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# Biodegradation of haloalkanes

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**Abstract**

Halogenated alkanes constitute a significant group among the organic pollutants of environmental concern. Their industrial and agricultural uses are extensive, but until 1978 they were considered to be non-biodegradable. In recent years, microorganisms were described that could degrade, partially or fully, singly or in consortia, many of the compounds tested. The first step in haloalkane degradation appears to be universal: removal of the halogen atom(s). This is mediated by a group of enzymes, generally known as dehalogenases, acting in most cases either as halidohydrolases or oxygenases. Nevertheless, information is still severely lacking regarding the biochemical pathways involved in these processes, as well as their genetic control.

A recently isolated *Pseudomonas* strain, named ES-2, was shown to possess a very wide degradative spectrum, and to contain at least one hydrolytic dehalogenase. The utilization by this organism of water-insoluble haloalkanes, such as 1-bromooctane, appears to consist of three phases: extracellular emulsification by a constitutively excreted surface active agent, periplasmic dehalogenation by an inducible dehalogenase, and intracellular degradation of the residual carbon skeleton.

## Introduction

Of the man-made chemicals currently in use in industry and agriculture, halogenated organic compounds have probably received the widest scientific attention. Used as herbicides, pesticides, refrigerants, fire retardants, solvents, degreasers and as various intermediates in organic synthesis, these compounds are often of environmental importance due to their toxicity and often limited biodegradability. Both of these characteristics are, in fact, often conveyed by the halogen(s) covalently bound to the carbon skeleton.

Of the numerous halogenated organics, the degradation of which has been studied, attention has been largely focused on chlorinated aromatics. Two other partially overlapping sub-groups have been relatively ignored: halogenated aliphatics and brominated compounds; their common component, the brominated aliphatics, have probably drawn minimal attention.

Of the various halogenated aliphatics in widespread use, the simplest configuration is that of the halogenated alkanes. These straight carbon chains offer simplified biochemical analysis to the scientist, and possibly facilitated biochemical solutions to the microorganisms involved in their biodegradation. They are therefore an ideal choice for studying and modeling microbial haloorganic metabolism.

While several excellent reviews have been published in recent years concerning the biodegradation of halogenated organic compounds (Knackmuss 1981; Lal & Saxener 1982; Slater & Bull 1982;

Leisinger 1983; Motosugi & Soda 1983; Ghosal et al. 1985; Alexander 1981, 1985; Reineke & Knackmuss 1988; Morgan & Watkinson 1989; Chaudhry & Chapalamadugu 1991), most of them mention haloalkanes only briefly or not at all. The purpose of the present review is to try and collate the available information concerning the biodegradation of haloalkanes, and amend it by new data from our laboratory concerning mostly bromoalkane metabolism.

## Occurrence and environmental significance of haloalkanes

In natural environments, the occurrence of haloaliphatic compounds may be of both biogenic and anthropogenic origin, with the latter category in most cases greatly outweighing the former. An exception may be the case of chloromethane; sources such as forest fires and decomposition of seaweeds appear to provide its major environmental input (Edwards et al. 1982; Lovelock 1975). Marine algae have also been shown to release other haloalkanes, among them 1-bromopentane (Gschwend et al. 1985).

Other known sources of haloalkanes are all of human origin; these compounds find their way into the environment mostly due to either agricultural uses or to improperly disposed industrial (and to some extent municipal) wastes. A third major anthropogenic source, mainly of trihalomethanes, is water chlorination. A number of organic precursors present in water, many of them humic in nature, are transformed to trihalomethanes in the presence of free chlorine (Pearson 1982). Both chloro- and bromohalomethanes are thus formed; the bromine is believed to originate from the bromide naturally present in the water, liberated as molecular bromine in the presence of excess chlorine. When effluents of sewage treatment are chlorinated, or when chlorine is used for the bleaching of textiles or paper pulp, the potential also exists for the formation of higher molecular weight halogenated compounds (Pearson 1982).

Various halogenated alkanes are used in agriculture, as fumigants, herbicides and pesticides, and in industry as solvents, intermediates in various synthetic processes, flame retardants and more. A list of some commonly used haloalkanes is presented in Table 1.

In view of these extensive uses, it is not surprising that haloalkanes, especially the short chain ones, are often found in all components of the biosphere – soil, water and air (Leisinger 1983). The toxicity and potential genotoxicity of many of these compounds (Veissman & Hammer 1985) turn this observation into an acute environmental problem. Consequently, haloaliphatics constitute a massive group in the EPA priority pollutants list (31 out of 114 organics). Of these, 19 are haloalkanes.

Since many of these compounds do not decompose readily in nature, understanding their biodegradation pathways is of utmost practical importance. While in most developed countries legislative measures now exist to curtail their use and release, remediation steps are needed in order to rehabilitate environments polluted over years of ignorance and/or neglect. Bioremediation procedures, increasingly implemented in many areas, are often hampered by a lack of basic understanding of many of the microbial processes involved. The present review, aimed at integrating much of what is known about haloalkane degradation, also serves to point out how much still remains unknown.

## Haloalkane metabolism

Reports on microbial degradation of haloalkanes may be roughly divided into three broad categories, often intermixed: (1) utilization of the organic substrate for growth, as a sole carbon and energy source; (2) various co-metabolic degradative pathways, and (3) partial degradation, not supporting growth, which often involves only dehalogenation.

### *Utilization of haloalkanes as single carbon and energy source*

Halogenated alkanes were considered to be non-

biodegradable, until microbial utilization was demonstrated by Omori & Alexander (1978a, 1978b). Since then the list of haloalkanes capable of supporting bacterial growth has grown; Table 2 lists these compounds, arranged by length of carbon chain.

