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Microbial dehalogenation

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1. Introduction

This dedicated volume of 'Biodegradation' contains nine papers dealing with aspects of microbial dehalogenation. It was conceived to mark the importance of our need to understand the microbial physiology, biochemistry, genetics and molecular biology of dehalogenating systems. We hope that from time to time similar volumes devoted to this subject will be justified on the grounds of new scientific discoveries and their significance to the issue of halogenated compounds in the biosphere. Furthermore we suggest that it may be an appropriate time to consider a small international meeting devoted to microbial dehalogenation. We would welcome further contributions, suggestions or opinions which might meet these objectives.

Physiologists and biochemists have known since the beginning of the twentieth century that the presence of halogenated compounds significantly affects metabolic processes. Halogenated analogues of intermediary metabolites are toxic because they inhibit key reactions. For example, fluoroacetate is a potent inhibitor of the tricarboxylic acid cycle because the lethal synthesis of fluorocitrate inhibits the TCA cycle enzyme aconitase (Peters 1952). In some cases the apparent recalcitrance of halogenated compounds is attributed to their general toxic properties, by whatever specific mechanism, which prevents the growth of microorganisms even though they may have the necessary mechanisms to degrade the compound.

Before the First World War, WJ Penfold discovered that a variety of chlorinated and brominated compounds, such as monochloroacetate, α-bromopropionic acid and monochlorohydrin, strongly inhibited the growth and sugar fermentation of *Bacterium coli* (Escherich) and *Bacterium lactis aerogenes*. Moreover, growth in the presence of these compounds led to the production of mutants which were resistant to the toxic effects of halogenated compounds (Penfold 1913).

The removal of halogens, particularly fluorine and chlorine, from organic molecules has fascinated chemists and microbiologists for many years since such mechanisms relieve inhibitory effects and provide alternative carbon and energy sources for growth. Den Dooren de Jong (1926) found that unlikely (then) compounds such as bromopropionate and bromosuccinate were catabolised by some bacteria and were used as sole carbon and energy sources for growth. We now know that many halogenated compounds are completely mineralised (Alexander 1981; Knackmuss 1981; Reineke & Knackmuss 1988; Hardman 1991; Chaudhry & Chapalamadugu 1991; Commandeur & Parsons 1994; Janssen et al. 1994; Slater 1994). Today the search continues for novel mechanisms for the catabolism of an ever-expanding range of halogenated compounds.

We also know that many halogenated compounds are, or appear to be, resistant to microbial attack. In general the greater the number of halogens per organic molecule, the more difficult it is to show degradation and find microbes capable of dehalogenating the compound either alone or in consort as part of a microbial community (consortium) (Commandeur & Parsons 1994). This is of considerable importance since many man-made (xenobiotic) compounds which have found their way into the biosphere in the last 50 years tend to be multihalogenated. Indeed, their utility as pesticides, dielectrics, flame retardants, preservatives or whatever, is often related to their chemical and biochemical inertness. We are now only too well aware (or should be) that this property is exactly what is not required in environmental terms, since an inability to degrade leads to the accumulation and persis-

tence of compounds which are frequently highly toxic. Moreover, if degradation does occur but the rate of degradation is too slow, then damage is done in the environment before the compound is degraded. It is of major practical importance to define the limits of microbial abilities to degrade unusual xenobiotic halogenated compounds: if, indeed, there are any ultimate limits.

2. The general range of microbial capabilities

Historically most research has focused on the selection and isolation of microbes or microbial communities which grow on halogenated compounds under aerobic conditions. A large number of commonly encountered microbes, such as *Pseudomonas, Alcaligenes, Arthrobacter, Rhodococcus, Hyphomicrobium* and many more genera, have the metabolic ability to dehalogenate a wide range of halogenated compounds (Slater 1994).

