Biodegradation of chlorinated and unsaturated hydrocarbons in relation to biological waste-gas treatment

Promotoren: dr. ir. J.A.M. de Bont,
hoogleraar in de industriële microbiologie

dr. ir. J. Tramper,
hoogleraar in de bioprocestechnologie

Biodegradation of chlorinated and unsaturated hydrocarbons in relation to biological waste-gas treatment

S. Hartmans

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen op
vrijdag 19 maart 1993
des namiddags te vier uur in de Aula
van de Landbouwuniversteit te Wageningen

ISBN 90-5485-098-1

STELLINGEN

1 Verbindingen die in het groeimedium ophopen zijn niet altijd intermediairen in de afbraakroute van het groeisubstraat (Utkin *et al.*, 1991; Shirai en Hisatsuka).

Utkin, I.B., M.M. Yakimov, L.N. Matveeva, E.I. Kozlyak, I.S. Rogozhin, Z.G. Solomon, and A.M. Bezborodov. 1991. Degradation of styrene and ethylbenzene by *Pseudomonas* species Y2. FEMS Microbiol. Lett. 77:237-242.
Shirai, K., and K. Hisatsuka. 1979a. Production of β-phenetyl alcohol from styrene by *Pseudomonas* 305-STR-1-4. Agric. Biol. Chem. 43:1399-1406.

2. De trichloorethyleentolerantie van isopreen-afbrekende bacteriën (Ewers *et al.*, 1990), wordt nogal eens ten onrechte geïnterpreteerd als ongevoeligheid voor het oxydatieprodukt van trichloorethyleen (Holliger, 1992).

Ewers, J., D. Freier-Schröder, and H.-J. Knackmuss. 1990. Selection of trichloroethene (TCE) degrading bacteria that resist inactivation by TCE. Arch. Microbiol. 154:410-413.
Holliger, C. 1992. Reductive dehalogenation by anaerobic bacteria. Thesis Wageningen Agricultural University, p. 80.

3. Phelps *et al.* (1991) verklaren de afname in vinylchloride in hun experimenten door aan te nemen dat dit door de Teflon septa verdwijnt. Deze afname is echter waarschijnlijk het gevolg van de fotochemische omzetting van vinylchloride.

Phelps, T.J., K. Malachowsky, R.M. Schram, and D.C. White. 1991. Aerobic mineralization of vinyl chloride by a bacterium of the order *Actinomycetales*. Appl. Environ. Microbiol. 57:1252-1254.

4. De frequentie waarmee revertanten van methaanmonooxygenase deficiënte mutanten optreden is circa een miljoen maal gevoeliger te bepalen door groei op methaan als criterium te gebruiken in plaats van de door Nicolaidis en Sargent (1987) gebruikte methode.

Nicolaidis, A.A., and A.W. Sargent. 1987. Isolation of methane monooxygenase-deficient mutants from *Methylosinus trichosporium* OB3b using dichloromethane. FEMS Microbiol. Lett. 41:47-52.

5. De verklaring van Diks en Ottengraf, dat de temperatuureffecten op de biologische reactiesnelheid en de verdelingscoëfficiënt van dichloormethaan elkaar compenseren bij de dichloormethaanverwijdering met een biologisch trickling filter in het diffusie gelimiteerde regime, is tegenstrijdig.

Diks, R.M.M., and S.P.P. Ottengraf. 1990. Verification studies of a simplified model for the removal of dichloromethane from waste gases using a biological trickling filter (Part II). Bioprocess Eng. 6:131-140.

6. Mehta *et al.* (1989) veronderstellen ten onrechte dat de opgeloste concentratie methaan in een chemostaat in evenwicht is met de gasfase concentratie. De door hen gerapporteerde verzadigingsconstante voor methaan is dan ook circa 1000 maal te hoog.

   Mehta, P.K., S. Mishra, and T.K. Ghose. 1989. Growth kinetics and methanol oxidation in *Methylosinus trichosporium* NCIB 11131. Biotechnol. Appl. Biochem. 11:328-335.

7. Het plaatsen van een advertentie voor een vacature binnen de LU, waarbij vrouwen zeer nadrukkelijk worden verzocht te reflecteren terwijl een zeer nauw omschreven pakket van vereisten wordt geformuleerd dat precies overeenkomt met de kwalificaties van een reeds in tijdelijke dienst zijnde man is om ethische, financiële en psychologische redenen ongewenst en komt de geloofwaardigheid van de LU niet ten goede.

8. Door slechts op de luchthaven van bestemming tax-free artikelen aan te bieden kan een bijdrage geleverd worden aan de reductie van de mondiale CO_2 emissie.

9. Berichten in het NRC Handelsblad over gebeurtenissen in Suriname verschijnen ten onrechte op de pagina binnenland van dit dagblad.

10. De uitdrukking "the grass is always greener on the other side of the fence" is op meer van toepassing dan op gras alleen. Desalniettemin berust zij veelal op gezichtsbedrog.

Stellingen behorende bij het proefschrift "Biodegradation of chlorinated and unsaturated hydrocarbons in relation to biological waste-gas treatment".

S. Hartmans
Wageningen, 19 maart 1993.

Aan Deborah
Aan mijn ouders

Contents

Chapter 1

GENERAL INTRODUCTION

The increase in material wealth during the last century, especially in the Western world, is very much correlated with an increase in the consumption of fossil fuels and an increase in the production and use of chemicals. As a consequence all kinds of wastes are being produced. These waste streams which can contain many different chemicals are, very often, discharged into the environment.

Gradually, mainly due to an increase in environmental concern of the general public, increasingly stringent environmental regulations are coming into force. These regulations require the development of techniques to reduce the amount of waste discharged into the environment.

Wastewater treatment is already applied on a large scale throughout the world and the bulk of the components in wastewater are actually removed using biological techniques. Treatment of waste gases and remediation of polluted groundwater and soils are less well established techniques. Especially in the last decade attention has focused on biological techniques to tackle these problems.

The biological treatment of waste gases is still relatively little applied, until now mainly for the treatment of odorous air (Ottengraf, 1987; Leson and Winer, 1991). The technique may, however, also be used to treat industrial waste gases. Waste gases almost invariably contain air and hence oxygen. Therefore, successful biological waste-gas treatment will require that aerobic microbial degradation of the contaminants of interest is possible.

In this introduction I will briefly review the gaseous industrial emission of organic chemicals with an emphasis on the situation in The Netherlands, the aerobic biodegradative potential of microorganisms focusing on unsaturated and chlorinated hydrocarbons, and biological waste-gas treatment.

Emissions of volatile and gaseous compounds

The emission of hydrocarbons is mainly due to industrial activity and traffic. The per capita emission of hydrocarbons of anthropogenic origin is the highest in the USA (Table 1).

TABLE 1. Anthropogenic airborne hydrocarbon emissions (kg per capita per year in 1985).

The Netherlands	24
European Community	31
Europe	34
USA	85
World	27

(Data from van den Hout, 1990)

Besides hydrocarbons there are several other major anthropogenic emissions. For sulphur dioxide, nitrogen oxides and carbon dioxide the world average anthropogenic emissions per capita per annum are estimated at 35, 24 and 4400 kg, respectively (van den Hout, 1990). Besides the emissions from anthropogenic sources the contribution from natural sources of certain compounds, e.g. methane, ethene, isoprene and methyl chloride, can be very significant. Although the major contribution of methane release is from natural wetlands (25-35%) very significant contributions are associated with human activities such as rice cultivation (20-25%), livestock breeding (20-25%) and the mining of fossil fuels (10%) (Tyler, 1991). As a consequence the atmospheric methane concentration has approximately doubled during the last 150 years, with an exponential increase during the last decennia (IPCC report 1990, Anonymous, 1991).