Utilization of the two compounds at the end of the list, 1-chlorohexadecane and 1-chlorooctadecane, is different from those preceding them in that they were shown to be incorporated, intact, into the membrane lipids of the *Mycobacterium* species studied (Murphy & Perry 1983). However, since these compounds appeared to promote growth in the absence of other organics, they must have also been degraded to allow carbon utilization by the cells. Except for the compounds degraded by *Arthrobacter* HA1 (Scholtz et al. 1987a, 1987b, 1988) and for one other mention of 1,10-dichlorododecane (Yokota et al. 1986), all compounds in Table 2 are 1 to 9 carbon atoms long, and appear to be aerobically utilized by a limited number of microbial isolates. The capabilities of *Arthrobacter* HA1 (Scholtz et al. 1987a, 1987b, 1988) were subjected to the most methodical screening. This isolate was originally shown to grow on $C_4$ to $C_8$ 1-chlorosubstituted, $C_2$ to $C_8$ 1-bromosubstituted and $C_2$ to $C_7$ 1-iodosubstituted alkanes. Later reports (Scholtz et al. 1988) expanded its biodegradative spectrum to $C_{12}$, $C_{14}$ and $C_{16}$ haloalkanes, though the growth rates were very slow. None of the $C_9$ alkanes tested was degraded by this organism. The highest specific growth rate measured was 0.14 $h^{-1}$ for 1-chloropentane and 1-chlorohexane; the corresponding growth yields were 5.2 and 5.5 g protein/mol C. For other isolates growing on various haloalkanes, the values, when reported, were similar. In no case was growth on haloalkanes observed at a generation time shorter than 4 hours. Interestingly, in some cases (Scholtz et al. 1987a), growth on haloalkanes was not paralleled by growth on the equivalent non-substituted alkanes. The hypothesis proposed by Omori & Alexander (1978b) that the capacity for dehalogenation of chloroalkanes is closely related to the process of metabolizing structurally similar alkanes, is therefore not necessarily universally true.

*Table 1.* Industrial and agricultural uses of various haloalkanes.

| Compound (synonym) | Uses |
|---|---|
| Chloromethane (methyl chloride) | Intermediate, refrigerant |
| Dichloromethane (methyl chloride) | Solvent |
| Trichloromethane (chloroform) | Intermediate, solvent |
| Chlorobromomethane | Intermediate (agrochemicals), fire extinguisher, solvent |
| Bromomethane (methyl bromide) | Soil and space fumigant, intermediate |
| Dibromomethane (methylene bromide) | Intermediate (agrochemicals and biocides) |
| Chloroethane (ethyl chloride) | Intermediate, solvent, refrigerant, alkylating agent |
| 1,2-Dichloroethane (ethylene dichloride) | Solvent, intermediate (dyes, perfumes, pharmaceuticals, lacquers) |
| Chloroethane (vinyl chloride) | Intermediate (PVC production) |
| 1,1,1-Trichloroethane (chlorothene) | Solvent |
| 1,1,2-Trichloroethane (vinyl trichloride) | Solvent |
| 1,1,2,2-Tetrachloroethane (cellon) | Solvent |
| Hexachloroethane (perchloroethane) | Solvent, explosives, rubber vulcanizing |
| Bromoethane (ethyl bromide) | Ethylating agent, refrigerant |
| Dibromoethane (ethylene dibromide, EDB) | Anti-knock additive, nematicidic soil fumigant, solvent |
| 1-Bromopropane (propyl bromide) | Intermediate (pharmaceuticals, insecticides, quarternary ammonium compounds, flavors and fragrances) |
| 1,2-Dichloropropane (propyl bromide) | Nematicidic soil fumigant, solvent |
| 1,2 Dibromo-3-chloropropane (DBCP) | Nematicidic soil fumigant |
| 1-Bromobutane (butyl bromide) | Intermediate (pharmaceuticals, quarternary ammonium compounds) |
| 1-Bromooctane (octyl bromide) | Intermediate (UV absorbers, quarternary ammonium compounds, encapsulated dyes) |

*Table 2.* Microbial utilization of haloalkanes as sole carbon and energy sources.