We have no doubt that microbes with more exacting growth requirements are not finding their way into laboratories for analysis because of the restricted range of selective conditions employed during isolation procedures. In particular this gives a biased perspective because of the difficulties of successfully culturing anaerobes, although it is encouraging to see that much more attention is now being paid to anaerobic systems. Haloalkanes are readily transformed under anaerobic conditions (Bouwer et al. 1981; Vogel & McCarty 1985; Fogel et al. 1986). Demonstrating the anaerobic degradation of aromatic compounds has also been problematic, but there is now widespread acceptance that many different compounds can be dehalogenated under anaerobic, reductive conditions (Horowitz et al. 1983; Quensen et al. 1988; Gibson & Sulflita 1990; Häggblom & Young 1990; Fathepure & Vogel 1991; Morris et al. 1992). To date, however, only one obligate anaerobe has been isolated capable of the reductive dehalogenation of halogenated aryl compounds, namely *Desulfomonile tiedjei* (DeWeerd et al. 1990). This no doubt reflects the difficulties of growing pure cultures under laboratory conditions when their natural mode of growth is as members of tightly coupled consortia.

A range of different mechanisms have evolved to catabolise halogenated compounds. The following discussion characterises those systems which we have most confidence about, but there are clearly many novel systems awaiting full descriptions. For example, the mechanisms for dehalogenating unsaturated haloacids, such as 3-chloroacrylic acid (van Hylckama Vlieg & Janssen 1991), various alkanoic acids substituted in positions other than carbon 3 (Hughes 1988; Kohler-Staub & Kohler 1989), and compounds such as trichloroacetic acid (Weightman et al. 1992) require complete elucidation. A dehydrodehalogenation mechanism is important for the growth of *Pseudomonas paucimobilis* strain UT26 on γ-hexachlorocyclohexane (Imai et al. 1991; Nagata et al. 1993). We have not included here for detailed consideration the oxygenase-type haloalkane dehalogenases (Higgins et al. 1979; Armfield et al. 1995).

3. Dehalogenating enzymes

Historically the first enzymes involved in dehalogenation were termed dehalogenases (Jensen 1951, 1957, 1959, 1960), and this name came to be used for enzymes involved in hydrolytic reactions for the dehalogenation of 2-haloalkanoic acids. However, this term is limited because a disparate range of substrates can be dehalogenated and different mechanisms are involved in the removal of a halogen. Initially specificity in naming dehalogenases came by either adjectival reference to different substrates (for example, aliphatic dehalogenase, haloalkane dehalogenase) or early mechanistic classifications (for example, halidohydrolase). Various attempts have been made to group dehalogenase on the basis of substrate affinities, reaction kinetics, detailed molecular mechanisms, sensitivities to inhibitory compounds and so on (Little & Williams 1971; Hardman 1991; Slater 1994).

In our opinion, the naming of different dehalogenases associated particularly with haloaliphatic compounds is confused, and perhaps the time has come to agree a uniform system for the common names for dehalogenases. This should reflect, where possible, the historical precedents which may be important, and be based on the recognition of the importance of different dehalogenation mechanisms. We suggest that naming dehalogenases after different substrates should be less important than recognising a specific mechanism. Furthermore, protein and DNA sequence data are emerging and patterns of significant similarities and differences are being recognised between various dehalogenases. In due course this will lead to complete descriptions of the genetic and evolutionary relationships and a classification of the different enzyme mechanisms which are undoubtedly involved in dehalogenation by

reference to significant tertiary structures (Leisinger & Bader 1993; Slater 1994; Janssen et al. 1994). At this stage, however, we would caution against placing too much emphasis on either sequence similarities since they might falsely indicate common dehalogenating mechanisms, or sequence differences since they may fail to reveal a common mechanism. In our view, therefore, the naming and grouping of different dehalogenases should primarily be on mechanisms, with sub-groups based on other factors, such as substrate specificities, and DNA or amino acid sequence information.

Accordingly, in the following sections we have used the names which we suggest should be adopted as the standard common names, and groupings which best describes the different dehalogenating mechanisms to date. We have not attempted to indicate synonyms or give other names used since we believe that from the context and the references given, the enzymes discussed are obvious and we seek to avoid confusion by giving alternative names.