Table 2 shows the emission of a number of hydrocarbons and chlorinated hydrocarbons in The Netherlands (41,160 km^2). It should be emphasized that the data in Table 2, especially for 1,1,1-trichloroethane, dichloromethane and tetrachloroethene are minimal estimations. The West-European market for chlorinated solvents (tetrachloroethene, trichloroethene, dichloromethane and 1,1,1,-trichloroethane) was more than 500,000 tons in 1990 and 620,000 tons in 1986 (European Chlorinated Solvent Association). As these compounds are only used as solvents the complete production volume is ultimately lost to the environment. If we make a conservative estimate of the Dutch share of the West-European market at 5%, this means that at least 25,000 tons of these chlorinated solvents are emitted in The Netherlands. This is twice as much as listed in Table 2 for these four compounds, and amounts to about 1.7 kg per capita per year. In the USA the use of these four solvents was about 3.5 kg per capita per year in 1988 (Wolf *et al.*, 1991).

From Table 2 it can be seen that for most of the non-chlorinated hydrocarbons

the industrial emissions are not the major source. In most cases traffic is the major source. Styrene is the exception with more than 60% of the emissions originating from industrial sources. For the chlorinated hydrocarbons the situation is less consistent. For a number of compounds the bulk of the emissions are, however, from industrial sources. Therefore a reduction of these emissions is possible without imposing strict regulations on the use of specific compounds or completely prohibiting their use as will be the case for a number of fluorocarbons.

TABLE 2. Gaseous emissions of a number volatile of organic compounds and chlorinated hydrocarbons in The Netherlands (1985-1987) in ton per annum (van den Hout, 1990).

Compound	Source				
Hydrocarbons	Industrial*		Other	Total	(%)#
Methane	7060	b	543000	550000	>99
Ethene	2740	a/b	15000	17700	>99
Benzene	1660	a	7570	9230	>99
Toluene	8290	c/d	20800	29100	>99
Styrene	987		502	1490	99
Chlorinated hydrocarbons					
CFK's	2710	c	7200	9910	>99
Chloromethane	538		nd	nd	nd
Dichloromethane	2090	c	2600	4690	95
Trichloromethane	180	a/b	10	190	94
Tetrachloromethane	354	b	40	390	nd
Chloroethene	323	a	≈0	323	nd
1,2-Dichloroethane	1300	a	15	1320	nd
Trichloroethene	569	b/c	140	709	nd
1,1,1-Trichloroethane	940	d	>1000	>1940	nd
Tetrachloroethene	954	c	3300	4250	nd

* Based on individual registration. The estimate of the emission by non registered industrial sources in comparison with the registered emission: a, less than about 10%; b, about 10 - 50%; c, in the same order of magnitude; d, significantly more.

\# Emission to the ambient air as percentage of the total emission to the environment.

nd, not determined.

Regulations and threshold levels

For specific compounds a reduction in the emission is required either because the compound is toxic, or even carcinogenic (e.g. vinyl chloride), or because it is a problem due to its odour characteristics. In Table 3 TLV (Threshold Limit Values) and odour threshold levels are given for a number of selected compounds to

illustrate this. For those compounds which are liquids at ambient temperature the vapour pressure is also shown, expressed in ppmv to allow simple comparison.

As can be seen in Table 3 some compounds, e.g. carbon monoxide and vinyl chloride, have much higher odour threshold levels than the TLV so that these compounds can be present at dangerous levels without being noticed. When working with these compounds particular care is required. In contrast a number of other compounds, e.g. hydrogen sulfide, acetaldehyde and styrene, have very low odour thresholds. These compounds can already cause an odour problem when present in waste gases at very low concentrations.

TABLE 3. TLV, volatility and odour threshold data of selected chemicals (Data from Amoore and Hautala, 1983).

	Threshold limit value (ppmv)	Volatility at 25°C (ppmv)	Air odour threshold (ppmv)
Acetaldehyde	100	g	0.05
Benzene	10	120000	12
Carbon monoxide	50	g	100000
1,2-Dichloroethane	10	110000	88
Dichloromethane	100	550000	250
Ethene	#	g	290
Hydrogen sulfide	10	g	0.008
Propene	#	g	76
Styrene	50	9600	0.3
Vinyl chloride	5	g	3000

No TLV established. g = gas at ambient pressure.

In 1986 the Technische Anleitung-Luft (TA-Luft), containing legislation on the maximal allowable concentrations of pollutants in industrial waste gases, was published in the Federal Republic of Germany (Anonymous, 1986). It is expected that the TA-Luft will form the basis for environmental legislation on air pollution in the European Community in the coming years. In the TA-Luft non-carcinogenic organic chemicals have been classified in three categories based on toxicological and environmental persistence data. If the total emission of compounds belonging to category I is more than 0.1 kg/h the total maximal allowable concentration of these compounds in the waste gas may not exceed 20 mg/m^3. For categories II and III the figures are 2 and 3 kg/h and 100 and 150 mg/m^3, respectively. Chlorinated hydrocarbons included in category I are methyl chloride, chloroform, tetrachloromethane and 1,2-dichloroethane. Category II includes trichloroethene, tetrachloroethene, 1,1,1-trichloroethane, toluene and styrene. Dichloromethane,

alkyl alcohols, alkyl acetates and ketones are in category III.

Carcinogenic compounds are also classified into three categories with respect to the maximal allowable emissions. Most organic carcinogenic compounds belong to category III implying that the maximal allowable concentration of these compounds in industrial waste gases may not exceed 5 mg/m^3 if the total emission exceeds 25 g/h. This category includes acrylonitrile, benzene, 1,3-butadiene, epichlorohydrin, epoxyethane, epoxypropane and vinyl chloride.

A number of measures can be taken to reduce emissions associated with industrial activity to the ambient air. Implementation of process improvements or the introduction of entirely new technologies, e.g. powder based coatings, can result in a significant reduction of the amount of chemicals discharged to the environment. However, in many cases waste gases will still be generated and will require treatment. In some cases a biological method may be the most economic alternative (Kok, 1991).

For an effective biological waste-gas treatment technology aerobic microorganisms capable of degrading the compounds of interest, as well as a suitable reactor, are required. In the following sections general aspects of biodegradation will be discussed as well as aspects of the degradation of specific compounds. Subsequently a short overview of biological waste-gas treatment will be given.

Biodegradation of hydrocarbons

There is general consensus that all natural compounds are biodegradable, i.e. they do not accumulate in nature. This does, however, not mean that all these compounds can be completely degraded by pure cultures of microorganisms, or under aerobic conditions. Nevertheless, many compounds, including xenobiotics, can be biodegraded by pure cultures of aerobic microorganisms.

When considering a specific (xenobiotic) compound in relation to its biodegradability very often terms as readily biodegradable, persistent, or recalcitrant are used (Fewson, 1988). These terms should, however, be used with some caution. An aspect which plays a very important role in this respect is the environment in which the specific compound is present (Fewson, 1988). A compound may be degraded, and may even be utilized as a single source of carbon and energy by a pure culture, in the laboratory at optimal pO_2, pH and nutrient availability, but may be quite recalcitrant in specific environments.

In view of developing stable systems for the removal of specific compounds from waste gases I have defined biodegradable as "capable of supporting aerobic growth of a pure culture as a sole source of carbon and energy". Therefore according to this definition a compound can either be classified as "biodegradable" or as "not yet demonstrated to be biodegradable". In most cases "biodegradable" is therefore synonymous with mineralization, i.e., the compound is completely degraded to carbon dioxide, water, anorganic salts and biomass. Fortuitous oxidation (Stirling

and Dalton, 1979) may play an important role in the degradation of a number of xenobiotics in Nature but will not be discussed here.

In the following two sections I will focus on the biodegradation of unsaturated and chlorinated hydrocarbons, with an emphasis on gaseous and volatile compounds.

BIODEGRADATION OF UNSATURATED HYDROCARBONS

Hydrocarbons containing an unsaturated carbon-carbon bond, may be metabolized by an initial attack on the unsaturated moiety of the molecule or, for compounds other than ethene, by an attack elsewhere on the molecule.

The following reactions may be observed at the carbon-carbon double bond of the molecule: (i) oxidation with hydrogen peroxide and halide ions by haloperoxidases, (ii) oxidation by monooxygenases using molecular oxygen as oxidant, (iii) the addition of water, and (iv) the reduction of the double bond. The addition of ammonia to an unsaturated carbon-carbon bond, e.g. the formation of aspartate from fumarate and ammonia by aspartate ammonia-lyase, will not be discussed.

