$).

The cultures were incubated overnight at 30°C after which the zones of inhibition were measured, that is, the mean distance between the edge of the well and the limit of the bacterial lawn.

2.9 SIZING THE WHOLE PLASMIDS

This was achieved by electrophoresing the isolated plasmids together with plasmids of known molecular weight (Table 2.2).

The organism possessing the plasmid of unknown size was cultured in a chemostat, (Section 2.1.3) at a dilution rate of 19% of its μ_{max} . The organisms carrying the plasmid standards were cultured in batch conditions growing on nutrient broth.

Sample preparation and the electrophoretic technique were as described in Section 2.6.2. The plasmid under investigation was electrophoresed in a central well, with plasmid standards on either side, for 1 h at 60 mA, stained and photographed (Section 2.6.2). The migration distances of the standards were plotted as \log_{10} values against \log_{10} molecular weight to produce a straight line. From this an approximate size for the unknown plasmid was determined.

2.10 RESTRICTION ENDONUCLEASE ANALYSIS

2.10.1 Preparative system of the isolation of plasmid DNA

The best fractions, that is those with the greatest amount of plasmid DNA and the least contaminating RNA, were obtained from cultures harvested from chemostats (Section 2.1.3). When it was necessary to take

TABLE 2.2.: Molecular weight of the plasmids used as standards in electrophoresis of whole plasmid preparations to determine the molecular weight of the isolated plasmids.
(Ap, ampicillin; Sm, streptomycin; Su, sulphonilamide; Tc, tetracycline; Km, kanamycin; Cm, chloramphenicol)

PLASMID	PHENOTYPE	SIZE (in Megadaltons)	SOURCE
COL		c.5	<i>Escherichia coli</i>
TP120	Ap.Sm.Su.Tc	32	<i>Escherichia coli</i> K12 IR713
TP113	Km	56.7	<i>Escherichia coli</i> K12 IR713 <i>lac</i> ⁻ (G. Willshaw, Colindale, London)
R1	Ap.Cm.Km.Su.Sm	62 - 65	<i>Escherichia coli</i> K12 J5-3 (D. Godwin, Warwick)
TOL	unknown	63	<i>Pseudomonas arvilla</i> strain PaW (P.A. Williams, Bangor)
TP125	Cm.Sm.Su.Tc	64	<i>Escherichia coli</i> K12 IR713 <i>lac</i> ⁻ (G. Willshaw, Colindale, London)
RA1	Su.Tc	86	<i>Escherichia coli</i> K12 IR713 <i>lac</i> ⁻ (G. Willshaw, Colindale, London)
TP116	Cu.Sm.Su	143.7	<i>Escherichia coli</i> K12 IR713 <i>lac</i> ⁻ (G. Willshaw, Colindale, London)

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RA1	Su.Tc	86	<i>Escherichia coli</i> K12 IR713 <i>lac</i> ⁻ (G. Willshaw, Colindale, London)
TP116	Cu.Sm.Su	143.7	<i>Escherichia coli</i> K12 IR713 <i>lac</i> ⁻ (G. Willshaw, Colindale, London)

samples from batch cultures, they were harvested in early exponential phase.

The procedure for preparing plasmid fractions was adapted from the method used by Wheatcroft & Williams (1981). Culture samples (40 to 50 ml) were harvested by centrifugation at 5,000g for 10 min, the supernatant discarded and the pellet drained of excess fluid. The pellet was resuspended in solution (1.6 ml), which contained: Trizma base, 50 mM; EDTA, 50 mM; xylene cyanol FF, 0.1 mgml⁻¹ and antifoam, 5% (vv⁻¹). The suspension was transferred into autoclaved glass tubes (5 ml) and solution B (0.4 ml) added. Solution B was a saturated solution of SDS in NaOH (1.0 M).

The contents of the tube were gently mixed by rolling the tubes for 1.0 min, after which they were vortex mixed for 5 min using a whirlimixer. Aliquots (0.7 ml) were loaded onto sucrose gradients (4.0 ml), prepared as described by Baxter-Gabbard (1972) by a process of freeze-thaw-freezing and thawing ready for use, a sucrose solution (20% wv⁻¹) at -20°C, which yielded sucrose density gradients of 14% to 26% (wv⁻¹).

The gradients, with samples loaded, were balanced by addition of further sample, placed into a 6 x 5.5 swing-out rotor and centrifuged at 30,000g for 1.0 h using a MSE PrepSpin 65. Ten drop aliquots (ca 0.35 ml) were siphoned from the bottom of the gradients into autoclaved Eppendorf vials and mixed by inversion to homogenise the gradients. The first four fractions were routinely discarded.

2.10.2 Primary gel electrophoresis

To determine which fractions contained the most plasmid DNA and the least contaminating material, a primary gel of all the collected

fractions was run. The gel was an 18 x 24 cm agarose gel (0.7% wv^{-1}) made in Trisborate buffer (89 mM), pH 8.2 (150 ml) and electrophoresed in an horizontal position as previously described (Section 2.6.2). The well-formers were positioned to give double-or triple-decker gels (Wheatcroft & Williams, 1981) to enable up to 48 samples to be examined on one gel.

Samples (15 μ l) of each fraction were loaded into the wells and electrophoresed at 60 mA for 45 to 60 min. Cyanol violet was placed into the empty wells to mark the migration distance of RNA in the samples, and before the marker reached the set of wells below the last set, or before the marker migrated off the bottom of the gel, the electrophoresis was terminated and the gels stained and photographed as described (Section 2.6.2).

The highest fractions, those from the top of the gradient, with the most plasmid material and the least RNA were further divided into 40 μ l samples in autoclaved Eppendorf vials and stored at $-20^{\circ}C$ until required for endonuclease digestion, the remaining fractions were discarded.

2.10.3 Restriction endonuclease digestion

Five restriction endonucleases were used: *Hind* III; *Pst* I; *Xho* I; *Bam* HI and *Eco* RI. These were prepared in buffers suggested by the manufacturers at concentrations of 4 units μ l $^{-1}$. *Hind* III (Boehringer Mannheim) was made up in: Tris-HCl buffer (6 mM), pH 7.5; NaCl, 50 mM; MgCl₂, 6 mM and BSA, 5 μ g - this buffer was suggested by Miles. *Pst* I (Boehringer Mannheim) in: Tris-HCl buffer (10 mM), pH 7.0; NaCl, 50 mM and MgCl₂, 10 mM. *Xho* I (Bethesda

Research Laboratories Ltd) in: Tris-HCl buffer (8 mM), pH 7.4; NaCl, 150 mM, mercaptoethanol, 6 mM and $MgCl_2$, 6 mM. *Eco* RI (Miles) in: Tris-HCl buffer (100 mM), pH 7.5; NaCl, 50 mM and $MgCl_2$, 10 mM and *Bam* HI (Miles) in: Tris-HCl buffer (100 mM), pH 7.5 and $MgCl_2$, 10 mM.

All the Eppendorf vials and micropipettes were autoclaved before use. The samples (40 μ l) were thawed and a 10 times concentrated solution (5 μ l) of the restriction buffer required, measured into each vial, and the contents whirlmixed for 5s. To each vial the complementary restriction enzyme (1 μ l) was added using a micropipette (1 μ l) mixed by blowing and stirring with the micropipette and whirlmixed for 5s. After ensuring that all the tops of the vials were secure, to prevent evaporation, they were floated in a water bath at 37°C for 2 h. The manufacturers of the enzymes recommend 1.0 h, but this was doubled to ensure complete digestion.

At the end of the incubation period the vials were transferred to a 60°C water bath and incubated for 10 min, to inactivate the enzymes. After which 5 μ l of a ficoll solution (10% wv^{-1}) was added to prevent trailing of the bands when electrophoresed.

The digested samples (35 to 40 μ l) were loaded onto an horizontal agarose gel (Section 2.10.2) together with an internal standard and electrophoresed at 40 mA for 2.5 or 3 h. On each gel five samples of the same plasmid, digested with the five enzymes were electrophoresed together with an *Hind* III digest of the phage lambda (λ) in an internal standard. For more direct comparison of different plasmids different plasmid samples, digested with the same restriction endonuclease, were electrophoresed side-by-side, again with the λ standard.

After a 15 min run-in period the gels were flooded with electrode buffer (ca 50 ml) and covered with a piece of high density polythene to prevent evaporation. The flooding was repeated halfway through the electrophoresis period. After 2.5 or 3 h the gels were stained and photographed as described (Section 2.6.2) and the digest patterns compared.

2.10.4 Sizing the plasmids from the endonuclease digest patterns

The *Hind* III digestion of the phage λ DNA produced bands of known molecular weight (Philippsen *et al.*, 1978) from which a standard curve of fragment size and migration distance was produced by a double \log_{10} plot. The fragment sizes resulting from the endonuclease digestion of the unknown plasmids were then calculated. Identification of bands from the digests was made easier by enlarging the negative and using different grades of photographic paper (grades 2 and 3) to increase the contrast.

Having sized the fragments according to their migration distance the size of the whole plasmid was determined by summation of the fragment sizes. Bands which were notably more intense than their immediate neighbours were considered to be two bands with almost identical migration rates, and hence of equal size, and were added to the total twice.

The sizing from each digest pattern was averaged to give an overall estimate of the plasmids' molecular weight.

2.11 MATERIALS

2.11.1 Materials used in enzymatic analysis

The defined medium components for both batch and continuous culture

were obtained from Fisons Ltd (Loughborough, Leicestershire) and BDH Chemicals (Poole, Dorset). Yeast extract and LabM agar supplied by London Analytical and Bacteriological Media Ltd (London). The chlorinated aliphatic acids were of the highest, commercially available purity: MCA, 3MCPA and TCA from Hopkins & Williams (Chadwell Heath, Essex); 2MCPA, 2MBBA, 2MBPA and DCA - from Koch-Light Laboratory Ltd (Colnbrook, Bucks); 3MBPA - from Aldrich Chemical Co Ltd. (Gillingham, Dorset); 2MCBA, from Fluorochem Ltd (Glossop, Derbyshire), 3MCBA - from Pfaltz & Baver Inc. (Stamford, Conn, U.S.A.); 4MCBA - from Ralph N. Emanuel Ltd; 22DCPA was provided by Dow Chemical Co Ltd. as the sodium salt - Dalapon and was further purified by D. Lovatt for enzyme assays.

The compounds for electrophoretic studies were supplied by: $(\text{NH}_4)_2\text{S}_2\text{O}_8$ and AgNO_3 Fisons Ltd (Loughborough, Leicestershire); NNMethylene-bis-acrylamide, TEMED, chromatographically homogeneous glycine, SDS, Page Blue G90, 2mercaptoethanol and acrylamide- BDH Chemicals Ltd. (Poole, Dorset); Glycerol - Hopkin & Williams Ltd (Chadwell Heath, Essex); D-L-Dithiothreitol, Bovine serum albumin, N tris(hydroxymethyl)methyl-2aminoethane sulphonic acid (Tes) and Trizma base - Sigma (Poole, Dorset); Bromophenol Blue and sodium potassium tartrate - May & Baker Ltd (Dagenham, Essex).

All the acids and methanol were obtained from Fisons Ltd (Loughborough, Leicestershire).

2.11.2 Materials used in plasmid identification and characterisation

All the antibiotics, lysozyme (egg white), ribonuclease A, agarose type II medium E.E.U, ethidium bromide and ficoll were supplied by Sigma (Poole, Dorset). Dow Corning Antifoam - Hopkin & Williams

(Chadwell Heath, Essex); Xylene cyanol FF and Na EDTA - BDH Chemicals Ltd (Poole, Dorset). The heavy metals were obtained from BDH Chemicals Ltd and Fisons Ltd (Poole, Dorset). Nutrient Broth from Oxoid (Basingstoke Hampshire).

The restriction endonucleases supplied by: Miles (Slough, Bucks); Boehringer Mannheim (London) and Bethesda Research Laboratories Ltd (Cambridge).

CHAPTER 3

ISOLATION AND IDENTIFICATION OF SOIL BACTERIA CAPABLE OF UTILIZING HALOGENATED ALKANOIC ACIDS AS THE SOLE SOURCE OF CARBON AND ENERGY

Many reports have indicated the existence of soil bacteria capable of utilizing halogenated compounds as their sole source of carbon and energy (Goldman, 1965, 1972; Goldman *et al.*, 1968; Little & Williams, 1971; Senior *et al.* 1976a & b; Slater *et al.*, 1979). Although all of the isolated organisms have possessed dehalogenases, each enzymic system has demonstrated different characteristics of induction, substrate specificity or sensitivity to thiol reagents. This suggests that a number of dehalogenase systems may exist; indeed Goldman *et al.* (1968) and Slater *et al.* (1979) have described systems involving two enzymes. This work was instigated to isolate a number of soil bacteria capable of utilization of halogenated substrates and to study their dehalogenating mechanisms.

3.1 ISOLATION METHODS

Two methods were used to isolate organisms capable of utilizing MCA, DCA, 2MCPA or 22DCPA as their sole carbon and energy sources (Section 2.2.1).

The first method involved suspending soil samples in sterile buffer and spreading samples into Petri dishes of solidified defined medium containing one of the four halogenated compounds. The second procedure involved a 7d enrichment of the soil sample under batch culture conditions followed by plating samples of the culture onto selective media as described (Section 2.2.1). Isolates capable of growth on these substrates were purified by repeated dilution-streaking

onto fresh selective media.

3.2 RESULTS FROM THE TWO ISOLATION-TECHNIQUES

The first procedure resulted in the isolation of a number of organisms which grew on the halogenated compounds as their sole source of carbon and energy. These isolates, described as isolates S1 to S88, were shown to be capable of growth on all four substrates, although the growth on the dihalogenated substrates was in general less vigorous than on the mono-substituted compounds. However, growth on all substrates was not extensive, producing colonies of no more than 1 mm in diameter after 48 h incubation. Furthermore, growth was poor in liquid cultures (Section 2.1.1) which suggested that the isolated organisms were not suited to these conditions. Repeated subculturing resulted in the loss of a number of the isolates. Growth was enhanced by the presence of yeast extract although not significantly in batch cultures. The presence of soil extract promoted the growth of organisms, especially fungi, but when these organisms were transferred to solid media, in the absence of soil extract, they failed to grow. This suggested that growth was promoted by carbon sources present in the soil extract.

The second procedure, involving the enrichment phase, resulted in the isolation of many organisms capable of rapid growth on the halogenated substrates. These organisms were named E1 to E30 and produced colonies 2 to 3 mm in diameter in 48 h. After purification a number of isolates were shown to grow in batch culture to give final absorbance values of 0.5 at 610 nm after 30 h. The presence of yeast extract, especially for the dihalogenated substrates, considerably enhanced the growth characteristics and the rate of chloride release by

the cultures. Maximal absorbances corresponded to chloride ion levels which indicated complete dehalogenation of the substrates.

Repeated subculturing of the E isolates did not result in the loss of growth capability as was found with the S isolates obtained by the first method. However, the addition of yeast extract to the growth media containing the dichlorinated substrates was required to maintain viability. Subsequent observations indicated that these compounds, especially DCA, were not ideal substrates and those organisms enriched on them grew more effectively when supplied with the mono-substituted equivalents.

3.3 GROWTH SUBSTRATE SPECIFICITIES

The ability to utilize the different halogenated substrates, other than the one on which the organism was isolated, was qualitatively assessed by inoculating media containing one of the four substrates and comparing the amount of growth after 48 h incubation. The ability to grow on the theoretical breakdown products of the halogenated substrates was also assessed in the same manner.

All of the isolates were capable of growth on MCA, DCA, 2MCPA and 22DCPA (Table 3.1) although visibly greater growth was observed on the mono-substituted compounds. In order to overcome any toxic effects of the substrates, as had previously been described (Slater *et al.*, 1979), four concentrations of each substrate were used; 0.25, 0.5 1.0 and 1.5 gCl^{-1} . In a number of cases the highest concentration of the substrate inhibited growth completely. For example MCA at 1.5 gCl^{-1} inhibited growth of isolates E2 to E8 inclusively and also E12, E15 and E17. Usually the 0.5 gCl^{-1} concentration enabled the maximum growth observed

TABLE 3.1 Substrate Specificities of a number of soil bacteria isolated by enrichment on halogenated substrates. Growth described qualitatively in order of increasing magnitude: +, ++, +++, +++++, -, no growth.

Isolate	Enrichment substrate	MCA (gCl ⁻¹)				Substrate DCA (gCl ⁻¹)				2MCPA (gCl ⁻¹)				22DCPA (gCl ⁻¹)			
		0.25	0.5	1.0	1.5	0.25	0.5	1.0	1.5	0.25	0.5	1.0	1.5	0.25	0.5	1.0	1.5
E1	2MCPA	++	+++	+++	+++	++	++	++	++	++	++	++	++	+	+	++	++
E2	2MCPA	++	+++	+++	-	+	++	++	++	++	+++	+++	+	+	+	+	+
E3	2MCPA	++	++	+	-	++	+	+	-	+	+	+	+	+	+	+	+
E4	2MCPA	++	+	+	-	+	+	+	+	+++	+++	+++	+	+	+	+	+
E5	2MCPA	+	+	+++	-	+	+	+	+	++	+++	+++	+	+	+	+	+
E6	2MCPA	+	+	++	-	++	+	+	+	++	+++	+++	+	+	+	+	+
E7	2MCPA	++	+	+	-	++	+	+	+	++	+++	+++	+	+	+	+	+
E8	2MCPA	+	+	+	-	+	+	+	+	++	+++	+++	+	+	+	+	+
E11	MCA	++	+++	+++	+++	++	+	+	+	++	+++	+++	+	+	+	+	+
E12	MCA	++	++	+++	-	++	+	+	+	++	+++	+++	+	+	+	+	+
E13	MCA	++	+++	+++	+++	+	+	+	+	++	+++	+++	+	+	+	+	+
E14	MCA	++	++	+++	+	++	+	+	+	++	+++	+++	+	+	+	+	+
E15	MCA	++	+++	+++	-	++	+	+	+	++	+++	+++	+	+	+	+	+
E16	MCA	+++	+++	+++	+++	+	+	+	+	++	+++	+++	+	+	+	+	+
E17	MCA	+++	+++	+++	-	++	+	+	+	++	+++	+++	+	+	+	+	+
E18	MCA	+	++	+++	+++	++	+	+	+	++	+++	+++	+	+	+	+	+

and this concentration was used in all subsequent experiments.

Growth on the theoretical breakdown products, glyoxylate, glycolate, succinate, pyruvate, acetate and lactate, indicated that all of the isolates could utilize all of the substrates. However, qualitative differences in growth between the various isolates suggested differing abilities to utilize the given substrates, which in turn indicated that there were a number of different types of organisms isolated.

3.4 IDENTIFICATION OF THE SOIL ISOLATES

A number of morphological and physiological tests were undertaken to identify the soil isolates. These included: Gram-stain, cell morphology, motility, catalase and oxidase activity, oxidation or fermentation of glucose, indole production, and acid production from glucose, lactose, mannitol, maltose, xylose or sucrose. However, it was not possible to segregate any of the isolates according to the results of these tests as each isolate produced very similar results. They were all Gram-negative, motile, rod-shaped bacteria which were catalase and oxidase positive, non-fermentative, indole negative and produced acid from all of the sugars. One exception was isolate E2 which was the only isolate not able to produce acid from glucose.

Samples of the isolates were sent to the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen for identification. Representatives were chosen from members of groups segregated in accordance with their dehalogenase activities (Chapter 4). The results are given in Tables 3.2 and 3.3. The great similarities observed in the original tests are reflected in these results, with isolates E3, E4 and E6 appearing closely related as do isolates E20 and E22. Isolate E2 belonged

TABLE 3.2 Identification of soil isolates (by courtesy of the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen)

Isolate	FIRST STAGE						Comments
	E2	E3	E4	E6	E20	E22	
0°C Incubation	30	30	30	30	30	30	
Gram	-ve	-ve	-ve	-ve	-ve	-ve	
Spores	none	none	none	none	none	none	
Motility	+	+	+	+	+	+	
Flagella (EM)	single polar	multi polar	multi polar	1-6 polar	peritrichous	peritrichous	
Colonial morphology	Off-white, low convex, smooth, entire, circular, translucent 2 d	Off-white, flat, smooth, entire, circular, translucent 2 d	as for E3	Off-white (slightly yellow) low convex, smooth, entire, circular, translucent 1 d	similar to E22	Off-white, low convex, smooth, entire, circular, translucent 3/4-1mm 1d	Growth on Oxoid CM3 Nutrient Agar
0°C Growth	37° + 41° -	50° + 30° + 37° -	50° + 30° + 37° -	50° + 37° + 41° -	37° + 41° -	37° + 41° -	
Catalase	+	+	+	+	+	+	
Oxidase, Kovacs	+	+	+	+	+	+	
O-F Glucose	oxidative (weak acid)	oxidative	oxidative	oxidative	oxidative	oxidative	E6 one stain regarded as negative other as oxidative.
First Stage Identification	All Gram negative, non-fermentatives.						

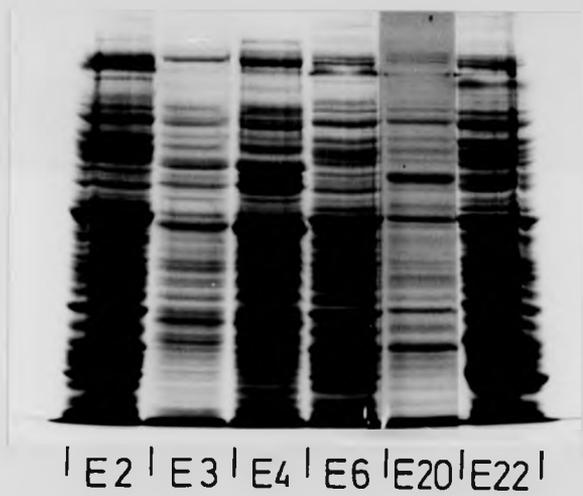
TABLE 3.3 Identification of soil isolates (by courtesy of the N.C.I.B. Torry Research Station, Aberdeen).

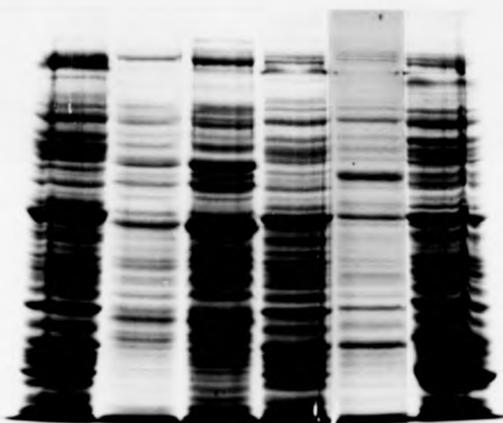
Isolate	SECOND STAGE						Comments
	E2	E3	E4	E6	E20	E22	
^o C Incubation	25	25	25	25	25	25	
Pyocyanin	-	-	-	-	-	-	
Fluorescence	-	-	-	-	-	-	NH ₄ -salts
DL-Arg CSU	-	+	+	-(+)	-	-	
Betaine CSU	-	-	+	-(+)	-	-	Medium
Glucose CSU	+	+	+	-(+)	-	-	(+) w.r.t. Second E6 strain
Lactate CSU	+	+	+	-(+ with met)	-	-	
Acetate CSU	+	+	+	-(+)	-	-	
Penicillin G	-	-	-	-	-	-	
Streptomycin	+	+	+	+	+	+	
Chloramphenicol	+	+	+	+	+	+	
Tetracycline	+	+	+	+	+	+	
Novobiocin	-	-	-	-	-	+	
Polymixin B	-	+	+	+	+	+	
O/129	-	-	-	-	-	-	
Levan	-	-	-	-	-	-	
Growth factor requirement	-	-	-	methio-nine	+(?)	+(?)	E20, E22 +? as negative CSU
Acid glucose	-	-	-	-	-	-	
Gas glucose	-	-	-	-	-	-	
ONPG	-	+	-	+	-	-	
Arg Møller	+	+	+	+	-	-	
Lys Møller	-	+	+	+	-	-	
Orn Møller	-	-	+(trace)	-	-	-	
NO ₃ ⁻ to NO ₂ ⁻	+	-	-	-	-	-	(+) w.r.t. second E6 strain
NO ₃ ⁻ to N ₂	+	+(weak)	+	-(+)	+	+	
DNA ase (20+25°C)	+	-	-	-	-	-	
Gel stab 20 ^o	-	+	+	+	-	-	(-) w.r.t. second E6 strain
Gel plate	-	-	-	+(-)	-	-	
Casein	-	-	-	+(-)	-	-	strain
Starch	-	-	-	-	-	-	
Lecith egg	-	-	-	-	-	-	(-) w.r.t. second E6 strain
Lipase egg	-	-	-	+(-)	-	-	
NH ₃	-	-	-	+(-)	-	-	strain
Indole	-	-	-	-	-	-	
H ₂ S	-	-	-	-	not determined		

TABLE 3.4 Identification of the soil isolates (E60 was the stock of E6 - see text for details)

Nomenclature used	Identification
E2	<i>Pseudomonas</i> sp. or <i>Alteromonas</i> sp.
E3)	<i>Pseudomonas</i> sp.
E4)	
E60)	
E6	<i>Pseudomonas maltophilia</i>
E20)	<i>Alcaligenes</i> sp.
E22)	

*Figure 3.1: S.D.S. polyacrylamide gel electrophoresis of
the protein constituents of the soil isolates.*





| E2 | E3 | E4 | E6 | E20 | E22 |

to a group on its own.

Table 3.4 indicates the isolates' identification as suggested by the N.C.I.B. and subsequently these organisms are to be described as *Pseudomonas* sp. strain E2; *Pseudomonas* sp. strains E3, E4 and E6 and *Alcaligenes* sp. strains E20 and E22.

Protein-SDS electrophoresis of cell-free extracts of each isolate enabled a comparison of the proteins possessed by each organism to be studied. The more closely related the organisms are the greater the similarity of the protein profiles. The definition on the resulting gels was not good, due to the large number of proteins present, however a large number of bands were common to all the isolates (Figure 3.1). It was difficult to distinguish any differences for the *Pseudomonas* sp. strains E3, E4 and E6. However, the number of differences between these and *Pseudomonas* sp. strain E2 and the *Alcaligenes* sp. strains E20 and E22 provided further evidence to suggest that the latter three strains were different organisms to the former three isolates.

3.5 DISCUSSION

Under batch enrichment conditions the substrate is in excess and hence selection is based solely on the maximum specific growth rate of the organisms present (Jannasch, 1967; Parkes, 1982). The organisms selected are characteristically capable of high growth rates at high substrate concentrations, that is, they are **zymogenous**.

The enrichment techniques resulted in the isolation of a number of bacteria capable of rapid and effective growth on the halogenated substrates. This growth was much more efficient than that of organisms isolated directly from the same soil. Apparently the period of mixed

culture growth in the enrichment cultures selected organisms which were more capable of utilizing the substrate than the parental strains used to inoculate the cultures originally.

It is possible that the presence of a mixed population of microorganisms as opposed to the 'pure cultures' on selective media, represented by the individual organisms and eventually individual colonies, provided the necessary additional metabolites to overcome either the toxic nature of the substrate or the gradual depletion of growth factors in the enrichment medium. The growth of larger populations under conditions not suitable for growth of individual organisms was described by Jannasch (1967**b**).

Hall and Zuzel (1980) investigated mutations of the *ebg* A gene of *Escherichia coli* and discovered that the mutations fall into two classes neither of which could utilize galactosylarabinose. However, when both classes of mutation were present in the same *ebg* A gene, the *ebg* enzyme acquired a specificity for this substrate. They suggested that their results showed that a new enzymatic function could evolve via recombination within the *ebg* A gene. They envisaged the situation in which mutants of the same gene, which had diverged under different selection pressures (Section 1.5), could recombine, when the population was remixed, to generate a new allele with a substrate specificity present in neither parent. Thus, the daughter organisms could exploit resources, in this case a carbon source, unavailable to either parent.

Work with *Pseudomonas putida* strain PP3 (Slater *et al.*, 1979) has indicated that the dehalogenases are the growth-rate limiting enzymes. Thus the selection of organisms capable of more efficient utilization

of the halogenated substrates during the batch enrichment stage suggests that the concentration of dehalogenase within these more efficient organisms was increased, so allowing more rapid growth. This could have been achieved by either selecting for constitutive mutants, or selecting for mutants with gene multiplication (Section 1.4.5) or selecting for strains with more than one different dehalogenase obtained through the activity of any gene transfer mechanism.

The increased growth-rate cannot be explained by the selection of constitutive mutants, as all the dehalogenase mechanisms in the isolates were demonstrated to be inducible. Their activities were absent in cultures grown on succinate as the sole carbon source yet when transferred back to halogenated substrate containing medium activity could be detected.

The apparent requirement for a period of mixed culture growth, in order to isolate bacteria capable of efficient growth on the halo-compounds, indicates that the selection process could involve the accumulation of different dehalogenase genes in the same organism from different parents. That is by a mechanism similar to the one envisaged by Hall and Zuzel (1980).

The inability of those isolates selected for growth on the halogenated substrates directly from soil samples to grow reliably on these compounds caused practical difficulties which culminated in a decision to concentrate further experimental effort towards the study of the bacteria isolated from the enrichment cultures (the E isolates). These organisms grew effectively in batch culture with MCA or 2MCPA as their sole carbon source. However, DCA was apparently relatively toxic to all the isolates, including those enriched with DCA as the carbon source, and growth on 22DCPA was restricted. Because of this further research centred around the following

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isolates: *Pseudomonas* sp. strain E2; *Pseudomonas* sp. strains E3, E4 and E6, all of which utilized 2MCPA as their sole carbon and energy source, and *Alcaligenes* sp. strains E20 and E22. The former *Alcaligenes* strain although initially capable of growth on MCA in batch culture, later became unable to do so yet it maintained the ability to utilize this compound in continuous-flow culture. *Alcaligenes* sp. strain E22, originally isolated from the MCA enrichment culture, later apparently lost the ability to grow on MCA in both batch and continuous-flow culture, yet was capable of growth on 2MCPA under either of the culture conditions. The reason for these changes is not understood but apparently MCA became toxic to these organisms such that even at 10 and 50-fold lower concentrations, that is 0.05 and 0.01 gC MCA ℓ^{-1} , growth was inhibited.

The results from the identification studies (Table 3.4) indicated a different result for the isolate E6 and isolate E60. The latter was from a stock culture of the other. The isolate E6 was tentatively identified as *Pseudomonas maltophilia* largely on its auxotrophic requirement for methionine, a requirement which isolate E60 did not exhibit. As they are known to be the same organism it is suggested that the isolate E6 should have been grouped along with isolates E3 and E4 and described as a *Pseudomonas* sp. especially as nearly all of the characteristics of isolate E6 are the same as isolates E3 and E4.