4. Removing halogens from aliphatic compounds

4.1 Hydrolytic dehalogenases

The most common dehalogenation mechanism appears to be a hydrolysis reaction. At the moment, two sub-groups reflecting different substrate specificities seem appropriate (sections 4.1.1. and 4.1.2). However, sequence data suggest substantial diversity within these sub-groups which may be associated with fundamental mechanistic differences. Resolution of the groupings must await a full correlation of mechanism details with key sequence information, such as the amino acids involved within active sites.

4.1.1 2-Haloalkanoic acid hydrolytic dehalogenases

On the basis of a number of factors, four classes of 2HAA hydrolytic dehalogenases are indicated (Table 1).

Class 1L. A significant group of nine 2HAA hydrolytic dehalogenases have been sequenced to varying degrees of completion and found to have substantial sequence similarities (Table 1). Class 1L dehalogenases remove halides from L-2-haloalkanoic acids, inverting the product configuration with respect to the substrate. Class 1L probably should include the halidohydrolases I and II of Goldman et al. (1968) and the dehalogenase described by Little & Williams (1971), but this is without the modern benefit of sequence data. How representative class 1L is of the general spectrum of dehalogenases and their importance and abundance in nature, remains to be seen, but it does seem to represent a core group of dehalogenases.

Class 1D. Two D-specific hydrolytic dehalogenases have been characterised from *P. putida* and a *Rhizobium* species (Table 1) and sequence data presently suggest no relationship with other dehalogenases. D-specific enzymes are apparently isolated infrequently suggesting that Class 1D dehalogenases are certainly less common in nature than the Group 1L enzymes.

Class 2R and 2I. Class 2 enzymes differ from Class 1 dehalogenases by an ability to dehalogenate both D- and L-isomers of 2-haloalkanoic acids (Table 1) with either retention (Class 2R) or inversion (Class 2I) of product configuration compared with the original substrate. No sequence data are available for the three enzymes which exhibit the Class 2I reaction and, so for the time being, we suggest that Class 2I enzymes are treated as a separate group (on the basis of substrate specificity) unless or until sequence data suggest other relationships.

The DNA and amino acid sequence data for dehalogenase I (DehI) from *Pseudomonas putida* strain PP3 shows that it is unrelated to any known dehalogenase (Topping 1992) and so Class 2R seems to represent another core dehalogenase type.

4.1.2 Haloalkane hydrolytic dehalogenases

A number of aerobic microbes are well characterised all showing hydrolytic mechanisms for halide removal, such as *Xanthobacter autotrophicus* strain GJ10 (Janssen et al. 1985; Keuning et al. 1985; Janssen et al. 1989), various *Corynebacterium* species (Yokota et al. 1986), *Arthrobacter* species (Scholtz et al. 1988a), *Rhodococcus* (Sallis et al. 1990) and *Ancylobacter aquaticus* strains (van den Wijngaard et al. 1992). On the basis of substrate specificity the enzymes from these organisms can be divided into two classes.

Class 3R. This class of HA hydrolytic dehalogenases, from Gram-negative bacteria, shows a fairly

Table 1. Classification of three groups of dehalogenases involved in the dehalogenation of haloaliphatic compounds.