| No. of carbon atoms | Compound | Organism | Specific growth rate ($h^{-1}$) | Growth yield[a] | Reference |
|---|---|---|---|---|---|
| 1 | Chloromethane | *Hyphomicrobium* sp. | 0.09 | 5.0 | Hartmans et al. (1986) |
| | Dichloromethane | *Pseudomonas* DM1 | 0.11 | 15.7[b] | Brunner et al. (1980) |
| | | *Hyphomicrobium* DM2 | 0.07 | – | Stucki et al. (1981) |
| 2 | 1,2-Dichloroethane | *Xanthobacter autotrophicus* | 0.11 | – | Janssen et al. (1985) |
| | | *Pseudomonas fluorescens* | – | – | Vandenberg & Kunka (1988) |
| | | unidentified, gram-negative rod, DE2 | 0.08 | – | Stucki et al. (1983) |
| | 1-Bromoethane | *X. autotrophicus* GJ10 | 0.03 | – | Janssen et al. (1985) |
| | | *Acinetobacter* sp. GJ70 | 0.06 | – | Janssen et al. (1987) |
| | | *Arthrobacter* sp. HA1 | – | – | Scholtz et al. (1987a) |
| | 1-Iodoethane | " | – | – | " |
| 3 | 1-Chloropropane | *X. autotrophicus* GJ10 | 0.12 | – | Janssen et al. (1985) |
| | 1,3-Dichloropropane | " | 0.09 | – | " |
| | 1,2-Dichloropropane | *P. fluorescens* | – | – | Vandenberg & Kunka (1988) |
| | 2,2-Dichloropropane | " | – | – | " |
| | 1-Bromopropane | *Arthrobacter* sp. HA1 | 0.12 | 4.0 | Scholtz et al. (1987a) |
| | | *Acinetobacter* sp. GJ70 | – | – | Janssen et al. (1987) |
| | 1-Iodopropane | *Arthrobacter* sp. HA1 | 0.09 | 4.2 | Scholtz et al. (1987a) |
| | | *Acinetobacter* sp. GJ70 | – | – | Janssen et al. (1987) |
| 4 | 1-Chlorobutane | *Corynebacterium* m15-3 | – | – | Yokota et al. (1986, 1987) |
| | | *Corynebacterium* m2c-32 | | | |
| | | *X. autotrophicus* GJ10 | 0.10 | – | Janssen et al. (1985) |
| | | *Arthrobacter* sp. HA-1 | 0.11 | 5.6 | Scholtz et al. (1987a, 1988) |
| | | *Acinetobacter* sp. GJ 70 | 0.15 | – | Janssen et al. (1987) |
| | 1,2-Dichlorobutane | *Corynebacterium* m15-3 | – | – | Yokota et al. (1986, 1987) |
| | | *Corynebacterium* m2c-32 | | | |
| | 1-Bromobutane | *Arthrobacter* sp. HA-1 | 0.11 | 4.1 | Scholtz et al. (1987a) |
| | 1-Iodobutane | " | – | – | " |
| 5 | 1-Chloropentane | *Corynebacterium* sp. | – | – | Yokota et al. (1986, 1987) |
| | | *Arthrobacter* sp. HA1 | 0.14 | 5.2 | Scholtz et al. (1987a) |
| | | *Acinetobacter* sp. GJ70 | 0.21 | – | Janssen et al. (1987) |
| | 1,5-Dichloropentane | *Pseudomonas* sp. | – | – | Omori & Alexander (1978) |
| | 1-Bromopentane | *Arthrobacter* sp. HA1 | – | – | Scholtz et al. (1987a) |
| | 1-Iodopentane | " | – | – | " |
| 6 | 1-Chlorohexane | *Arthrobacter* sp. HA1 | 0.14 | 5.5 | Scholtz et al. (1987a) |
| | 1,6-Dichlorohexane | *Acinetobacter* sp. GJ70 | – | – | Janssen et al. (1987) |
| | | *Pseudomonas* sp. | – | – | Omori & Alexander (1978) |
| | 1-Bromohexane | *Arthrobacter* sp. HA1 | – | – | Scholtz et al. (1987a) |
| | 1-Iodohexane | " | – | – | " |
| 7 | 1-Chloroheptane | *Arthrobacter* sp. HA1 | – | – | Scholtz et al. (1987a) |
| | | *Pseudomonas* sp. | – | – | Omori & Alesander (1978) |
| | 1-Bromoheptane | *Arthrobacter* sp. HA1 | – | – | Scholtz et al. (1987a) |
| | | *Pseudomonas* sp. | – | – | Omori & Alexander (1978) |
| | 1-Iodoheptane | *Arthrobacter* sp. HA1 | – | – | Scholtz et al. (1987a) |
| | | *Pseudomonas* sp. | – | – | Omori & Alexander (1978) |
| 8 | 1-Chlorooctane | *Arthrobacter* sp. HA1 | – | – | Scholtz et al. (1987a) |
| | 1-Bromooctane | " | – | – | " |
| 9 | 1,9-Dichlorononane | "1,9-dichlorononane-utilizing" H10-2, H15-1 | – | – | Yokota et al. (1986) |

*Table 2.* Continued.

| No. of carbon atoms | Compound | Organism | Specific growth rate ($h^{-1}$) | Growth yield[a] | Reference |
|---|---|---|---|---|---|
| | 1,9-Dichlorononane | *Acinoetobacter* sp. GJ70 | – | – | Janssen et al. (1987) |
| | | *Pseudomonas* sp. | – | – | Omori & Alexander (1978) |
| 10 | 1-Chlorodecane | *Arthrobacter* sp. HA1 | slow[c] | – | Scholtz et al. (1988) |
| | 1-Bromodecane | " | very slow[d] | – | " |
| 12 | 1-Chlorododecane | *Arthrobacter* sp. HA1 | slow | – | Scholtz et al. (1988) |
| | 1-Bromododecane | " | very slow | – | " |
| | 1,10-Dichlorododecane | "1,9-dichlorononane-utilizing" H10-2, H15-1 | – | – | Yokota et al. (1986) |
| 14 | 1-Bromotetradecane | *Arthrobacter* sp. HA1 | very slow | – | Scholtz et al. (1988) |
| 16 | 1-Chlorohexadecane | *Arthrobacter* sp. HA1 | slow | – | Scholtz et al. (1988) |
| | 1-Bromohexadecane | " | slow | – | " |
| | 1-Chlorohexadecane | *Mycobacterium vaccae (M. convolutum)* | – | – | Murphy & Perry (1983) |
| 18 | 1-Chlorooctadecane | *Mycobacterium vaccae (M. convolutum)* | – | – | " |

[a] g protein·mol$^{-1}$ C, except where mentioned.
[b] g dry weight·mol$^{-1}$ substrate.
[c] Growth period – 7 days.
[d] Growth period – 14 days.