Oxidation of unsaturated carbon-carbon bonds

Oxidation by haloperoxidases

Haloperoxidases catalyze the formation of α,β-halohydrins from alkenes (Geigert *et al.*, 1983) in the presence of halide ion and hydrogen peroxide. In the absence of halide ions, the action of a chloroperoxidase may also result in the formation of epoxides (Geigert *et al.*, 1986). It is, however, unlikely that these enzymes are involved in the metabolism of short-chain unsaturated hydrocarbons.

Oxidation by monooxygenases

Alkane metabolism has been studied for many years and several times alkenes have been proposed as intermediates in the degradation of long-chain alkanes. This assumption was based on the detection of trace amounts of 1-alkenes after growth with the corresponding alkanes (see Gallo *et al.*, (1973) for references) and the formation of 1-decene from decane by cell-free extracts of *Candida rugosa* (Iizuka *et al.*, 1968). Reduction of NAD^+ in the presence of alkane by crude extracts of *Candida tropicalis* was later shown to be caused by impurities in the alkanes used (Gallo *et al.*, 1973). Arguments against the involvement of 1-alkenes in the degradation pathway of alkanes to primary alcohols and subsequently carboxylic acids are, however, abundant (Klug and Markovetz, 1971) and it is now generally accepted that aerobic degradation of aliphatic hydrocarbons proceeds via an initial oxidation by a monooxygenase yielding primary or secondary alcohols (Britton, 1984).

Recently anaerobic oxidation of hexadecane by a sulfate-reducing bacterium was reported (Aeckersberg *et al.*, 1991) indicating that an alternative mode of initial attack of saturated hydrocarbons is possible. Furthermore desaturation of

hexadecane and several other hydrocarbons by a *Rhodococcus* mutant was reported recently (Takeuchi *et al.*, 1990) indicating that desaturation of saturated hydrocarbons is indeed possible.

The first evidence for oxidation of the double bond of an alkene was the isolation of 1,2-dihydroxyhexadecane from cultures of *Candida lipolytica* growing on 1-hexadecene (Bruyn, 1954). Later it was shown that a large amount of the oxygen in the diol was derived from molecular oxygen (Ishikura and Foster, 1961). Using heptane-grown *Pseudomonas* cells van der Linden (1963) demonstrated the formation of 1,2-epoxyheptane from 1-heptene. Subsequently, many more bacteria were found that possessed monooxygenases capable of epoxidating alkenes (see May (1979) for a review). Although alkane- and alkene-grown cells generally can epoxidate alkenes, reports concerning the metabolism of 1-alkenes with a chain-length longer than C_5 show that the epoxide is not a major intermediate in the degradative pathways, which generally proceed via oxidation of the saturated terminal methyl group. The first reports of an epoxidation reaction actually participating in the complete metabolism of an alkene were on ethene metabolism in *Mycobacterium* E20 (de Bont and Harder, 1978; de Bont *et al.*, 1979).

Hydration of unsaturated carbon-carbon bonds

Hydration of fumarate to malic acid in the citric acid cycle is probably the best known biochemical hydration of a double carbon-carbon bond. A similar hydration reaction was reported by Wallen *et al.* who demonstrated formation of 10-hydroxystearic acid from oleic acid by a *Pseudomonas* sp. (Wallen *et al.*, 1962). Further work by Schoepfer (Schoepfer, 1966) revealed that the 10-hydroxystearic acid produced was optically active and had the R-configuration. In a 3-chloroacrylic acid-degrading bacterium, we have recently detected two 3-chloroacrylic acid hydratases, acting on either the *cis*- or the *trans*-isomer of this acid (Hartmans *et al.*, 1991). A *Pseudomonas picketti* that hydrates acrylic acid directly to 3-hydroxypropionic acid is presently being studied in our laboratory (van der Werf, pers. comm.).

Hydratases acting on alkenes are less well studied. Iida and Iizuka reported the enzymatic conversion of 1-decene to 1-decanol in crude extracts of a decane-grown *Candida rugosa*, although it is not clear if this reaction participates in decane catabolism (Iida and Izuka, 1971). There are two reports in which the anaerobic degradation of aliphatic alkenes is described. In 1-hexadecene degradation by a methanogenic enrichment culture (Schink, 1985) and in the anaerobic degradation of 1-heptadecene by a denitrifying bacterium (Gilewicz, 1991) the corresponding primary alcohols are implicated as intermediates in the catabolic pathway. Biotransformation studies of acyclic terpenoids with *Fusarium solani* revealed that hydration of the inner double bond takes place (Abraham and Arfmann, 1989). Enzymes capable of adding water to the double bond of olefins may therefore exist in nature in larger numbers than anticipated until now.

Reduction of unsaturated carbon-carbon bonds

Apart from the reduction of the 2,3-*trans* double bond in fatty acid synthesis, examples of reductases acting on double bonds are scarce. One example is the hydrogenation of unsaturated fatty acids by anaerobic rumen bacteria (Kellens *et al.*, 1986). Until now there is no evidence that a reduction of unsaturated carbon-carbon bonds is involved in the mineralization of unsaturated hydrocarbons.

SPECIFIC BIODEGRADATION PATHWAYS

Ethene

Ethene (ethylene), the simplest olefin, can be oxidized to epoxyethane by several types of microorganisms, including methanotrophs (Stirling and Dalton, 1979), alkane-utilizers (Hou *et al.*, 1983) and *Nitrosomonas europaea* (Hyman and Wood, 1984). These organisms, however, are not able to grow with ethene, but other bacteria have been isolated which utilize ethene as the sole source of carbon and energy.

Ishikura and Foster (1961) isolated an orange-yellow pigmented "ethylene bacterium" from soil. It was a Gram-positive, motile, non-sporulating rod that was also capable of growth with ethanediol. In 1976 both Heyer and de Bont reported the isolation of several mycobacteria capable of growth with ethene as sole carbon and energy source. Subsequent enrichment cultures with ethene have always resulted in the isolation of mycobacteria.

Using propene or 1-butene as the carbon source in enrichment cultures, van Ginkel *et al.* (1987a) isolated xanthobacters in most cases. These *Xanthobacter* strains, along with *Nocardia* H8 which was isolated with 1-hexene as a carbon source, were all capable of growth with ethene. However, the growth rates of these strains with ethene as substrate were lower than those of the ethene-utilizing mycobacteria. This could explain why mycobacteria have almost always been isolated when ethene is used as carbon source in batch-type enrichment cultures.

Growth of the "ethylene bacterium" on ethene in the presence of labelled oxygen resulted in incorporation of significant amounts of ^{18}O in cell material as compared to growth with acetate under the same conditions (Ishikura and Foster, 1961). This would implicate the involvement of a monooxygenase type of reaction in the assimilation of ethene into cell material. More specific evidence for the involvement of such an enzyme was obtained in studies with *Mycobacterium* E20. In this ethene-utilizing *Mycobacterium* it is possible to accumulate epoxyethane from ethene when whole cells are incubated in the presence of fluoroacetate (de Bont and Harder, 1978), implicating that ethene is metabolized via epoxyethane in a reaction catalyzed by a monooxygenase.

When similarly inhibited cells were incubated with either $^{18}O_2$ or $H_2{}^{18}O$ it was established that the oxygen atom in epoxyethane was indeed derived from molecular oxygen (de Bont *et al.*, 1979). Monooxygenase activity with ethene was confirmed using cell-free extracts of ethene-grown *Mycobacterium* E20. The reaction was

shown to be O_2- and NADH-dependent. NADH could be replaced by NADPH, although this resulted in a lower specific activity.