Group	Sub-group	Class	Characteristic	Example	Reference
hydrolytic dehalogenase	2-haloalkanoic acid hydrolytic dehalogenase	1D	D-isomer specific-Inverts substrate-product configuration	HadD-*P. putida* strain AJ1	Barth et al. (1992)
				HadD-*Rhizobium*	Smith et al. (1990)
					Cairns (1994)
		1L	L-isomer specific-Inverts substrate-product configuration	HadL-*P. putida* strain AJ1	Jones et al. (1992)
				DehCI-*Pseudomonas* sp. strain CBS3	Schneider et al. (1991)
				DehCII-*Pseudomonas* sp. strain CBS3	Schneider et al. (1991)
				DhlB-*Xanthobacter autotrophicus* strain GJ10	van der Ploeg et al. (1991)
				Deh-109-*P. putida* strain 109	Kawasaki et al. (1994)
				HdlIVa-*P. cepacia* strain MBA4	Murdiyatmo et al. (1992)
				DehH-2-*Moraxella* species strain B	Kawasaki et al. (1992)
				HadL-*Rhizobium*	Cairns (1994)
				L-DEX-*Pseudomonas* species strain YL	Nardi-Dei et al. (1994)
		2I	D- and L-isomers as substrates. Inverts substrate-product configuration	DehII-*P. putida* strain PP3	Weightman et al. (1982); Topping (1992)
				Dehalogenase from *Pseudomonas*strain 113	Motosugi et al. (1982a, b)
				Dehalogenase II from *Rhizobium*	Leigh et al. (1988)
		2R	D- and L-isomers as substrates. Retains substrate-product configuration.	DehI-*P. Putida* strain PP3	Weightman et al. (1982); Topping (1992)
				DhlC-*Alcaligenes xylosooxidans*	Brokamp & Schmidt (1991)
				HdlV - isolate K37	Murdiyatmo (1991)
	haloalkane hydrolytic dehalogenase	3R	restricted range of substrates	DhlA-*Xanthobacter autotrophicus* strain GJ10	Janssen et al. (1989)
				Dehalogenases from *Ancylobacter aquaticus* strains AD20 and AD25	van den Wijngaard et al. (1992)
				Dehalogenase from *Xanthobacter autotrophicus* strain GJ11	van den Wijngaard et al. (1992)
				LinB-*P. paucimobilis* strain UT26	Nagata et al. (1992)
				Dehalogenase from *Pseudomonas* species strain E4M	G. Nyandoroh et al. unpublished observations
		3B	broad substrate specificity	Dehalogenase from *Rhodococcus erythropolis* strain Y2	Sallis et al. (1990)
				Dehalogenase from *Rhodococcus erythropolis* strain CP9	G. Nyandoroh et al. unpublished observations

Table 1. Continued.

Group	Sub-group	Class	Characteristic	Example	Reference
				Dehalogenase from *Arthrobacter* species strain HA1	Scholtz et al. (1987)
				Dehalogenase from *Corynebacterium* species strain m15-3	Yokata et al. (1987)
				Dehalogenase from '*Xanthobacter autotrophicus*' strain GJ 70	Janssen et al. (1988)
haloalcohol dehalogenase		4S	broad substrate specificities; simple multimeric proteins	I_a-*Corynebacterium* species strain N-1074	Nakamura et al. (1992)
				Dehalogenase from *Arthrobacter* species strain AD2	van den Wijngaard et al. (1991)
		4C	narrow substrate specificities; complex multimeric proteins	Dehalogenase from *Corynebacterium* species strain N-1074	Nakamura et al. (1994)
				DehA-*Arthrobacter erithii* strain H10a	HMS Assis et al. unpublished observations
co-factor-dependent dehalogenase	glutathione (GSH)-dependent dehalogenase			Dehalogenase from *Hyphomicrobium* species strain DM2	Stucki et al. (1981)
				DcmA-*Methylobacterium* species strain DM4	Kohler-Staub et al. (1986); La Roche & Leisinger (1990)

restricted range of substrate specificities and is best exemplified by the highly characterised enzyme from *Xanthobacter autotrophicus* strain GJ10 (Janssen et al. 1989; Keuning et al. 1985). The tertiary structure of the enzyme has been determined (Franken et al. 1991; Verschueren et al. 1993a) and the mechanism described in detail with the importance of three amino acids residues – Asp124, His289 and Asp260 – in the hydrolysis reaction being recognised (Verschueren et al. 1993b). Sequence data from *Ancylobacter aquaticus* shows that the enzyme from this organism is identical to that from *Xanthobacter autotrophicus* (van den Wijngaard et al. 1992). N-terminal amino acid sequence data sets this class of enzymes apart from Class 3B haloalkane dehalogenases (see below).