*Cometabolism of haloalkanes*

Out of 500 soil enrichments carried out by Omori & Alexander (1978a), only three have yielded microorganisms capable of growth on 1,9-dichlorononane as a single carbon source, while many of the isolates dehalogenated this molecule. One of their conclusions (Omori & Alexander 1978b) was that halogenated compounds may be acted on in nature by cometabolism. Indeed, many examples were reported in which halogenated alkanes, as well as other haloorganics, underwent transformations in the presence of a growth substrate. Many of these instances involve cooxidations by monoxigenases of methane-oxidizing (Perry 1979; Dalton & Stirling 1982; Little et al. 1988; Oldenhuis et al. 1989) or nitrifying (Rasche et al. 1990; Hyman et al. 1988) bacteria. These reports deal almost exclusively with cooxidations of short (1–3 carbon atoms) haloalkanes; however, indications for cometabolism of longer haloalkanes also exist. For instance, Scholtz et al. (1988) present a long list of substrates up to 7 carbon atoms in length dehalogenated by *Arthrobacter* HA1 in the presence of butanol. Another example of cometabolism is a bromoethanol-resistant mutant of *Acinetobacter* sp. GJ70, possibly with impaired alcohol dehydrogenase activity (Janssen et al. 1987). This mutant lost the ability to grow on bromoethane, 1-bromopropane, 1-chlorobutane or 1-chloropentane as a sole carbon source as well as the equivalent alcohols. However, it could well cometabolize 1,2 dibromoethane in the presence of citrate. Worthy of special mention, though not directly involving haloalkanes, is the description of codegradation of chlorinated paraffins with n-hexadecane by bacterial isolates capable of dehalogenating several haloalkanes (Omori et al. 1987). Other interesting examples are the anaerobic transformations of carbon tetrachloride and brominated trihalomethanes under methanogenic (Bouwer & McCarty 1983a) and denitrifying (Bouwer & McCarty 1983b) conditions.

As shown in many of these examples, the cometabolic requirements may be very specific in

nature. This may be of special significance when bioremediation measures are considered, for instance, for contaminated soils and aquifers. Indeed, it has been shown that addition of non-selective carbon sources, such as glucose or amino acids, inhibited mineralization in subsurface samples of xenobiotic substrates, among them dibromoethane (Swindoll et al. 1988). Apparently, the easily degradable organic amendments were preferentially utilized by indigenous bacterial populations. Nevertheless, many reports exist (see Morgan & Watkinson 1989, for review) describing microbially mediated removal of haloalkanes, as well as other contaminants, from environments such as soils (Castro & Belser 1968; Pignatello 1986), aquifers (Baldauf 1981; Werner 1989) and wastewater (Omori & Alexander 1978b). It is impossible to determine whether in these cases degradation was carried out cometabolically, but it is probably safe to assume that it involved the synergistic activity of mixed bacterial consortia.

*Dehalogenation*

An essential step in any microbial transformation of halogenated compounds is the removal of the halogen from the organic molecule, a step known as dehalogenation. The process may occur abiotically, as demonstrated by halide release into the medium under sterile conditions (Omori & Alexander 1978a; Yokota et al. 1986; Janssen et al. 1987); however, in most cases dehalogenation is enzymatically driven. The enzymes catalyzing the process are generally known as 'dehalogenases' (Jensen 1960). As will be described below, these include several enzymes highly varied in their mode of action.

The number of halogenated compounds which were shown to be microbially dehalogenated far exceeds the number of such compounds actually supporting growth. In many cases dehalogenations were observed and the reaction products were identified, but there were no indications of further uses the microorganisms involved may make of those compounds. Dehalogenases, therefore, possess a wider specificity towards their substrates than the subsequent metabolic machinery needed to utilize the residual carbon skeleton. One of the possible reasons for this may be that for the longer alkanes, dehalogenation takes place on the outside of the cell membrane; the end product may still be too large to penetrate into the cell.

The environmental importance of the degradation of haloorganics, as well as other potential biotechnological applications of this process, have led to extensive studies of dehalogenation mechanisms. While aromatic chlorides were the major group investigated, substantial information concerning haloalkanes has also accumulated. Table 3 lists some of the relevant findings concerning intact cell and cell-free activities of various dehalogenases. In order to allow some generalizations to be made, only data from the better characterized systems, using pure bacterial strains only, were selected.

As can be seen from the data in Table 3, as well as from additional information not included therein, haloalkane dehalogenases may be generally grouped into the following three major categories:

1. Oxygenases, leading to the formation of the corresponding aldehyde. Further dehydrogenation yields the acid species (Yokota et al. 1986):

$$^1/_2O_2 + \text{R-CH}_2\text{X} \xrightarrow[\text{oxygenase}]{} \text{R-CHOHX} \xrightarrow[\text{spontaneous}]{} \text{HX} + \text{R-CHO} \xrightarrow[\text{dehydrogenase}]{} \text{R-COOH} \quad (1)$$

This type of sequence was reported for the 1-chlorobutane grown *Corynebacterium* m15-3 (Yokota et al. 1986, 1987). Similar activities were described for the methane- and ammonia-monoxygenases (Oldenhuis et al. 1989; Rasche et al. 1990, respectively).

2. Halidohydrolases, substituting the halide with a hydroxyl:

$$\text{H}_2\text{O} + \text{R-CH}_2\text{X} \xrightarrow[\text{hydrolase}]{} \text{R-CH}_2\text{OH} + \text{HX} \quad (2)$$

This reaction, invariably leading to medium acidification, appears to be shared by many of the organisms in Table 2, although this does not neces-

*Table 3.* Microbial dehalogenations of haloalkanes.