Whole cells of ethene-grown *Mycobacterium* E20 also oxidized ethane to some extent but this capacity was not present in crude extracts of ethene-grown cells. Ethane-grown cells oxidized ethane and ethene, but it was not possible to detect any monooxygenase activity in crude extracts of these cells. Apparently *Mycobacterium* E20 is capable of synthesizing two different monooxygenases: a soluble alkene monooxygenase during growth on ethene and an unstable alkane monooxygenase during growth on ethane. This dependency of the monooxygenase induction on the growth substrate was further confirmed by determining the enantiomeric composition of the epoxypropane produced by ethene- and ethane-grown cells of *Mycobacterium* E20 (Habets-Crützen *et al.*, 1985). From this study, as well as from the work by Weijers *et al.* (1988b), it appears that alkene monooxygenases form epoxides with a higher optical purity than alkane monooxygenases. Another obvious difference between these two classes of monooxygenases is the substrate specificity. Alkane type monooxygenases can oxidize alkanes and alkenes, whereas alkene monooxygenase only oxidizes alkenes (Fig. 1).

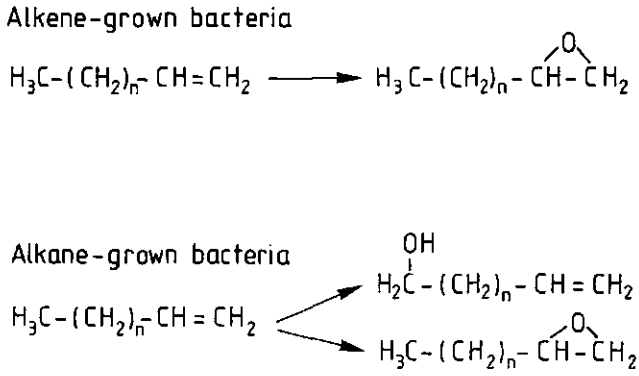

FIG. 1. General modes of oxidative attack of hydrocarbons by alkene- and alkane-grown bacteria.

Although it has been known for some time that microorganisms can oxidize alkenes to the corresponding epoxyalkanes (van der Linden, 1963), very little is known about the further metabolism of these compounds. Microbial metabolism of epoxides has recently been reviewed by Weijers *et al.* (1988a).

The metabolism of epoxyethane, the simplest epoxide, was studied in *Mycobacterium* E20. When ethane-grown *Mycobacterium* E20 was incubated with ethane, in the presence of fluoroacetate, acetate accumulated analogous to epoxyethane accumulation from ethene. This could implicate that epoxyethane, analogous to acetate in ethane metabolism, is metabolized in a CoA-dependent reaction. Cell-free extracts of ethene-grown cells of *Mycobacterium* E20 were able

to catalyze the oxidation of epoxyethane. The reaction was completely dependent upon the presence of NAD^+ and CoA, and the epoxyethane degradation rate was approximately doubled by adding FAD to the reaction mixture (de Bont and Harder, 1978). Besides NAD^+, CoA and FAD, a fourth unknown dissociable cofactor was involved in the enzymic conversion of epoxyethane. This dialysable, heat-stable cofactor was present in ethene-grown cells, but not in ethanol-grown cells. The nature of the unknown cofactor was not elucidated. Evidence that the product of the epoxyethane dehydrogenase reaction was acetyl-CoA was sought in experiments using [^{14}C]epoxyethane. Incubation of cell-free extract with the radioactive epoxide and the required cofactors along with citrate synthase, oxaloacetate and fluorocitrate resulted in radioactivity in ether-extracts which co-chromatographed with citrate. Omission of citrate synthase or oxaloacetate from the complete reaction mixture resulted in almost no radioactivity in the citrate spot. It is not clear whether one single enzyme or an enzyme complex is responsible for the oxidation of epoxyethane and much remains to be elucidated concerning this enzymic activity.

Anaerobic transformation of ethene was recently reported to occur in one anaerobic enrichment culture (de Bruin *et al.*, 1992), as part of the sequential reductive transformation of tetrachloroethene to ethane. Ethanol formation due to hydration of the double-bond does not appear to occur.

Propene and 1-butene

Analogous to the situation with ethene, many bacteria are capable of oxidizing propene to 1,2-epoxypropane (Stirling and Dalton, 1979; Hou *et al.*, 1983). In contrast to ethene, however, propene is an asymmetric molecule with an unsaturated and a saturated carbon-carbon bond, allowing more than one possibility for initial enzymic attack.

With propane-grown cells of *Mycobacterium convolutum* acrylic acid was identified as oxidation product of propene, indicating the initial formation of 3-hydroxy-1-propene (Cerniglia *et al.*, 1976). But with other bacteria grown on propane, epoxypropane was reported as an oxidation product of propene with only trace amounts of 3-hydroxy-1-propene being detected (Hou *et al.*, 1983). With *Pseudomonas fluorescens* NNRL B-1244 the propene consumption rate was 20% higher than the 1,2-epoxypropane formation rate. The 1,2-epoxypropane degradation rate was not reported so that only a minimal ratio of epoxidation versus hydroxylation of 6 can be calculated. Although the presence of two different enzymes could not be ruled out, both hydroxylation and epoxidation of propene to 3-hydroxy-1-propene and 1,2-epoxypropane, respectively, is probably effected by the same enzyme system in both *Pseudomonas fluorescens* NRRL B-1244 and *Brevibacterium* sp. strain CRL56 (Hou *et al.*, 1983). This situation has also been encountered in *Nitrosomonas europaea* (Hyman *et al.*, 1988).

The utilization of propene as a carbon and energy source is less frequently

described. The first report of an organism capable of growth with this gaseous compound was a "Methanbakterium" isolated in 1930 (Tausz and Donath, 1930). Cerniglia *et al.* (1976) also isolated a propene-utilizing organism. They proposed, on the basis of isocitrate lyase activities and the fatty acid composition of their strain PL-1 after growth with different substrates that propene was metabolized via initial attack at the double bond resulting in a C_2 + C_1 cleavage. Experiments with *Mycobacterium* Py1 (de Bont *et al.*, 1980; 1983), and *Xanthobacter* Py2 (van Ginkel and de Bont, 1986) which were both isolated with propene as carbon source, and *Nocardia* By1 and *Xanthobacter* By2 (van Ginkel *et al.*, 1987a) which were enriched with 1-butene, revealed that in these strains 1-alkenes were epoxidized to the corresponding 1,2-epoxyalkanes. In the strains Py1 and Py2, a NADH-dependent propene-monooxygenase activity was detected in crude cell-free extracts. 1,2-Epoxypropane, the product of the monooxygenase reaction, was also utilized as a growth substrate. Both strains can also utilize 1-butene as a growth substrate. In contrast to strain Py1, strain Py2 also grows on ethene although the growth rate is low.

In *Mycobacterium* Py1 isocitrate lyase activity was induced after growth on propene, 1,2-propanediol and acetate, indicating that these substrates are metabolized via acetyl-CoA. Growth on 1-butene and propionic acid did not result in isocitrate lyase induction. These results correspond with the results obtained by Cerniglia *et al.* (1976), but further indications as to how 1,2-epoxypropane is metabolized are still lacking. An analogous reaction as was proposed in ethene-metabolism (de Bont and Harder, 1978) in which epoxyethane is oxidized to acetyl-CoA seems very unlikely in view of the increased isocitrate lyase activities after growth with propene.

Another possibility for the further metabolism of 1,2-epoxypropane in *Mycobacterium* Py1 via 1,2-propanediol and propionaldehyde as was shown in *Nocardia* A60 (de Bont *et al.*, 1982) was ruled out by carrying out simultaneous adaptation experiments with propene- and 1,2-propanediol-grown cells (Hartmans, unpublished results). 1,2-Propanediol metabolism in *Mycobacterium* Py1 proceeds via acetol, which is subsequently cleaved into acetate and formaldehyde by acetol monooxygenase (Hartmans and de Bont, 1986). Although this explains the induction of isocitrate lyase after growth with 1,2-propanediol in *Mycobacterium* Py1, the metabolic pathway of propene degradation via 1,2-epoxypropane to acetyl-CoA still remained to be elucidated. Recently we have, however, detected 1,2-epoxypropane carboxylase activity in extracts of strain Py1. The product of this carboxylation reaction is probably acetoacetic acid which would be in accordance with the induction of isocitrate lyase after growth with propene.