The haloalkane dehalogenases from *Pseudomonas paucimobilis* strain UT2 active against γ-hexachlorocyclohexane (Nagata et al. 1993) and a mutant of *Pseudomonas* species strain E4 (Hardman & Slater 1981) capable of growth on 1-chlorobutane as a result of gene decryptification (G Nyandoroh, V Vienravi, S Armfield, J Damborsky, AT Bull & DJ Hardman, unpublished observations) also belong to this class. Janssen et al. (1994) have recently suggested, on the basis of limited sequence similarity, that the dehalogenase Deh-H1 from *Moraxella* species strain B, an enzyme which hydrolyses chloroacetate and may be more closely related to 2HAA hydrolytic dehalogenases (Kawasaki et al. 1992), could come within this class of dehalogenases. However, since this enzyme also shares sequence similarity with 2-hydroxymuconic acid semialdehyde hydrolase (DmpD) of *P. putida*; 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienate hydrolase (BphD) of *Pseudomonas* species strain KKS102; and tropinesterase (Tpes) from *P. putida*, the exact significance of the apparent relationship between these two dehalogenases, certainly in mechanistic terms, has yet to be resolved. Perhaps the short sequences are conserved and shared between different enzymes because the sequence has been successful in evolutionary terms and has been incorporated into many different enzymes which are fundamentally unrelated at the ancestral level. Alternatively these short sequences may have evolved more than one because they have the required

properties within, say, the active site of any enzyme carrying out a hydrolysis reaction.

Class 3B. The second class of haloalkane hydrolytic dehalogenases is represented by enzymes isolated from various rhodococci and closely related Gram-positive bacteria, namely *Rhodococcus erythropolis* strain Y2 (Sallis et al. 1990), *Rhodococcus erythropolis* strain CP9 (G Nyandoroh, AT Bull & DJ Hardman, unpublished observations), *Arthrobacter* species strain HA1 (Scholtz et al. 1987), and *Corynebacterium* species strain m15-3 (Yokata et al. 1987). They demonstrated much broader substrate specificities and all had the same N-terminal amino acid sequences. Strain GJ70 about which there have been doubts about its classification but is now thought to be an Gram-positive actinomycete-type organisms (Janssen et al. 1988), also possesses an enzyme which is closely related to other Gram-positive organisms. It remains to be seen if there is any real significance to the two classes of haloalkane hydrolytic dehalogenases proposed here, reflecting evolution from two different ancestral genes.

4.2 Haloalcohol dehalogenases

Dehalogenation of haloalcohols has been known for some time (Castro & Bartnicki 1965) but the present information is limited. Overall the pathway for dehalogenation of haloalcohols is a hydrolysis but in the examples to date the actual dehalogenation reactions involves the internal rearrangement of the molecule with the simultaneous elimination of a proton with the halide and the formation of an epoxide as a consequence. The enzymes which catalyses this reaction were designated halohydrin epoxidases (Castro & Bartnicki 1968), haloalcohol dehalogenases and haloalcohol halogen-halide lyases (van den Wijngaard et al. 1989, 1991) and halohydrin hydrogen-halide lyases (Nagasawa et al. 1992). In the pathway the resulting epoxides are then hydrolysed by an epoxide hydrolase.

Haloalcohol dehalogenases are active towards haloalcohols where the halogen is vicinal to the hydroxyl group, and possibly a keto group as in chloroacetone (van den Wijngaard et al. 1991). Five alcohol dehalogenases have been described to date (Kasai et al. 1990; Nagasawa et al. 1992; van den Wijngaard et al. 1991), and in our laboratory we have identified six electrophorectically distinct haloalcohol dehalogenases (Bull et al. 1992; M Huxley, DJ Scherr, AT Bull, JH Slater & DJ Hardman, unpublished observations). Much more work is required on the biochemistry and molecular biology of the haloalcohol dehalogenases in order to define the diversity of the enzymes and the mechanistic details. It appears to date that there are at least two classes of alcohol dehalogenase.