Although isolates E20 and E22 were classed as strains of *Alcaligenes* sp. it was suggested that with different tests for glucose utilization (Tatum *et al.*, 1974) both isolates may have been classed as *Achromobacter* sp. strains.

CHAPTER 4

DEHALOGENASE COMPLEMENT UNDER BATCH-CULTURE CONDITIONS

Considerable variation has been recorded in the catalytic-activity of several dehalogenases from a number of species of micro-organisms (Davies & Evans, 1962; Little & Williams, 1971; Goldman *et al.*, 1968; Berry *et al.*, 1979; Slater *et al.*, 1979). These enzymes demonstrate different substrate specificities; for example, Jensen (1960) noted that the MCA-induced dehalogenase of *Pseudomonas dehalogenans* attacked MCA and DCA, when the organism belonged to groups I or II, but if a group III organism, the substrate range was increased to include 22DCPA. Several investigations on different bacterial strains (Goldman *et al.*, 1968; Weightman *et al.*, 1979; Weightman & Slater, 1980) have suggested that more than one dehalogenase was present. This was also shown for the fungus *Trichoderma viride* (Jensen, 1960).

Although a number of dehalogenase systems have been described, dehalogenase variation has not been systematically studied. The dehalogenase activities of soil bacteria, isolated by batch-enrichment, have been investigated and the enzyme profiles studied using polyacrylamide gel electrophoresis.

4.1 RELATIVE DEHALOGENASE ACTIVITIES

The activity of dehalogenating enzymes enables microorganisms to utilize halogenated compounds as sources of carbon and energy. The concomitant release of chloride ions observed when soil isolates were growing on such halogenated substrates suggested the presence of one or more dehalogenating mechanisms.

Slater *et al.* (1979) demonstrated the presence of dehalogenase

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Slater *et al.* (1979) demonstrated the presence of dehalogenase

activity in cell-free extracts prepared from *Pseudomonas putida* strain PP3. Evidence for dehalogenase activity in cell-free extracts from the twenty soil isolates was obtained by detecting chloride ion release using a chloro-counter (Section 2.3.1). The extracts were studied for activity towards the four substrates on which the organisms had been originally isolated, MCA, DCA, 2MCPA and 22DCPA. All the cell-free extracts possessed the ability to dehalogenate all the substrates, although activity towards each substrate differed with respect to the rate of dechlorination.

The varying abilities of the different cell-free extracts to dehalogenate the four substrates made it possible to group sixteen of these isolates according to their relative activities. The dehalogenation rates towards DCA, 2MCPA and 22DCPA were standardized to the MCA dehalogenation rate, and the rates achieved by each isolate compared (Table 4.1). The determination of the rate of chloride release accounted for the presence of two chloride atoms per molecule in the disubstituted compounds.

The isolates were placed into five groups in accordance with their relative dehalogenase activities.

Group A organisms were segregated on the basis of the dominant MCA dehalogenase activity followed by DCA, 2MCPA and 22DCPA in order of decreasing activity. The rate of dehalogenation of DCA was in general 10 to 20% of the MCA rate. All of these organisms, with the exception of isolate E1, were isolated by enrichment on MCA and it might be expected that the activity towards MCA would be the highest. Indeed, the exception, isolate E1, may not belong to this group as it exhibited

TABLE 4.1 Relative dehalogenase activity (standardized with respect to the MCA dehalogenating rate) in cell-free extracts of sixteen soil bacterial isolates. The errors quoted are standard deviations

Group	Isolate	Enrichment substrate	Relative Dehalogenase Activity			
			MCA	DCA	2MCPA	22DCPA
A	E1	2MCPA	1.0	0.43 ^{-0.02}	0.28	0.17
	E12	MCA	1.0	0.12	0.11	0.07
	E13	MCA	1.0	0.13 ^{+0.02}	0.09 ^{+0.02}	0.07 ^{+0.02}
	E14	MCA	1.0	0.21 ^{+0.07}	0.15 ^{+0.03}	0.10 ^{+0.02}
	E15	MCA	1.0	0.20 ^{+0.06}	0.17 ^{+0.06}	0.11 ^{+0.04}
	E16	MCA	1.0	0.08 ^{+0.01}	0.14 ^{+0.03}	0.07 ^{+0.02}
	E17	MCA	1.0	0.21 ^{+0.04}	0.16 ^{+0.02}	0.14 ^{+0.02}
	E20	MCA	1.0	0.18 ^{+0.01}	0.17 ^{+0.04}	0.13 ^{+0.04}
	E22	MCA	1.0	0.18	0.11	0.08
B	E4	2MCPA	1.0	2.11 ^{+0.18}	0.26 ^{+0.02}	0.11
	E5	2MCPA	1.0	1.50 ^{+0.05}	0.28 ^{+0.06}	0.14 ^{+0.02}
	E7	2MCPA	1.0	1.70 ^{+0.21}	0.22 ^{+0.07}	0.14 ^{+0.03}
	E8	2MCPA	1.0	2.23 ^{+0.06}	0.26 ^{+0.07}	0.02 ^{+0.03}
C	E2	2MCPA	1.0	0.20	0.21	0.03
D	E3	2MCPA	1.0	1.96	0.35	0.22
E	E6	2MCPA	1.0	0.70 ^{+0.06}	0.16 ^{+0.03}	0.08 ^{+0.01}

relatively greater DCA and 2MCPA activity than the other members of the group. Isolate E16 also showed atypical activity towards DCA demonstrating only 50% of the relative DCA activity exhibited by the other members of the group.

The correlation between enrichment substrate and maximal enzymatic activity towards the substrate did not hold for the other groups, all of which were isolated from 2MCPA enrichment cultures, yet showed greater activity towards MCA or DCA. That is, none showed highest activity towards 2MCPA.

Group B isolates demonstrated the highest DCA relative activities, which were 1.5 to 2.5 times greater than the MCA dehalogenation rates, and were 10 to 20 times higher than the relative DCA dehalogenation rate of group A organisms. In comparison with the group A organisms the group B isolates exhibited significantly higher relative 2MCPA and 22DCPA dehalogenating activities. Isolate E1 again represented an anomaly in group A as its 2MCPA activity was more comparable to the activity exhibited by group B organisms, as is also true for its 22DCPA relative activity. However, the DCA activity of isolate E1 precludes it from group B as the relative activity towards this substrate is 4 to 5 times lower than the relative activities exhibited by the group B isolates.

Isolate E2 (*Pseudomonas* sp. strain E2) was placed in a category of its own because of its significantly higher 2MCPA dehalogenating ability in comparison with group A isolates, and the 10-fold lower activity towards DCA than the group B organisms. The unique 22DCPA relative dehalogenase activity of this organism, being approximately 7

times lower than the 2MCPA relative dehalogenation rate, contrasted with the 22DCPA relative activity of all the other isolates which demonstrated a 2-fold difference between 2MCPA and 22DCPA relative activities.

Group D was composed of isolate E3 (*Pseudomonas* sp. strain E3) as it possessed a greater 2MCPA relative activity and a high DCA dehalogenating ability.

Finally isolate E6 (*Pseudomonas* sp. strain E6) was placed into a separate group, group E, because of its intermediate DCA relative activity, which was 3 to 5 times higher than the same relative activities seen for group A organisms.

4.2 SPECIFIC DEHALOGENASE ACTIVITIES

Examination of the specific activities of the cell-free extracts (Table 4.2) suggested that isolate groupings could be achieved, as expected, which were very similar to those obtained from the relative activity data.

As indicated by the relative activities, the specific activities towards the individual substrates did not correlate with the substrate on which the isolate was enriched and cultured. *Pseudomonas* sp. strain E2 and *Pseudomonas* sp. strains E3 and E4 were all isolated and maintained on 2MCPA yet cell-free extracts of *Pseudomonas* sp. strains E2 and E3 demonstrated greatest specific activity towards MCA with a specific activity 3 to 4 times greater towards MCA than 2MCPA and *Pseudomonas* sp. strain E4 demonstrating a specific activity

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TABLE 4.2 Dehalogenase specific activities in cell-free extracts of sixteen soil bacteria grown in closed culture

Group	Isolate	Enrichment substrate	Dehalogenase Specific Activity ($\mu\text{mol substrate converted}(\text{mg protein})^{-1}\text{min}^{-1}$)			
			MCA	DCA	2MCPA	22DCPA
A	E1	2MCPA	0.90	0.38	0.18	0.07
	E12	MCA	0.54	0.07	0.06	0.04
	E13	MCA	2.75	0.30	0.20	0.14
	E14	MCA	1.56	0.25	0.15	0.05
	E15	MCA	0.16	0.26	0.20	0.13
	E16	MCA	2.38	0.17	0.33	0.13
	E17	MCA	0.67	0.15	0.12	0.10
	E20	MCA	0.42	0.08	0.07	0.05
	E22	MCA	0.39	0.12	0.07	0.05
B	E4	2MCPA	0.27	0.40	0.05	0.02
	E5	2MCPA	0.25	0.36	0.07	0.04
	E7	2MCPA	0.30	0.54	0.07	0.04
	E8	2MCPA	0.28	0.62	0.07	0.05
C	E2	2MCPA	1.26	0.26	0.27	0.03
D	E3	2MCPA	0.95	0.55	0.17	0.05
E	E6	2MCPA	0.64	0.46	0.11	0.05

towards DCA which was 8 times greater than towards 2MCPA.

A comparison of the specific activities of isolate E1 with those of other members of group A, suggested that this organism did belong to this group, although the DCA ability was significantly greater than most of the other isolates.

The specific and relative activities of the members of groups A and B showed significantly different degrees of variation. In group A, the specific activity towards MCA varied from 0.39 to 2.75 μmol substrate converted $(\text{mg protein})^{-1} \text{min}^{-1}$ whereas in group B, the activities varied from 0.25 to 0.30 μmol substrate converted $(\text{mg protein})^{-1} \text{min}^{-1}$.

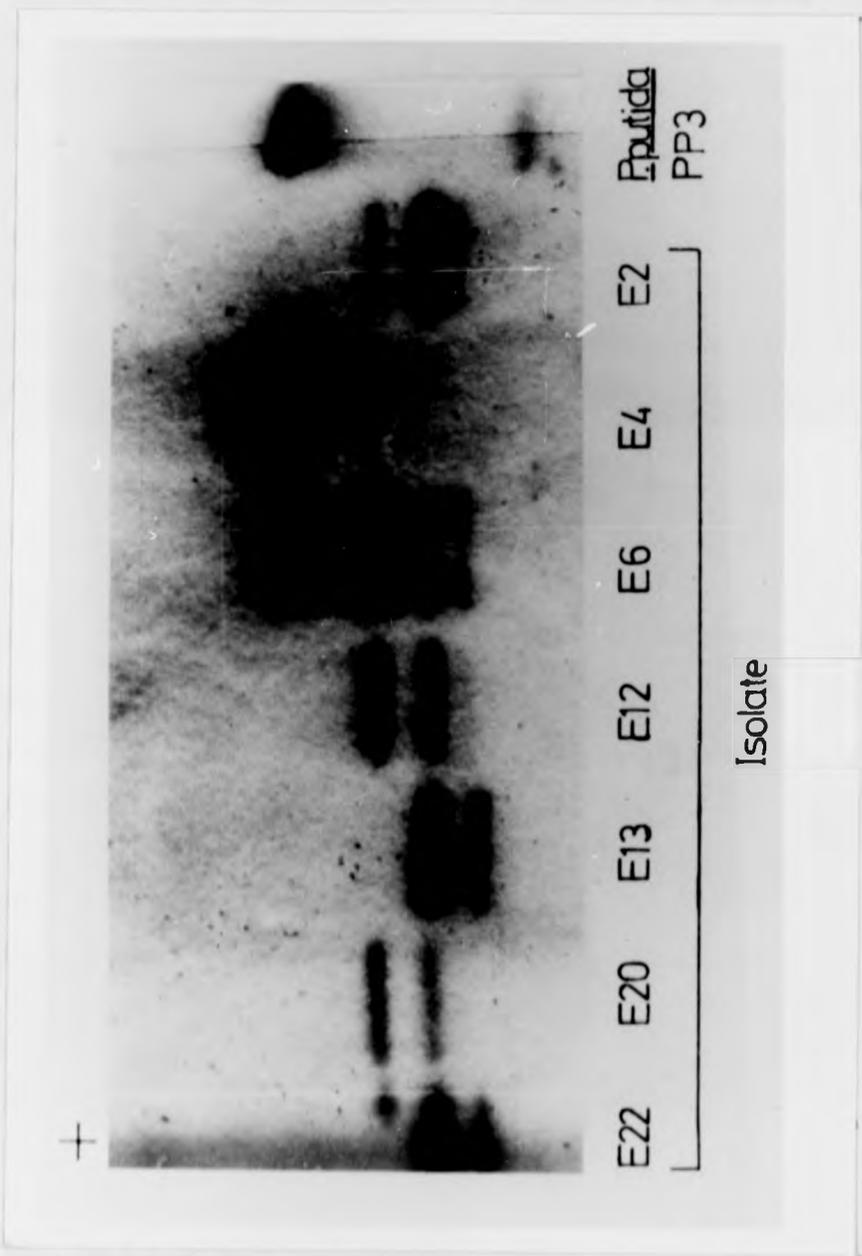
The variations observed between each isolate were also seen for different cell-free extracts of the same organism over repeated assays, although not for repeated assays using the same cell-free extract. These differences were less for group B and D organisms than for the other groups. The variations for each cell-free extract, usually 4 or 5 extracts, are noted as standard deviations seen on Table 4.1, the values quoted represent average relative activities of the 4 or 5 assays.

4.3 ELECTROPHORETIC STUDIES OF THE DEHALOGENASE MECHANISMS

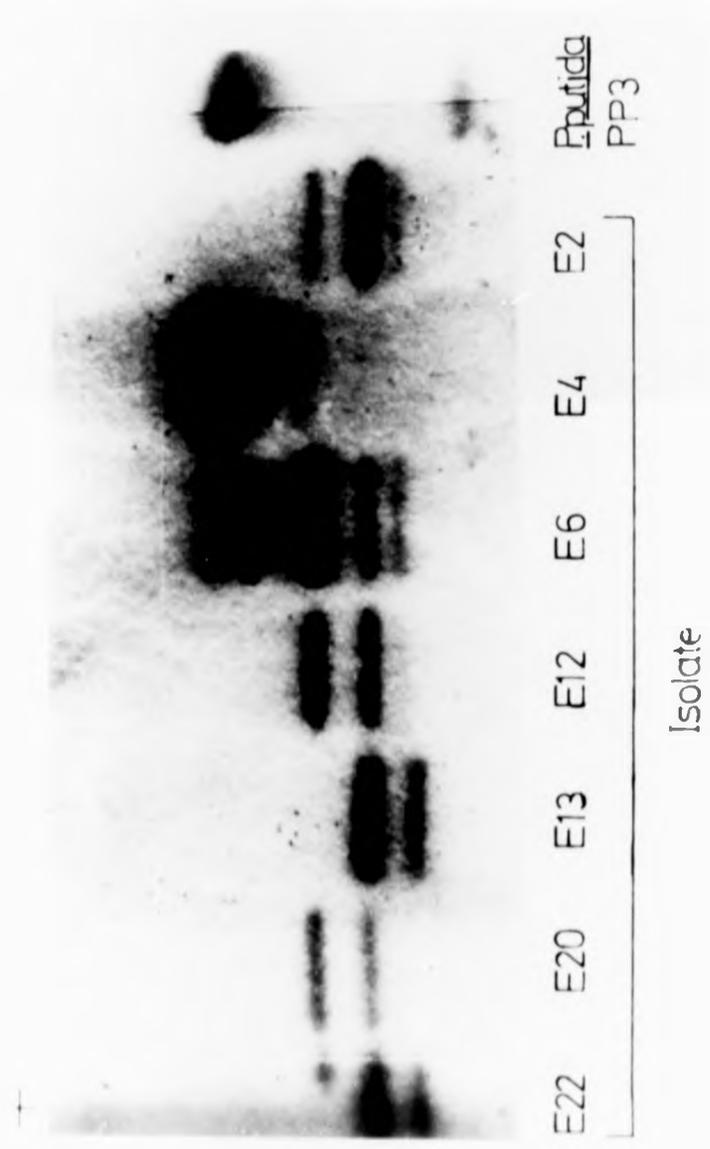
A polyacrylamide gel electrophoresis system developed by Weightman and Slater (1980) provided a method of visualizing dehalogenase activity after electrophoretic separation of the proteins present in cell-free extracts. The chloride released by enzymic dehalogenation of the chlorinated substrates, were used to precipitate silver ions as bands of silver chloride which thus located regions of the gel containing dehalogenase activity.

Figure 4.1: Distribution of five dehalogenases from 16 of the soil bacteria and Pseudomonas putida strain PP3, after separation by polyacrylamide gel electrophoresis.

soil
separation



the soil
separation



Cell-free extracts of the members of each group were prepared and electrophoresed on polyacrylamide gels (Section 2.4). The initial results indicated that all the groups, with the exception of group B, consisted of organisms possessing one or more of four dehalogenases in a number of permutations (Figure 4.1). The group B organisms failed to show any activity on the gels even though assays of the cell-free extracts showed that enzyme activity was present. This suggested that: either the enzyme system was not sufficiently stable to remain active during the 6 h electrophoresis period; some cofactor was separated from the enzyme system during electrophoresis, so inactivating it, or one or more of the gel components was specifically inhibiting the enzyme activity of this group of isolates. In order to discover the reason for the failure to visualize the enzyme system the effects of the individual components of the electrophoretic system were studied.

The stability of the enzyme system: The effect of storage of cell-free extracts of members of group B at 4°C for 26 h was studied by the enzyme assay procedure. A temperature of 4°C was used as the electrophoretic separation was achieved at this temperature. After storage at 4°C for 26 h the dehalogenase activity towards MCA and DCA, the substrates originally used to locate the dehalogenases on the polyacrylamide gels, was determined and compared to the activity of fresh extract (100% activity). After storage, activity towards MCA was still 100%. For DCA, in the presence of dithiothreitol, the dehalogenase activity had fallen to 86%, and in the absence of the thiol reagent, the activity was 67% of that in fresh extract. Thus, the 6 h period of electrophoresis appeared not to be the cause of loss of activity.

pH: The pH value of the gel was 8.8 as compared to the usual enzyme assay pH of 7.9. However, as described in Section 4.7. *Pseudomonas* sp. strain E4 showed greater activity at pH 9.0 than it did at pH 8.0, towards all the substrates. So the different pH of the electrophoretic system should not have interfered with dehalogenase activity of the group B isolates.

Acrylamide: In its monomeric form acrylamide caused 100% inhibition of dehalogenase activity at concentrations comparable to those in the formation of the gel matrix (80.0 g l^{-1}). Although the gel is composed of the much less reactive polymeric form, it is possible that the matrix contained some unpolymerized monomers which might have resulted in the loss of activity within the gel.

Ammonium persulphate: At the concentration used in the formation of the acrylamide gel (1.0 g l^{-1}), ammonium persulphate caused 30% inhibition of the dehalogenase activity.

The acrylamide monomers, ammonium sulphate and TEMED, which has also been shown to inhibit activity of some enzyme systems, may be removed by pre-running the gel overnight (Section 2.4.1). When cell-free extracts of the group B organisms were loaded onto the pre-run gel and electrophoresed, activity was observed in the form of two bands which had previously not been seen in this combination (Figure 4.1). However, when the group A isolates were electrophoresed under those conditions the bands of dehalogenase activity travelled only a third to a half of the distance they did on the gels which had not been pre-run. It seems likely that the structure of the gel has been altered in some way, for although the front, as indicated by the marker dye, migrated more quickly in the pre-run gels (reaching the bottom of the

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gel in 2 to 2.5 h) the proteins migrated at the same speed as in non-pre-run gels. This resulted in R_F values of approximately a third to a half of the R_F values obtained from the gels which were not pre-run. Consequently, these differences made it impossible to compare the enzyme systems of the other groups and those of group B directly. Thus, in order to compare all the R_F values all future electrophoresis was carried out using pre-run gels.

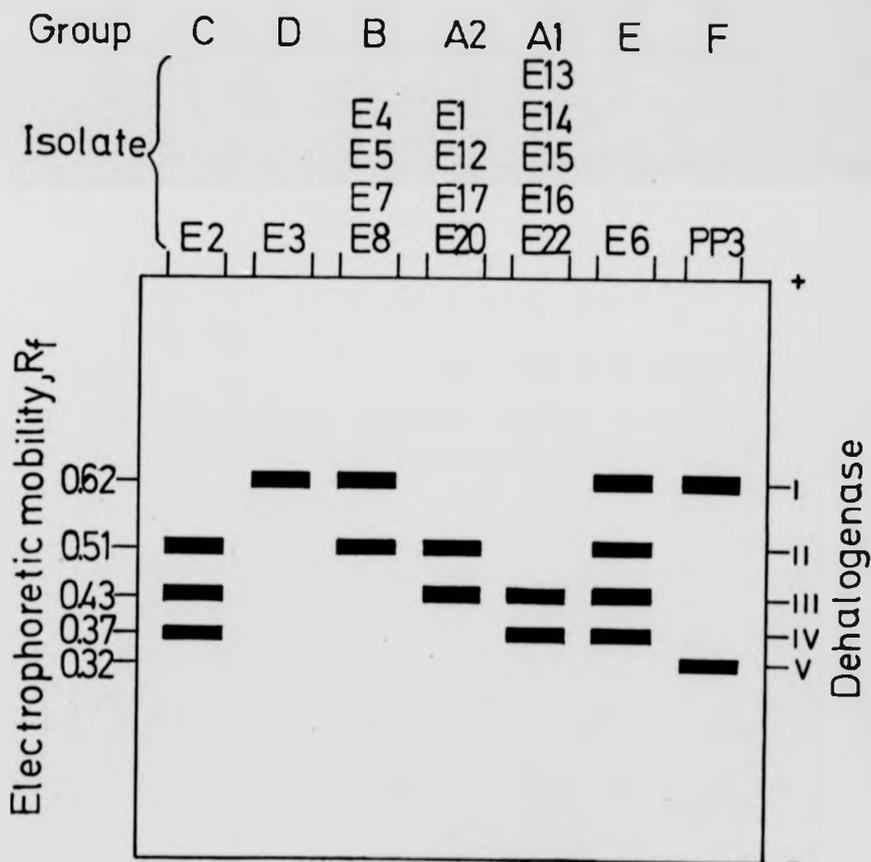
One consequence of pre-running the gels was that the protein bands were less defined. That is, the dehalogenase activity, as identified by silver chloride precipitation, was more diffuse, probably as a result of the equilibration of the pH gradient between the stacking and running gels, negating the stacking effects of the initial part of the gel. However, as the different dehalogenases exhibited substantially different electrophoretic mobilities, the slight loss of resolution did not impair the separation enough to prevent identification of each enzymic form.

4.4 DEHALOGENASE PROFILES OF THE DIFFERENT ISOLATES

Four dehalogenases were identified, possessing unique electrophoretic mobilities. The different enzymes were designated dehalogenase I, II, III and IV in accordance with their electrophoretic mobility (IUPAC-IUB, 1971). The dehalogenase profiles observed, are shown in Figure 4.1 and are summarized diagrammatically in Figure 4.2.

Dehalogenase I possessed the greatest anodal mobility, that is the highest R_F (0.62) and dehalogenase IV the lowest ($R_F = 0.37$). It should be noted that the R_F varied slightly between different gels although within the same gel the relative R_F values for the four enzymes

Figure 4.2: Distribution of five dehalogenases from 16 of the soil bacteria and Pseudomonas putida strain PP3, after separation by polyacrylamide gel electrophoresis. Bars represent bands of silver ohloride precipitation of chloride ions resulting from the dehalogenation of MCA and DCA, with no reference to substrate specificity.



the soil
separation
present bands
resulting
reference.

remained constant.

On the basis of electrophoretic separation and visualization after dehalogenase activity with respect to the substrates MCA and DCA, group A organisms were subdivided into groups A1 and A2. The former consisted of organisms possessing dehalogenases III ($R_F = 0.43$) and IV ($R_F = 0.37$) and the latter dehalogenases II ($R_F = 0.51$) and III ($R_F = 0.43$).

Isolate E2 (*Pseudomonas* sp. strain E2) possessed three enzymes, dehalogenases II, III and IV; dehalogenase III was more active or present in larger amounts, than the other two and frequently dehalogenase IV was not observed. Either this was due to levels of enzyme which were below the limit of resolution of the visualization procedure or the synthesis of the enzyme was variable.

Isolate E4 (*Pseudomonas* sp. strain E4) possessed two enzymes, with dehalogenase I ($R_F = 0.62$) the dominant form and dehalogenase II present at much lower levels, often below the threshold of resolution.

Isolate E6 (*Pseudomonas* sp. strain E6) possessed all four enzymes with dehalogenases I and II the most active under the staining regime used.

The possession of only one enzyme, dehalogenase I by isolate E3 (*Pseudomonas* sp. strain E3) made it unique from all the other isolates.

To compare these enzymes with those from a different organism cell-free extract of *Pseudomonas putida* strain PP3 was electrophoresed along with the other isolates. *P. putida* strain PP3 has been shown to possess two dehalogenases (Slater *et al.*, 1979; Weightman & Slater, 1980) these being described by the authors as fraction I and fraction II dehalogenases. As seen in Figure 4.2 the fraction II dehalogenase

corresponded to the dehalogenase I of the *Pseudomonas* sp. strains E4 and E6, with the same electrophoretic mobility. However, their fraction I dehalogenase had a lower R_F value (0.32) than the dehalogenase IV of *Pseudomonas* sp. strains E2 and E6 and the *Alcaligenes* sp. strain E22 (isolate E22), because of this, in this description (Figure 4.2) the fraction I of Weightman and Slater (1980) was designated as dehalogenase V.

4.5 SUBSTRATE SPECIFICITIES OF THE ISOLATED ENZYMES

The work described in Section 4.4 used both MCA and DCA as the enzyme substrates, simply to enable visualization and did not completely indicate substrate specificities of the different enzymes. However, results of cell-free extract studies of *Pseudomonas* sp. strain E6 (Figure 4.1) indicated some specificities may have existed. This strain demonstrated much greater dehalogenase I and II activity, that is the silver chloride banding was much more dense, than dehalogenase III and IV activity. This may have indicated either the presence of more of the enzymes I and II or else that dehalogenase I and II were more active towards MCA and DCA than dehalogenases III and IV.

The determination of overall specific activities (Table 4.2) indicated that the dehalogenase systems did demonstrate some degree of specificity, with greater activity in general towards the substituted acetic acids than towards the propionic acids.

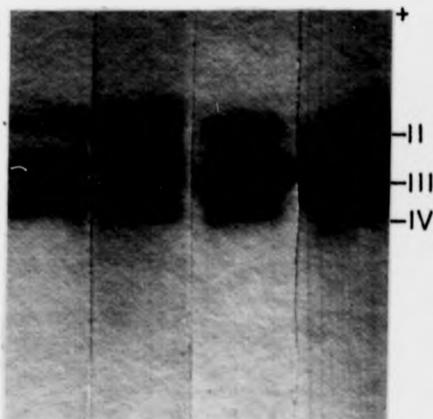
To study the specificities of each dehalogenase, four samples of cell-free extracts of each organism were electrophoresed as described in Section 2.4. Then the gels were cut into sections and one of each sample of cell-free extract incubated in buffer containing one of

the four halogenated substrates, MCA, DCA, 2MCPA and 22DCPA. Activity was then visualized (Section 2.4.3) and the activities of the different dehalogenases qualitatively compared. Such judgement of the significance of the density of each band was attempted cautiously since the degree of silver chloride precipitation depended on the number of chloride ions per substrate molecule. The density was also dependent on the amount of cell extract loaded onto the gels as extracts with a higher protein content would have possessed higher enzyme levels. Also the amount of synthesis of each enzyme, which could vary proportionally as a function of the stage of growth or the growth environment, would affect the relative densities of the bands. Precautions were taken to sample batch cultures at the same period, late exponential phase, for each isolate to standardize these variables.

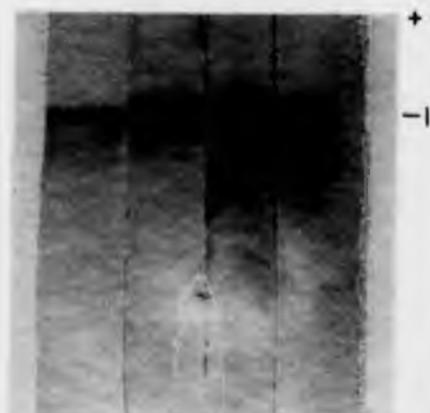
In general the heaviest precipitation of silver chloride, indicating the highest enzyme activity, was obtained when the gels had been incubated in the chloroacetate-containing buffers, which was consistent with the specific activities previously described (Table 4.2). This was shown to be true even for those organisms isolated after 2MCPA enrichment which was again consistent with the enzyme assay results (Tables 4.1 and 4.2). Figures 4.3 and diagrammatically in Figure 4.4 b and c show this for isolates E3 and E4 (*Pseudomonas* sp. strains E3 and E4).

In general a given enzyme, present in the cell-free extract of one of the isolates, demonstrated specificities towards one or two of the substrates. *Pseudomonas* sp. strain E2 possessed three enzymes with dehalogenase II and III demonstrating specificity for the chloroacetates and dehalogenase IV, only showing low activity towards all four

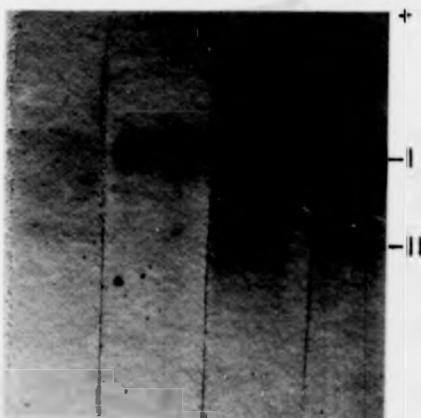
Figure 4.3: Distribution of dehalogenase activity towards four substrates - 2,2-DCPA, 2-MCPA, DCA and MCA - for six soil bacteria selected as representatives of different characteristic groups (brackets).