2-Butene

In contrast with ethene, propene and 1-butene, 2-butene is an internal alkene thus possessing only saturated terminal carbon atoms. This difference in chemical

structure is reflected in the ability of organisms to degrade the compound. It is not utilized as a growth substrate by at least six different 1-alkene-utilizers tested, including ethene-, propene-or 1-butene-utilizers (van Ginkel *et al.*, 1987a). Fujii *et al.* (1985), however, have described two mycobacteria which were isolated with propene and 1-butene, respectively, that were also able to grow on the C_2 to C_4 saturated hydrocarbons and both isomers of 2-butene. Both strains grew very poorly with ethene and did not grow with 1,3-butadiene as a carbon source.

Enrichment cultures with *trans*-2-butene as the carbon-source resulted in three bacterial isolates, two strains of the genus *Nocardia* and one *Mycobacterium* (van Ginkel *et al.*, 1987a). One of the *Nocardia* strains, a red-pigmented bacterium designated as *Nocardia* TB1 was chosen to study *trans*-butene degradation (van Ginkel *et al.*, 1987b). Strain TB1 was also capable of growing on the C_3 to C_6 alkanes but did not grow with methane, ethane, 1,3-butadiene or the C_2 to C_6 1-alkenes. Growth with *cis*-butene was extremely slow and doubling times on butane and *trans*-butene were 6 and 30 hours, respectively.

Using arsenite as inhibitor, butyric acid and crotonic acid accumulated when butane- or *trans*-butene-grown cells were incubated with their respective growth substrates.

Surprisingly, *trans*-butene- and butane-grown cells oxidized *trans*-butene at a higher rate than butane, the substrate which supports the faster growth. *cis*-Butene was degraded at the same rate as *trans*-butene.

Based on enzymic activities of *trans*-butene, butane and succinate grown cells a degradative pathway as shown in Fig. 2 was proposed (van Ginkel *et al.*, 1987b).

Although 2,3-epoxybutane was degraded by *trans*-butene-grown cells of strain TB1, it was not considered to be an intermediate in *trans*-butene metabolism as its degradation did not result in an increased CO_2-formation by washed cells, whereas the oxidation of crotonic alcohol and *trans*-butene itself did. Degradation of 2,3-epoxybutane resulted in the excretion of an unidentified product, probably originating from a hydroxylation reaction by the monooxygenase.

Further evidence that *Nocardia* TB1 contains an alkane-type monooxygenase was obtained from the enantiomeric composition of the epoxides formed by both butane- and *trans*-butene grown cells (Weijers *et al.*, 1988b).

Butadiene and isoprene

Isoprene (2-methylbutadiene) is a naturally occurring compound, whereas its non-methylated analogue butadiene, to our knowledge, is not formed biologically (van Ginkel *et al.*, 1987c). Butadiene can be compared to ethene, with respect to the unsaturated character of its carbon atoms, with the two double bonds probably behaving as a conjugated system.

Microbial utilization of butadiene has been reported by Watkinson and Somerville (1976) who isolated a *Nocardia* species from enrichment cultures with butadiene as the sole carbon and energy source. Respiration rates of butadiene-

grown cells with butadiene and 1,2-epoxy-3-butene as substrate were similar. This oxidative capacity was absent from acetate-grown cells. Based on isocitrate lyase activities butadiene metabolism in *Nocardia* sp. 249 was thought to proceed via acetate. The degradation pathway that was proposed for butadiene metabolism was

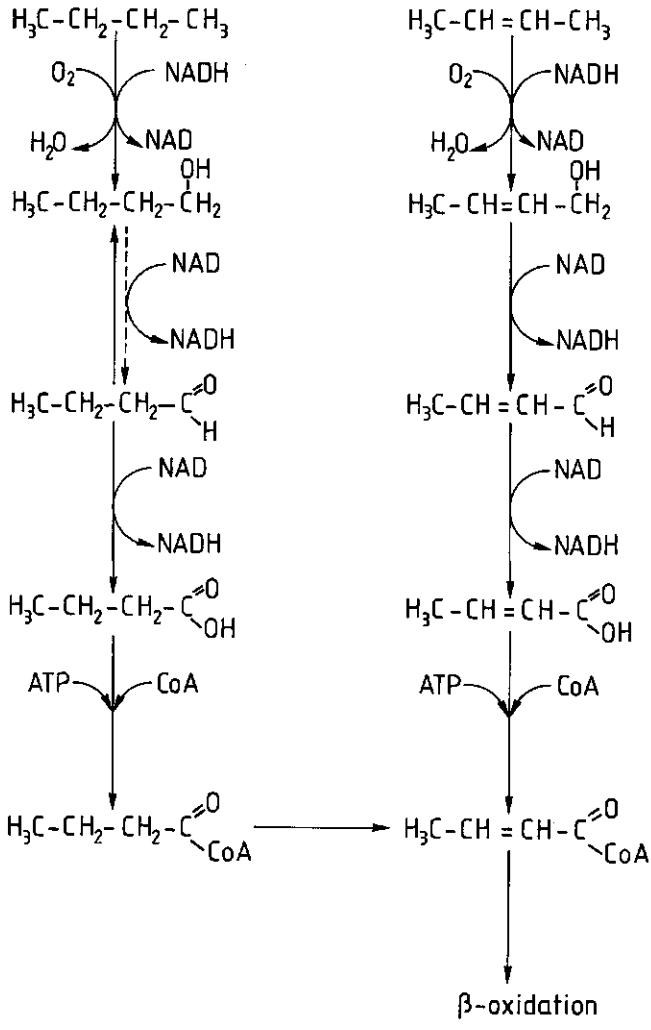

FIG. 2. Proposed degradative pathway of butane and *trans*-butene in *Nocardia* TB1 (van Ginkel *et al.*, 1987b).

very speculative, and not based on the measurement of enzymic activities or the identification of possible intermediates. It was suggested that butadiene is epoxidized to 1,2-epoxy-3-butene, which subsequently would be hydrolyzed to the

corresponding diol and oxidized to 2-oxo-3-butenoic acid. Oxidative decarboxylation to acrylic acid followed by hydration to lactate and oxidation to pyruvate would, after decarboxylation, eventually result in acetate formation. Interestingly, oxidation of racemic 1,2-epoxy-3-butene was not complete, and it was shown that the remaining epoxide material was optically active, thus implicating that the epoxide degrading enzymic activity was stereoselective. Based on this observation it was concluded that the epoxidation of butadiene by the *Nocardia* species is stereospecific (Watkinson and Somerville, 1976).

Enrichments using different soil samples and butadiene or isoprene as a carbon source in all cases resulted in the isolation of pink-pigmented bacteria belonging to the genus *Nocardia* (van Ginkel *et al.*, 1987a,c). All isolates were capable of growth on both substrates, possibly suggesting a connection between the degradation pathway of the two alkadienes. In cell-free extracts of alkadiene-grown *Nocardia* IP1 oxidation of these compounds was NADH- and oxygen-dependent, indicating that these compounds are degraded by a monooxygenase (van Ginkel *et al.*, 1987c). Incubation of washed cell suspensions of alkadiene-grown *Nocardia* TB1 with the respective growth substrates in the presence of 1,2-epoxyalkanes as competitive inhibitors of epoxide degradation, all the possible mono- and diepoxides of butadiene and isoprene could be detected. Although it was proposed that the initial step in the metabolism of both compounds *Nocardia* TB1 probably was the formation of an epoxide, the degradation pathways of these alkadienes remain to be elucidated.

Styrene

Although styrene contains an aromatic nucleus, it can also be classified as a substituted alkene (Fig. 3). Styrene can be oxidized by *Methylosinus trichosporium* OB3b (Higgins *et al.*, 1979) and *Nocardia corallina* B-276 (Furuhashi *et al.*, 1986). In both cases the alkenic moiety of the molecule is attacked, resulting in the formation of styrene oxide (7,8-epoxyethylbenzene or phenyloxirane).