Class 4S. This class of enzymes show broad substrate specificities and are structurally simpler than the Class 4C enzymes (see below) being comprised of simple multimeric proteins formed from a single type of polypeptide subunit. This class is exemplified by the aloalcohol dehalogenase I_a from *Corynebacterium* species strain N-1074 (Nakamura et al. 1992) and *Arthrobacter* species strain AD2 (van den Wijngaard et al. 1991).

Class 4C. These enzymes are more complex multimeric proteins being composed of 4–6 subunits with at least two different polypeptide subunits, and having much narrower substrate specificities than the Class 4S enzymes. A second haloalcohol dehalogenase from *Corynebacterium* species strain N-1074 described by Nakamura et al. (1994) and one isolated from *Arthrobacter erithii* strain H10a (HMS Assis, PJ Sallis, AT Bull and DJ Hardman, unpublished observations) are examples of this enzyme type.

4.3 Co-factor-dependent dehalogenases

Stucki et al. (1981) isolated a *Hyphomicrobium* species able to dechlorinate dichloromethane (DCM) and it was found that hydrolysis of DCM was glutathione dependent. Chloride removal was catalysed by a GSH-dependent S-transferase. Subsequently other facultative methylotrophs were found which synthesised similar GSH-dependent dehalogenases (Kohler-Staub et al. 1986). The purified enzyme was an hexameric protein with a narrow substrate specificity (Kohler-Staub & Leisinger 1985). La Roche & Leisinger (1990) sequenced the dehalogenase from *Methylobacterium* species strain DM4 and found that it was closely related to similar transferases found in higher organisms. Scholtz et al. (1988b) isolated another DCM-utilising microbe and showed that the dehalogenase's N-terminal amino acid sequence was different to those previously described, indicating that more than one class of GSH-dependent dehalogenase may exist.

5. Removing halogens from aromatic compounds

5.1 Oxidative dehalogenations

Most frequently the mechanism of arylhalides dehalogenation involves removal of aromaticity and labilisation of the halogenated molecule by the introduction of oxygen. Halide removal is spontaneous after aromatic ring cleavage, and often it appears to be a fortuitous event associated with normal aromatic catabolism. These are referred to as oxidative dehalogenations because dehalogenation is associated with oxygenase activity (Knackmuss 1981; Harayama et al. 1992; Commandeur & Parsons 1994).

5.2 Hydrolytic dehalogenations

However, specific haloaromatic hydrolytic dehalogenations have been discovered in which a halogen is replaced by a hydroxyl from water, although these seem to be rare mechanisms by comparison with the oxidative dehalogenations (section 5.1.). Overall it has been shown that the initial degradation of 4-chlorobenzoate occurs by the replacement of the halide by the hydroxyl from water, and this can occur under both aerobic and anaerobic (denitrifying) conditions (Müller et al. 1984, 1988). The enzyme system in *Pseudomonas* species strain CBS3 has been characterised in great detail, and shown to be comprised of three different enzymes. Initially a 4-chlorobenzoate-CoA ligase adenylates the carboxyl group in a reaction requiring ATP, followed by the replacement of AMP with CoA and the formation of a thioester. This intermediate is sufficiently energised to facilitate the nucleophilic attack by a hydroxyl group at the C4 position resulting in the displacement of the chloride ion. Finally, the third enzyme, 4-hydroxybenzoyl-CoA thioesterase, removes the CoA, and the hydroxylated benzoate can then be metabolised by standard aromatic pathways (Elsner et al. 1991; Scholten et al. 1991; Chang et al. 1992; Löffler et al. 1992). A similar system has been described in *Arthrobacter* species strain SU (Schmitz et al. 1992).

5.3. Reductive dehalogenations

Under anaerobic conditions halogenated aromatic compounds can serve as terminal electron acceptors as a result of anaerobic growth on simple organic molecules. As a consequence these anaerobes, such as *Desulfominile tiedje*, cause the elimination of the halide from the aromatic compound (DeWeerd et al. 1990).

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