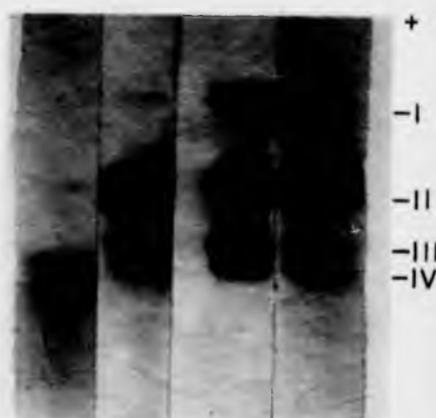
DCPA | MCPA | DCA | MCA
E2



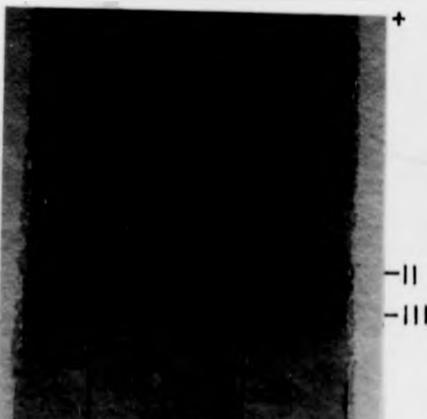
DCPA | MCPA | DCA | MCA
E3



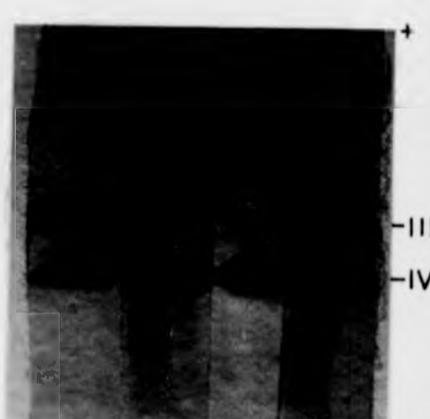
DCPA | MCPA | DCA | MCA
E4



DCPA | MCPA | DCA | MCA
E6

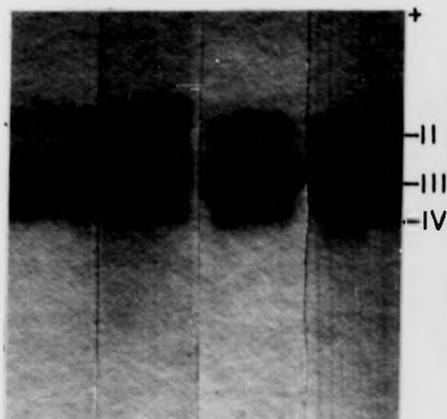


DCPA | MCPA | DCA | MCA
E20

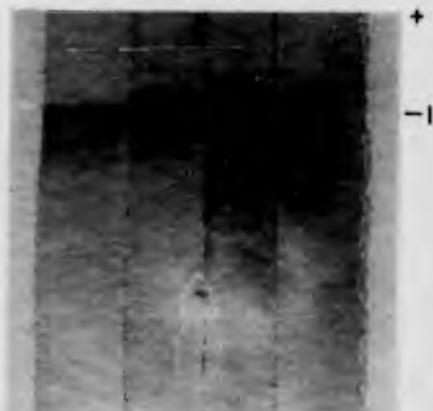


DCPA | MCPA | DCA | MCA
E22

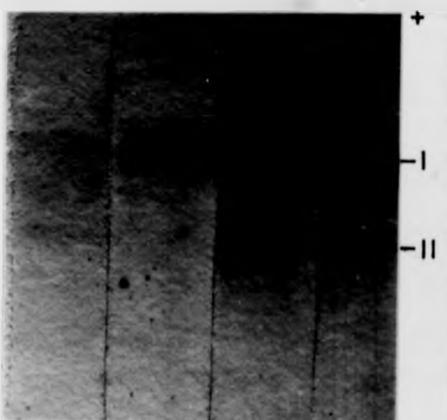
substrates -
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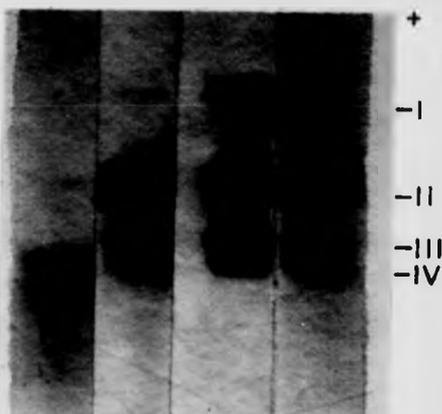
DCPA | MCPA | DCA | MCA
E2



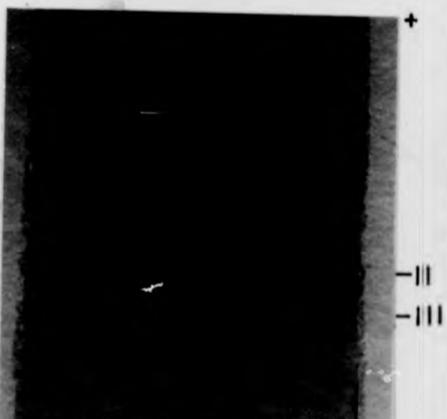
DCPA | MCPA | DCA | MCA
E3



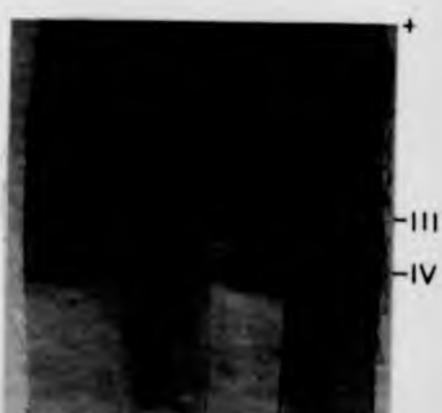
DCPA | MCPA | DCA | MCA
E4



DCPA | MCPA | DCA | MCA
E6



DCPA | MCPA | DCA | MCA
E20



DCPA | MCPA | DCA | MCA
E22

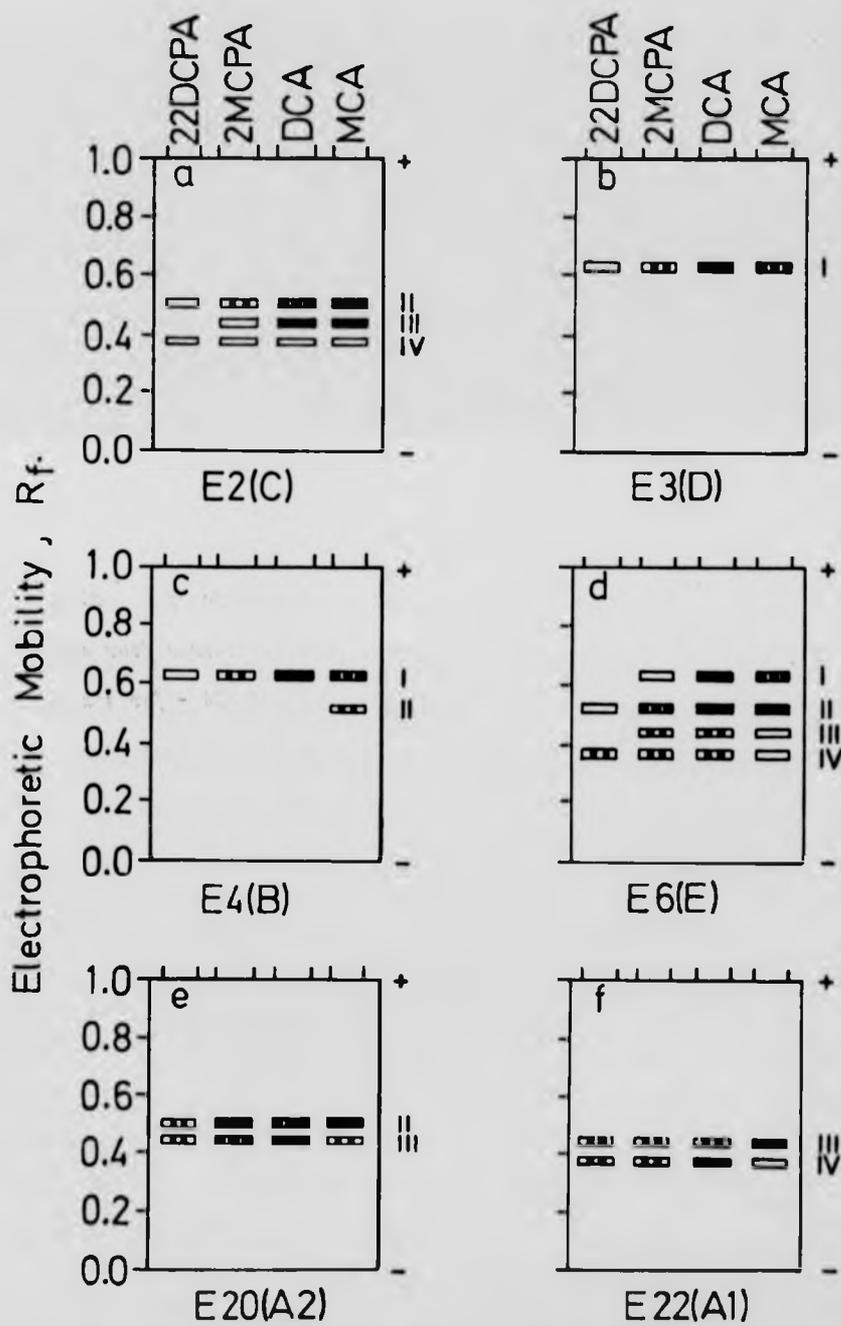
substrates -

ria selected

groups

Figure 4.4: Diagrammatic representation of the distribution of dehalogenase activity towards four substrates :- 2,2-DCPA, 2-MCPA, DCA and MCA - for six soil bacteria selected as representatives of different characteristic group (brackets). The bars represent semi-quantitative assessments of enzyme activity towards a particular substrate ranging from slight to great in the sequence:





selected

mp

assesse-

strate

substrates (Figure 4.4a). The dehalogenase I present in cell-free extracts of *Pseudomonas* sp. strain E3 (Figure 4.4b) showed specificity for the chloroacetic acids, with the heaviest silver chloride precipitation present after dechlorination of DCA. However, the significance of this must be judged cautiously as the specific activity is a comparison of activity as μmol substrate converted, which in the case of DCA required the removal of two chloride atoms but for MCA only required the removal of one.

The dehalogenase I of *Pseudomonas* sp. strain E4 (Figure 4.4c) possessed the same specificities as that of dehalogenase I in *Pseudomonas* sp. strain E3. However, strain E4 also possessed an enzyme, dehalogenase II, which was specifically active towards MCA, even though it was cultured with 2MCPA as the carbon source.

The dehalogenases I and II of *Pseudomonas* sp. strain E6 (Figure 4.4d) demonstrated the same specificities as those enzymes in isolates E3 and E2, but the dehalogenases III and IV were apparently more active towards the chloropropionic acids.

The *Aloaligenes* sp. strain E20 possessed two enzymes which apparently complemented the activities of each other (Figure 4.4e) with dehalogenase II possessing greater activity towards the monochloro-substituted compounds and dehalogenase III showing greater specificity for DCA.

Aloaligenes sp. strain E22 (Figure 4.4.f) possessed dehalogenases III and IV, with III demonstrating greatest activity towards MCA and dehalogenase IV towards DCA.

The profiles described were similar for each organism on all the

cell-free extracts studied, although the levels of enzymic activity varied slightly, such that those enzymes showing little activity, for example, dehalogenase IV of *Pseudomonas* sp. strain E2, failed to be observed on a number of occasions.

However, when the enzymes possessing identical electrophoretic mobilities were compared for substrate specificities in the different organisms some apparent differences and similarities were observed. Dehalogenase I in *Pseudomonas* sp. strains E3 and E4 showed the same specificities (Figure 4.4.b & c). *Pseudomonas* sp. strain E6 also contained an enzyme which corresponded to the dehalogenase I of isolates E3 and E4 (Figure 4.4.d) but this enzyme failed to show activity towards 22DCPA. Although the density of the DCA band was very similar to those observed in isolates E3 and E4, the overall density of precipitation was not as great as in the other strains, which may suggest that the level of dehalogenase I in isolate E6 was not as great as in isolates E3 and E4, which depended on this enzyme for the dehalogenation of the substrate.

The lower level of dehalogenase I in isolate E6 could have explained the apparent lack of activity towards 22DCPA, which even in strains E3 and E4 failed to show great precipitation of silver chloride. None of the other isolates expressed dehalogenase I activity even though it was apparently the most active of the four enzymes in those organisms which possessed it.

The dehalogenase II profile was very similar in isolates E2, E6 and E20, yet in isolate E4 it only showed activity towards MCA. The precipitation of silver chloride after enzymic activity towards MCA was

not as great as in the other strains which possibly indicates that the enzyme level was too low to enable visualization of the dehalogenating activities towards the other substrates. In *Aloaligenes* sp. strain E20 dehalogenase II demonstrated greater activity towards 2MCPA than in isolates E2 and E6, which was puzzling, as this strain was cultured with MCA as the carbon source whereas the others used 2MCPA.

Dehalogenase III in isolates E2 and E6 were similar, with the absence of activity towards 22DCPA, although activity towards MCA and DCA in strain E6 was much less than in strain E2. The differences in dehalogenase III activity towards 22DCPA in isolates E2 and E6 and E20 and E22 could be explained by being the result of lower levels of dehalogenase III in the former, the differences between the activities in E20 and E22 are less easily explained. In isolate E20 the heaviest precipitation occurred with DCA whereas it was MCA which caused this in isolate E22 (Figure 4.4e & f).

The differences in the profiles exhibited by dehalogenase IV may be explained as being the result of different levels of the enzyme in each isolate. However, the high DCA activity exhibited by the enzyme in isolate E22 (Figure 4.4f) is possibly significantly different from the other organisms.

4.6 MAXIMUM SPECIFIC GROWTH RATES

The maximum specific growth rates of a number of the soil isolates from each group were determined in batch culture using the substrates on which each organism was enriched. The results (Table 4.3) indicated that the organisms did differ from each other, with respect to growth rate and that the rates of different members of each group, determined by

TABLE 4.3 Maximum specific growth rates for representatives of six characteristic groups of soil bacteria, segregated according to the dehalogenation profiles.

Group	Isolate	Substrate	Maximum Specific Growth Rate (h^{-1})
A	E12	MCA	0.12
	E16	MCA	0.10
	E20	MCA	0.12
	E22	MCA	0.11
B	E4	2MCPA	0.21
	E5	2MCPA	0.21
C	E2	2MCPA	0.11
D	E3	2MCPA	0.17
E	E6	2MCPA	0.16

enzyme assay studies, were very similar.

The group B organisms showed the fastest growth ($\mu_{\max} = 0.21\text{h}^{-1}$) which was double that of *Pseudomonas* sp. strain E2 ($\mu_{\max} = 0.11\text{h}^{-1}$). The correlation between dehalogenase profile and growth rate is striking, though the number of dehalogenases does not seem to affect the rate of growth as *Pseudomonas* sp. strain E4, with the highest specific growth rate, possessed only two enzymes and *Pseudomonas* sp. strains E3 and E6, which demonstrated almost identical growth rates, possessed one and four enzymes respectively.

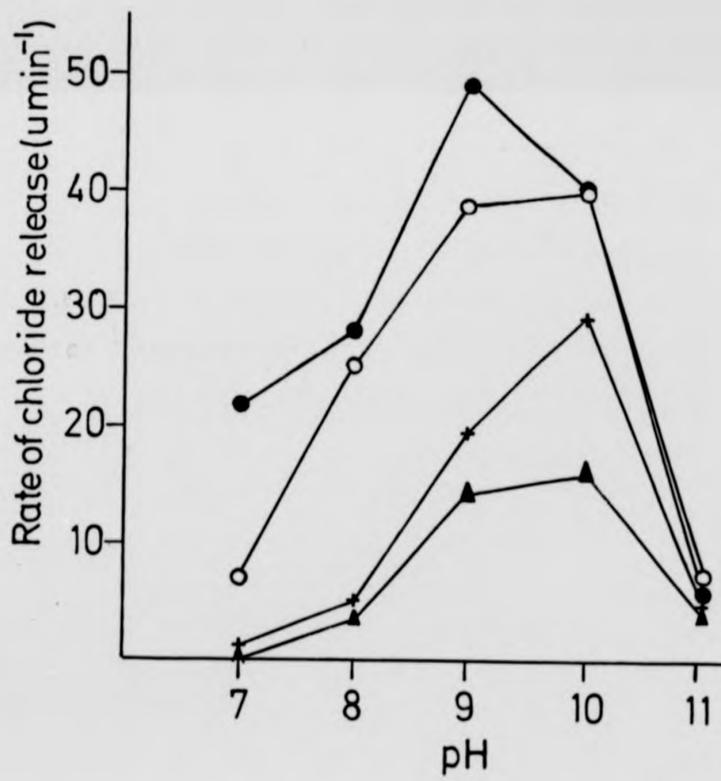
4.7 PHYSICAL PROPERTIES OF THE DEHALOGENASE SYSTEM OF *PSEUDOMONAS* SP STRAIN E4

Preliminary studies were undertaken using cell-free extracts of *Pseudomonas* sp. strain E4 to characterize some of the dehalogenase systems' physical properties. The results may represent the physical properties of dehalogenase I only as the dehalogenase II activity previously observed after electrophoresis (Section 4.4 and 4.5) failed to show any activity in the cell-free extracts used for these experiments. However, it should not be assumed that all these properties were just possessed by dehalogenase I, as the second enzyme may have been present at sub-visualization levels. Thus, although enzyme characterization ideally requires purified enzymes, these studies were aimed at studying the enzyme system as a whole.

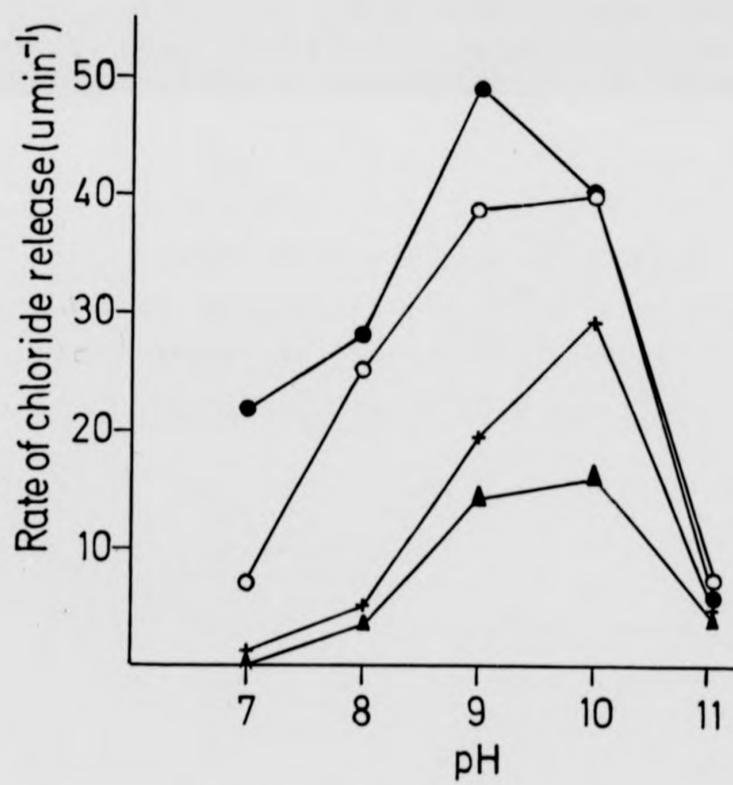
pH profile

Initial results indicated that the dehalogenase activity was maintained over a pH range of 7.0 to 11.0. TES buffer was used in these experiments as its buffering capacity covered the complete pH range.

Figure 4.5: pH profiles of dehalogenase activity in a cell-free extract of Pseudomonas sp. strain E4. Rate of chloride release in arbitrary units min^{-1} (○) MCA; (●) DCA (+) 2MCPA; (▲) 2,2DCPA



ride
CA



ride
CA

Initial experiments used the assay procedure previously described (Section 2.3.2) which used a final buffer concentration of 0.1 M. However, it was found that for pH values above 10.0 the acid produced by the reaction overloaded the buffering capacity, resulting in a decrease in the pH as the assay proceeded. To overcome this problem the buffer was adjusted to give a final concentration of 0.2 M.

The pH profile (Figure 4.5) indicated a pH optimum for activity towards all four substrates between pH 9.0 and 10.0. However, the effect on the activity of the enzyme towards each substrate differed markedly. If the rate of chloride release at pH 8.0 was taken as 100%, then at pH 10.0 the activities towards MCA, DCA, 2MCPA and 22DCPA were 159%, 145%, 566% and 481% respectively. That is, the activity of the dehalogenase system towards the chlorinated acetic acid increased by a half as much again over pH 8.0 values, whereas towards the chlorinated propionic acids the activity increased 5-fold. The stability of the substrates at the highest and lowest pH values were tested to ensure that the extreme pH values caused no spontaneous dehalogenation of the substrate. This proved not to be the case.

When the relative activity ratios of the extracts of each pH were compared, the increased activity towards the substituted propionic acids was reflected in changes in the relative activities. At pH 8.0 the MCA:DCA:2MCPA:22DCPA ratio was 1.0:1.12:0.21:0.14, whereas at pH 10.0 the ratio was 1.0:1.02:0.74:0.41. One explanation for this effect is that a second enzyme, which showed a greater specificity towards substituted propionates than towards acetates, became more active at pH 10.0

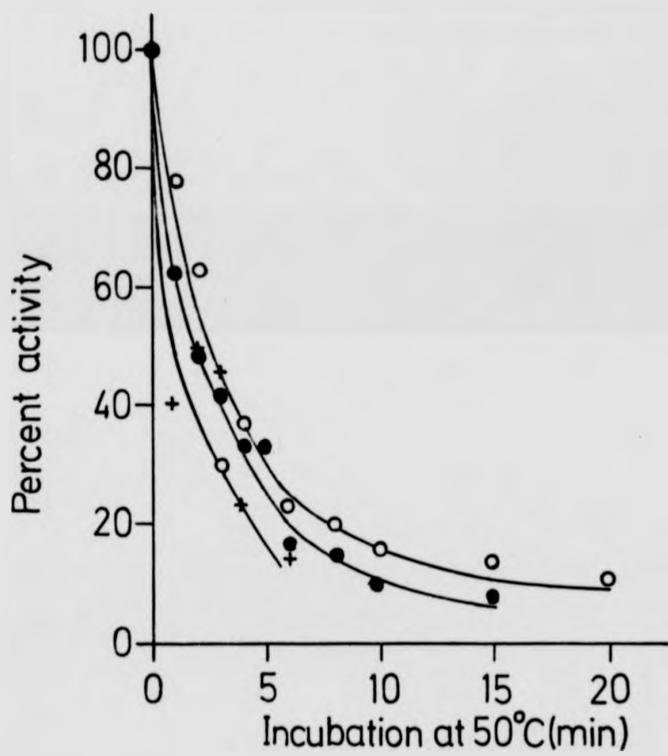
To investigate this possibility a number of cell-free extracts were electrophoresed as described (Section 2.4.) then placed into a strongly buffered incubation solution at pH 10.0. It was considered that after electrophoresis the buffering capacity of the buffer in the gel would have been depleted and thus, a strong buffer, at a different pH, would change the enzymes' environment to a value close to or equal to the pH of the new buffer. To this end the gel was incubated in glycine-NaOH buffer (0.5 M), pH 10.0 (Gomori, 1955), containing one of the four substrates for 30 min. Qualitative observation of the dehalogenase I activity, as determined by silver chloride precipitation, demonstrated an increase in activity of the enzyme at the higher pH as compared to the activity in gels incubated at pH 7.9. The increased activity, that is the difference in band density resulting from enzyme activity at the two pH values, was most noticeable when the chlorinated propionic acid substrates were used. No other enzymic activity, which could account for the change in relative activity ratios, was observed on the gels.

It is believed that the apparent greater effect of pH on dehalogenase I activity towards 2MCPA and 22DCPA was due to a change in the enzyme specificity at the higher pH, possibly the result of conformational changes, which resulted in a greater activity towards the chloropropionates than to the chloroacetates.

Thermal stability

The thermal stability of the dehalogenase system was studied at intervals of up to 40 min at 50°C. The results (Figure 4.6) show an asymptotic decrease in activity with increased incubation time. Figure 4.7 demonstrates the effect of incubation at 50°C on the dehalogenase I

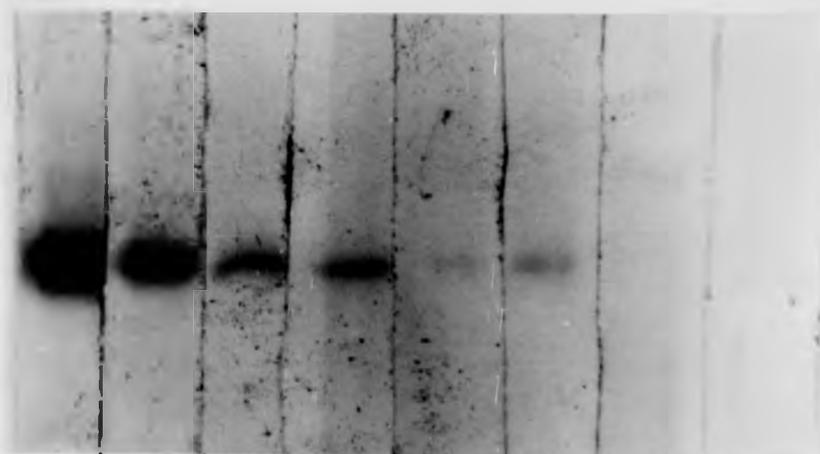
Figure 4.6: Stability of dehalogenase activity in a cell-free extract of Pseudomonas sp. strain E4 at 50°C: (○) MCA; (●) DCA; (+) 2MCPA.



extract

(●) DCA;

Figure 4.7: Electrophoretic study on the effect of incubation at 50°C on dehalogenase activity of cell-free extracts of Pseudomonas sp. strain E4. After electrophoresis the polyacrylamide gels were incubated at 50°C for the time intervals shown then placed into buffer containing a 50 mM mixture of MCA and DCA at 30°C for 30 min, after which dehalogenase activity was visualized using AgNO₃.



0 | 5 | 10 | 15 | 20 | 25 | 30 | 40

Incubation at 50°C (min)

at 50°C

the

time

a 50 mM

rich

activity more directly. Multiple samples of cell-free extract were electrophoresed then the gel sectioned and placed into Tris-sulphate buffer (0.2 M), pH 7.9 at 50°C for the indicated times. After which each section was placed into ice-cold buffer before being placed into the reaction buffer at 30°C as normal (Section 2.4.3), with MCA as the substrate. The results (Figure 4.7) showed a gradual loss of activity from 0 to 30 min. The apparently greater stability of the electrophoresed samples over the assay results suggests that the gel may help to stabilize the protein initially.

Effect of N-ethylmaleimide on dehalogenase activity

The effect of the sulphhydryl reducing agent, N-ethylmaleimide (NEM) on the dehalogenase I in cell-free extracts of *Pseudomonas* sp. strain E4 indicated that this enzyme belonged to the group of enzymes sensitive to this compound (Figure 4.8). After 5 min incubation in NEM (1.0 mM) the activity had fallen to 40% of the control activity and by 20 min incubation the activity of the extract was less than 10% the control. These results were also visualized by electrophoretic separation of the enzymes followed by incubation with NEM (1.0 mM), after which the gel was incubated in the reaction buffer before visualization of enzymic activity by treatment with silver nitrate (Section 2.4.3). The decrease in activity with increased incubation time can be seen in Figure 4.9.

Substrate specificity of the *Pseudomonas* sp. strain E4 dehalogenase system

The four chlorinated alkanolic acids, MCA, DCA, 2MCPA and 22DCPA were used to characterize the dehalogenase systems described. However, previous studies on the enzyme specificities of the *Pseudomonas putida*

activity more directly. Multiple samples of cell-free extract were electrophoresed then the gel sectioned and placed into Tris-sulphate buffer (0.2 M), pH 7.9 at 50°C for the indicated times. After which each section was placed into ice-cold buffer before being placed into the reaction buffer at 30°C as normal (Section 2.4.3), with MCA as the substrate. The results (Figure 4.7) showed a gradual loss of activity from 0 to 30 min. The apparently greater stability of the electrophoresed samples over the assay results suggests that the gel may help to stabilize the protein initially.

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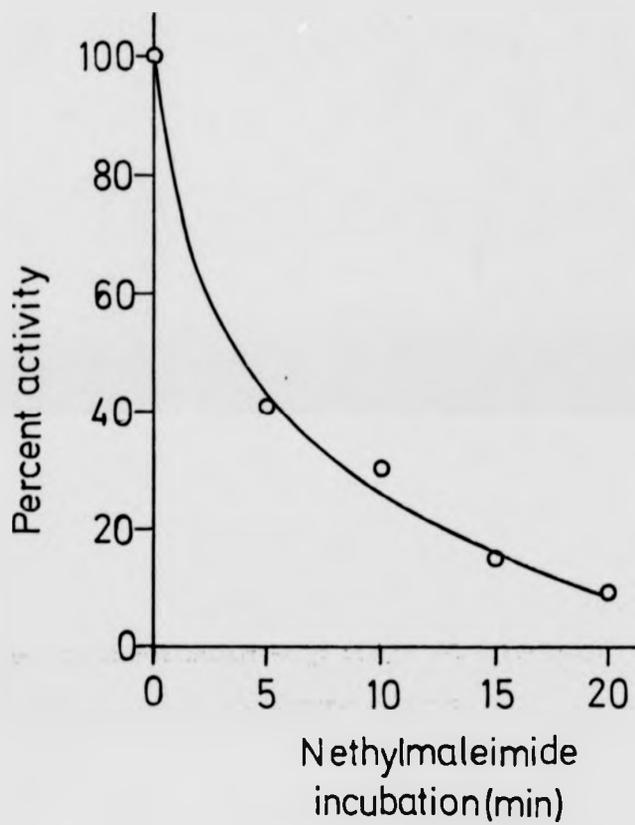
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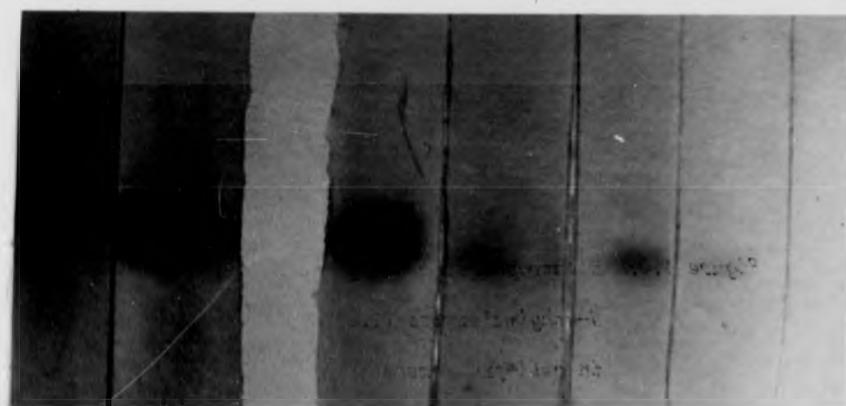
Figure 4.9: The effect of *N*-ethylmaleimide (1.0 mM) on dehalogenase activity towards MCA by cell-free extracts of *Pseudomonas* sp. strain E4.