Omori *et al.* (1975) were, to our knowledge, the first to attempt the isolation of styrene-utilizers. They tested 101 soil samples without success, probably because the concentration of styrene that was used in the enrichment cultures was too high (2% w/v). Sielicki *et al.* (1978) using a concentration of 1% (w/v), which is still much more than the solubility of styrene in water, obtained a mixed culture utilizing styrene. In ether extracts from styrene cultures phenylacetate and 2-phenylethanol were identified. Using a pure culture of a styrene-utilizing *Pseudomonas*, Shirai and Hisatsuka (1979) also demonstrated accumulation of 2-phenylethanol from both styrene and styrene oxide. Based on these results it was proposed that styrene oxide is an intermediate in the transformation of styrene to 2-phenylethanol. Baggi *et al.* (1983) isolated phenylacetic acid and 2-hydroxyphenylacetic acid from styrene-grown cultures of a *Pseudomonas fluorescens*, once more indicating that styrene metabolism involves initial attack of the ethylenic bond.

Using low concentrations of styrene we recently demonstrated (Hartmans *et al*, 1990) that styrene utilizers are very abundant. From all samples tested, bacterial strains were isolated that could utilize styrene as a sole carbon and energy source. In several of these strains an oxygen- and NADH-dependent styrene-degrading enzymic activity was present in cell-free extracts after growth with styrene. The further metabolism of the probable oxidation product styrene oxide, was in some cases via phenylacetaldehyde which was formed by a styrene oxide isomerase activity (Hartmans *et al.*, 1989). Reduction or oxidation of the phenylacetaldehyde thus formed could result in the formation of either 2-phenylethanol or phenylacetic acid, the intermediates previously isolated by other groups (Shirai and Hisatsuka, 1979; Baggi *et al.*, 1983).

2-Phenylethanol formation from styrene could also result from a hydration reaction. Indeed recently anaerobic isolates with styrene as sole carbon and energy source have been shown to produce 2-phenylethanol as an intermediate in styrene degradation (Grbić-Galić *et al.*, 1990).

From the above we can conclude that there are at least two different modes of initial attack of styrene involving the alkenic bond.

BIODEGRADATION OF CHLORINATED ALIPHATIC HYDROCARBONS

The biodegradation of chlorinated hydrocarbons has been studied intensively, mainly in the last ten years as it became evident that a number of these compounds appeared to accumulate in the environment, predominantly in groundwater.

TABLE 4. Aerobic biodegradation of common C_1 and C_2 chlorinated hydrocarbons by pure cultures of microorganisms.

	Mineralization	Oxidation
Chloromethane (Methyl chloride)	+	+
Dichloromethane (Methylene chloride)	+	+
Trichloromethane (Chloroform)	−	+
Tetrachloromethane (Carbon tetrachloride)	−	+
Chloroethene (Vinyl chloride)	+	+
1,1-Dichloroethene (Vinylidene chloride)	−	+
Trichloroethene (Tri)	−	+
Tetrachloroethene (Per)	−	−
1,2-Dichloroethane (Ethylene dichloride)	+	+
1,1,1,-Trichloroethane (Methyl chloroform)	−	+

In this introduction I will only describe those aliphatic chlorinated hydrocarbons that are biodegradable under aerobic conditions, i.e. compounds that can be utilized

as growth substrate by a pure culture. Other reviews concerning the biodegradation of chlorinated, including aromatic, hydrocarbons are by Vogel *et al.* (1987), Müller and Lingens (1988), Cook *et al.* (1988), Janssen *et al.* (1989a). For a recent review of the anaerobic degradation of chlorinated aliphatic hydrocarbons I refer to the thesis of Holliger (1992).

Table 4 gives an overview of de biodegradability of the C_1 and C_2 chlorinated hydrocarbons. The compounds that can support growth of pure cultures are further discussed in this introduction.

The most interesting step in the biodegradation of chlorinated aliphatic hydrocarbons is the dehalogenation reaction. Besides reductive dehalogenation mechanisms (Holliger, 1992), hydrolytic and oxidative dehalogenation reactions can be involved in the biodegradation of these compounds.

A number of hydrolytic dehalogenases have been studied. The 2-halocarboxylic acid dehalogenases were the first dehalogenase recognized and mainly studied in relation to the biodegradation of 2,2-dichloropropionic acid, the active ingredient of the herbicide Dalapon. These studies revealed that several classes of 2-halocarboxylic acid dehalogenases could be distinguished on the basis of the stereospecificity with which 2-chloropropionic acid was transformed to 2-hydroxypropionic acid (lactic acid) (Weightman *et al.*, 1982). Two other hydrolytic dehalogenases that have been well studied are dichloromethane dehalogenase (Kohler-Staub and Leisinger, 1985) and 1,2-dichloroethane dehalogenase (Keuning *et al.*, 1985). These enzymes will be discussed below. Longer chain haloalkane dehalogenases have also been reported (Scholtz, 1987; 1988a; Sallis, 1990).

Oxidative dehalogenation (Yokota *et al.*, 1986) is less well studied due to the fact that the enzymes involved are more difficult to purify than the hydrolytic dehalogenases, and also because the dehalogenating capacity probably is a fortuitous property (Stirling and Dalton, 1979).

In the following sections the four compounds shown in Table 4 to support growth of pure cultures will be discussed in more detail.

Methyl chloride

Methyl chloride is the simplest chlorinated hydrocarbon. It is a gas at ambient temperature and pressure and is produced in the chemicals industry mainly for use as a methylating agent. It is, however, also produced in significant amounts in nature, and is therefore a natural compound.

Aerobic degradation of methyl chloride is described in chapter 2 of this thesis. *Hyphomicrobium* MC1 can utilize methyl chloride as sole source of carbon and energy, but the degradation pathway has not been elucidated.

Hydrolytic cleavage of the carbon-chlorine bond yielding methanol, as can be achieved with haloalkane dehalogenase of *Xanthobacter autotrophicus* GJ10 (Janssen *et al.*, 1985) does not occur with strain MC1. This is based on the growth yield of strain MC1, which is higher with methanol than with methyl chloride, and

furthermore, methyl chloride degradation by washed cells of strain MC1 is oxygen dependent. Although *Xanthobacter autotrophicus* GJ10 is capable of growth on methanol and forms the haloalkane dehalogenase constitutively it was reported not to grow on methyl chloride as sole source of carbon and energy (Janssen *et al.*, 1985), possibly because the methanol concentration generated from methyl chloride was too low to induce the methanol biodegradative pathway.

Oxidation of methyl chloride by a methanotroph has been observed (Stirling and Dalton, 1979), and is probably due to the methane monooxygenase activity. Methyl chloride has also been tested as a substrate for mercaptan oxidase of a *Hyphomicrobium* sp., but was not oxidized by this enzyme (Suylen *et al.*, 1987).

Very recently a strict anaerobic bacterium, strain MC, capable of growth with methyl chloride was isolated and characterized (Traunecker *et al.*, 1991). In contrast to the situation for the aerobic degradation of methyl chloride and methanol the growth yield of the strict anaerobe is higher with methyl chloride than with methanol (Traunecker *et al.*, 1991).

Dichloromethane

Dichloromethane was considered to be non-biodegradable for many years (Klečka, 1982). However, starting in 1980 aerobic biodegradation of dichloromethane by activated sludge (Rittman and McCarty, 1980; Klečka, 1982) and by pure cultures (Brunner *et al.*, 1980; Stucki *et al.*, 1981a; Gälli and Leisinger, 1985) was reported. Recently anaerobic biodegradation and utilization as a growth substrate was also reported (Freedman and Gosset, 1991).

Dehalogenation of dichloromethane by the aerobic isolates was shown to be accomplished by a glutathione dependent dehalogenase yielding formaldehyde (Stucki *et al.*, 1981b). Subsequently this inducible dehalogenase was purified from *Hyphomicrobium* DM2 (Kohler-Staub and Leisinger, 1985). In induced cells 15-20% of the soluble protein consists of the dichloromethane dehalogenase. 1,2-Dichloroethane was the only non-dihalomethane of the different substrates tested that was dehalogenated although rate was very low, approximately a 1,000 times lower than dichloromethane. Interestingly the dehalogenase was also induced by 1,1-dichloroethane and 1,2-dichloroethane although these compounds are not dehalogenated at an appreciable rate (Kohler-Staub *et al.*, 1986). The dichloromethane dehalogenases (group A) from several other dichloromethane utilizers isolated from different environments have also been characterized and were shown to have the same immunological properties and identical N-terminal amino acid sequences (Kohler-Staub *et al.*, 1986). This could be an indication that this enzyme has evolved only very recently.