| Organism | Mode of enzyme activity | Induction[a] | Location[b] | Substrate[c] | Product | Growth[d] | Comments | Reference |
|---|---|---|---|---|---|---|---|---|
| Methylotroph DM1 (*Pseudomonas* sp.) | hydrolytic (?) | + | s | Dichloromethane | formaldehyde | + | monoxygenase not likely | Brunner et al. (1980) |
| *Xanthobacter autotrophicus GJ10* | hydrolytic | – | s (?) | bromoethane | ethanol | + | a different enzyme | Janssen et al. (1985) |
| | | | | 1-chlorobutane | butanol | + | exists for alkanoic | Kuening et al. (1985) |
| | | | | 1,2 dichloroethane | 2-chloroethanol | + | acids | |
| | | | | 1,3 dichloropropane | 3-chloropropanol | – | – | |
| | | | | 1,2 dibromoethane | 2-bromoethanol | | | |
| *Acinetobacter* sp. GJ70 | hydrolytic | ? | ? | 1-chlorobutane | butanol | + | | Janssen et al. (1987) |
| | | | | 1-iodopropane | propanol | + | | |
| | | | | 1-chloropropane | ? | – | | |
| | | | | 1,2 dibromoethane | 2-bromoethanol | – | | |
| *Hyphomicrobium* sp. DM2 | [e] | + | s | dichloromethane | formaldehyde | + | | Kohler-Staub & Leisinger (1985) Stucki et al. (1981) |
| *Methylosinus trichosporium* OB3b | methane monooxygenase | + | s | trichloroethylene | trichloroethylene oxide (?) | +/– | induction by copper starvation; other $C_1$-$C_3$ haloalkanes at least partially degraded | Oldhenius et al. (1989) |
| *Nitrosomonas europaea* | ammonia monooxygenase | ? | m | 1,2dichloropropane bromoethane | ? formaldehyde | +/– | other $C_1$-$C_3$ fumigants degraded | Rasche et al. (1990) |
| *Arthrobacter* sp. HA1 | 1) hydrolytic | + | s | $C_2$-$C_{10}$ 1-chloroalkanes | alcohols[f] | + | other haloalkanes including many | Scholtz et al. (1987a,b) |
| | | | | $C_2$-$C_9$ 1-bromoalkanes | alcohols[f] | + | dihaloalkanes, also dehalogenated; | |
| | | | | $C_1$-$C_7$ 1-iodoalkanes | alcohols[f] | + | induced by 1-chlorobutane | |
| | 2) hydrolytic | + | s | $C_{10,12,16}$ 1-chloroalkanes | prob. alcohols[f] | + (slow) | induced by 1-bromohexadecane | Scholtz et al. (1988) |
| | | | | $C_{10\ 12,14,16}$ 1-bromoalkanes | prob. alcohols[f] | + (slow) | | |
| | 3) hydrolytic | + | s | 1-bromoalkanes | ? | + | unstable enzyme, induced by 1-chlorobutane | Scholtz et al. (1987b) |
| *Corynebacterium* m-15-3 | 1)hydrolytic | + | s | 1-chlorobutane | n-butanol | + | anaerobic | Yokota et al. (1986, 1987) |
| | 2) oxygenase | ? | ? | 1-chlorobutane | butyric acid | + | aerobic | Yokota et al. (1986) |
| | | | | 1,3 dichloropropane | 3-chloropropinic acid | ? | aerobic | Yokota et al. (1986) |
| Methylotroph H-2 | oxygenase | ? | ? | 1,2 dichloroethane | 2-chloroacetic acid | | | Yokota et al. (1986) |

[a] (+), an inducible enzyme; (–), a constitutive enzyme; (?), data not available.
[b] (s), soluble; (m), membrane bound; (?), data not available.
[c] For some broad-spectrum enzymes or microorganisms, only representative substrates are presented.
[d] (+), growth substrate, (–), non-growth substrate; (+/–), cosubstrate.
[e] Glutathione-dependent nucleophilic substitution, leading to a non-enzymatic hydrolysis.
[f] When determined.

sarily imply a uniformity in enzyme structure and function. In fact, in several cases, the presence of more than one enzyme form has been reported within a single microbial species. In *Arthrobacter* HA1, for instance, the existence of four different halidohydrolases was postulated (Scholtz et al. 1988), three of them haloalkane dehalogenating. Selective induction of the different enzymes was shown to be controlled by the haloalkane serving as a carbon source for growth. This organism was also the first to be reported to hydrolyze the carbon-halogen bond in long-chain 1-haloalkanes. *Xantobacter autotrophicus* GJ10 is another organism containing more than one dehalogenase. In addition to the haloalkanes-specific enzyme mentioned in Table 2, it also contains an alkanoic acid-specific halidohydrolase (Janssen et al. 1985; Kuening et al. 1985). These two enzymes appear to have a non-overlapping activity spectrum. In all tested cases, the haloalkane halidohydrolases did not dehalogenate the haloacids, even those with the same number of carbon atoms.

When the halogen position on the alkane chain is not terminal, dehalogenation appears to be preceded by oxidation of the terminal methyl group to 2- or 3-haloaliphatic acids (Yokota et al. 1986). In the case of a halogen substituted in the second position, it is then removed from the halo-acid by a 2-haloacid-type dehalogenase (Motosugi et al. 1982):

$$\text{R-CHX-CH}_3 \longrightarrow \text{R-CHX-COOH} \xrightarrow{\text{H}_2\text{O}} \text{R-CHOH-COOH} + \text{HX} \quad (3)$$

For the 3-halogenated acid formed by oxidation of the 3-haloalkanes, halogen removal appears to be mediated by a membrane-bound enzyme system, such as the one involved in β-oxidation of fatty acids:

$$\text{R-CHX-CH}_2\text{-CH}_3 \rightarrow \text{R-CHX-CH}_2\text{-COOH} \rightarrow \text{R-CH}_2\text{-CH}_2\text{-COOH} + \text{X}^- \quad (4)$$

While all of these reactions are aerobic in nature, only those mediated by the oxygenase (see Eq. 1) appear to be obligately aerobic. The halidohydrolase reaction was shown to proceed also anaerobically, although sometimes at slower rates.

3. Reductases, leading directly to the formation of the corresponding alkane:

$$\text{H}_2 + \text{RX} \rightarrow \text{RH} + \text{HX} \quad (5)$$

This type of mechanism has been described for various haloaromatic compounds (Kobayashi & Rittman 1982; Lal & Saxena 1982; Reineke & Knackmuss 1988); to my knowledge, however, it has not been described for haloalkanes except for an unconfirmed report by Lang et al. (Ann. Meet. Am. Water Works Assoc. 1981). There are, however, some indications of reductive dehalogenations of C1 and C2 halogenated aliphatics under methanogenic and nitrifying conditions (Bouwer & McCarty 1983a, 1983b). The actual metabolic pathways in these two cases have not been resolved.