Algenase

Pseudomonas

Figure 4.9: Electrophoretic study visualizing the effect of
N-ethylmaleimide (1.0 mM) on dehalogenase I activity,
in cell-free extracts of Pseudomonas sp. strain E4.



Control Control | 5 | 7 | 10 | 15 | 20
Nethylmaleimide incubation (min)

ivity,
Ed.

strain PP3 dehalogenases (Slater *et al.*, 1979) indicated that this type of enzyme can show a broad substrate specificity.

The ability of the enzyme system of *Pseudomonas* sp. strain E4 to dehalogenate other halogenated compounds was investigated using a range of halogenated alkanolic acids in the assay procedure previously described (Section 2.3.2). Table 4.4 indicates that the dehalogenase system of *Pseudomonas* sp. strain E4 was capable of dehalogenating a number of other halogen-substituted compounds. For example: monobromoacetic acid; 2-monobromopropionic acid and 2-monobromobutyric acid.

The greater ability to dehalogenate substituted acetic acid compounds also applied to the debromination of monobromoacetic acid (MBA) which was twice that of the relative rate of 2MCPA dehalogenation and five times higher than the 2,2-DCPA relative rate. However the lower rate of dehalogenation of trichloroacetic acid (TCA) suggested that too much substitution restricted the enzyme's activity.

The rates of halogen release from 2MCPA and 2MBPA were 1.1 and 2.5 $\mu\text{M Halogen}^- \text{ released min}^{-1}$ which indicated that with the propionate molecule bromide was more effectively removed than chloride by the dehalogenase system. This preference was also seen for the dehalogenation of 2MCBA ($0.1 \mu\text{mol Cl}^- \text{ min}^{-1}$) and 2MBBA ($0.4 \mu\text{mol Br}^- \text{ min}^{-1}$).

The rate of dehalogenation of 2-monochlorobutanoic acid (2MCBA) was 10-fold less than that of 2MCPA, suggesting that the enzyme system was more effective at dehalogenating the short chain alkanolic acids. This may also be reflected in the 3-fold difference in the dehalogenation rate between MCA and 2MCPA and the 10-fold difference between DCA and 2,2-DCPA.

TABLE 4.4 Dehalogenase substrate specificity in cell-free extracts of *Pseudomonas* sp. strain E4

TCA, trichloroacetic acid; MBA, monobromoacetic acid; 3MCPA, 3-monochloropropionic acid; 2MBPA, 2-monobromopropionic acid; 3MCBA, 3-monochlorobutanoic acid; 4MCBA, 4-monochlorobutanoic acid; 2MBBA, 2-monobromobutanoic acid; 2MCBA, 2-monochlorobutanoic acid

Substrate	MCA	DCA	2MCPA	22DCPA	TCA	MBA	3MCBA	2MBPA	3MCBA	4MCBA	2MBBA	2MCBA
Rate of Halogen release (chloro-coupler counts min ⁻¹)	9.33	12.0	2.83	1.04	0.18	5.42	0.33	6.25	0.38	0	0.93	0.35
$\mu\text{mol Halogen}^{-1} \text{ min}^{-1}$	3.7	4.8	1.1	0.4	<0.1	2.2	0.1	2.5	0.15	0	0.40	0.10
Relative dehalogenase activities	1.0	1.29	0.30	0.11	0.02	0.58	0.04	0.67	0.04	0	0.10	0.04

The enzyme system was also apparently specific for molecules substituted on the second carbon rather than the third or fourth. The activity towards 3-monochloropropionic acid (3MCPA), $0.1 \mu\text{mol Cl}^- \text{min}^{-1}$, was 10-fold less than that towards 2MCPA, $1.1 \mu\text{mol Cl}^- \text{min}^{-1}$ and no activity could be demonstrated towards 4-monochlorobutanoic acid (4MCBA).

4.8 DISCUSSION

From the twenty soil bacteria isolated from liquid enrichment cultures with either MCA or 2MCPA as the growth substrate, it was possible to group sixteen of them into 5 groups on the basis of dehalogenase relative and specific activities.

Electrophoretic analysis of cell-free extracts from these organisms showed that the characteristic dehalogenase activities were the result of possessing one or more of four dehalogenases in a number of permutations. These enzymes were separated by electrophoresis because of different anodal mobilities, which proved to be a constant property of each enzyme form. Of the 5 groups, A to E, the dehalogenase profiles split group A into two, A1 and A2 but the other groups were maintained.

Goldman *et al.* (1968) reported the presence of two dehalogenases in a soil pseudomonad, isolated by growth on DCA. Slater *et al.* (1979) and Weightman and Slater (1980) demonstrated the activity of two dehalogenases in *Pseudomonas putida* strain PP3 and Kawasaki *et al.* (1981a) isolated a *Moraxella* species also with two dehalogenases. Other workers have described organisms with a single dehalogenase. (Davies & Evans, 1962; Goldman, 1965; Tonomura *et al.*, 1965; Little & Williams, 1971; Berry *et al.*, 1979). However, the literature does not provide any

examples of microorganisms possessing three or four enzymes.

Thirteen of the sixteen organisms placed into the six groups possessed two dehalogenases; dehalogenases I and II, II and IV or III and IV. As these organisms were chosen at random from the selection cultures inoculated from the liquid enrichment cultures, it appeared that the possession of two enzymes was the most common, although the reason for this is not known.

Although the presence of more than one enzyme catalysing the same reaction could be explained by differing substrate specificities, the electrophoresis results (Figure 4.4) suggest that the enzymes show a wide range of substrate specificities being active towards MCA, DCA, 2MCPA and 22DCPA in nearly all cases. The exceptions, dehalogenase II of *Pseudomonas* sp. strain E4, dehalogenase I in *Pseudomonas* sp. strains E2 and E6, may in reality be able to dehalogenate the other substrates but due to the lack of sensitivity of the visualization technique were not seen.

In those isolates possessing two or more enzymes the single substrate, on which the organism was cultured induced all the enzymes, which suggested that inducer specificity was different from substrate specificity. Jensen (1960) reported that the MCA-induced dehalogenase of *Pseudomonas dehalogenans* was, in two groups of organisms, active towards MCA and DCA, whilst in a third group the substrate range was increased to include 22DCPA as well. Whereas Goldman *et al.* (1968) isolated two dehalogenases, one of which was constitutive and showed greatest activity towards MCA than 2MCPA, iodoacetate (IDA) and DCA whilst the second enzyme was induced by DCA and demonstrated twice as

much activity towards DCA as towards MCA. The dehalogenase identified by Tonomura *et al.* (1965) provided a further example of a dehalogenase system in which inducer and substrate specificity were the same. Their system was induced solely by fluoroacetate and showed a specific activity of $50 \mu\text{MF}^{-1} (\text{mg protein})^{-1} \text{h}^{-1}$ towards this substrate and only $1.75 \mu\text{MF}^{-1} (\text{mg protein})^{-1} \text{h}^{-1}$ towards fluoropropionate.

The electrophoretic mobilities of corresponding enzyme forms in each isolate remained the same, suggesting that the enzyme labelled dehalogenase I in *Pseudomonas* sp. strain E4 was the same as that in strains E3 and E6. The substrate specificities of dehalogenase I in each of these isolates were generally the same, the lack of activity of the strain E6 enzyme being explained by the enzyme being present at lower levels than in the other two strains. Dehalogenase II also demonstrated similar levels of activity towards each substrate. However, dehalogenase III in the *Alcaligenes* sp. strains E20 and E22 appeared to possess a different distribution of activity towards the four substrates in each isolate. In strain E20 the greatest activity was towards DCA, showing also a higher than normal activity towards 2MCPA, but in isolate E22 it was MCA which was dehalogenated the most.

One possible explanation for this effect is that the presence of other enzymes affect the activity of dehalogenase III. In strain E20 dehalogenase III was paired with dehalogenase II, whilst in strain E22 it was paired with dehalogenase IV. Dehalogenases II and IV exhibit different activity profiles and in both cases dehalogenase III apparently complements the other enzyme, in being more active towards the substrate towards which the other enzyme is less active, DCA in strain E20 and MCA in strain E22.

This effect can possibly also be observed for *Pseudomonas* sp. strain E6 which possessed all four enzymes. In *Pseudomonas* sp. strains E3 and E4 the dehalogenase I was the only major dehalogenating enzyme and proved very effective at catalysing this reaction. However, the same enzyme in isolate E6 was not as active, this may have been due to the presence of the other three enzymes. There is however, an additional factor to be considered when comparing and contrasting the dehalogenases of each group of organisms. Although there are apparent similarities between the dehalogenase I of strains E3 and E4 and between the dehalogenase II of strains E2 and E6, it was necessary to take special steps to ensure visualization of the enzymes of the group B strain E4 and group E, strain E6, after polyacrylamide gel electrophoresis. The gels had to be pre-run to show the activities of the dehalogenases:- strains E4 and E6 which was not necessary for strains E2 and E3. Thus, there are apparently other differences in the dehalogenases apart from their specific activities.

The presence of multiple dehalogenase forms in these isolates may be a reflection of the importance of these enzymes to them. Under the conditions described it has been demonstrated that the dehalogenase system is the growth-rate limiting factor (Slater *et al.*, 1979). Enzyme polymorphism with each form effective under slightly different environmental conditions, can enable organisms to adapt to their changing environment over short time intervals (Gillespie & Kojima, 1968). Substrate affinities of enzymes can alter with changes in the physiological environment such that under one set of conditions the enzymic form possessing the lowest K_{11} dominates the activity and so controls the rate of growth of the organism, but under different conditions the substrate

affinity of this enzyme may decrease, such that a second enzyme, which under the new conditions has an increased affinity for the substrate, becomes the rate limiting enzyme.

The polymorphism observed for the dehalogenases in these organisms may reflect the resulting balance of polymorphism due to selection of strains most capable of utilizing the halogenated substrates in the changing environment of the batch enrichment cultures used in their isolation.

If it is assumed that the observed multi-enzyme systems represent the selection of the most suitable enzymes for each organism under the given environmental conditions, these results would suggest a difference between 'most suitable' and fastest growth rate. *Pseudomonas* sp. strain E4 possessed the highest growth rate on 2MCPA ($\mu_{\max} = 0.21\text{h}^{-1}$) and produced dehalogenases I and II. Although dehalogenase II was, from these results, of no use to the organism when growing on 2MCPA, the dehalogenase system of this strain enabled a faster growth rate than the organism, *Pseudomonas* sp. strain E3, which only possessed dehalogenase I. This possibly provides further evidence that the second dehalogenase in strain E4 is in fact active towards all the substrates. The growth rate of *Pseudomonas* sp. strain E2 was only half that of strain E4 ($\mu_{\max} = 0.11\text{h}^{-1}$). It did not possess dehalogenase I which, when present, appeared to be the most effective dehalogenating enzyme. Although growing on MCA, not 2MCPA, the group A organisms, *Aloaligenes* sp. strains E20 and E22, showed a similar μ_{\max} value to strain E2 (0.12 and 0.11 respectively). These strains also lacked dehalogenase I which was seen to be the most efficient enzyme at dehalogenating the chloroacetic acids.

These results suggest that strains E2, E20 and E22 are not ideally suited for the utilization of their substrates. Possibly they are adapted to make the best use of the enzyme systems they possess, that is they are incapable of producing dehalogenase I, but by the use of the enzymes they do possess can maintain themselves in the environment.

By using the more general description suggested by Markert (1975) the multiple enzymic forms of the dehalogenases could be described as isoenzymes. However, as to whether they are primary isoenzymes, that is of common genetic origin, or secondary isoenzymes, that is due to posttranslational modification, no further conclusions can be reached from this work.

Blackburn *et al.* (1972) demonstrated a class of pseudo-isoenzymes which resulted from oxidation or reduction of sulphhydryl groups. It is not believed that this is the cause of the isoenzymic forms of these dehalogenases because dithiothreitol was always present in the extracts, to protect the sulphhydryl groups, and also the pattern of isoenzymes remained constant over a period of months which Hopkinson (1975) showed not to be the case when sulphhydryl groups were oxidized, as it led to an increase in anodal mobilities with increased storage time.

This work does not negate the possibility that the isoenzyme patterns are the result of protease action on a single enzyme. However, the consistency of these results together with the results reported in the next section would appear to invalidate this possibility.

The existence of the apparent number of isoenzymes of the dehalogenase may be the result of enzyme recruitment, or that the silver chloride banding is the result of the action of non-specific hydrolases which fortuitously

dechlorinate and debrominate the substrates. However, the substrate specificities and rates of reaction seem to make such an explanation unlikely, especially for the major enzymes dehalogenase I, II and III, although apart from its activity in *Aloaligene* sp. strain E22 dehalogenase IV shows only low activities towards these substrates.

At this stage of the work it was believed that there were two possible explanations for the existence of the isoenzymic forms. Firstly, as the concentration of the dehalogenase in the cell directly influences the organisms' specific growth rate (Slater *et al.*, 1979), in order for the organism to grow more rapidly it must synthesize more dehalogenase protein. As none of the isolates produced the enzyme constitutively (Section 1.4.3) two possible mechanisms may have allowed this increase; gene duplication (Cullum & Saedler, 1981; Section 1.4.5) or selecting for organisms with more than one different dehalogenase obtained through the activity of any gene transfer mechanism.

Batch enrichment was necessary to obtain isolates capable of rapid growth on these substrates. During this period of mixed growth dehalogenase genes may have accumulated in individual organisms from different parents, enabling the more efficient growth. If this were the case it could explain the inability to select reliably, organisms able to grow on MCA and 2MCPA directly from soil samples. The involvement of this mechanism could explain the different dehalogenase profiles, as being the result of the chance accumulation of one or more of the four genes specifying dehalogenase activity.

The second possibility was that the broad specificity of the dehalogenases might represent organisms at an intermediate stage in their evolution. Koch (1972) suggested that gene duplication (Section 1.4.2) was an

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important pre-requisite for the divergence of a single parent enzyme into new species capable of significantly different catabolic function. If this mechanism was involved in evolution it would lead at some stage of the process, to organisms possessing two or more enzymes with broadly similar activities towards different substrates. Should this mechanism be involved, long term experiments may enable a study of this evolutionary process which would eventually lead to the elimination of the inefficient enzymes, possibly dehalogenase IV, and lead to the selection of strains demonstrating greater abilities at utilizing these substrates.

The study of the physical properties of the dehalogenase system of *Pseudomonas* sp. strain E4 enabled a comparison of this mechanism with other dehalogenase systems.

The pH profile of this enzyme system showed there to be an optimum pH between 9.0 and 10.0 in common with a number of other mechanisms studied. The enzyme of *Pseudomonas dehalogenans* demonstrated a pH optimum of 9.5 towards MCA (Davies & Evans, 1962) and Little and Williams (1971) suggested an optimum of pH 9.4 for the same organism; the pH curves for the two enzymes isolated from a soil pseudomonad by Goldman *et al.* (1968) showed a broad optima in the range 9.0 to 9.4 which agreed with Goldman (1965) for the activity of haloacetate halidohydrolase. Kawasaki *et al.* (1981b) found an optimum value of 9.5 for the haloacetate halidohydrolase of a *Moraxella* sp.

There does however appear to be a group of enzymes which possess a lower pH optimal value. Kearney *et al.* (1964) studied the dehalogenation of 22DCPA by an *Arthrobacter* sp. which, as a cell-free extract, showed a sharp pH optimum at pH 8.0, a value also seen by Bollag and Alexander (1971)

for the dehalogenase system of *Micrococcus denitrificans*.

Slater *et al.* (1979) demonstrated that the pH optimum for the dehalogenase system of *Pseudomonas putida* strain PP3 had two markedly different pH profiles for the four major substrates. For MCA and DCA the pH optima lay between 7.9 and 8.1 and the activity declined rapidly on either side of the optimal values, whilst towards 2MCPA and 22DCPA the optima was explained by the presence of two enzymes with different substrate specificities and pH optima.

The differences in the effect of pH on activity of the enzyme dependent on substrates was also seen for *Pseudomonas* sp. strain E4. In this case, however the effect was apparently not a difference in pH optima, as only one enzyme was shown to be active, but a change in ability to dehalogenate the substrate. At a pH 9.0 to 10.0 the dehalogenase was able to dehalogenate the chloropropionic acids much more effectively, a 5-fold increase, than at pH 8.0. The reason for this was interpreted as conformational changes in the enzyme or substrate molecules.

It should however be noted that the type of buffer can affect the optimal pH value. Senior (1977) found that the different buffers, Tris-maleate, glycine-NaOH or phosphate-phosphate, gave differing pH optima ranging from 7.6 to 9.6 and to 10.0 in one case for the *Pseudomonas* sp. strain P3. Thus a comparison of the pH optima of the different dehalogenase mechanisms should also account for the different buffering systems used.

The thermal stability profile of strain E4 showed a half-life of 2 to 4 min for the dehalogenase system at 50 °C. The rate of loss of activity towards MCA, DCA and 2MCPA were very similar, providing further

evidence for the presence of only one enzyme in the dehalogenase system. Slater *et al.* (1979) found that the denaturation curves of cell-free extracts of *Pseudomonas putida* strain PP3 varied depending on the substrate, which, they believed, provided further evidence that this organism possessed more than one dehalogenase which Weightman and Slater (1980) later demonstrated by electrophoretic studies.

The haloacetate halohydrolyase from a *Moraxella* sp. identified by Kawasaki *et al.* (1981b) was apparently more stable at high temperatures as after 10 min incubation at 50 and 60°C activity remained completely, exhibiting the same activity as those extracts incubated at 0 to 40°C. However, at 70°C or above the activity rapidly diminished.

The effect of N-ethylmaleimide on isoenzymic activity has been suggested as a method of characterizing each form (Hopkinson, 1975). Dehalogenases can be segregated into two groups by the relative inhibitory effects of NEM on their activities (Little & Williams, 1971). The enzymes isolated by Davies and Evans (1962) and by Goldman (1965) were completely inhibited by this thiol reagent, while the two enzymes from the soil pseudomonad isolated by Goldman *et al.* (1968) were virtually unaffected by NEM at 1 mM concentrations. The dehalogenase of *Pseudomonas* sp. strain E4 apparently belonged to the first group in that NEM (1 mM) rapidly inhibited activity.

In common with most of the dehalogenase systems identified the system of *Pseudomonas* sp. strain E4 was specific for halogen substituents on the second carbon of the molecule, with activity towards 3MCPA and 3MCBA only a tenth of the activity towards 2MCPA. Although it should be noted that enzymes active towards the compounds substituted on the 3 carbon have been isolated (Castro & Bartnicki, 1965; Bollag & Alexander, 1971).

Another feature the strain E4 dehalogenase system has in common with a number of other dehalogenase mechanisms is the decrease in activity as the carbon chain length of the alkanolic acids is extended. Little and Williams (1971) found that *Pseudomonas dehalogenans* showed much greater activity towards halogenated acetates than propionates. This effect was also apparently regardless of whether the halogen was chlorine bromine or iodine. Slater *et al.* (1979) found the same with *Pseudomonas putida* strain PP3, as did Kawasaki *et al.* (1981b). Although it should be noted that Berry *et al.* (1979) found that the dehalogenase system of a *Rhizobium* sp. was more active towards chloropropionate than chloroacetates.

The three chlorine atoms of TCA apparently inhibit activity of the enzyme in strain E4 a factor also seen for haloacetate halohydrolyase II of the *Moraxella* sp. identified by Kawasaki *et al.* (1981b). Indeed this enzyme was very specific for monohalogenated compounds. Demonstrating a relative activity of 100% towards MCA, 3.3% towards DCA and 0% towards TCA.

Pseudomonas sp. strain E4 demonstrated the ability to debrominate a number of compounds very efficiently. The rate of debromination of MBA being the third fastest behind DCA and MCA. It also more readily dehalogenated 2MBPA ($0.25 \mu\text{mol Br}^- \text{min}^{-1}$) than 2MCPA ($0.11 \mu\text{mol Cl}^- \text{min}^{-1}$) and 2MBBA ($0.04 \mu\text{mol Br}^- \text{min}^{-1}$) was dehalogenated four times faster than 2MCBA ($0.01 \mu\text{mol Cl}^- \text{min}^{-1}$). Kawasaki *et al.* (1981b) also found that the *Moraxella* sp. system debrominated MBA far more effectively than it dechlorinated MCA, the relative activities being 160% and 100% respectively.

Thus, the dehalogenase mechanism of *Pseudomonas* sp. strain E4 apparently demonstrates a number of features common to other dehalogenating

mechanisms. Purification of the four enzymes present in these soil isolates and a study of their physical profiles could provide an explanation for the presence of these four isoenzymes in these organisms.

CHAPTER 5

DEHALOGENASE COMPLEMENT IN CONTINUOUS-FLOW CULTURE

During the batch culture experiments the relative activity ratios and specific activities for each organism were seen to vary about a median value. The isolates possessing more than one dehalogenase showed greater variation than those with a single enzyme. The inducible nature of these enzymes may have been the cause of these fluctuations, reflecting differing degrees of induction of the different isoenzymes, which possess different specific activity profiles.

As described in Section 1.2.3, the regulation of enzyme activity in response to environmental parameters is easily studied in steady-state chemostat cultures. The selective pressure of growth under carbon-limitation in a chemostat culture would select those organisms capable of the most efficient use of the carbon source. In these experiments, with a halogenated carbon source, those organisms capable of the most efficient dehalogenation should be selected.

5.1 DEHALOGENASE COMPLEMENT OF *PSEUDOMONAS* SP. STRAIN E4 IN CONTINUOUS-FLOW CULTURE WITH MCA OR 2MCPA AS THE SOLE CARBON SOURCE

Pseudomonas sp. strain E4 possessed two dehalogenases, I and II, in batch culture (Section 4.4). Dehalogenase I demonstrated activity towards all four halogenated substrates whereas dehalogenase II only showed activity towards MCA.

Under conditions of carbon limitation with MCA as the carbon source it might be expected that both enzymes would be expressed as they both showed activity towards this substrate. Strains with elevated levels of dehalogenase II, which appeared to be specific for this substrate,

might outcompete other strains and so become the dominant force in the culture.

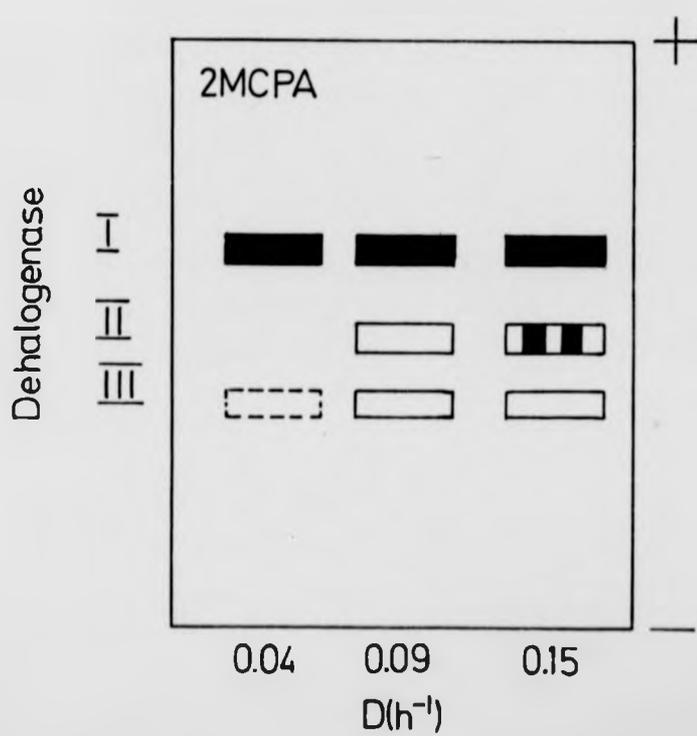
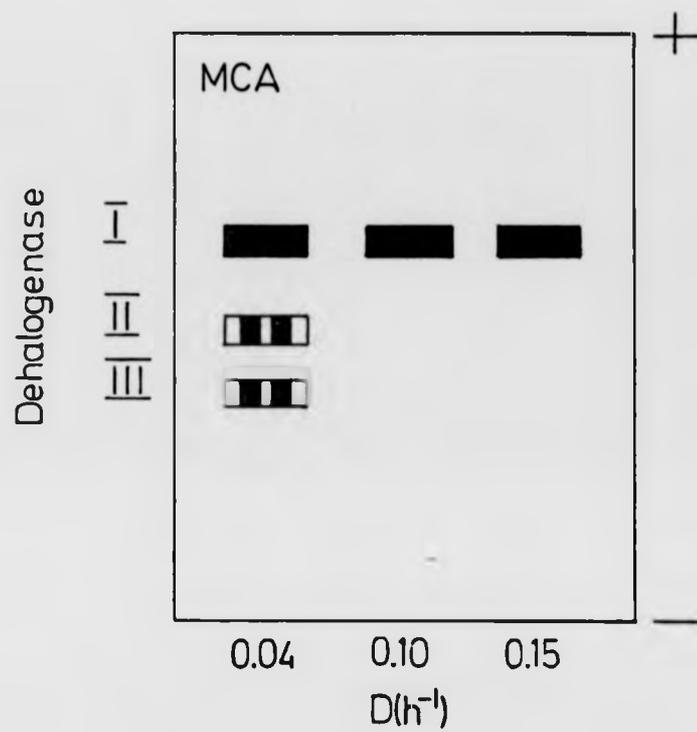
If however, the carbon source was 2MCPA, then the production of dehalogenase II would represent a metabolic drain on the organism, because metabolites would be used in producing an enzyme which was of no benefit to the organism as it could not dehalogenate the carbon source. Under these conditions strains only producing dehalogenase I would outcompete strains producing both enzymes.

With MCA as the carbon source in a chemostat culture ($D = 0.1 \text{ h}^{-1}$) strain E4 failed to show the expected dehalogenase profile with dehalogenases I and II. Instead, the initial cell-free extracts demonstrated dehalogenases II and III and subsequent cell-free extracts showed dehalogenase I only.

With 2MCPA as the carbon source ($D = 0.1 \text{ h}^{-1}$) the results also varied from the expected profile. The first electrophoresis gel showed only dehalogenase I the second dehalogenases I, II and III and subsequent gels I and III.

Apparently this organism possessed complex regulatory controls over the production of its dehalogenases. The different results obtained from the batch cultures and the initial chemostat cultures suggested the organisms' environment and growth rate affected the production of the dehalogenases. To investigate this further chemostat cultures were subjected to different dilution rates, with either MCA or 2MCPA as the carbon source, and enzyme assays and electrophoretic studies of cell-free extracts undertaken at each dilution rate. Three dilution rates were used corresponding to 19%, 48% and 86% μ_{max} (as determined in batch culture, Section 4.6).

Figure 5.1: Summary of the initial results from cell-free extracts prepared from chemostat cultures of Pseudomonas sp. strain E4 with MCA (A) or 2MCPA (B) as the growth-limiting substrate. Dehalogenase profiles identified after electrophoresis.



The effect of dilution rate on the dehalogenase profiles was shown to be dependent upon the carbon source. Dehalogenase I, the major enzyme in batch culture, was the only enzyme present at a dilution rate of 0.1 h^{-1} for strain E4 with MCA as the carbon source (Figure 5.1). With 2MCPA as the substrate although the major enzyme remained as dehalogenase I, dehalogenases II and III were also detected (Figure 5.1).

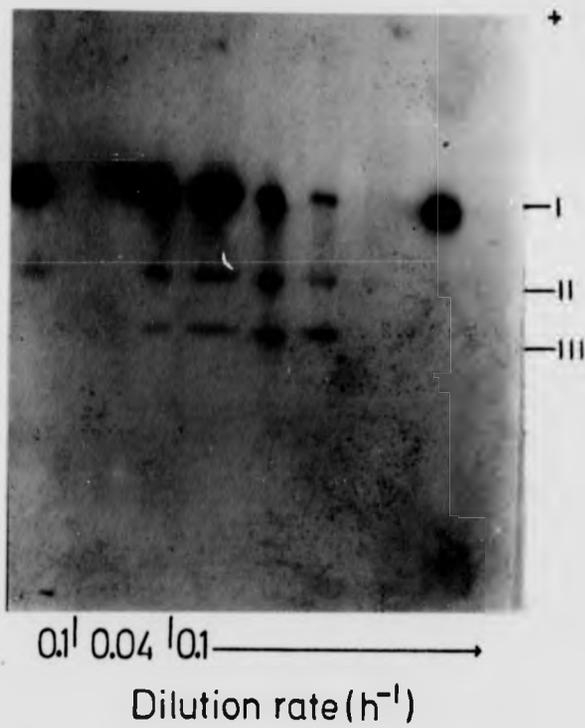
A number of steady state cultures were examined from both cultures at this dilution rate. The expression of dehalogenases II and III in the 2MCPA-limited culture was shown to be variable. Although both enzymes were usually detected, on a number of occasions only one of them could be visualized. However, the activity of these two enzymes was low, as compared to the activity of dehalogenase I, thus at times their activity may have been below the threshold of the visualization system, so the possibility that both enzymes were always present even when not observed, cannot be ruled out.

In both the MCA and 2MCPA-limited cultures changes in the dilution rate resulted in different dehalogenase profiles, although the nature of these changes was dependent on the growth rate limiting substrate.

Increasing the dilution rate of the MCA-limited culture from 0.1 h^{-1} to $0.15\text{-}0.18 \text{ h}^{-1}$, that is 48 to 86% μ_{max} , resulted in no change in the dehalogenase profile, dehalogenase I remained the only active form (Figure 5.1). However, by decreasing the dilution rate from 0.1 h^{-1} to 0.04 h^{-1} (19% μ_{max}) the profile changed and dehalogenase II and III were also expressed together with dehalogenase I. The latter isoenzyme remained the major enzyme, but the activity of the other two isoenzymes was significantly greater than when the organism was grown under 2MCPA-

Figure 5.2 : The gradual change in dehalogenase profile resulting from a shift in the dilution rate from 0.04 h^{-1} to 0.1 h^{-1} in cell-free extracts of Pseudomonas sp. strain E4 with MCA as the growth-limiting substrate.

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limitation at a dilution rate of 0.1 h^{-1} .