A novel dichloromethane dehalogenase (group B) has recently been characterized (Sholtz *et al.*, 1988b) which had only one of the 15 amino acids of the N-terminus in the same position as in the group A enzyme. The group B enzyme has a higher specific activity than the group A enzyme but represents a lower fraction of total

protein when compared with organisms having the group A enzyme. Nevertheless, the new isolate grew at a higher rate (0.22 h^{-1}) with dichloromethane than the strains with the group A enzyme (0.07 to 0.10 h^{-1}).

Although several plasmids were detected in the dichloromethane-utilizing *Methylobacterium* DM4 the dichloromethane-utilization genes were shown to be located on the chromosome or on a megaplasmid (Gälli and Leisinger, 1988). The structural (*dcmA*) and regulatory (*dcmR*) genes for dichloromethane dehalogenase from strain DM4 have now been isolated and analyzed (La Roche and Leisinger, 1990;1991). These studies revealed a relationship between the deduced amino acid sequences of the glutathione dependent dichloromethane dehalogenase and eucaryotic glutathione *S*-transferases, indicating that *dcmA* is a member of the glutathione *S*-transferase supergene family (La Roche and Leisinger, 1990). A tentative mechanism for the regulation of dehalogenase synthesis was proposed in which it is assumed that the *dcmR* gene encodes a repressor which binds to the promoter region of the *dcmA* gene in the absence of dichloromethane (La Roche and Leisinger, 1991).

1,2-Dichloroethane

Biological transformation of 1,2-dichloroethane by activated sludge was already reported in 1954 (at the Purdue Conference). Attempts to isolate pure cultures capable of degrading 1,2-dichloroethane were initially unsuccessful (Stucki *et al.*, 1981a). In 1984 Janssen *et al.* described the isolation of a bacterial strain (GJ10) capable of growth with 1,2-dichloroethane. This isolate was subsequently identified as a *Xanthobacter autotrophicus* (Janssen *et al.*, 1985). This strain constitutively produces two dehalogenases, one specific for haloalkanes and one specific for halogenated carboxylic acids. Degradation of 1,2-dichloroethane is initiated by haloalkane dehalogenase yielding chloroethanol, which subsequently is oxidized to chloroacetaldehyde and chloroacetic acid (Janssen *et al.*, 1985). Dehalogenation of 2-chloroacetic acid by the second dehalogenase yields glycollic acid. The haloalkane dehalogenase has been purified and characterized (Keuning *et al.*, 1985). The enzyme exhibits dehalogenase activity with quite a number of different halogenated alkanes but not with the common groundwater contaminant 1,2-dichloropropane. The K_M of the purified enzyme for 1,2-dichloroethane has been determined at 1.1 mM (Keuning *et al.*, 1985) and 0.63 mM (van den Wijngaard *et al.*, 1991). This is rather high in view of a possible application in bioremediation. Interestingly new 1,2-dichloroethane-utilizing strains of *Ancylobacter aquaticus* were described recently which contain the same dehalogenase although at higher levels (van den Wijngaard *et al.*, 1992). This resulted in an apparent K_S value for one of these new strains for 1,2-dichloroethane of 0.029 mM, compared with 0.26 mM for strain GJ10 (van den Wijngaard *et al.*, 1991).

The structural gene of the haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10, *dhl*A, has been isolated and sequenced (Janssen *et al.*, 1989b).

The dehalogenase sequence did not show any overall similarity to other proteins although a short stretch is significantly similar to several mammalian epoxide hydrolases (Janssen *et al.*, 1989b). Just recently it was shown that the structural gene of the dehalogenase in *Xanthobacter autotrophicus* GJ10 is plasmid encoded (Tardiff *et al.*, 1991). This possibly explains why other bacteria have been isolated with the same dehalogenase (van den Wijngaard *et al.*, 1992).

Vinyl chloride

Aerobic vinyl chloride transformation has been observed with a wide range of microorganisms exhibiting monooxygenase activity. Methane- (Fogel *et al.*, 1986; Tsien *et al.*, 1989), propane- (Wackett *et al.*, 1989), isoprene- (Ewers *et al.*, 1990) and ammonia- (Vannelli *et al.*, 1990) utilizing bacteria have been shown to oxidize vinyl chloride but no oxidation products were identified. Using purified soluble methane monooxygenase from *Methylosinus trichosporium* OB3b the oxidation product of vinyl chloride was identified as chlorooxirane (Fox *et al.*, 1990).

Aerobic mineralization of vinyl chloride in groundwater (Davis and Carpenter, 1990) and by a Gram-positive propane-grown bacterium (Phelps *et al.*, 1991) has been reported recently. To our knowledge, however, *Mycobacterium* strain L1 (Hartmans *et al.*, 1985) is the only bacterial strain described so far which utilizes vinyl chloride aerobically as a sole source of carbon and energy. *Mycobacterium* strain L1 was isolated from soil that had been contaminated with vinyl chloride containing water for a number of years. Subsequently three additional strains were isolated, all *Mycobacterium aurum* species (see Chapter 3). Metabolism of vinyl chloride in strain L1 is also initiated by a monooxygenase (see Chapters 3 and 4), but the fate of the chlorooxirane during growth of strain L1 on vinyl chloride is still unknown.

BIOLOGICAL WASTE-GAS TREATMENT

There are a number of approaches which can be followed to reduce the emission of chemicals to the ambient air. Preferably a reduction in the emissions is achieved by process improvement or the development of alternative processes.

If this is not possible there are two fundamentally different approaches to treat waste gases, either recovery or oxidation of the contaminants (Table 5).

For reviews on physical and physico-chemical waste-gas treatment techniques, also in relation to biological waste-gas treatment, see Heck *et al.* (1988) and Diks and Ottengraf (1991).

Recovery processes are generally only applicable for waste gases containing one or only a limited number of compounds, preferably at relatively high concentrations. However, due to new developments in membrane technology the number of potential applications for the recovery of organic vapours by selective membrane permeation is increasing (Paul *et al.*, 1988; Kimmerle *et al.*, 1988).

More complex mixtures can generally most economically be oxidized. Incineration

is often the most economic alternative for waste gases with high concentrations of combustible contaminants. For waste gases with lower concentrations adsorption on activated carbon and biological oxidation are often claimed as the most economic alternatives (Diks and Ottengraf, 1991; Kok, 1991).

TABLE 5. Waste-gas treatment technologies.

Recovery processes	Oxidation processes
Condensation	Incineration
Activated carbon	Catalytic oxidation
Absorption	Activated carbon
Membrane permeation	Biological oxidation

Although biological waste-gas treatment is often claimed to be the cheapest technology, it is not applied as much as one could expect based on this claim. Presumably this is because besides the costs involved, also the long-term reliability of the process plays an important role in the ultimate selection of a technology.

In this respect it is a pity that the majority of the publications concerning biological waste-gas treatment do not mention the duration of the experiments described. In the following I will briefly discus the different bioreactors that are presently applied in waste-gas treatment. I also refer to the excellent reviews by Brauer (1984), Ottengraf (1986;1987) and Diks and Ottengraf (1991).

Biofilter

A biofilter is essentially a packed bed reactor through which the waste gas is forced. The first biofilters were actually soil beds of variable height and were applied to treat the odorous air from wastewater treatment plants. In the subsequent development of the biofilter attention focused on the packing material and humidity control. The choice of packing material is very important in view of the pressure drop over the filter. Composted bark mixed with compost and a mixture of compost and polystyrene particles are the two packing materials now applied most often. These packing materials are generally applied in closed biofilters allowing a better control of the physical conditions within the biofilter, especially the humidity. Humidity control is a very important factor as this parameter very strongly affects biological activity. Usually the air is saturated with water before it enters the biofilter. However, if biodegradation takes place the air leaving the filter will have a slightly higher temperature due to the oxidation (= combustion) of the contaminants, and consequently will no longer be saturated with water. Therefore additional humidification control is required. A new development in this respect is the operation of the biofilter in the down-flow mode (van Lith, see

Hartmans, 1990). In this way the water is applied on to the top of the packing material where, due to the relatively high inlet concentrations of contaminants, most of the heat formation and water evaporation take place.