Finally, a dehalogenation reaction which deserves special mention is that reported for *Hyphomicrobium* DM-2 (Kohler-Staub & Leisinger 1985; Stucki et al. 1981). Dichloromethane dehalogenation by this organism was demonstrated to be a glutathione-dependent nucleophilic substitution. It was proposed that an S-chloromethyl glutathione conjugate is formed enzymatically, which is then hydrolyzed non-enzymatically to an unstable 2-hydroxymethyl glutathione; the latter compound decomposes to formaldehyde, thus regenerating reduced glutathione.

## Genetics of haloalkane degradation

As pointed out in the introduction, most of the research into the biodegradation of halogenated organics has been focused on aromatic chlorides. It is not surprising, therefore, that available information concerning the genetics of haloalkane degradation is scant. This is in sharp contrast to the constantly increasing genetic information regarding the degradation of halogenated aromatics (see Chaudhry & Chapalamadugu 1991, for a recent review). In fact, in all the literature cited to this point, including the articles from which the data for

the compilation of Tables 2 and 3 were derived, the subject is hardly mentioned. It is even unclear, for instance, whether the genetic information for dehalogenation and degradation of the haloalkanes mentioned in these tables is plasmid- or chromosome- encoded. In the *Pseudomonas* strain described later in this review (ES-2), which is capable of degrading a wide spectrum of haloalkanes, repeated attempts to show the presence of plasmids have failed.

In contrast to haloalkanes, several reports describe plasmids involved in the degradation of haloalkanoic acids. Hardman et al. (1986) have examined four *Pseudomonas* and two *Alcaligenes* species, capable of growth on 2-chloropropionic acid and chloroacetic acid. All their isolates contained a single plasmid (pUU204). When the *Pseudomonas* isolates were cured of their plasmids, dehalogenase activities were lost. However, attempts to reintroduce the plasmid to the cured original strains or to new hosts have failed. Two different plasmids, involved in the degradation of haloacetate, pU01 and pU011, were isolated by Kawasaki et al. (1981) from *Moraxella* strain B. Both plasmids also code for mercury resistance, but no further genetic information is available.

It should be remembered that, as mentioned above, in all cases examined the dehalogenase acting on haloacids was different from that involved in dehalogenating haloalkanes. The information presented above, in addition to being very preliminary, is thus also only partially relevant to haloalkane degradation. It was included in this review since, to my knowledge, this is the only genetic information concerning the biodegradation of halogenated aliphatics in general.

## Recent advances

We have recently studied several bacterial isolates from an experimental reactor system for the treatment of chemical industrial wastewater, rich in haloorganics, including many brominated com-

*Table 4.* Degradation[a] of nonsubstituted and substituted alkanes by strain ES-2.

| Chain length (No of C atoms) | Non-substituted | 1-chloro | 1-bromo | 1-iodo | 1-fluoro | 1-alcohol | 1-acid |
|---|---|---|---|---|---|---|---|
| 1 | | | | | | − | − |
| 2 | | | | | | + | + |
| 3 | | | +/−[b] | | | + | + |
| 4 | | − | +/−[b] | | | + | + |
| 6 | − | − | + | | − | − | − |
| 7 | | | + | | | | |
| 8 | − | − | + | + | | + | + |
| 9 | | | + | | − | | |
| 10 | − | + | + | | | + | + |
| 11 | | | | | | | + |
| 12 | | | + | | | | + |
| 13 | | | + | | | | |
| 14 | | | + | | | | |
| 16 | − | + | + | | | + | + |
| 17 | | | | | | | + |
| 18 | − | + | + | | | + | + |
| 20 | | | | | | | − |
| 22 | | | | | | | − |
| 26 | | | | | | | − |

[a] Utilization of the substrate as a single carbon and energy source.
[b] Dehalogenation but no growth.

pounds. One of these isolates, a gram negative motile rod named ES-2 (Shochat et al., in preparation) is probably a *Pseudomonas* sp., and will be briefly described here. Under optimal growth conditions, with 1-bromooctane as a sole carbon and energy source, its generation time is 113 min. Its dehalogenase activity is inducible, and it is capable of growth on a variety of haloalkanes.

*Biodegradation and dehalogenation*

The biodegradation spectrum of this strain appears to be wider than any previously described for a haloalkane degrading organism (Table 4), except possibly *Arthrobacter* HA1 (Scholtz et al. 1987a, 1987b, 1988). Originally isolated on 1-bromooctane as a sole carbon and energy source, ES-2 is capable of growth on all the 1-bromoalkanes tested in the $C_6$–$C_{18}$ range. In addition, it can debrominate 1-bromopropane and 1-bromobutane, but it could not utilize the latter two compounds for growth. It grows also on 1-chloroalkanes, though only at the $C_{10}$–$C_{18}$ range. $C_2$–$C_{18}$ fatty acids and alcohols also support growth, with the exception of the $C_6$ species - caproic acid and hexanol. It grew on a variety of sugars, tricarboxylic and amino acids (not shown), but *not* on the non-substituted alkanes ($C_6$–$C_{18}$), chloroacetate and aromatics (benzene, phenol, bromophenol) tested.