To see if these results were reversible, the dilution rate was returned to 0.1 h^{-1} , samples taken at intervals and the dehalogenase profile studied. Figure 5.2 shows the gradual disappearance of dehalogenase III, such that by 136 h the activity had disappeared, and also the activity of dehalogenase II had significantly decreased. After a further period the single dehalogenase profile seen on the right-hand side of the figure was obtained.

Under 2MCPA-limitation the results were different. At the low dilution rate (0.04 h^{-1}) only dehalogenase I was seen although on a number of occasions dehalogenase III was detected on the gels (Figure 5.1). When the dilution rate was increased from 0.1 to $0.15-0.18 \text{ h}^{-1}$ the profile remained unchanged from that of the middle dilution rate, dehalogenases I, II and III were detected. The levels of dehalogenases II and III were higher at $D = 0.15-0.18$, and the activity of II was always significantly higher than that of III, while remaining less active than dehalogenase I.

The significant differences in the responses to dilution rate and the differing effects the dilution rates had with different substrates, were also reflected in the specific and relative activities of the enzymes in cell-free extracts.

With MCA as the growth-limiting substrate the dehalogenase specific activities increased with decreasing dilution rate (Table 5.1). The specific activity towards MCA exhibited a 30-fold increase as the dilution rate was decreased from $0.15-0.18 \text{ h}^{-1}$ to 0.04 h^{-1} , whilst the activity towards DCA increased 37-fold, 2MCPA activity increased 49-fold

TABLE 5.1 The effect of dilution rate on the dehalogenase specific activities in steady state culture of *Pseudomonas* sp. strain E4 with MCA as the growth limiting substrate

Length of steady state (h)	Diltn rate (h ⁻¹)	Percent μ_{\max}	Dehalogenase specific activity ($\mu\text{mole substrate converted (mg protein)}^{-1} \text{ min}^{-1}$)			
			MCA	DCA	2MCPA	22DCPA
70	0.04	19	1.75	3.46	0.56	Not dtmd
			0.68	2.03	0.48	0.32
			1.26	3.94	0.44	Not dtmd
			1.16	2.43	0.49	0.33
			Average	1.21	2.97	0.49
390	0.10	48	0.50	0.70	0.17	0.10
			0.05	0.21	0.02	0.01
			0.11	0.40	0.04	0.01
			0.08	0.23	0.02	0.01
			0.29	0.96	0.08	0.03
Average	0.21	0.50	0.07	0.03		
160	0.15- 0.18	71- 86	0.07	0.10	0.01	< 0.01
			0.02	0.04	0.01	< 0.01
			0.04	0.10	0.01	< 0.01
			Average	0.04	0.08	0.01

TABLE 5.2 The effect of dilution rate on the dehalogenase specific activities in steady state cultures of *Pseudomonas* sp. strain E4 with 2MCPA as the growth-limiting substrate

Length of steady state (h)	Dln rate (h ⁻¹)	Percent μ_{max}	Dehalogenase specific activity ($\mu\text{mol substrate converted (mg protein)}^{-1} \text{ min}^{-1}$)				
			MCA	DCA	2MCPA	22DCPA	
80	0.04	19	0.44	0.58	0.12	0.07	
			0.41	0.46	0.17	0.08	
			0.41	0.55	0.16	0.09	
			0.48	0.49	0.20	0.08	
			Average	0.44	0.52	0.16	0.08
170	0.09	43	0.73	0.73	0.14	0.08	
			0.60	0.73	0.15	0.08	
			0.46	0.63	0.11	0.08	
			0.41	0.61	0.09	0.08	
			Average	0.55	0.68	0.12	0.08
50	0.18	86	0.27	0.63	0.04	0.02	
			0.73	1.74	Nt dtmd	0.07	
			0.47	1.13	0.08	0.03	
			Average	0.49	1.17	0.06	0.04

and that towards 22DCPA increased by at least 33-fold. A more exact value for the latter increase in activity was not possible as the specific activity at the highest dilution rate was less than $0.01 \mu\text{mol substrate converted (mg protein)}^{-1} \text{ min}^{-1}$ which was below the resolution of the assay procedure.

The time at each dilution rate, after the theoretical period of adjustment to the new steady state conditions, was extensive, however the specific activities show a high degree of variation. This was found to be a property of the different cell-free extracts and not a lack of consistency in the assay procedure.

Table 5.2 describes the specific activities from cell-free extracts obtained from the 2MCPA-limited culture. The effects of dilution rate on this culture were much less marked than for the MCA-limited culture. The specific activity towards MCA remained almost constant and activity towards DCA increased by 2-fold as the dilution rate was increased from 0.04 h^{-1} to 0.18 h^{-1} . The increase in the activity towards DCA may have been caused by the expression of dehalogenase II and III activity (Figure 5.1). Dehalogenase II showed greater activity towards MCA and DCA than did dehalogenase III (Figure 4.4) thus this may represent an enzyme with greater specificity for DCA than the other isoenzyme.

The change in specific activity towards 2MCPA and 22DCPA, with dilution rate, was the opposite to that observed for DCA, in that the activity towards these two substrates increased with decreasing dilution rate by 2 to 2.5-fold.

The change in the dehalogenase profile was also reflected in changes in the relative activities of the cell-free extracts towards the different

TABLE 5.3 The effect of dilution rate on the relative dehalogenase activities in steady state cultures of *Pseudomonas* sp. strain E4 with MCA as the growth-limiting substrate

Length of steady state (h)	Dltn rate (h^{-1})	Percent μ_{max}	Relative dehalogenase activity			
			MCA	DCA	2MCPA	22DCPA
70	0.04	19	1.0	1.98	0.32	Nt dtmd
			1.0	2.99	0.71	0.47
			1.0	3.13	0.35	Nt dtmd
			1.0	2.09	0.42	0.28
			Average	1.0	2.55	0.45
390	0.10	48	1.0	1.42	0.35	0.20
			1.0	4.29	0.39	0.15
			1.0	3.63	0.34	0.12
			1.0	2.69	0.27	0.10
			1.0	3.26	0.26	0.10
Average	1.0	3.06	0.32	0.13		
160	0.15- 0.18	71- 86	1.0	1.43	0.14	0.01
			1.0	2.00	0.50	0.13
			1.0	2.50	0.25	0.10
		Average	1.0	1.98	0.30	0.08

TABLE 5.4 The effect of dilution rate on the relative dehalogenase activities in steady state cultures of *Pseudomonas* sp. strain E4 with 2MCPA as the growth-limiting substrate

Length of steady state (h)	Diltn rate (h^{-1})	Percent μ_{max}	Relative dehalogenase activity				
			MCA	DCA	2MCPA	22DCPA	
80	0.04	19	1.0	1.31	0.27	0.16	
			1.0	1.13	0.41	0.19	
			1.0	1.33	0.38	0.23	
			1.0	1.05	0.43	0.16	
			Average	1.0	1.21	0.37	0.19
170	0.09	43	1.0	1.00	0.19	0.11	
			1.0	1.22	0.25	0.13	
			1.0	1.37	0.24	0.17	
			1.0	1.49	0.22	0.20	
			Average	1.0	1.27	0.23	0.15
50	0.18	86	1.0	2.33	0.15	0.07	
			1.0	2.38	Nt dtmd	0.10	
			1.0	2.40	0.17	0.06	
			Average	1.0	2.37	0.16	0.08

substrates. Such changes confirm the evidence in Figure 5.1, that the changes in specific activities are associated with activity of different enzymes with unique substrate specificities, and not just due to increased levels of one enzyme.

For the MCA-limited cultures the relative activities of 2MCPA and 22DCPA, with respect to MCA increased with decreasing dilution rate (Table 5.3). The response to DCA was variable reaching a maximum at the middle dilution rate (0.1 h^{-1}) and showing a minimum at the highest rate.

Under 2MCPA-limitation (Table 5.4) the relative activities varied in a similar manner to the changes, in the specific activities, with a 2-fold increase in the relative activity towards 22DCPA as the dilution rate was decreased, and a 2 to 3-fold increase towards 2MCPA. The converse increase in DCA specific activity with increased dilution rate was also observed in the activity ratio results, the relative activities showing a 2-fold increase with increased dilution rate, 0.04 h^{-1} to 0.18 h^{-1} .

5.2 DEHALOGENASE COMPLEMENT OF *PSEUDOMONAS* SP. STRAIN E2 IN CONTINUOUS-FLOW CULTURE AT DIFFERENT DILUTION RATES

The effect of dilution rate on the dehalogenase complement of *Pseudomonas* sp. strain E4 and the expression of isoenzymes not seen under batch culture led onto a study of the other isolates to see if they also possessed the capability of producing isoenzyme forms which they did not express under batch culture conditions.

Pseudomonas sp. strain E2 showed an increase in specific activities towards all four substrates with increasing dilution rate (Table 5.5). The

TABLE 5.5 A comparison of the effect of dilution rate on the dehalogenase specific activity of five soil isolates. Strains E2, E3, E4 and E6 were cultured under 2MCPA carbon-limited conditions and E20 with MCA carbon limitation.

Steady state growth rate as a percentage of μ_{max}	Bacterial strain																			
	E2		E3		E4		E6		E20											
	MCA	2MCPA	MCA	DCA	2MCPA	22DCPA	MCA	DCA	2MCPA	22DCPA	MCA	DCA	2MCPA	22DCPA						
19 - 23	0.12	0.13	0.06	0.04	0.05	0.14	0.02	-0.01	0.44	0.52	0.16	0.08	0.18	0.32	0.10	0.08	0.47	0.10	0.09	0.06
43 - 48	0.15	0.16	0.06	0.04	0.31	0.50	0.06	0.05	0.55	0.68	0.12	0.08	1.27	0.43	0.27	0.20	0.31	0.10	0.08	0.04
71-86	0.61	0.80	0.28	0.19	0.75	0.90	0.15	0.12	0.49	1.17	0.06	0.04	1.33	0.11	0.12	0.07	0.46	0.13	0.16	0.08

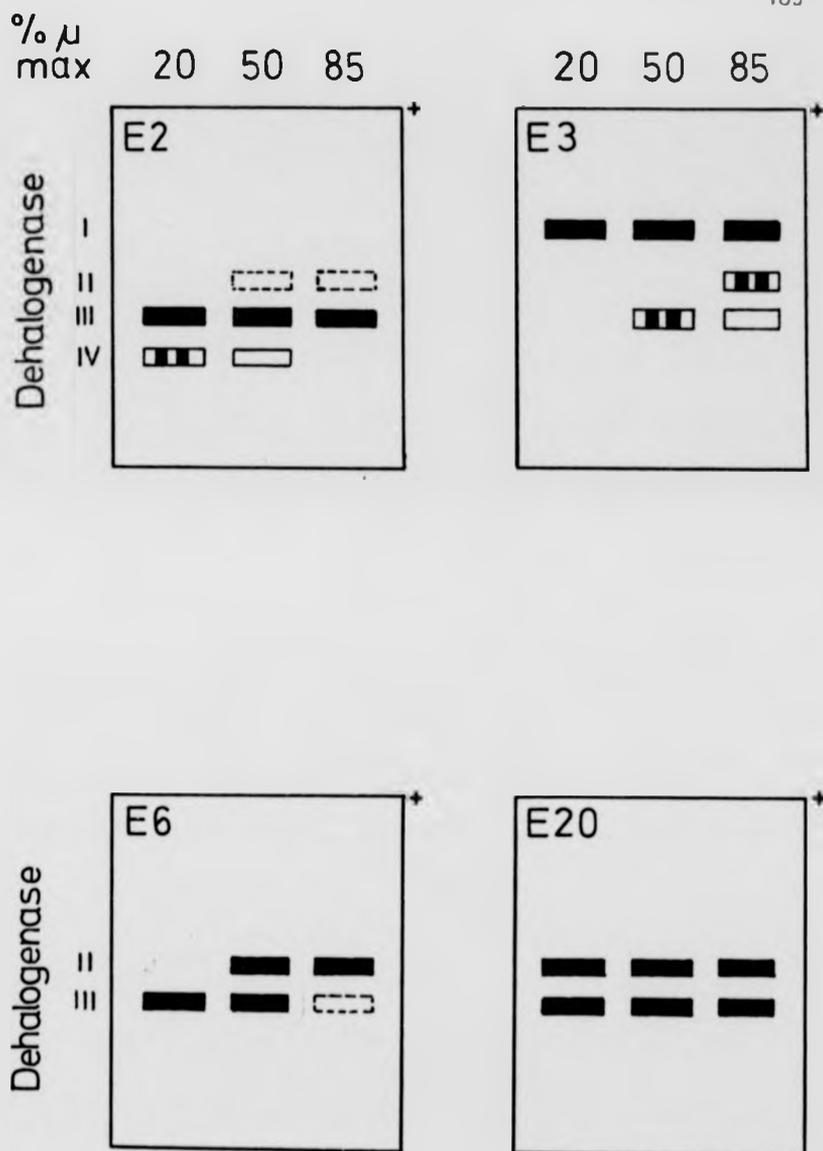
¹ Determined from 4 or 5 assays

TABLE 5.6 A comparison of the effect of dilution rate on the relative dehalogenase activity of five soil isolates. Strains E2, E3, E4 and E6 were cultured under 2MCPA carbon-limited conditions and E20 with MCA carbon limitation

Steady state growth rate as a percentage of μ_{max}	Bacterial strain																			
	E2		E3		E4		E5		E6											
	MCA	DCA	MCA	DCA	MCA	DCA	MCA	DCA	MCA	DCA	MCA	DCA								
19-23	1.0	1.18	0.52	0.34	1.0	2.48	0.32	0.07	1.0	1.21	0.37	0.19	1.0	1.79	0.60	0.51	1.0	0.22	0.18	0.13
43-48	1.0	1.11	0.35	0.28	1.0	1.73	0.21	0.18	1.0	1.27	0.23	0.15	1.0	0.37	0.22	0.16	1.0	0.31	0.25	0.13
71-86	1.0	1.42	0.54	0.35	1.0	1.20	0.20	0.16	1.0	2.37	0.16	0.08	1.0	0.09	0.09	0.06	1.0	0.22	0.27	0.13

¹Determined from 4 or 5 assays

Figure 5.3: The effect of growth rate on the dehalogenase profiles of the soil isolates: Pseudomonas/Alteromonas sp. strain E2; Pseudomonas sp. strains E3 and E6 and Alcaligenes sp. strain E20. The bars represent a semi-quantitative assessment of enzyme activity towards each substrate ranging from slight to great.  The bars represented by  indicate presence in some extracts but not all.



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magnitude of these changes was comparable to those observed for strain E4 under 2MCPA-limitation. However, the increases were constant for all the substrates, unlike strain E4, with a 5 to 6-fold increase in activity towards all four substrates when the dilution rate was increased from the lowest to the highest values examined.

In common with the results obtained for strain E4 the effect of changing from the low to medium dilution rate had a lesser effect on the specific activities than when the medium rate was changed to the highest rate. Towards all four substrates, most of the 5 to 6-fold increase in activity occurred in the second increase in dilution rate.

The relative activity ratios (Table 5.6) showed little overall change for this organism which suggested that most of the increased specific activity was due to an increase in the levels of existing enzymes, rather than the induction of others. However, Figure 5.3 demonstrates that this was not the case. The consistently high levels of dehalogenase III, as compared with the low and changing levels of dehalogenases II and IV may explain the relative stability of the activity ratios (Table 5.6).

5.3 DEHALOGENASE COMPLEMENT OF *PSEUDOMONAS* SP. STRAIN E3 IN CONTINUOUS-FLOW CULTURE AT DIFFERENT DILUTION RATES.

Pseudomonas sp. strain E3 also demonstrated an increase in the specific activities towards the four substrates as the dilution rate was increased (Table 5.5). The activity towards MCA increased 15-fold as the dilution rate was increased from the lowest to highest values, with a 6 to 7-fold increase in the activity at the intermediary dilution rate. This was unlike the results obtained for *Pseudomonas* sp.

strain E2 which showed only a slight increase in activity between the lowest and medium dilution rates.

The activity towards DCA increased 6 to 7-fold between the two extreme dilution rates, 2MCPA activity increased by 7.5-fold and 22DCPA activity increased by at least 12-fold. As with the MCA activity the activity towards each substrate increased by half the overall amount when the median dilution rate was applied.

Table 5.6 indicated that the relative activities of the dehalogenases in this organism changed with dilution rate, indicating a change in the dehalogenase profile. Figure 5.3 shows that this was the case. The changes observed were similar to those demonstrated by *Pseudomonas* sp. strain E4 under 2MCPA-limitation. Although at the median dilution rate dehalogenase II was not observed and the level of dehalogenase III was greater than that observed for strain E4.

5.4 DEHALOGENASE COMPLEMENT OF *PSEUDOMONAS* SP. STRAIN E6 IN CONTINUOUS-FLOW CULTURE AT DIFFERENT DILUTION RATES

The initial results obtained for *Pseudomonas* sp. strain E6 in continuous culture provided further evidence for the belief that the expression of dehalogenase activities was under a complex regulatory control.

This organism consistently produced all four dehalogenases in batch culture (Section 4.4), however, when placed in continuous culture at a dilution rate of half the u_{max} of the organism it briefly expressed dehalogenase I and II before reaching a steady-state in which only dehalogenase I was present. When the effect of dilution rate was studied different results were seen.

Strain E6 demonstrated a novel reaction to increased dilution rate,

not previously observed in the other organisms. The specific activity towards MCA increased 7-fold as the dilution rate was increased from the lowest to the median values and then only a slight increase as the dilution rate was switched to the highest value producing an overall increase of 7.4-fold from the lowest to the highest dilution rate (Table 5.5). The activity towards the other three substrates reached a maximum at the median dilution rate, although the maximum only represented

a 1.3-fold increase in DCA activity, a 3-fold increase in 2MCPA activity and a 2 to 3-fold increase in 22DCPA activity. At the highest dilution rate the activity fell back to, or below, the activities exhibited by the extracts from the lowest dilution rate cultures.

As one would expect from the specific activity results, the relative activities towards DCA, 2MCPA and 22DCPA decreased with respect to MCA activity as the dilution rate was increased. The relative rate of DCA activity decreased 20-fold, 2MCPA activity by 7-fold and the 22DCPA activity by 8.5-fold (Table 5.6).

These differences were reflected, or were reflections of, the change in the dehalogenase profile over the three dilution rates (Figure 5.3). At the lowest dilution rate the organism produced only dehalogenase III, but at the highest rate only dehalogenase II was produced. The median dilution rate represented a mid-point in the dehalogenase profile shift, with both dehalogenase II and III being present. The low level of dehalogenase III present in some of the cell-free extracts at the high dilution rate suggested that this enzyme was present at sub-threshold levels, at the high rate.

5.5 DEHALOGENASE COMPLEMENT OF *ALCALIGENES* SP. STRAIN E20 IN CONTINUOUS-FLOW CULTURE AT DIFFERENT DILUTION RATES

Unlike all the other isolates, *Alcaligenes* sp. strain E20 demonstrated a dehalogenase activity divorced from the effects of dilution rate. Both specific activities (Table 5.5) and relative activity ratios (Table 5.6) remained almost constant. This reflected the constant dehalogenase profile of the cell-free extracts at all three dilution rates which was the same as the batch-culture profile, that is dehalogenase II and III were produced (Figure 5.3).

5.6 DISCUSSION

Previous studies, with other systems, have demonstrated considerable variations in enzyme activity in response to variations in dilution rates and the nature of the growth-limiting substrate (Clarke & Lilly, 1969; Bolton & Dean, 1972; Almengor-Hecht & Bull, 1978; Bull & Brown, 1979).

Five classical responses for enzyme activity changes in dilution rate were identified by Dean (1972): activity remains constant; activity passes through a maximum or a minimum at a given dilution rate; or activity increases or decreases as the dilution rate increases.

The dehalogenase system in *Pseudomonas putida* strain PP3 has been shown previously to exhibit activity which passed through a maximum as the dilution rate was increased (Slater *et al.*, 1979), a response considered to be characteristic of catabolic enzymes (Clarke & Lilly, 1969; Dean, 1972).

Cultures of *Pseudomonas* sp. strain E4 under carbon-limitation with MCA or 2MCPA as the substrate, demonstrated variations in enzyme levels

in response to both dilution rate and the growth-limiting substrate.

Growth of strain E4 in a chemostat made it possible for the organism to grow on MCA, which inhibited growth of the organism in batch culture conditions. Chemostat culturing prevents the accumulation of toxic levels of the substrate so overcoming the inhibitory effect seen in batch culture due to the necessary high initial concentrations of the substrate.

In the MCA-limited culture the highest dilution rate was described as 0.15 to 0.18 h^{-1} this was due to the culture washing out at the higher dilution rate and the necessary decrease in flow-rate to the lower values for 24 to 48 h to enable the culture to recover. Although 0.18 h^{-1} only represents 86% of the μ_{max} for the organism, this value was derived from growth experiments with 2MCPA as the carbon source, as MCA was toxic under such conditions. Thus the μ_{max} of strain E4 growing on MCA may well have been lower than 0.13 h^{-1} , possibly just over 0.15 h^{-1} .

A major observation from these results is that growth under substrate-limited conditions resulted in the synthesis of a third dehalogenase, which had not been observed in the analysis of *Pseudomonas* sp. strain E4 grown on 2MCPA in closed culture. This result demonstrates the problems associated with the analysis of enzyme systems solely on the basis of results obtained with organisms grown in closed culture. The third dehalogenase demonstrated an electrophoretic mobility identical to that of the dehalogenase III isoenzyme seen in batch cultures of *Pseudomonas* sp. strains E2 and E6 and *Aloaligenes* sp. strains E20 and E22.

This result indicated that the regulation of synthesis and expression

of dehalogenase III in strain E4 had been modified, such that if the four genes for dehalogenase activity had been collected in various permutations into the six groups of isolates the expression and synthesis of each gene was not controlled in the same way in the different isolates. As such, these isolates could provide model systems of not only the evolutionary relationships between the four dehalogenases, but also of the regulatory control mechanisms involved.

The levels of enzymic activity expressed at equivalent growth rates was seen to be dependent on the nature of the growth-limiting substrate. At the lowest dilution rate (0.04 h^{-1}) the MCA and 2MCPA dehalogenation rates were approximately 3 times greater with MCA as the growth-limiting substrate compared with 2MCPA (Tables 5.1 and 5.2). At the highest dilution rate (0.18 h^{-1}) the rate of MCA dehalogenation was 12 times lower for MCA-limited organisms than the same rate for 2MCPA-limited ones, and the 2MCPA rate was 6 times lower.

The different responses to dilution rate when utilizing the different carbon sources were also seen as a difference in the overall change in specific activities towards the different substrates.

When MCA was the limiting substrate there was an overall increase in specific activities with decreasing dilution rate. However, the possibility that at a dilution rate lower than 0.04 h^{-1} a decrease in activity might have been observed, thereby providing a maximum in the activity at a low dilution rate cannot be ruled out.

With 2MCPA as the limiting substrate the response varied and depended on the substrate examined.

The differences in response to dilution rate of the two cultures

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can be explained by the different variations in the dehalogenase profiles. Although the increases in activities towards the four substrates in the MCA-limited culture could have resulted from an increase in the level of a single enzyme, this would have resulted in nearly constant relative activities (Table 5.3). This was not the case and electrophoretic studies (Figure 5.1) proved that the responses to changes in dilution rate in both the MCA and the 2MCPA-limited cultures were the result of changes in the dehalogenase profiles. The observed overall rate of dehalogenation measured in cell-free extracts varied because it depended not only on the amount of each dehalogenase present but also on their individual kinetic properties.

Although the dilution rates were kept constant for a period of time which should theoretically have allowed steady state conditions to have been reached, approximately 30 h at a dilution rate of 0.1 h^{-1} , considerable variations in the specific activities of the cell-free extracts were observed. This was especially so when more than one enzyme was present.

The oscillatory nature of enzyme systems in steady state cultures has been described in other systems. The levels of a number of enzymes have been shown to oscillate about an average value (MacLeod *et al.*, 1975; Sikyta & Fenc1, 1976). The oscillation of biomass and amidase levels in a chemostat culture of *Pseudomonas aeruginosa* strain 8602 led Boddy *et al.* (1967) to suggest that the oscillations were a reflection of changes in concentration of the inducer or catabolite repressor.

The balance between induction and repression of an enzyme can lead

to a peak in enzyme activity at a given dilution rate at a point where growth rate is such that enzyme induction is at its greatest but catabolite repressor concentrations are not sufficient to inhibit activity (Clarke *et al.*, 1968).

The response of *Pseudomonas* sp. strain E4 to growth on MCA appears to be more readily explained than its response to growth on 2MCPA. Dehalogenase I has been shown (Section 4.5, Table 4.4) to more readily dehalogenate chloroacetates than chloropropionates, and was seen to be the major enzyme active in dehalogenation. The minimum activity, exhibited at high growth rates, possibly under catabolite repression (Clarke *et al.*, 1968) is comparable to the minimum activities observed for *Pseudomonas putida* PP3 (Slater *et al.*, 1979) and are sufficient to account for the growth rate observed.

As the dilution rate decreased the concentration of unmetabolized MCA was decreased, this would result in greater enzyme synthesis, hence the higher specific activities, and the derepression of other dehalogenases which may increase the rate at which the slowly growing organism can scavenge the limiting amounts of MCA present in its environment.

The changes observed in strain E4 under 2MCPA-limitation cannot be explained by such a mechanism. It is at the high dilution rates that more isoenzymic forms are induced, under conditions of higher substrate concentration. This was particularly puzzling for dehalogenase II since it was previously shown to exhibit very little activity towards 2MCPA. One possible explanation for the 2MCPA system is that the induction of other isoenzymic forms at the higher substrate concentrations represents a detoxification response. At the higher substrate levels, even

enzymes exhibiting a low specificity for a substrate may cause sufficient breakdown of the substrate to help to prevent a build-up of the substrate to toxic concentrations. This would assume that the organism can maintain a suitable growth rate at low dilution rates with the single dehalogenase, dehalogenase I. If indeed this response was a detoxification one, it may explain why MCA inhibits growth of strain E4 at the higher concentrations found in batch cultures, since it cannot overcome catabolite repression sufficiently to detoxify its environment.

However, for whatever reason, it was apparent that *Pseudomonas* sp. strain E4 demonstrates complex control mechanisms over the synthesis and expression of its dehalogenase complement.

It was equally apparent that the other isolates also possessed such complex control mechanisms, but these differed from those of *Pseudomonas* sp. strain E4 in ways which resulted in the different responses to the environmental conditions which were observed.

Pseudomonas sp. strain E2 demonstrated an increase in specific activity which increased with dilution rate. The 5 to 6-fold increase in activity was seen towards all substrates which contrasts with results obtained for *Pseudomonas* sp. strain E4 which showed variable changes in activities in response to different substrates. It is of note that the increase in specific activity with increase in dilution rate is completely opposite to the results of *Pseudomonas* sp. strain E4 under MCA-limitation. However, in this case the overall increase in specific activity and the observed consistency of the relative activities are consistent with an increase in the level of a single enzyme as the dilution rate increased.

However, when the dehalogenase profile was observed (Figure 5.1) the levels of two isoenzymic forms were seen to vary. It is believed that the apparent consistency of dehalogenase profile suggested by the enzyme assay results can be explained as being due to an increase in the level of dehalogenase III, which throughout proved to be the major enzyme in this organism. The switch between producing dehalogenase III and IV at low dilution rates, to producing dehalogenase II and III at higher rates indicates a strict regulatory control over the production of these enzymes, although the reason for this switch is not known.

Thus, dehalogenase III was the rate-determining enzyme in *Pseudomonas* sp. strain E2 and this enzyme apparently increased in concentration as the dilution rate was increased, so allowing faster growth of the organism.

Unlike *Pseudomonas* sp. strain E4, strain E2 produced no isoenzymes that were not identified in batch cultures, although the level of dehalogenase II was much lower in the chemostat culture than in batch ones.

Dehalogenase I has been shown to be the most effective dehalogenating enzyme. Yet in *Pseudomonas* sp. strain E2 dehalogenase III has become the dominant enzyme. This would indicate that strain E2 does not possess the ability to produce dehalogenase I. From the growth rate experiments it would seem that in not possessing this ability, strain E2 and also the *Aloaligenes* sp. strains E20 and E22 are less competitive than the other isolates which possess this isoenzyme. The μ_{\max} of strain E2 is only half that for *Pseudomonas* sp. strain E4 which possess dehalogenase I.

Pseudomonas sp. strain E3 showed a dehalogenase profile which changed with dilution rate in a similar manner to that of *Pseudomonas* sp. strain E4 under 2MCPA-limited conditions. However, the relative and specific activities towards 22DCPA were apparently oppositely affected by the increased dilution rate.

As with strain E4, strain E3 produced dehalogenase III in continuous culture, even though it was also never observed under batch culture conditions. Additionally this organism had not shown any dehalogenase II activity in batch systems. However, even in continuous culture dehalogenase II was not observed until the highest dilution rate, which possibly indicates that the enzyme was under more strict repressive control in strain E3 than it was in E4.

Pseudomonas sp. strain E6 demonstrated an increase in activity towards MCA as the dilution rate was increased, with a concomitant decrease in activity towards the other substrates. This corresponded to a change in the dehalogenase profile from producing dehalogenase III to predominantly producing dehalogenase II. Although in batch culture experiments dehalogenase II possessed a high activity towards MCA, it also was very active towards DCA, thus the definite fall in the DCA specific activity with increasing levels of dehalogenase II is difficult to explain. The median dilution rate produced the greatest activity towards DCA, 2MCPA and 22DCPA, suggesting that the combined activity of the two enzymes produced greater activity towards these three substrates than either of the enzymes were capable of on their own. If this effect was due purely to the concentration of enzyme one would also have expected a peak in the MCA dehalogenating ability of the cell-free extracts. Further work with purified isoenzymes would be necessary to explain these results.

Strain E6 was originally placed in a group on its own because of its ability to produce all four dehalogenases. However, during the course of this work it has apparently lost the ability to produce dehalogenases I and IV. When any of the other isolates were inoculated into batch culture from the chemostat cultures, at any dilution rate, the dehalogenase profile reverted to the one originally observed in batch culture. This was not the case for strain E6. However, when the stock cultures were screened for dehalogenase activity these too had apparently lost the ability to produce the four enzymes. This suggests that either a mutation has occurred in two of the dehalogenase genes or that during the course of this investigation the regulatory controls for dehalogenase activity in strain E6 have altered.

The *Aloaligenes* sp. strain E20 possessed the same dehalogenase profile at all dilution rates and this was the same as the one seen in batch culture. The slight differences observed in the specific and relative activities of the enzyme system were presumably due to fluctuations in the levels of the two enzymes.

As dehalogenase I shows a high degree of specificity for MCA, the substrate on which strain E20 was growing, it can only be presumed that this organism does not possess the ability to produce this isoenzyme as evidence from *Pseudomonas* sp. strain E4 suggests it would grow more efficiently if it produced this enzyme.

The biological significance of isoenzymes as a mechanism which allows an organism to quickly adapt to a changing environment has been widely discussed (Gillespie & Kojima, 1968; Darrow & Knotts, 1977; Fuchs & Keister, 1980). The evidence from the continuous culture of

these five soil isolates suggests that the control of the expression of their dehalogenase complement may be a mechanism allowing them to utilize the carbon source in their environment more efficiently.

CHAPTER 6

EVIDENCE FOR THE ASSOCIATION OF DEHALOGENASE ACTIVITIES WITH THE PRESENCE OF A NUMBER OF PLASMIDS IN THE SOIL ISOLATES

The bacterial strains isolated were obtained after a period of mixed culture enrichment in closed systems. It was suggested that during the course of the enrichment, strains might arise containing more than one dehalogenase through a gene transfer and aggregation mechanism. This could operate through the transfer of plasmids between different microbial populations, mobilizing dehalogenase genes onto the transferring plasmid.

The apparent similarity of the dehalogenases in the two bacterial genera isolated, *Pseudomonas* and *Alcaligenes*, adds further to the theory of plasmid transfer, which could explain the apparently identical genes in two different genera. Plasmid mediated transfer of catabolic enzymes' genes has been shown to occur freely within the *Pseudomonas* genus and to other genera (Chakrabarty *et al.*, 1973; Benson & Shapiro, 1978; Don & Pemberton, 1981).

6.1 DETERMINATION OF PLASMID PRESENCE IN THE SOIL ISOLATES

The presence of plasmids in *Pseudomonas* sp. strains E2, E3, E4 and E6 and *Alcaligenes* sp. strains E20 and E22, was revealed by the rapid screening procedure (Section 2.6.2). Although all of these strains were shown to contain a large plasmid, with a molecular weight of 90 to 100 Md or more, the initial agarose gels indicated that they were not all of equal size. The largest plasmid occurred in *Pseudomonas* sp. strain E4, whilst the smallest occurred in *Pseudomonas* sp. strains E3 and E6.

Although most of the screening used bacteria cultured under batch conditions, it was found that preparations from organisms grown in

continuous-flow culture produced significantly more distinct bands when electrophoresed on agarose gels and stained with ethidium bromide.

6.2 PLASMID NOMENCLATURE

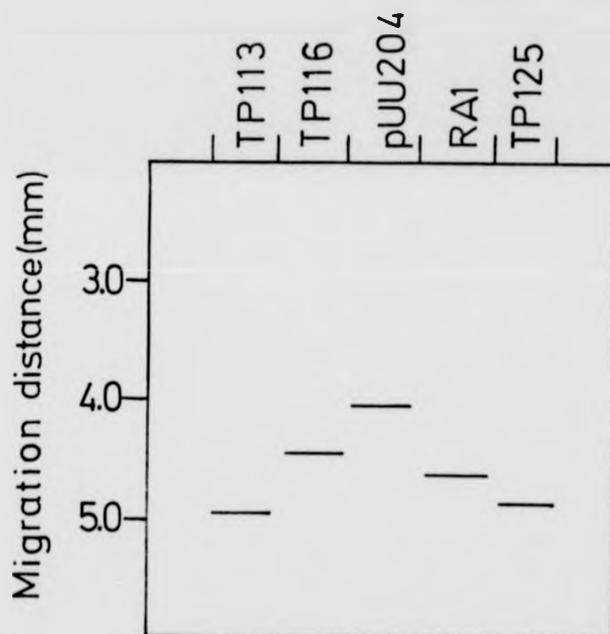
In order to avoid confusion the following nomenclature was used to describe the individual plasmids. The naming of these plasmids was achieved after reference to restriction endonuclease digest patterns of each plasmid (Section 6.5). pUU 202 present in *Pseudomonas* sp. strain E2; pUU 204 present in *Pseudomonas* sp. strain E4; pUU 247 present in *Pseudomonas* sp. strain E47 (a derivative of strain E4); pUU 206 present in *Pseudomonas* sp. strains E3 and E6; pUU 220 present in *Alcaligenes* sp. strain E20 and pUU 222 present in *Alcaligenes* sp. strain E22. Other plasmids derived from these used other plasmids and will be described in context.

6.3 SIZING THE WHOLE PLASMIDS

The migration distances of the plasmids on the screening gels indicated that the plasmids were large. Sizing of a whole plasmid was attempted for pUU 204 using the standard electrophoretic procedure with preparations of plasmids of known molecular weight electrophoresed alongside. The size of the plasmid was determined from a double logarithmic plot of migration distances against molecular weight of the standard plasmids. However, due to the logarithmic nature of the standard curve, minor differences in migration distance produced a major difference in the size estimation with such large plasmids.

Due to these problems it was not possible to determine the molecular weight of pUU 204 by this procedure. From results such as those described in Figure 6.1, it was concluded that the molecular weight of this plasmid was greater than pTP116, which was the largest molecular weight standard

Figure 6.1 Agarose gel electrophoresis demonstrating the relative mobilities of pUU204 with respect to various plasmids of known molecular weight TP 113, 56.7 Md; TP 116, 143.7 Md; RA1, 86 Md and TP 125, 64 Md.



used, thus pUU 204 had a molecular weight in excess of 143.7 Md.

6.4 SIZING OF THE PLASMIDS BY SUMMATION OF THE MOLECULAR WEIGHTS OF RESTRICTION ENDONUCLEASE FRAGMENTS

As noted with the whole plasmid studies, the best restriction endonuclease digest patterns, that is those with the least background staining, were obtained from cultures grown in continuous-flow culture at a low dilution rate (0.04 h^{-1}).

It was apparent from the first restriction endonuclease digest patterns that a large number of restriction sites were present on these plasmids, as each digest generated numerous fragments. There were a different number of restriction sites for each of five restriction endonucleases chosen to fragment the plasmid. By obtaining five different patterns for each plasmid a relatively accurate estimate of the plasmid's molecular weight could be achieved by summation of the molecular weights of the individual fragments. A number of these endonuclease digests were repeated and an average value for each molecular weight determined. The values determined for the molecular weights of the seven plasmids are shown in Table 6.1.

The determination of molecular weight was achieved by reference to a standard curve produced for each gel from an internal standard, which consisted of a *Hind* III restriction endonuclease digest of lambda (λ) DNA. The individual molecular weights of each fragment were determined, then, before totalling, to avoid any bias, the bands showing more dense staining were identified and, for determination of molecular weights, considered to be two bands of very similar or identical molecular weight and were added into the total molecular weight twice. This determination

TABLE 6.1 Restriction endonuclease size determination (nd = not determined)

Strain	Plasmid nomenclature	Restriction endonuclease sizing-sum of digest fragments in kilobases							Mean size kilobases	Mean size megadaltons
		<i>Hind</i> III	<i>Pst</i> I	<i>Eco</i> RI	<i>Bam</i> HI	<i>Xho</i>				
<i>Pseudomonas</i> sp strain E2	pUU 202	≈230	nd	nd	nd	nd	nd	≈230.0	≈150.0	
<i>Pseudomonas</i> sp strain E3	pUU 206	156	150	nd	nd	150		152.0	98.8	
<i>Pseudomonas</i> sp strain E4	pUU 204	287.2	298.9	298.4	292.2	291.2		293.6	190.8	
<i>Pseudomonas</i> sp strain E47	pUU 247	21.5	nd	nd	nd	27.7		24.6	16.0	
<i>Pseudomonas</i> sp strain E6	pUU 206	164.1	168.8	150.5	182.7	172.1		167.6	109.0	
<i>Alcaligenes</i> sp strain E20	pUU 220	287.0	135.5	178.7	317.1	183.1		nd	nd	
<i>Alcaligenes</i> sp strain E22	pUU 222	264.2	202.4	222.2	nd	197.2		nd	nd	

involved comparing the degree of fluorescence, or brightness on the photographs, of individual fragments with their immediate neighbours above and below. By doing this smaller fragments, which were not as bright as the large fragments higher up the gel, were identified as doubles even though they were not as bright as some of the single bands higher up the gel. It is possible that a number of the bands represented bands of more than two fragments but determination of this was not possible.

Sizing was made more easy by enlarging the Land camera negatives and developing segments of the photographs in different ways, and on different grades of paper, to increase resolution.

The restriction endonuclease digests of pUU 202 consistently failed to yield adequate resolution of the fragments from the overall background level of stain. Treatment of the plasmid preparation with RNAase, before electrophoresis, failed to increase the resolution and attempts to pellet contaminating material by ultra-centrifugation at 100,000g in sucrose gradient only improved the resolution slightly.

The value of 230 Kb derived from the fragment patterns of a more distinct *Hind* III digest gave an approximate value. The value was only approximate because of the impossibility of identifying the smaller fragments, which, by the nature of the staining technique, are less densely stained than larger fragments, in the high background staining of contaminating material.

The results shown in Table 6.1 indicated that the plasmids differed markedly, with molecular weights ranging from 99 or 109 Md up to 191 Md. The agreement in the molecular weights for the *Pseudomonas* sp. plasmids determined from the various restriction endonuclease patterns was good.

However, the values for the *Alcaligenes* sp. plasmids pUU 220 and pUU 222, were not in such close agreement. It is believed from these results that the molecular weights of these plasmids were in the region of 100 to 150 Md, that is smaller than pUU 202 and pUU 204 but larger than pUU 206.

pUU 247 failed to show any restriction sites for *Pst* I, *Eco* RI or *Bam* HI but the fragment patterns from *Hind* III and *Xho* I endonuclease digestions suggested a molecular weight of approximately 16 Md.

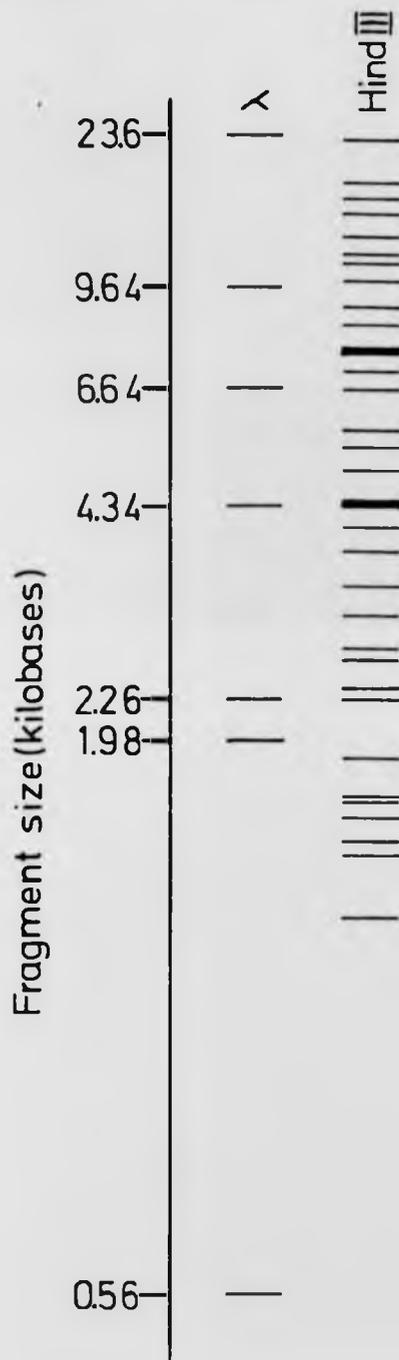
6.5 PLASMID CHARACTERIZATION BY RESTRICTION ENDONUCLEASE ANALYSIS

The restriction endonuclease digest patterns clearly showed that the various plasmids not only differed in total molecular weights but also in the number, and position, of the specific restriction sites of the five restriction endonucleases used. The fragment patterns are shown in Figure 6.2 to 6.9.

The plasmids from the six bacterial strains produced five different fragment patterns. The fragment patterns of the plasmids isolated from *Pseudomonas* sp. strains E3 and E6, after each endonuclease digestion with one of the five enzymes, were almost identical. A comparison of the *Hind* III, *Pst* I and *Xho* I endonuclease digest patterns of the plasmids from both isolates after electrophoresis on the same agarose gel showed that all the resulting fragments match almost identically. As a consequence of these results the two *Pseudomonas* sp. strains were considered to possess the same plasmid, pUU 206.

The largest plasmid, pUU 204, yielded the largest number of fragments after *Eco* RI endonuclease digestion, 49 fragments being identified (Figures 6.3 and 6.4). Whilst the smallest plasmid, pUU 206, produced only

Figure 6.2: Hind III restriction endonuclease digest pattern of the Pseudomonas sp. strain E 2 plasmid, pUU 202, with a Lambda (λ) DNA standard. The thick bands represent fragments of identical or very similar molecular weights.



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Figure 6.3: Restriction endonuclease digest patterns of the Pseudomonas sp. strain E 4 plasmid, pUU 204, with λ standards digested with Hind III restriction endonuclease

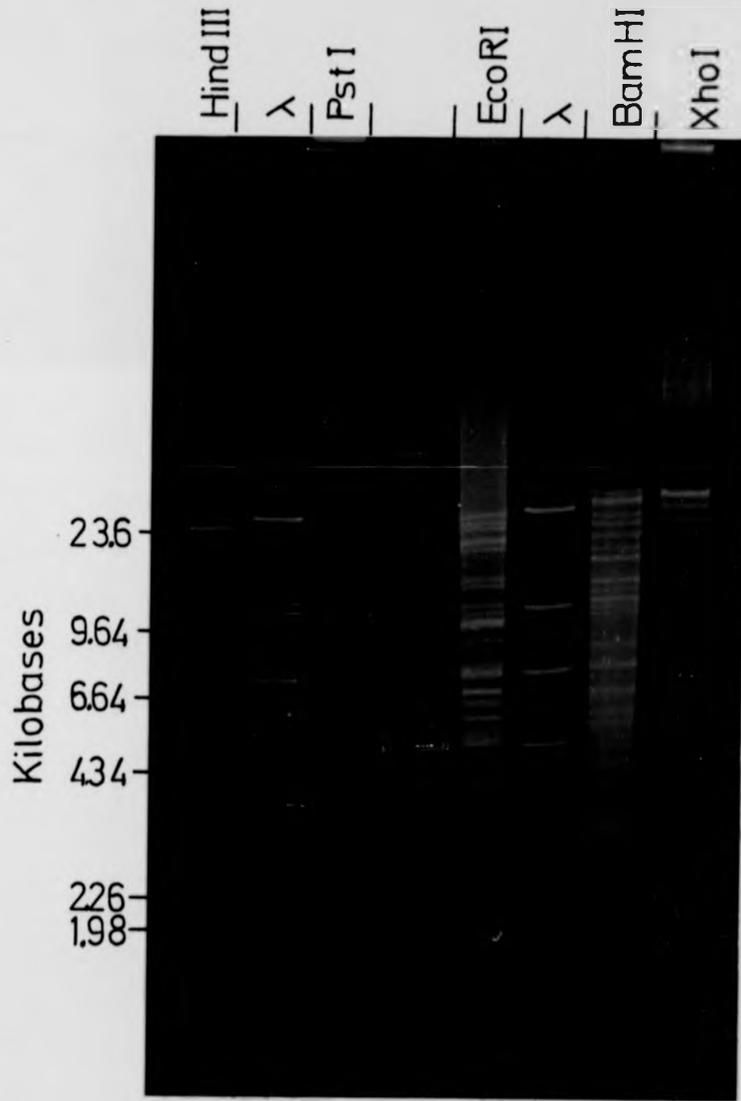


Figure 6.4: Restriction endonuclease digest patterns for the Pseudomonas sp. strain E4 plasmid, pUU 204 (see legend of Figure 6.2 for further details).

Fragment size (kilobases)

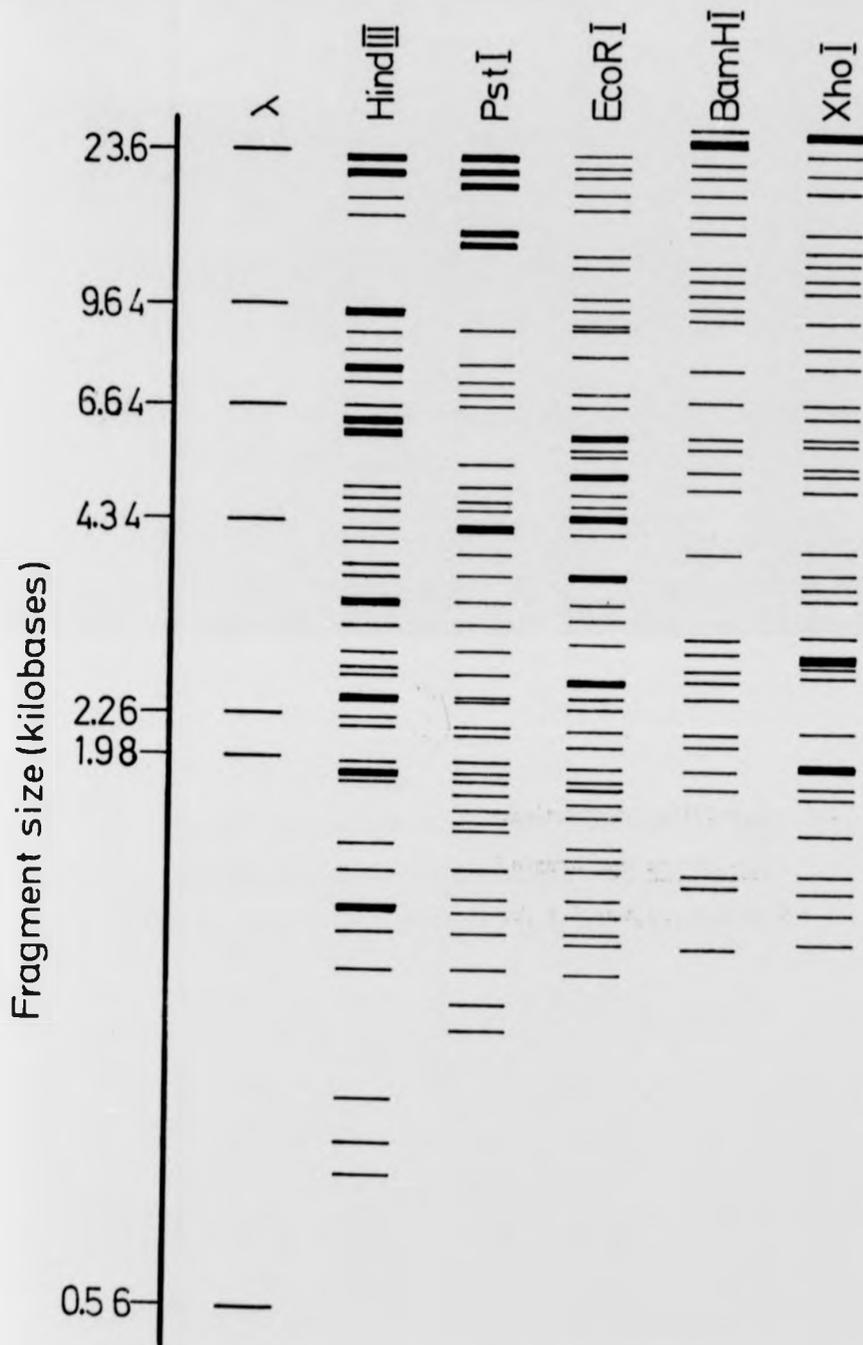
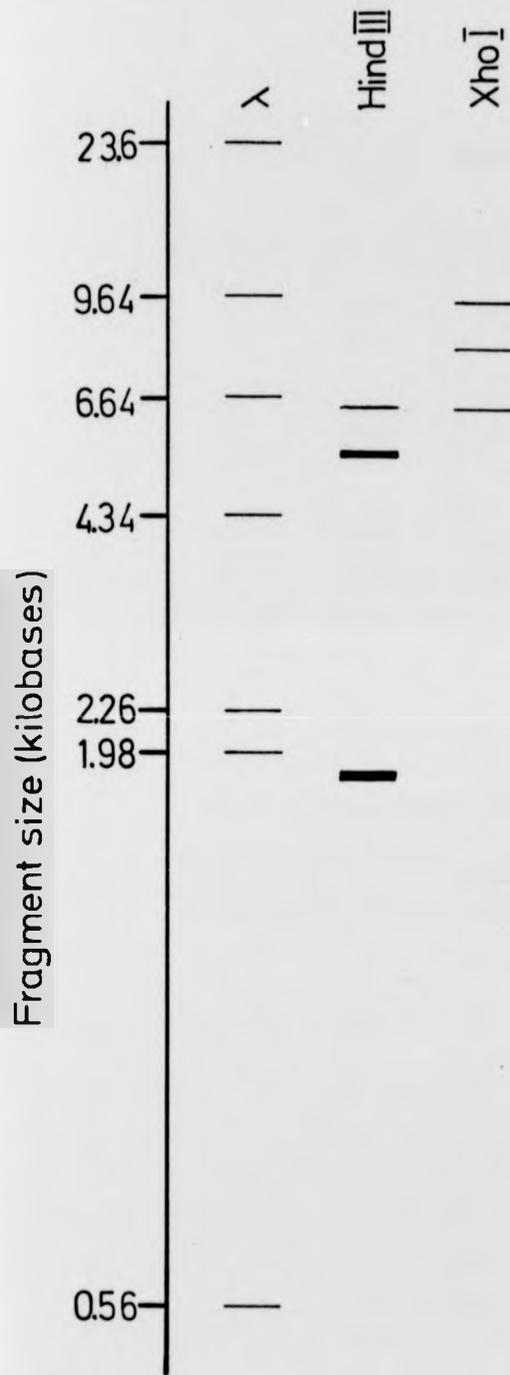


Figure 6.5: Hind III and Xho I restriction endonuclease digest patterns of the Pseudomonas sp. strain E47 plasmid pUU 247 (see legend of Figure 6.2 for further details).

Fragment size (kilobases)



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id
tails).

Figure 6.6: Restriction endonuclease digest patterns of the
Pseudomonas sp. strains E3 and E6 plasmid, pUU 206.
(see legend to Figure 6.2 for further details).

Fragment size (kilobases)

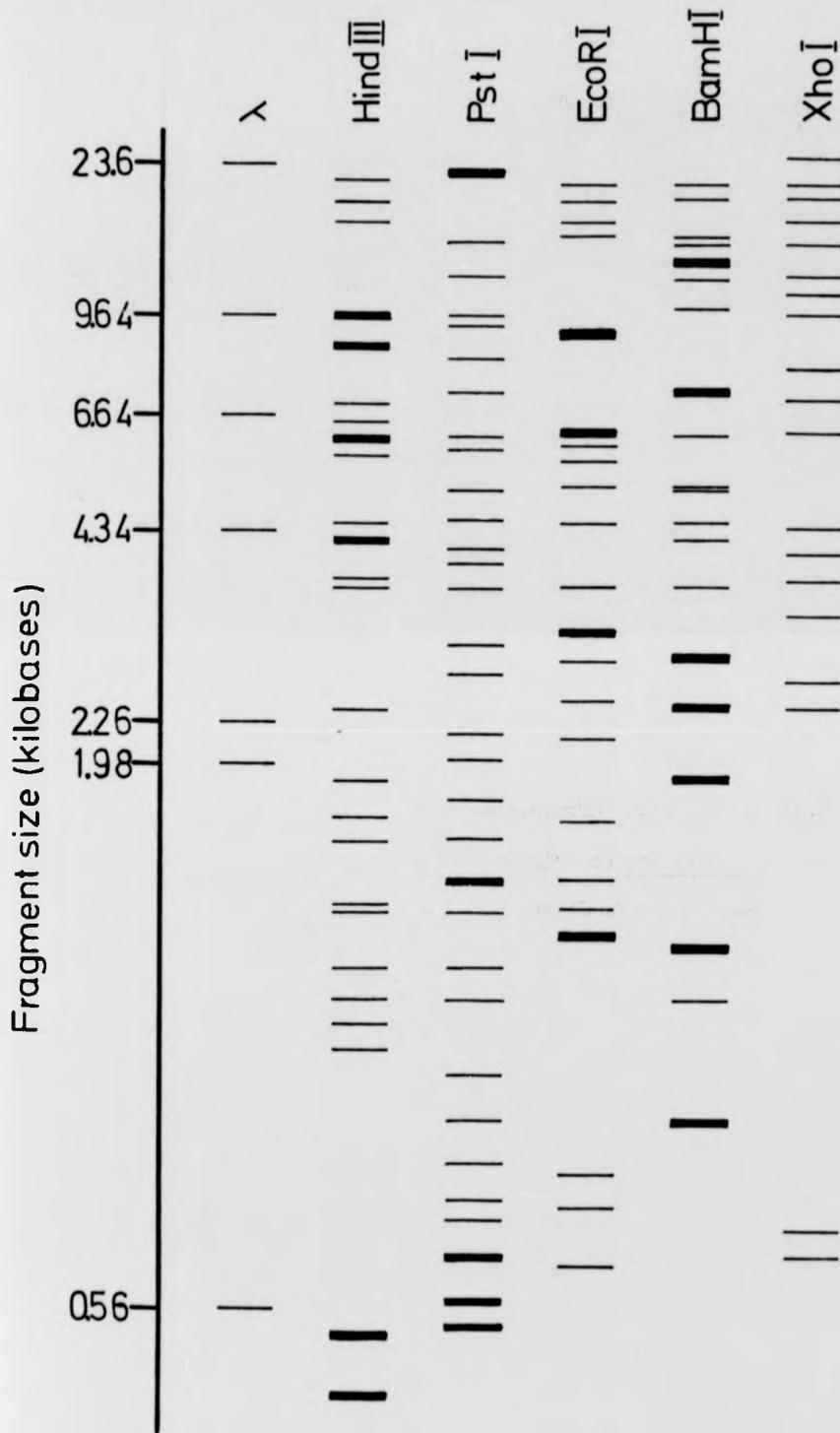


Figure 6.7: Restriction endonuclease digest patterns for the Aloaligenes sp. strain E 20 plasmid, pUU 220 (see legend to Figure 6.2 for further details).

Fragment size (kilobases)

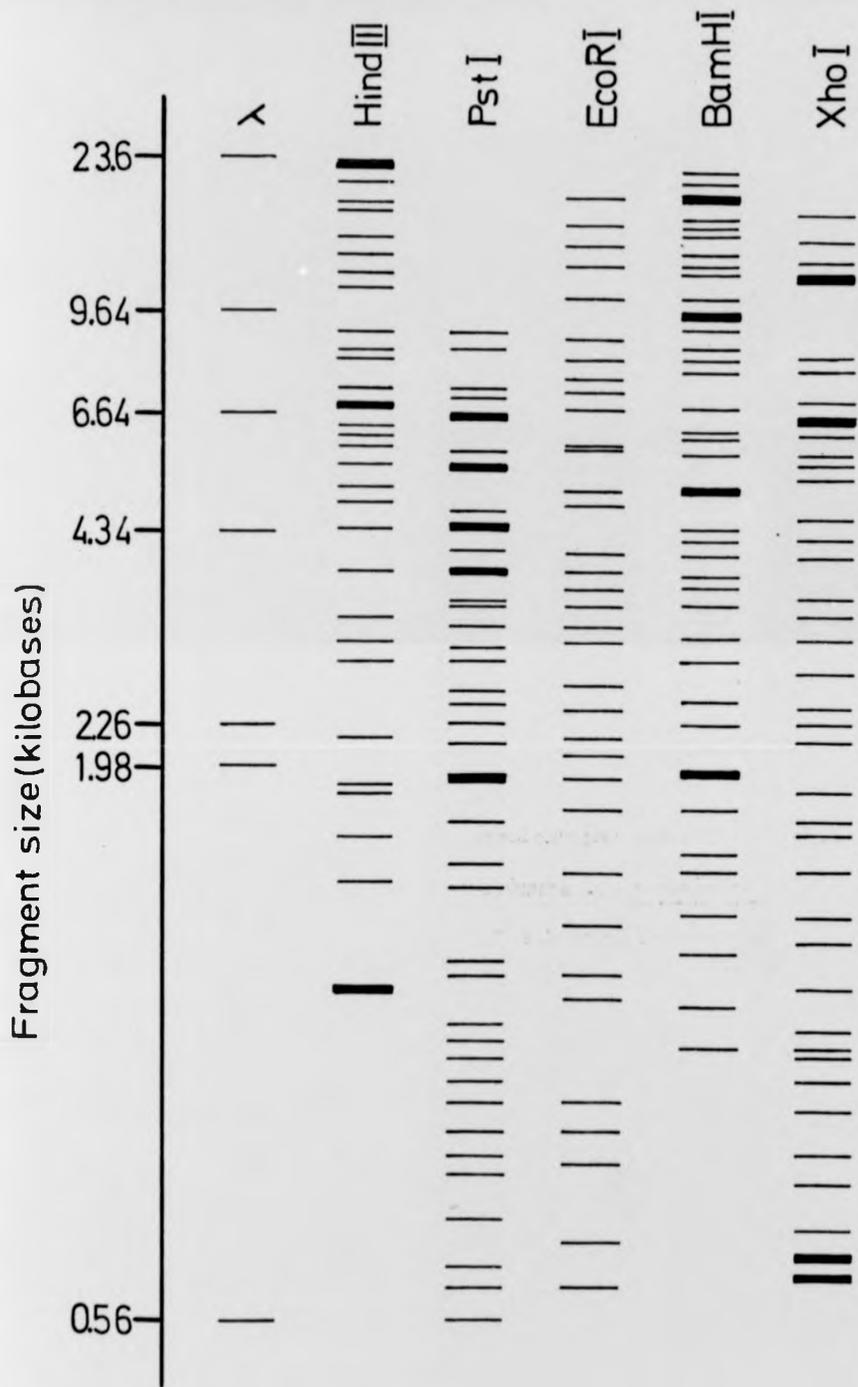
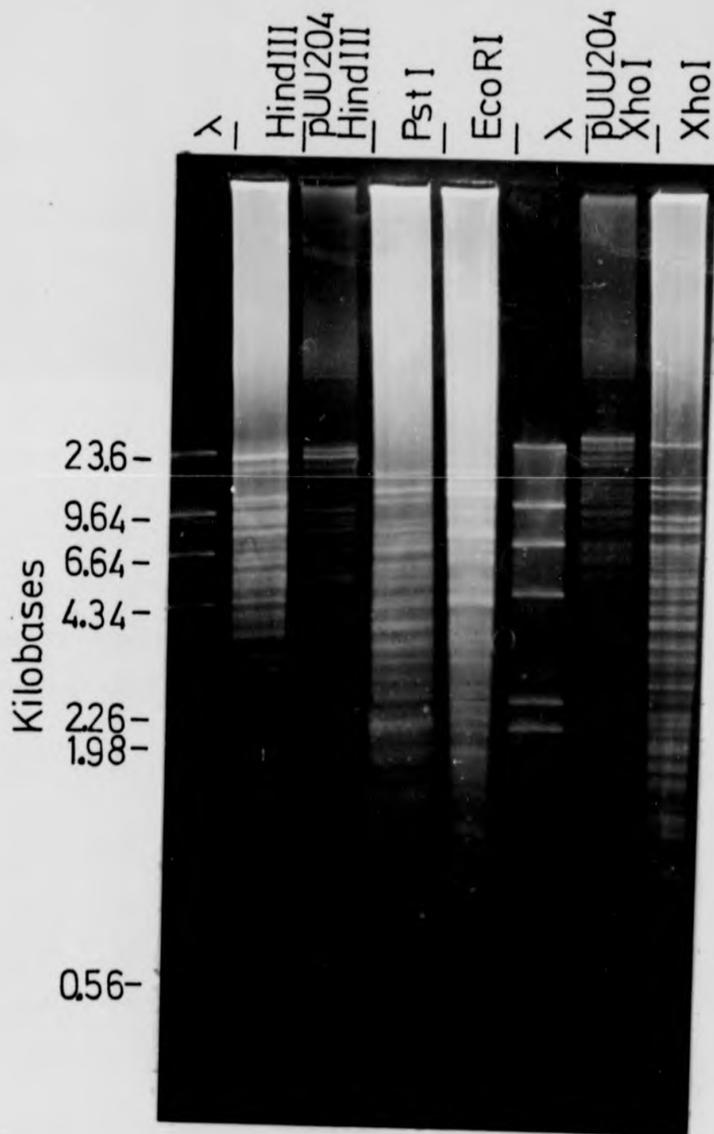
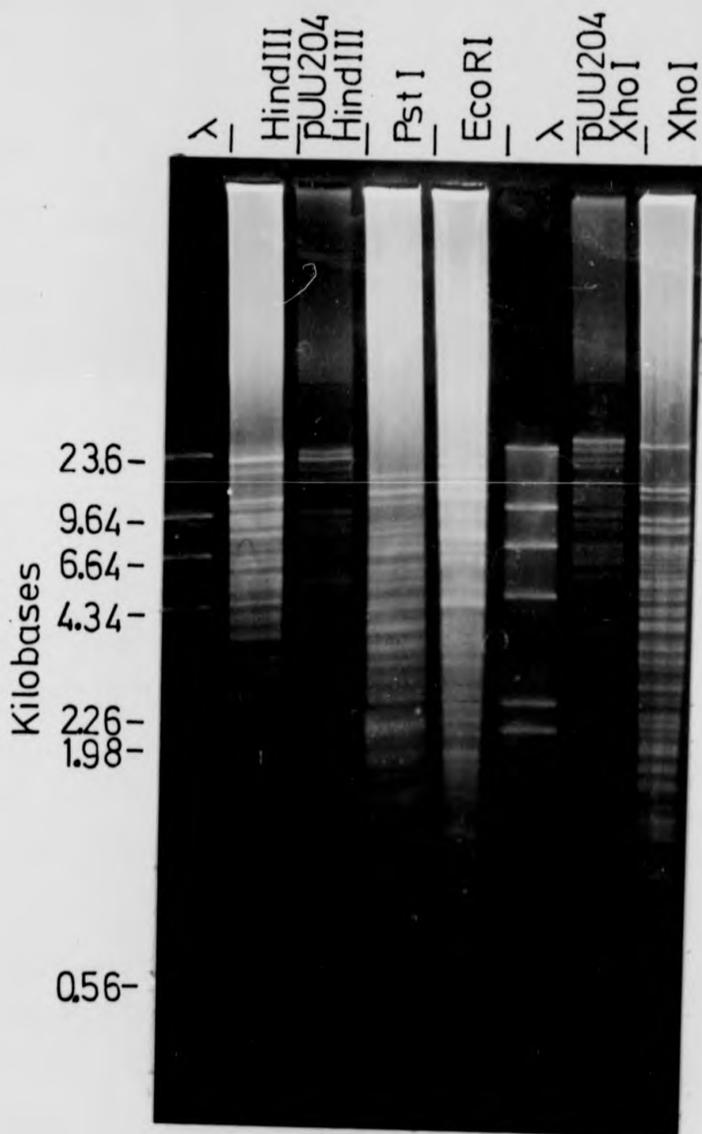


Figure 6.8: Restriction endonuclease digest patterns of the pUU 222 plasmid using 4 endonucleases and a comparison with the BlnI and XhoI digest patterns of the plasmid pUU 204.