One of the interesting observations regarding the ES-2 degradation spectrum (Table 4) is that the organism exhibited a wider specificity for bromides than for chlorides. It dehalogenated $C_3$ to $C_{18}$ bromoalkanes, utilizing all of them but bromopropane and bromobutane for growth, while only chloroalkanes of 10 carbon atoms or longer were metabolized. The dehalogenase, acting on bromopropane through bromooctane, was incapable of removing the chloride from the parallel chloroalkanes. For alkanes $C_{10}$ and longer, both halogens were removed; but it is unclear whether this latter activity was mediated by the same enzyme dehalogenating the shorter bromoalkanes. It is difficult to envisage how a dehalogenase would lose its bromide-specificity with longer substrates; it is therefore likely that there are at least two dehalogenases involved. There are several other indications for the presence of more than one dehalogenase in ES-2 cells. One of them is the puzzling observation that ES-2 can utilize bromooctane, bromohexane, and octanol for growth, but not hexanol. The first identified product of bromooctane degradation was octanol (Table 5), indicating that dehalogenation is carried out hydrolytically. If the same enzyme would act on bromohexane, the hexanol formed would not be able to support growth. Thus, bromohexane (and possibly lower bromoalkanes) is probably dehalogenated by a different enzyme. Nevertheless, hexanol is produced from 1-bromohexane by resting cells of ES-2 (Table 5). However, unlike the octanol produced from bromooctane, which is found only inside the cells, the hexanol produced from bromohexane is detectable only in the medium. It

*Table 5.* ES-2 degradation products of some haloalkanes.

| Substrate | Growth[a] | Location[b] | Product[c] | Growth[d] | Location[b] |
|---|---|---|---|---|---|
| 1-Bromobutane | – | EC | ? | –[e] | EC |
| 1-Bromohexane | + | EC | 1-hexanol | – | EC |
| 1-Bromooctane | + | EC | 1-octanol | + | IC |
| 1-Bromodecane | + | EC | 1-decanol | + | IC |
| 1-Chlorodecane | + | EC | 1-decanol | + | IC |

[a] Use of substrate as a single carbon and energy source for growth.

[b] Cells were separated from the medium and disrupted by vigorous shaking with glass beads. Both medium and the cleared supernatant of the broken cells were extracted with ethyl acetate, and assayed by gas chromatography. EC – extracellular; IC – intracellular.

[c] Identified by gas chromatography, using an SPB-1 column and a flame ionization detector.

[d] Known use of product as a single carbon and energy source for growth.

[e] Since ES-2 is incapable of growth on 1-bromobutane, the same is likely to be true for its yet unidentified degradation product.

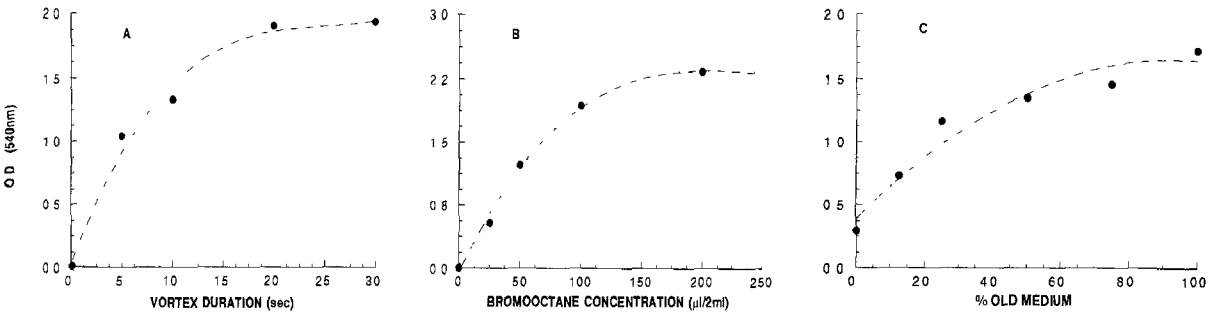


*Fig. 1.* A rapid method for determination of emulsification activity. A 24-hour cell-free growth medium was used. (A), 2 ml medium and 100 µl 1-bromooctane vortexed for different durations; (B), different concentrations of 1-bromooctane in 2 ml medium, vortexed for 30 sec; (C), different ratios of 24-hour to fresh medium (2 ml total volume), vortexed for 30 sec with 100 µl 1-bromooctane. In all cases, optical density at 540 nm was determined immediately following the mixing.

is therefore unclear at present how bromohexane is metabolized and utilized for growth. A degradation product of 1 bromobutane was also found extracellularly; it has not been identified yet, but it was shown not to be butanol (Table 5).

*Emulsification*

One of the reasons contributing to the fact that most biodegradation studies involving haloalkanes have been restricted to short chain compounds may be attributed to the insolubility in water of most longer haloalkanes. However, even the reports on the dehalogenation or degradation of clearly insoluble halogenated aliphatics generally do not mention this obviously significant factor. For instance, Omori & Alexander (1978a) in their pioneering work on haloalkane degradation mention that the substrate, 1,9-dichlorononane, was separated from the medium by centrifugation; however, no mention is made of the obvious accessibility problems of the substrate to the bacteria. Similarly, Janssen et al. (1987) mention that some substrates were poorly soluble, thus explaining low utilization efficiency. Conversely, Scholtz et al. (1987b) presumed that the rate of dissolution of the sparingly soluble chloroalkanes ($C_6$ and longer) was higher than the rate of hydrolysis, and therefore not rate limiting for dehalogenation by the cell-free enzyme of Arthrobacter HA1. Such an assumption, however, cannot be justified for intact cells. In all cases where water-insoluble haloalkanes were partially or fully utilized (see Tables 2 and 3), a solubilization step must have taken place before any metabolic activity.

Emulsification of insoluble organic compounds has been investigated in detail in the past (see Rosenberg 1986, for review), mostly with regard to petroleum hydrocarbons. Many microorganisms produce surface active materials, ranging in structure from low molecular weight glycolipids, via polyanionic heteropolysaccharides containing covalently linked hydrophobic side chains, to polysaccharide-protein complexes. In most cases, the biosurfactants are extracellular; they were shown to be involved in cell adhesion, emulsification, dispersion, flocculation, and aggregation phenomena (Rosenberg 1986). All of these activities are significant for the physiology and ecology of the biosurfactant-producing strains; in addition, they present the potential for various industrial applications. Both of these aspects – the natural and the industrial – are manifested in the case of the emulsifiers produced by haloalkane-degrading microorganisms. For both reasons, therefore, more effort should be directed towards their research.