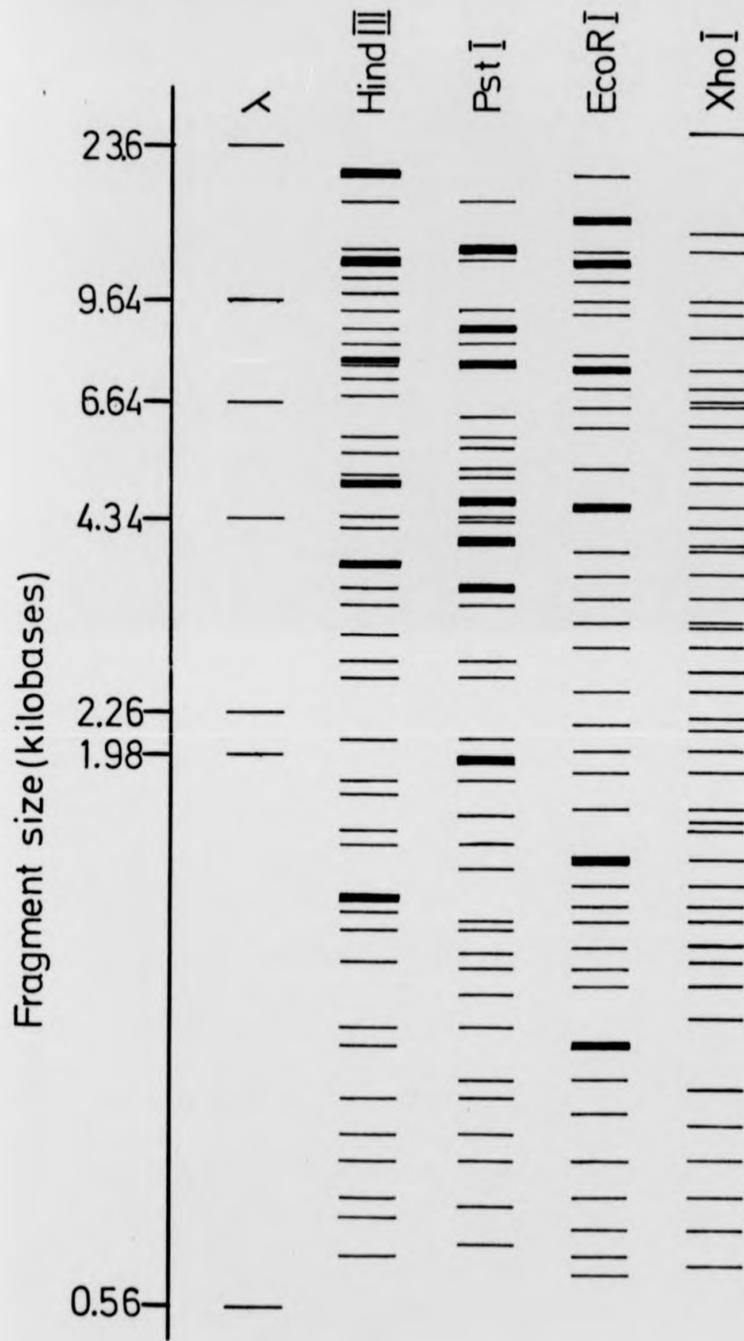
pUU 222

with the

pUU 204.

Figure 6.9: Restriction endonuclease digest patterns of the Alcaligenes sp. strain E 22 plasmid, pUU 222 (see legend to Figure 6.2 for further details).

Fragment size (kilobases)



19 fragments with *Xho* I endonuclease digestion (Figure 6.6). Such differences in fragment number indicated numerous differences in the structures of the individual plasmids.

The plasmid pUU 247, isolated after ethidium bromide treatment of *Pseudomonas* sp. strain E4 (Section 6.6) yielded the digestion patterns shown in Figure 6.5. It can be seen to have been considerably simplified, its' fragments when summed, produce a total molecular weight of 16 Md. With the exception of the smallest two fragments, which were seen as a double band, of the *Hind* III endonuclease digest, none of the fragments correspond to any of the fragments exhibited by pUU 204 after *Hind* III or *Xho* I endonuclease digestion.

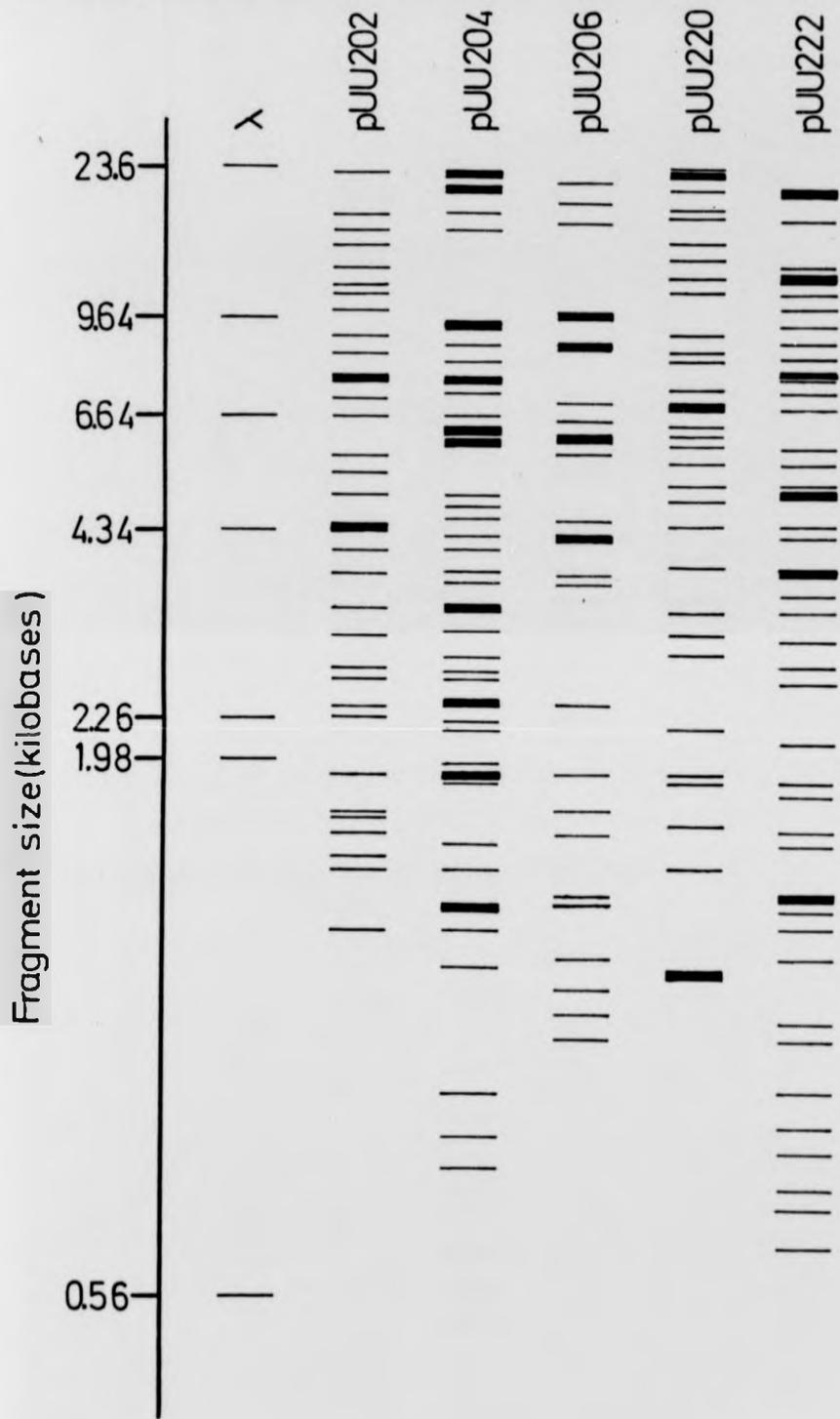
Attempts to recognise common fragments shared by the five plasmids after restriction endonuclease digestion did not provide satisfactory results. Even when drawn, as are the Figures 6.2 to 6.9, with the individual fragment's molecular weights converted to standard migration distances by reference to a single standard curve, common fragments could not be identified.

Towards the top of the gel such recognition of identical fragments becomes increasingly difficult because although fragments may migrate at very similar rates, and hence distances, any slight variations are difficult to detect. These problems are compounded because small differences in migratory distances represent considerable size differences due to the logarithmic nature of the separation technique.

A number of the smaller fragments did appear to migrate to the same place on the agarose gels, however, owing to the large number of fragments, it is not possible to discover whether such similarities represent

Figure 6.10: Summary of the digest patterns obtained for pUU 202, pUU 204, pUU 206, pUU 220 and pUU 222 using the restriction endonuclease Hind III in all cases. The λ DNA standard was also digested with Hind III.

Fragment size(kilobases)



302,

The

Figure 6.11: Summary of the digest patterns obtained for pUU 204, pUU206, pUU 220 and pUU 222 using the restriction endonuclease Pst I. The λ DNA was digested with Hind III.

Fragment size (kilobases)

Fragment size (kilobases)

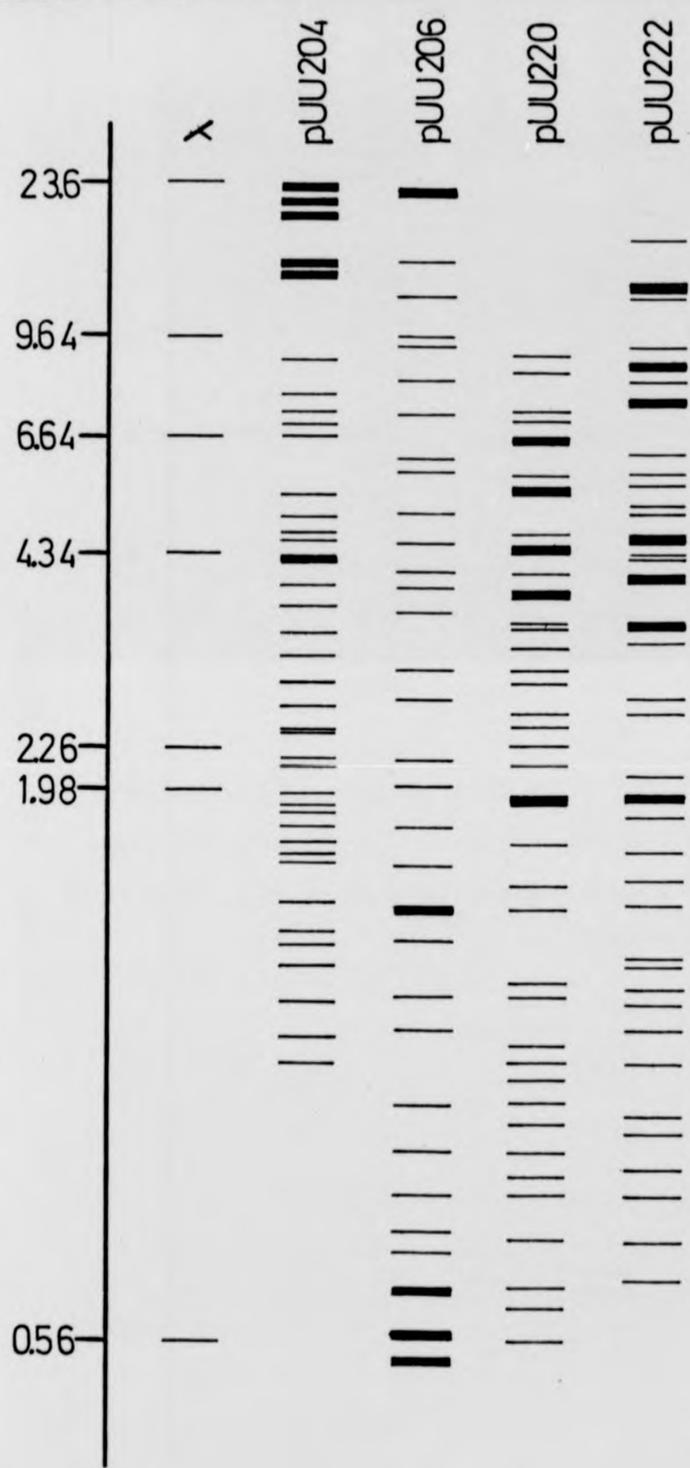
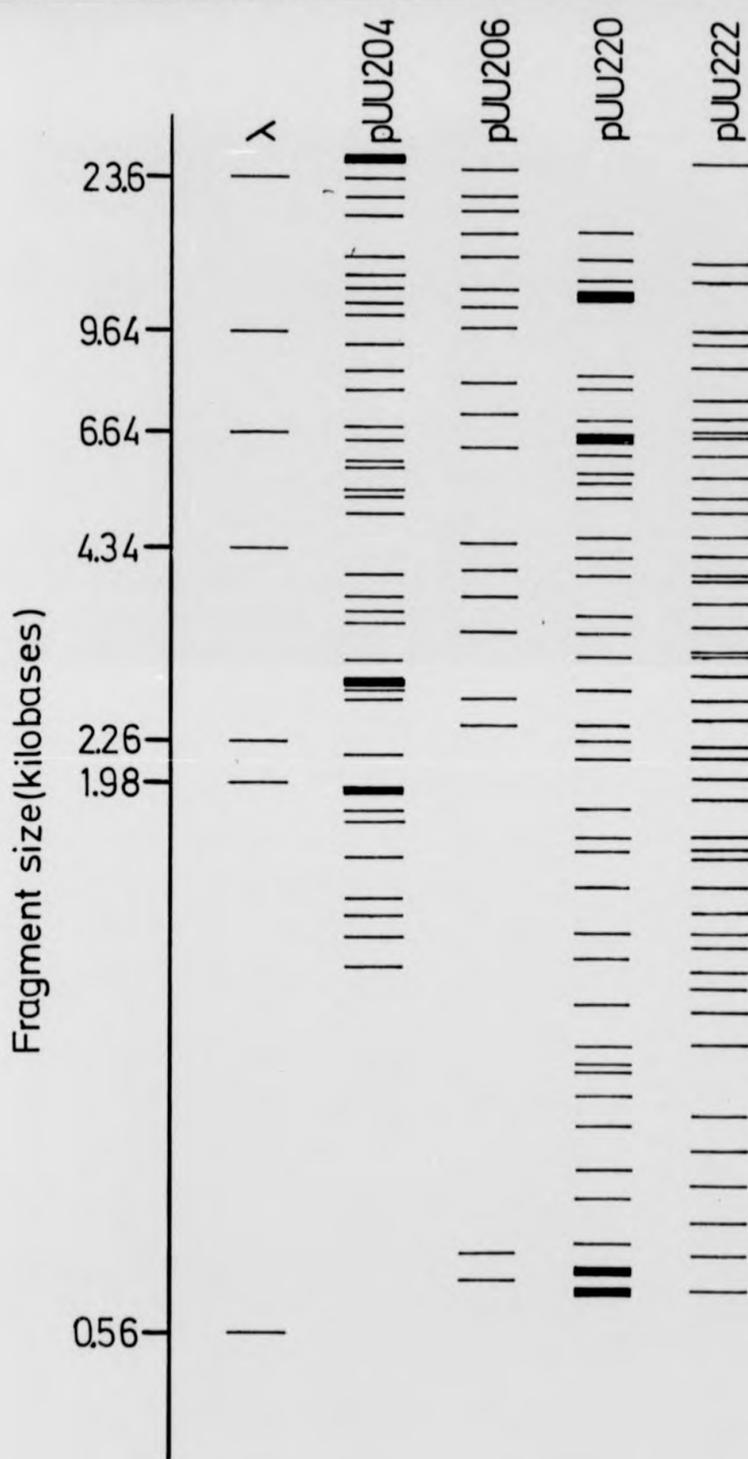


Figure 6.12: Summary of the digest patterns obtained for pUU 204, pUU 206, pUU 220 and pUU 222 using the restriction endonuclease Xho I. The λ DNA standard was digested with Hind III.



homologous restriction sites or just coincidence.

The distribution of those fluorescent bands described as representing at least two fragments are conspicuously different in all five plasmids. Very few correspond to double bands in other plasmids restricted by the same enzyme. It is possible that the top double of pUU 202 corresponds to a double band in pUU 204, the top double of pUU 204 with the top double in pUU 220 in the *Hind* III endonuclease digest patterns, (Figure 6.10). Also the bottom double bands of the pUU 220 and pUU 222 *Pst* I endonuclease digest may correspond to each other (Figure 6.11).

Conspicuous differences were also observed in the fragment size distribution. The plasmids pUU 204 and pUU 206 exhibited no bands between 16 and 10 Kb in the *Hind* III endonuclease digest whereas the other plasmids do. (Figure 6.10). Yet pUU 206 differed from pUU 204 in exhibiting no fragments between 3.5 and 2.4 Kb. The *Pst* I endonuclease digest of pUU 204 and pUU 206 and pUU 220 and pUU 222 (Figure 6.11) differ in the larger fragments in that the largest fragments of pUU 204 and pUU 206 were 22.39 and 21.88 Kb respectively, whereas the largest pUU 220 and pUU 222 fragments were 9.55 and 19.95. The latter two exhibited differences in plasmid form in the same genera.

The most obvious difference in the *Xho* I endonuclease digest patterns (Figure 6.12) was the lack of fragments between 2.40 Kb and 0.66 Kb for pUU 206, in which range the other plasmids showed a number of fragments.

6.6 PLASMID CURING EXPERIMENTS

The possibility that one or more of the genes encoding for dehalogenase activity were carried on the plasmids identified was examined by curing the bacteria of the plasmid and determining the effect on the capacity to grow

on 2MCPA.

The curing experiments used various concentrations of ethidium bromide on all of the six isolates. However, the growth of *Alcaligenes* sp. strains E20 and E22 and *Pseudomonas* sp. strain E6 was completely inhibited by the lowest concentration of ethidium bromide used ($10 \mu\text{gml}^{-1}$). The data obtained for *Pseudomonas* sp. strain E4 are presented in Table 6.2. They indicate a substantial increase in the loss of the ability to grow on 2MCPA was associated with growth on succinate with ethidium bromide concentrations in excess of $80 \mu\text{gml}^{-1}$. A comparison of the colony numbers on the succinate recovery plates indicated only a 3-fold decrease in the number of viable organisms recovered with the $100 \mu\text{gml}^{-1}$ ethidium bromide culture as compared with the control culture. Yet when the colony counts on 2MCPA recovery plates were compared a 430-fold decrease was seen. This suggested a selective loss of the ability to grow on 2MCPA which was not caused by a generalized effect of ethidium bromide on this organism.

Some colonies (100), from the succinate recovery medium were transferred in duplicate onto fresh succinate recovery medium and also onto 2MCPA containing medium. After incubation 100% of the selected colonies had grown on the succinate medium, but none grew on the 2MCPA medium.

The colonies present on the original recovery medium were either large or small. Representatives of each type were transferred onto succinate recovery medium and incubated. After which these, and a number of the 100 succinate colonies were cultured overnight in succinate defined liquid medium (Section 2.1.1) and screened for the presence of a plasmid. The cultures from the large colonies on the 2MCPA recovery plates possessed a large plasmid which appeared to be of the same size as the plasmid in

TABLE 6.2 The effect of ethidium bromide on the viability of an overnight culture of *Pseudomonas* sp. E4 plated onto a defined recovery medium with either succinate or 2MCPA as the carbon and energy source

Ethidium bromide concentration ($\mu\text{g ml}^{-1}$)	Viable counts (organisms ml^{-1}) on defined recovery medium		% decrease on 2MCPA recovery medium
	Succinate	2MCPA	
0	1.70×10^9	1.05×10^9	62
40	6.00×10^8	2.63×10^8	44
80	5.30×10^8	8.80×10^5	< 0.002
100	5.00×10^8	2.40×10^6	< 0.005

the parental strain, *Pseudomonas* sp. strain E4. However, in all the other cultures the plasmid was not detected, although one of the strains, designated *Pseudomonas* sp. strain E47 possessed a small plasmid which showed a greater mobility than that of the fragmented chromosomal DNA.

As a further test for the loss of dehalogenase activity, overnight succinate-grown cultures of the plasmid-minus and parental strains were split 50:50 into fresh sterile medium containing 2MCPA, and the chloride release monitored. The parental strain released chloride into the culture medium to a level concomitant with complete breakdown of the halogenated substrate, whereas all the plasmid deficient strains, including *Pseudomonas* sp. strain E47, failed to show any chloride release after 24 h.

If the loss of the dehalogenase activity were associated with the loss of the large plasmid no reversion to dehalogenase active strains should be possible. However, if the ethidium bromide treatment had resulted in a point mutation in the chromosome, which caused the loss of dehalogenase activity, then a reversion rate of approximately 1 in 10^5 cells would be expected. In order that all the dehalogenase isoenzymes would be inactivated it would be necessary for the point mutation to have occurred in a regulatory gene common to the expression of all the isoenzymic forms.

The reversion rate was studied using overnight cultures of a number of the *Pseudomonas* sp. strain E4 dehalogenase deficient strains by serially plating onto succinate and 2MCPA defined medium aliquots (0.1 ml) of the culture. After incubation the colonies were counted on each plate. The succinate count was 4×10^8 organisms ml^{-1} and the

2MCPA count was nil, only an overall background of small colonies were present. These were of similar size to those small colonies on the original 2MCPA recovery media used in the ethidium bromide experiments.

The experiment was repeated and still no revertants were observed, also after many subcultures the progeny were again tested for reversion with negative results. This suggests that the loss of the dehalogenase activity was associated with the loss of genetic material rather than due to a point mutation.

Similar curing experiments were undertaken with *Pseudomonas* sp. strains E2 and E3. The treatment with ethidium bromide resulted in the loss of dehalogenase activity at a very high frequency, comparable to that observed with *Pseudomonas* sp. strain E4, and the loss was shown to be stable. When the dehalogenase deficient strains of strain E2 were screened for the presence of a plasmid a number were seen to possess a plasmid much smaller than the parental plasmid, although it was larger than the *Pseudomonas* sp. strain E47 plasmid, pUU 247.

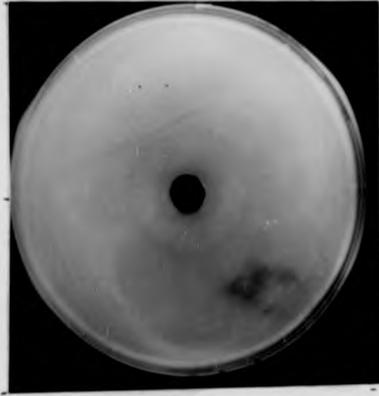
6.7 HEAVY METAL RESISTANCE

The inhibition of growth of the bacterial strains, with or without the native plasmids, by heavy metals was investigated (Figure 6.13). This suggested that these plasmids conferred some degree of resistance to a number of heavy metals on their hosts. Table 6.3 presents the data as regions of inhibition around a central well cut into the agar and filled with 175 μ l of a heavy metal solution.

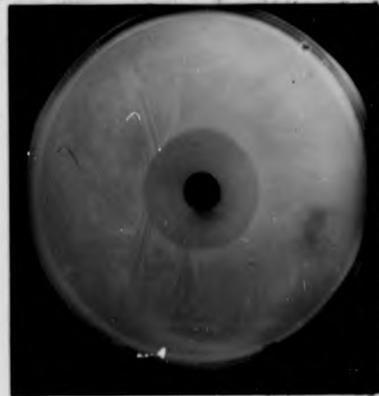
If the resistance patterns of *Pseudomonas* sp. strains E4, E41 and E47 are compared, the plasmid bearing strain E4 shows a greater resistance to mercury, selenium and tellurium than either of its cured derivatives

Figure 6.13: Inhibition of Pseudomonas sp. strains E4 (pUU 204) and E41 (plasmid-free) growth by mercury, tellurium and selenium, as measured by growth inhibition zones in bacterial lawns.

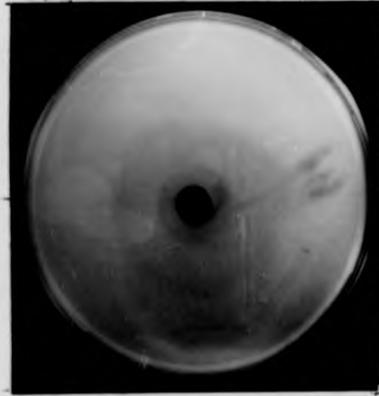
) and
nd selenium,
al laws.



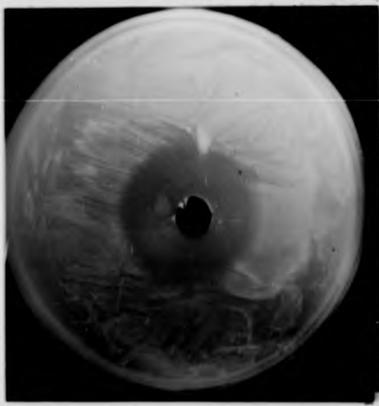
Pseudomonas sp E4 Te



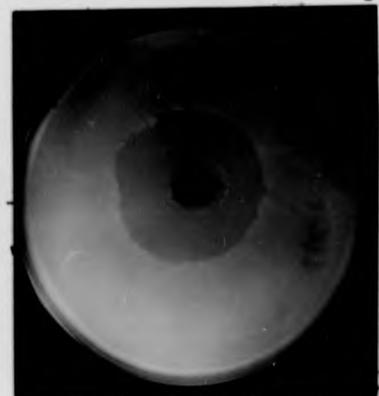
Pseudomonas sp E41 Te



Pseudomonas sp E4 Hg



Pseudomonas sp E41 Se



Pseudomonas sp E41 Hg

TABLE 6.3 Resistance to heavy metals by plasmid-containing and plasmid-free strains of *Pseudomonas* and *Alcaligenes* species (nd = not determined)

Strain	Plasmid	Metal-resistance, Growth-inhibition zones (mm)											
		Hg 1mM	Hg 100mM	Se 100mM	Te 1mM	Zn 100mM	As 100mM	Cd 100mM	Co 100mM	Cu 100mM	Ni 100mM	Pb 100mM	
<i>Pseudomonas</i> sp strain E2	pUU 202	20	>30	12	0	6	0	12	15	18	15	1	
<i>Pseudomonas</i> sp strain E235	none	20	nd	15	13	nd	nd	10	nd	nd	nd	nd	
<i>Pseudomonas</i> sp strain E4	pUU 204	4	19	0	3	4	0	10	16	11	16	2	
<i>Pseudomonas</i> sp strain E41	none	15	27	10	12	4	0	nd	17	13	17	3	
<i>Pseudomonas</i> sp strain E47	pUU 247	16	27	14	12	4	0	7	16	13	17	3	
<i>Pseudomonas</i> sp strain E3	pUU 206	3	20	0	2	4	0	12	15	13	16	3	
<i>Pseudomonas</i> sp strain E6	pUU 206	3	20	0	0	4	0	11	15	13	7	3	
<i>Alcaligenes</i> sp strain E20	pUU 220	24	> 30	15	3	7	nd	13	18	18	18	nd	
<i>Alcaligenes</i> sp strain E22	pUU 222	20	30	11	0	9	0	11	18	17	17	3	

strain E41, plasmid free, and E47 containing pUU 247. However, the presence or absence of pUU 204 did not affect the response towards the other seven metals tested.