During growth, strain ES-2 produces an extracellular surface-active compound which leads to emulsification of the substrate. When bromooctane is added to a culture of this bacterium, it initially rests as a single large droplet at the bottom of the incubation vessel. After agitation and growth, at least part of it is transformed into an emulsion of fine droplets. Excretion of the emulsifying agent is observed immediately upon inoc-

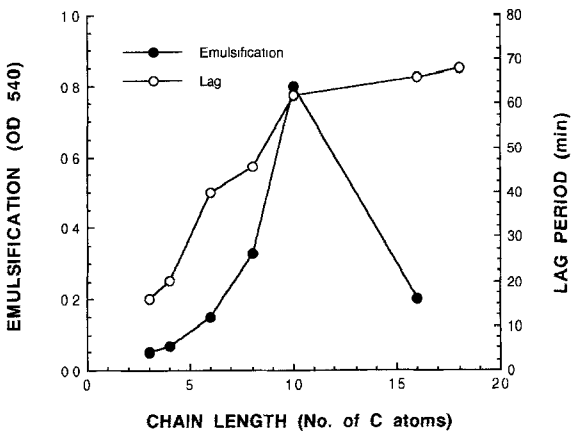


*Fig. 2.* Emulsification of 1-bromoalkanes by strain ES-2. (•), emulsifying activity determined with 2 ml of a 24-hour medium of a 1-bromooctane grown culture and 100 $\mu$l of the tested 1-bromoalkane; (○), length of lag period preceding debromination in a concentrated cell suspension, with 20 mM of the tested 1-bromoalkane.

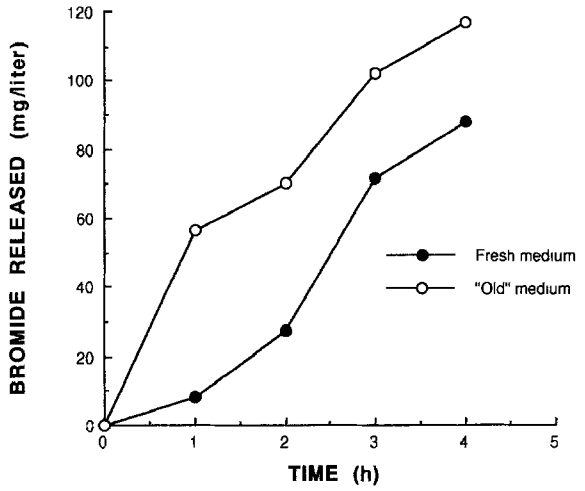


*Fig. 3.* Importance of emulsifier for bromooctane dehalogenation. Late exponential phase cells were harvested by centrifugation, washed with fresh medium, and resuspended in either fresh (•) or "old" (○) medium. 1-bromooctane (20 mM) was added, and bromide release was followed for 4 hours.

ulation, and proceeds throughout the lag and exponential growth phases. Emulsifier production was observed irrespectively of the solubility of the growth substrate. For example, emulsification activities determined in a growth medium of acetate-grown or bromooctane-grown cells were similar. This may mean that the emulsifier also plays a yet undetermined role beyond accessing water-insoluble compounds.

The chemical nature of the ES-2 emulsifier has not been determined. Either due to a different mode of action or to a relatively low potency, its activity could not be measured by the methods described by Rosenberg et al. (1979) and by Sar & Rosenberg (1983, 1989). A different methodology was therefore developed which allowed a rapid assessment of relative emulsification activity, as described in Fig. 1. The method involved a 30-sec intensive mixing (by vortex) of a 2-ml sample with 100 $\mu$l of substrate, and an immediate determination of the optical density at 540 nm. Using this approach, the specificity of the ES-2 emulsifier to various bromoalkanes was determined. The results are shown in Fig. 2, along with another parameter affected by carbon chain length – the length of the lag period preceding bromide release. While maximal emulsification values were obtained for 1-bromodecane, the length of the lag period preceding bromide release by resting cells increased with increasing chain length; up to $C_{10}$, at least, the correlation to emulsifier activity was actually negative. Debromination, therefore, is apparently also restricted by other parameters, possibly steric in nature, on top of the insolubility of the substrate. However, as demonstrated in Fig. 3, the presence of the emulsifier was essential for dehalogenation. When cells were suspended in a 24-h old medium supplemented with fresh bromooctane, bromide release proceeded from time zero, as compared to nearly a 1-h lag in fresh medium.

### *Location of bromooctane degradation*

As mentioned above, extracellular emulsification was the first step in bromooctane utilization. The following stage, dehalogenation, appears to take place in the periplasmic space. This assumption is based on the observation that bromooctane could not be found intracellularly, whereas the product, octanol, was not found in the medium (Table 5). The subsequent degradation of octanol, possibly

by dehydrogenations to octyl aldehyde and octanoic acid, followed by β-oxidation, is thus believed to be intracellular.

Utilization of 1-bromooctane is thus a three-step process, taking place in separate locales:

1) extracellular emulsification by a constitutively excreted emulsifier
2) periplasmic dehalogenation by an inducible halidohydrolase
3) intracellular degradation

It remains to be determined how universal this sequence of events is for other substrates.

## Concluding remarks

Two main conclusions may be drawn from the data presented in this review. The first is that, contrary to earlier beliefs, many haloalkanes are biodegradable, either partially or fully, by a variety of microorganisms. The second conclusion is that the study of this industrially, agriculturally and – primarily – environmentally important group of organic chemicals is still only in the preliminary stages. While an appreciable amount of data has accumulated concerning their environmental distribution, available information regarding degradative biochemical pathways is lacking, and that concerning genetic control is practically nonexistent. Thus, based upon these two conclusions, it may be stated that in many cases, environmental hazards involving haloalkanes may be potentially biologically solved or at least neutralized. On the other hand, further work is needed in order to allow a better understanding of the processes involved, and hence facilitate the implementation of such measures.

## Acknowledgements

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