Comparison of the *Pseudomonas* sp. strains E2 and E235 suggested that pUU 202 conferred resistance to tellurium only, with the response to mercury, selenium and the other seven metals unaffected by the presence or absence of the plasmid. This provided further evidence for the existence of differences between pUU 202 and pUU 204.

Although plasmid-minus strains of the other three strains were not tested by analogy to the results obtained from pUU 202 and pUU 204, it could be seen that pUU 206 conferred resistance to mercury, selenium and tellurium. Indeed the pattern of inhibition of growth was identical to that observed for pUU 204. The resistances exhibited by strains E3 and E6 being identical provided further evidence for the belief that both strains carry the pUU 206 plasmid.

The results for the pUU 220 and pU 222 containing organisms suggest a similar conference of tellurium resistance only as seen with the pUU 202 plasmid. Such similarities may have been apparent in the *Hind* III digest patterns (Figure 6.10).

Whilst using this method to study resistance to heavy metals, the procedure was also used to see if resistance to any antibiotics was encoded for on pUU 204. Multodisk sensitivity tests had previously indicated that all the isolates were resistant to a large number of antibiotics. For instance *Pseudomonas* sp. strain E4 demonstrated resistance to cephaloridine, sulphurazole, ampicillin, carbenicillin, sulphamethexate and trimethoprim, chloramphenicol, nitrofurantoin,

nalidixic acid, erythromycin, cloxacillin and penicillin.

However, when the plasmid bearing and plasmid-free strains were plated as for the metal resistance experiments and antibiotics placed in the wells no differences were observed in the sensitivity or resistance to any of the eight antibiotics tested, suggesting the plasmid did not carry the genetic information for antibiotic resistances.

6.8 CONJUGAL TRANSFER OF THE PLASMIDS FROM THE *PSEUDOMONAS* SPP

To provide additional evidence that the genes for dehalogenase activity and heavy metal resistances were plasmid encoded, these transfer experiments were undertaken. Despite repeated attempts the appropriate transconjugants, with phenotypes consistent with the transfer of the dehalogenase plasmid were not isolated.

Although transfer experiments were attempted with all the strains most of the work was attempted using *Pseudomonas* sp. strain E4 as the donor. Two approaches were used to transfer the plasmid into a recipient organism.

The first was to attempt to transfer the pUU 204 plasmid from *Pseudomonas* sp. strain E4 into an unrelated host. A series of recipients were used (see Section 2.7.2.1., Table 2.7), with different antibiotic resistance markers, in order to distinguish transconjugants by the ability to utilize 2MCPA as the carbon source and to have resistance to the two selected antibiotics. Those experiments using rifampicin as the selective pressure against the donor failed because resistant mutants of the donor were selected for in large numbers, such that the resulting colonies from the strain E4 x *P. aeruginosa* 8602/6 were rifampicin resistant yet failed to produce the green colouration on Kings medium.

One possible explanation for the failure to obtain transconjugants from the attempted matings was that restriction mechanisms in *P. aeruginosa* strains 8602/6 and PA03R and *Pseudomonas* sp. strain JRB1 prevented the transfer of the plasmid. In order to overcome this problem two restriction-minus mutants were used, *P. putida* KT2440 and *P. aeruginosa* PA01162. However, no transconjugants were obtained.

The second approach represented an attempt to overcome the restriction problem. This was to use the cured strain of *Pseudomonas* sp. strain E4, strain E41 as the recipient organism. Doubly resistant mutants of the recipient were selected and the mating experiments attempted. Again these experiments failed to yield any transconjugants.

6.9 THE USE OF PLASMID R68-45 TO CO-TRANSFER THE DEHALOGENASE PLASMID

Knowing that R68-45 was readily transferable and was capable of co-transferring genetic information, an experiment was conducted to determine whether or not it could co-transfer the dehalogenase plasmid from *Pseudomonas* sp. strain E4.

In the initial transfer, to introduce R68-45 from *P. aeruginosa* into *Pseudomonas* sp. strain E4 a single transconjugant was isolated which possessed activity towards 2MCPA, utilizing it as a carbon source, and was resistant to tetracycline, kanomycin and ampicillin, which were the drug resistances conferred by the presence of R68-45. Growth on Kings A medium failed to produce the green colouration characteristic of *P. aeruginosa* strains. The organism was also capable of prototrophic growth, whereas *P. aeruginosa* PA08 was an isoleucine auxotroph. This suggested that the transconjugant was a derivative of *Pseudomonas* sp. strain E4 and was accordingly designated *Pseudomonas* sp. strain E4R.

It grew in liquid batch culture on 2MCPA in the presence of the three antibiotics and gel electrophoresis of crude extracts demonstrated the presence of dehalogenase I, an observation consistent with the previous dehalogenase studies (Section 4.4).

Agarose gel electrophoresis of plasmid preparations by the rapid screening method (Section 2.6.2) identified a single plasmid in *Pseudomonas* sp. strain E4R with a migration distance very similar to R68-45. Plasmid pUU 204 was not detected.

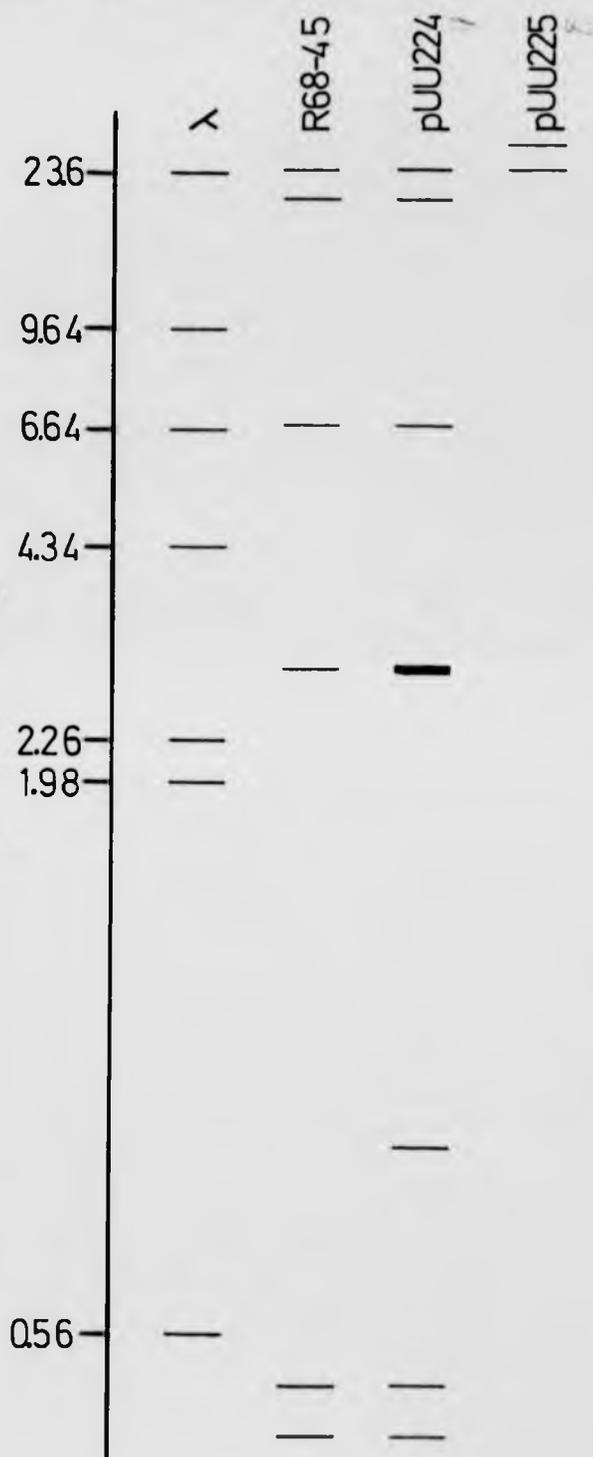
Succinate cultures of *Pseudomonas* sp. strain E4R containing a range of ethidium bromide concentrations (10 to 100 $\mu\text{g ml}^{-1}$) were incubated overnight then serially plated onto succinate and 2MCPA recovery plates. Although there was no significant difference in the colony counts on either type of plate (contrary to the *Pseudomonas* sp. strain E4 experiment - Section 6.6) when 50 colonies from the succinate recovery plates were transferred onto succinate and 2MCPA plates only three of the colonies retained the capacity to grow on 2MCPA. Thus the dehalogenating mechanism appeared to be associated with the presence of the R68-45 plasmid or its derivative.

When screening the *Pseudomonas* sp. strain E4R ethidium bromide treated isolates, one which had lost dehalogenase activity was shown to still possess a plasmid of similar size to the parental organism. This strain was designated *Pseudomonas* sp. strain E4R13.

The plasmids in the *Pseudomonas* sp. strains E4R and E4R13 were designated pUU 224 and pUU 225 respectively. Plasmid preparations from these strains were digested with *Pst* I restriction endonuclease and electrophoresed alongside a similar endonuclease digest of R68-45

Figure 6.14: A comparison of the Pst I restriction endonuclease digest patterns of the plasmid R68-45 from Pseudomonas aeruginosa PA08, the plasmid pUU 224 from Pseudomonas sp strain E4R and the plasmid pUU 225 from Pseudomonas strain E4R13, together with an Hind III restriction endonuclease digest of λ DNA as the standard. The thick band represents fragments with identical or very similar molecular weights.

Fragment size (kilobases)



lease

PseudomonasPseudomonas spPseudomonas strain

endonuclease

and represents

molecular weights.

from *P. aeruginosa* PA08. The resulting digest patterns are described in Figure 6.14.

The results indicated that the plasmid from *Pseudomonas* sp. strain E4R contained two more fragments than the R68-45 fragment pattern digest. This additional material represented an increased molecular weight of 3.71 Kb (2.41 Md) over that of R68-45 (54.17 Kb) giving a total of 57.88 Kb (37.62 Md).

However the pUU 225 plasmid, although not coding for dehalogenase activity, did not revert to the R68-45 digest pattern, but possessed only 2 restriction sites so producing 2 fragments which when totalled represented a molecular weight of 50.98 Kb (33.14 Md). This was 4.48 Md less than pUU 224 and 2.07 Md less than R68-45.

Transfer of pUU 224 into a recipient strain *P. aeruginosa* PA01162 was achieved readily conferring the ability to utilize 2MCPA on the recipient. In the mating experiment the donor (*Pseudomonas* sp. strain E4R) was present at a concentration of 1.7×10^7 organisms ml^{-1} and the recipient at 2.5×10^{10} organisms ml^{-1} . After incubation on the selection medium colony counts of 2.2×10^4 ml^{-1} were seen, indicating a transfer frequency approaching 1×10^{-3} producing transconjugants which grew on 2MCPA in the presence of leucine and developed a green pigment when cultured on Kings A medium.

6.10 DISCUSSION

The results represented in Chapter 6 show that *Pseudomonas* sp strains E2, E3, E4 and E6 and the *Aloaligenes* sp. strains E20 and E22 each possess a large plasmid which, for *Pseudomonas* sp. strains E2, E3 and E4, when lost results in the loss of ability to utilize 2MCPA.

This suggests, but does not conclusively prove, that the plasmids encoded for the four dehalogenases previously described.

The dehalogenase plasmids ranged in size from 109 to 190 Md, a size which is characteristic of several other types of degradative plasmids which exceed a molecular weight of 100 Md (Gunsalus *et al.*, 1975; Chakrabarty, 1976; Duggleby *et al.*, 1977; Hansen & Olsen, 1978; Sakaguchi & Okanishi, 1980).

All of the plasmids have been shown to code for more than one dehalogenase, so providing an example of two or more isoenzyme forms encoded together on a single plasmid. The presence of a second isoenzyme of α -galactosidase in *Escherichia coli* K-12 which was specified by a transmissible plasmid, while the other isoenzymic form was demonstrated by Schmid and Schmitt (1976) to be chromosomally encoded. This was interesting because this isolate with a plasmid-encoded second isoenzyme represented the only example in bacteria of an organism producing isoenzymes of α -galactosidase. Such combined chromosomal and plasmid encoded control of enzymic function was also observed in *Pseudomonas putida* for the degradation of phenol (Wong & Dunn, 1976) and in *Pseudomonas alcaligenes* a number of isofunctional enzymes used in cresol and 2,5 xylenol degradation were seen to be coded for by both chromosomal and plasmid genes (Poh & Bayly, 1980).

However, it would appear from the results presented that all the dehalogenase isoenzymes are encoded for on the plasmids. This would seem to be so because after the plasmid was cured from *Pseudomonas* sp. strain E4 no dehalogenase activity was observed. The ability to produce all three dehalogenases was lost.

These observations are consistent with the hypothesis that in order to improve the overall dehalogenase specific activity, the dehalogenase genes were aggregated together to improve the populations growth rate. Their location on a plasmid is interesting since it seems probable that the frequency of aggregation of the different genes would be greater in a plasmid mediated process, compared to similar events which only involved the chromosome.

Presumably, the various dehalogenase genes were once located in different organisms, or else direct plating of soil samples onto solidified-defined media containing halogenated substrates would have resulted in the immediate isolation of dependable utilizers (Section 3.2). If this were so, then for the aggregation of these genes onto a given plasmid, it would seem necessary for the plasmid to be transmissible, capable of moving between different genera, as was suggested by the isolation of *Pseudomonas* and *Alcaligenes* spp. and possibly capable of mobilizing sections of the bacterial chromosome, such as seen with the plasmid R68-45 (Rieß *et al.*, 1980; Willetts *et al.*, 1981). The work with the plasmid R68-45 demonstrated that the gene coding for dehalogenase I, at least, could be mobilized and transferred to other strains of *Pseudomonas*. However, it cannot be determined whether the gene was mobilized from the plasmid pUU 204 or from the *Pseudomonas* sp. strain E4 chromosome.

However, the unsuccessful attempts to conjugal transfer of the present plasmids suggests that they are non-transmissible. By analogy with most degradative plasmids so far described, which are self-transmissible, it seems unlikely that the dehalogenase plasmids are truly

non-transmissible. The plasmids were certainly large enough to code for transfer functions.

Although most of the degradative plasmids do encode for their own transfer mechanisms it has been shown that on transfer to different hosts these plasmids can dissociate into a number of smaller plasmids. The TOL plasmid when transferred from *Pseudomonas putida*, *Pseudomonas aeruginosa* PAO dissociates into two plasmids (Chakrabarty *et al.*, 1978). The plasmid dissociates into a non-infectious toluene degrading plasmid and a transfer plasmid.

It is possible that the observed small plasmid, pUU 247, was involved in transfer function, however, no transfer experiments were attempted with this plasmid. Formation of such a recombinant was postulated by Kameda *et al.* (1969) to explain the transfer of a non-transmissible R-factor involving recombination with a transfer factor (T). If such an involvement were to be the case, one would have expected to have seen this plasmid in the original *Pseudomonas* sp. strain E4 before treatment with ethidium bromide. Observations made from the primary gels used to determine which sucrose gradients were to be used (Section 2.10.2) showed two plasmids to be present in pUU 204, pUU 220 and pUU 222 preparations; although none of the screening gels showed the presence of this second plasmid.

In all three instances the agarose gels separated a large plasmid, assumed to be the major plasmid, the dehalogenase plasmid, and a smaller one which possessed far greater mobility than the chromosomal DNA debris to a position comparable to that of pUU 247.

If the aggregation of dehalogenase genes on a plasmid was the

mechanism involved in the selection procedure then the observed differences in the plasmids isolated and the differences in translation of the dehalogenase genes need to be explained. Two theories could be advanced to explain this: Firstly there were originally four plasmids which exchanged genetic information by recombination (Kopecko & Cohen, 1975) such that the genes for dehalogenase activity were distributed among the plasmids in a way such as to produce the dehalogenase profiles observed. This would be enhanced if the dehalogenase genes represented a readily mobilized transposon. The second possibility is that a single plasmid on which all the dehalogenase genes had aggregated was distributed throughout the bacterial population and that owing to the various restriction mechanisms of the different isolates and the large number of restriction sites known to be present on those plasmids, various segments of the original plasmid were lost. The type of restriction would then determine the final dehalogenase profile.

After transfer to a different host, *Pseudomonas aeruginosa*, the TOL plasmid from *Pseudomonas putida* has been shown to apparently lose the ability to degrade (Chakrabarty *et al.*, 1978), but when transfer in the opposite direction was achieved the plasmid again expressed toluene degrading activity. Possibly all the genes for the dehalogenase isoenzymes are present on all four plasmids, but due to the nature of the plasmid's hosts they are not all translated into functioning isoenzymes. This could explain why the seemingly identical plasmids in *Pseudomonas* sp. strains E3 and E6, pUU 206, apparently code for different dehalogenase profiles.

Kawasaki and his colleagues have described a plasmid, pU01 which

codes for two haloacetate halohydrolyses in a *Moraxella* sp. (Tonomura *et al.*, 1980; Kawasaki, *et al.*, 1981a,b,c) suggesting again that two isoenzymic forms of an enzyme are encoded for on a plasmid. This plasmid was considerably smaller than the ones isolated in this investigation, having a molecular weight of 43.7 Md.

It is interesting to note that spontaneous mutants, deficient in the second dehalogenase were isolated during the cultivation of the wild strain. These mutants were shown to possess a plasmid, pU011, which had a molecular weight of 3.6 Md less than the original plasmid, pU01 (Kawasaki, *et al.*, 1981b). Such a deletion of genetic information from the plasmid, resulting in the loss of one dehalogenase function, may represent an example of the events which took place during the evolution of the dehalogenase plasmids isolated from the *Pseudomonas* and *Alcaligenes* spp.

Kawasaki *et al.* (1981b) tentatively suggested that the 3.6 Md DNA segment was a transposon carrying one of the dehalogenase genes. The plasmid pUU 224 was seen to possess a molecular weight 2.41 Md greater than the R68-45 plasmids. The results suggest that the 2.41 Md segment, possibly from pUU 204, encodes for dehalogenase I activity, a possibility enhanced by the observed smaller plasmid pUU 225 which was isolated after ethidium bromide treatment of *Pseudomonas* sp. strain E4R. The pUU 225 plasmid was 4.48 Md less than pUU 224 and 2.07 Md less than R68-45.

It is possible that this mobilized segment of DNA, 2.41 Md or 4.48 Md, represents a transposon which may have originally been mobilized by R68-45 from pUU 204.

The *Pseudomonas* and *Alcaligenes* spp. plasmids were also shown to code for resistance to heavy metals. pUU 204 and pUU 206 coding for resistance to mercury, selenium and tellurium and pUU 202, pUU 220 and pUU 222 coding for resistance to tellurium. Resistance to all the heavy metals tested in the experiments described has previously been shown, in some cases, to be plasmid mediated (Hedges & Baumberg, 1973; Kondo *et al.*, 1974; Summers & Silver, 1977; Summers & Jacoby, 1977; Chakrabarty, 1978; Tezuka & Tonomura, 1978; Silver *et al.*, 1981).

The association of heavy metal resistance with degradative plasmids has often been seen, indeed Kawasaki *et al.* (1981b) demonstrated mercuric reductase was mediated by the pU01 plasmid in a *Moraxella* sp.

As plasmids are studied in greater detail, so it is becoming apparent that nearly all of the plasmid's genetic information is transcribed, little or none of it remains silent. If this is true then it is difficult to explain what the majority of the genetic information in the plasmids isolated encodes for. Assuming the dehalogenase and metal resistance genes are present on the plasmid there is still a large amount of potential genetic information present. From the plasmid curing experiments the loss of the plasmid prevented growth on 2MCPA but the organism appeared to grow perfectly well on succinate defined medium, suggesting that under the culture-conditions provided the plasmid was not necessary for growth of the organism.

CHAPTER 7

DISCUSSION AND GENERAL REMARKS

This work provides evidence of the existence of four isoenzymic forms of the carbon-halogen-bond-cleaving enzyme dehalogenase. It is believed to be the first time that four isoenzymes of this enzyme have been demonstrated in a single organism. Two dehalogenases have previously been reported in *Pseudomonas* species by Goldman *et al.* (1968) and by Slater *et al.* (1979). It has been suggested (Bull *et al.*, 1976; Weightman & Slater, 1980) that this group of enzymes might be suitable as a system for exploring the evolution of catalytic activities. The initial experiments (Section 3.2) suggested that this may be true. The apparent requirement for a period of mixed growth, which resulted in the isolation of a number of organisms capable of rapid growth on the halogenated substrates, and the inability to isolate reliable utilizers by direct sampling of soils, suggested some form of selection took place in the batch enrichment cultures.

The suitability as a model system was further advanced by the discovery that the various isolates, each containing one or more of four dehalogenases, belonged to different genera, *Pseudomonas* and *Alcaligenes* and possibly *Alteromonas* (Section 3.4). The presence of electrophoretically identical enzymes in different genera indicated that a gene transfer mechanism may have been involved in the enrichment procedure. The detection of plasmids in all of the strains (Chapter 6) provided evidence of a gene transfer mechanism.

It is thus suggested that these organisms provide a model on which to study the evolution of catabolic isoenzymes. However, the results discussed in Section 3.3 provide a further factor for the proposed model.

That is one of the evolution of the regulatory control mechanisms.

The suggestion that the possession of isoenzymes enables organisms to respond over short periods of time to a changing environment has caused a great deal of discussion (Gillespie & Kojima, 1968; Somero & Hochachka, 1971; Powers & Power, 1975). *Rhizobium* nitrogen metabolism apparently responds to changes in the levels of environmental ammonia by expression of one or other of two isoenzymic forms of glutamine synthetase (Darrow & Knotts, 1977).

It is possible that the reason for the possession of more than one dehalogenase per organism, which demonstrate a wide substrate and inducer specificity, is that they enable the organisms to more effectively utilize changing levels of the halogenated compounds in their environment.

The chemostat culturing of a number of the isolates indicated that this may indeed be the case. In batch culture *Pseudomonas* sp. strains E3 and E4 only demonstrated one major dehalogenase, dehalogenase I, however, when placed into a chemostat dehalogenases II and III were also identified. This suggests that although the bacteria were capable of producing the other two isoenzymes, their requirements for carbon and energy were adequately met by the activity of dehalogenase I only. However, under the different, more stressful environmental conditions of continuous culture, the other two isoenzymes were produced.

Although the other isolates, *Pseudomonas* sp. strains E2 and E6, and *Aloaligenes* sp. strain E20 did not produce any dehalogenases which were not seen in batch culture, all but strain E20 demonstrated changes in the levels of activity of each isoenzyme form as the environmental conditions were changed. Also when the conditions were reverted, either to batch culture or

to the previous dilution rate, the dehalogenase profile also reverted to its original form.

It is possible that evolutionary changes in this model system have already been observed. *Pseudomonas* sp. strain E6, originally placed into group E because of its novel dehalogenase activities and profile, after a number of months apparently lost the ability to produce two of the four dehalogenases it originally possessed (Sections 4.4 and 5.4). In *Pseudomonas* sp. strains E3 and E4 and originally in strain E6, dehalogenase I was apparently the major isoenzyme, demonstrating greater activity towards all the four substrates, especially the chloroacetates, than the other isoenzyme forms. However, this isoenzyme was apparently lost by strain E6. Even under chemostat culture at a high dilution rate, during which strains E3 and E4 appeared to increase the levels of all their isoenzymes, strain E6 failed to produce the isoenzymes which it had earlier demonstrated to be its' most efficient.

This would suggest a mutation or deletion had occurred either in the dehalogenase gene itself or in the regulatory control mechanisms.

The apparently involved control mechanisms which govern the expression of dehalogenase activity represents an example of the complex mechanisms which control the catabolic enzymes in soil bacteria and as thus could provide further information of these mechanisms.

The importance of continuous-flow culture in examining physiological and biochemical parameters of bacteria is demonstrated by these results, and high-lights the care which must be exercised when studying these organisms solely on the basis of growth under closed culture conditions. Results obtained from the batch culture of these soil isolates (Chapter 4)

failed to indicate the presences of the regulatory control mechanisms. Although with hind-sight it could be said that the apparent specificity of dehalogenase II for MCA in cell-free extracts from batch cultured *Pseudomonas* sp. strain E4 and the variations in the relative and specific activities of the cell-free extracts suggested that such mechanisms existed.

Plasmid-mediated transfer of the genes specifying catabolic enzymes has been shown to occur freely within the *Pseudomonas* genus and between this and other genera (Benson & Shapiro, 1978; Don & Pemberton, 1981). Indeed the work of Don and Pemberton (1981) is particularly relevant to this discussion as the genes encoded for on the plasmid isolated in that work were involved in the catabolism of the halogen substituted compounds 2,4-dichloro- and 2,4,5-trichlorophenoxyacetic acids.

As such transfer is known to occur and on the basis of the results obtained during the course of this work the following hypothetical model for the evolution of dehalogenase activity in the organisms identified is suggested. However, it should be emphasised that a crucial factor in this hypothetical evolutionary process is the ability of plasmids to transfer readily between hosts. To date the transfer of the pUU plasmids has not been demonstrated after numerous attempts. Most of the large catabolic plasmids so far identified possess self-transfer capabilities, thus it would seem likely that these plasmids, in the size range 109 to 190 Md, do possess this ability which will hopefully soon be demonstrated.

Also, the original soil organisms were shown to possess ineffective dehalogenase mechanisms, as they failed to grow well on these substrates. For the initial parts of the selection process to start there must first

have been changes, mutational events effecting structural or regulatory genes or possibly even permease systems in order that the genes which coded for the observed dehalogenases were indeed present in the microbial population.

In the original soil inoculum, placed into the batch enrichment vessel, there were bacteria which possessed dehalogenase genes which were either chromosomally or plasmid encoded. Numerous soil bacteria have been seen to possess plasmids thus it is likely that a number of different plasmids were present in the bacterial population. It is suggested that gradually the plasmids or possibly a single type of plasmid collected together the four dehalogenase genes, either by recombinational events between it and the chromosome of a number of organisms or between it and other plasmids, or by aggregation of a number of smaller plasmids containing the dehalogenase genes forming a plasmid aggregate. This, necessarily promiscuous plasmid was then replicated and transferred to a number of different bacterial strains, so providing the necessary genes for dehalogenase production.

To explain the different dehalogenase profiles three possible theories could be advanced: firstly, that once the single plasmid was present in the given strain it was attacked by the restriction mechanisms of the organism which resulted in deletion of fragments of DNA from the plasmid, some of which encoded for one or two of the dehalogenase isoenzymes. This would also explain the different restriction analysis results obtained for each organism (Section 6.5); secondly, a mechanism similar to that observed for the TOL plasmid in a novel host (Chakrabarty *et al.*, 1978) caused the incomplete expression of the potential dehalogenase activities encoded for on the plasmid. As such this could not explain the different endonuclease digestion patterns; or thirdly a different number of the original

dehalogenase genes were incorporated into five different plasmids present in the six isolates examined.

Continued changes in the regulatory mechanisms either associated with the plasmid or the host could eventually result in the observed dehalogenase profiles, and further mutations could lead to additional variations, as seen in *Pseudomonas* sp. strain E6.

Such degradative plasmids may have evolved as a rapid means of spreading genes encoding detoxification systems within a microbial population. Thus, the observed dehalogenase systems may represent a response of the microbial community to a challenge by toxic compounds, in this case the chlorinated alkanolic acids.

In order to investigate this hypothetical evolutionary pathway, a number of experiments could be undertaken.

The initial enrichment interactions could be further studied by using a mixed population of those organisms classed as the 'S' isolates in this work. That is, those organisms isolated directly from soil samples could be placed into liquid culture in the presence of one of the halogenated substrates and enriched as previously described. If the ability to effectively utilize these substrates originated from these organisms then after enrichment reliable utilizers should be observed. It may also result in the isolation of organisms with some of the other possible permutations of the isoenzymes not isolated in this study.

Obviously the result of major importance would be the demonstration of plasmid transfer and associated conference of dehalogenase activity on the recipient. In all of the conjugal mating experiments undertaken so far the ability to dehalogenate 2MCPA has represented the selection against the

recipient strain. It may be possible to use one of the heavy metal resistances to mediate this selection.

After such transfer had been achieved, either by conjugation or transformation, it would be of considerable interest to see if the dehalogenase profile exhibited by the transconjugant was the same as that of the donor. Also, whether the plasmid demonstrated the same results after endonuclease restriction analysis.

Further study is required into the origin of the plasmid found in *Pseudomonas* sp. strain E47, pUU 247, and of the other small plasmids identified on the primary screening gels (Section 6.10), as it is possible that these represent smaller plasmids which exist as part of the larger plasmid.

The R68-45 derivative, pUU 224, isolated from *Pseudomonas* sp. strain E4R showed the ability to produce dehalogenase I, and confer dehalogenating ability on a recipient organism, *Pseudomonas aeruginosa* PA01162.

This plasmid possessed a DNA segment of 2.41 Md which was not present in R68-45 and a derivative of pUU 224, pUU 225 which was present in *Pseudomonas* sp. strain E4R13, an organism lacking dehalogenase activity, was 4.48 Md smaller than pUU 224. It was tentatively suggested that this DNA segment represented a dehalogenase I transposon. If this were the case then *Pseudomonas* sp. strain E4R which demonstrated dehalogenase I activity in batch culture, would not be capable of producing the other dehalogenases, dehalogenase II and III, exhibited by the parental strain *Pseudomonas* sp. strain E4 when cultured in a chemostat. Such an experiment could help to understand the dehalogenase activity conferred on the host by pUU 224.

Further study of the properties of the dehalogenase isoenzymes is required. Purification of the isoenzymes by column chromatography would enable detailed analysis of the substrate specificities, physical properties and inhibition characteristics to be determined. This would enable a more detailed comparison of these enzymes with those isolated in other studies.

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