Biofilters are successfully applied on a large scale for the removal of odour (Leson and Winer, 1991). The actual degradation rates taking place in the packing material are, however, fairly low. Although elimination rates as high as 64 g carbon m^{-3} packing material per hour have been reported (Ottengraf *et al.*, 1986), these high rates can not be maintained for a long period of time. Maintaining a sufficient humidity will become a problem and nutrients in the compost will probably become exhausted. The effect of the nutrient content of the compost in a biofilter on the elimination capacity of a mixture of solvents was studied by Beyreitz *et al.* (1989). These experiments lasted for about one year. The packing material used was bark mixed with a nutrient-rich compost or a nutrient-poor compost. Elimination rates of about 35 and 25 g C m^{-3} h^{-1} were determined, respectively, thus illustrating the effect of the nutrient levels. We have observed a similar effect in styrene elimination with a compost-polystyrene biofilter. Initially 55 g styrene m^{-3} h^{-1} was eliminated. However, after several days the elimination dropped significantly, probably due to a lack of available nutrients.

Beyreitz *et al.*, (1989) also compared a bioscrubber using the same waste gas as they used in the biofilter study mentioned above. The percentage of the solvents removed was somewhat lower, especially for those compounds with a lower water solubility.

Based on these, and other data, it is clear that the biofilter is especially effective for the treatment of waste gases with relatively low concentrations of contaminants, which need not necessarily be very water soluble.

Other bioreactors

For the elimination of higher concentrations of contaminants, especially contaminants with a higher water solubility, or rather a low Henry coefficient, bioscrubbers appear to be more efficient. Bioscrubbers essentially comprise of a liquid scrubber in combination with a regeneration tank, which can be compared with a traditional aerobic wastewater treatment facility. The water phase which is circulated can contain high concentrations of suspended biomass.

Very similar to the bioscrubber with suspended biomass is the trickle-bed bioreactor or trickling filter. It is essentially the same as the trickling filters already being applied in wastewater treatment. In the wastewater treatment applictaion the water flows over a packed bed covered with a biofilm which degrades the contaminants. Oxygen is supplied by diffusion through the flowing water layer and by natural convection of air through the filter due to the temperature gradient over the filter (Grady, 1983). For the treatment of waste gas the liquid phase is circulated. In the trickle-bed bioreactor the environment of the biofilm can be controlled to some extent by the circulating water phase. Nutrients can be supplied

and the pH can be controlled. Therefore, in contrast to the biofilter, it is possible to treat waste gases containing compounds like ammonia and chlorinated hydrocarbons which result in acid production upon biodegradation. As an example dichloromethane elimination has been demonstrated (Chapter 9; Diks, 1992). Dichloromethane is reasonably water soluble, and was shown to be eliminated with a sufficient efficiency over a long period of time.

The trickle-bed bioreactor is, however, less effective in the removal of compounds with a higher Henry coefficient i.e. a lower water solubility. In view of this aspect we are now studying a membrane bioreactor. In this reactor type a porous hydrophobic membrane is used to create a large specific interface with a low mass-transfer resistance (Hartmans, 1991; Hartmans *et al.*, 1992). In this reactor configuration it should be possible to combine the advantages of the low mass-transfer resistance and high specific surface area of the biofilter with the possibility to control the environment of the biofilm via the circulating water phase like in the trickle-bed bioreactor.

Scope of this thesis

Initially the subject of research for this thesis was to study vinyl chloride biodegradation and to develop a process for the biological removal of vinyl chloride from waste gases of a PVC production facility. As it became evident that a process using the available vinyl chloride-degrading bacterium was not a very realistic option, the scope of the research was slightly expanded. The biodegradation of other chlorinated and unsaturated hydrocarbons was studied to expand our understanding of the aerobic biodegradative potential of microorganisms. Furthermore, a bioreactor was developed for the treatment of air contaminated with dichloromethane.

Chapter 2 describes the isolation and initial characterization of a *Hyphomicrobium* species which grows on the simplest chlorinated hydrocarbon methyl chloride. In Chapter 3 the initial step of the vinyl chloride degradation pathway in *Mycobacterium aurum* L1 is described. Initial characterization of alkene monooxygenase, the enzyme that also catalyzes the oxidation of vinyl chloride is described in Chapter 4. Alkene monooxygenase was shown to be a multicomponent enzyme. In Chapter 5 a method is described to isolate mutants which are no longer capable of growth on alkenes by using vinyl chloride as a specific mutagen.

Chapters 6 and 7 describe the isolation of bacteria which degrade compounds which are structurally related to vinyl chloride. 3-Chloroacrylic acid (Fig. 3), e.g. vinyl chloride substituted with a carboxyl group, was shown to be degraded by an initial hydration reaction resulting in dechlorination (Chapter 6). Styrene (vinyl benzene), which can be viewed as ethene substituted with the bulky phenyl group analogous to the chlorine group in vinyl chloride (Fig. 3), was shown to be oxidized by a monooxygenase (Chapter 7). Styrene monooxygenase was quite different from alkene monooxygenase. Neither the chloroacrylic acid- nor the styrene-utilizing

bacteria studied could, however, transform vinyl chloride.

In Chapter 8 the possibilities of biological removal of vinyl chloride and 1,2-dichloroethane (the vinyl chloride precursor in the major production process) from waste gas using *M. aurum* L1 and *Xanthobacter autotrophicus* GJ10 are studied. Based on the experimental results such a process does not seem very promising. Chapter 9 describes the trickle-bed bioreactor for the removal of dichloromethane from waste gases. Finally in Chapter 10 a few general conclusions are discussed.

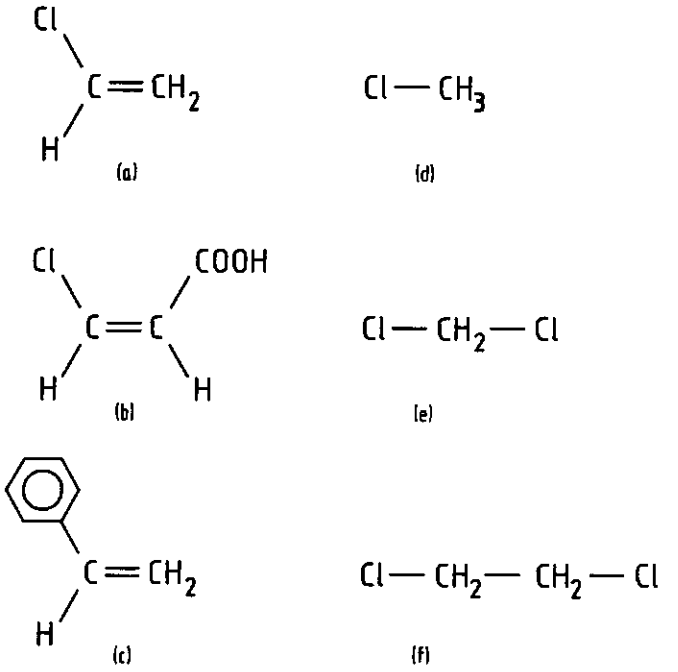

FIG. 3. Chemical structures of the compounds discussed in this thesis; (a) vinyl chloride; (b) *trans*-3-chloroacrylic acid; (c) styrene; (d) methyl chloride; (e) dichloromethane; (f) 1,2-dichloroethane.

REFERENCES

Abraham, W.-R., and H.-A. Arfmann. 1989. Addition of water to acyclic terpenoids by *Fusarium solani*. Appl. Microbiol. Biotechnol. 32:295-298.

Aeckersberg, F., F. Bak, and F. Widdel. 1991. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. Arch. Microbiol. 156:5-14.

Amoore, J.E., and E. Hautala. 1983. Odor as an aid to chemical safety: odor thresholds compared with threshold limit values and volatilities for 214 industrial chemicals in air and water dilution. J. Appl. Toxicol. 3:272-290.

Anonymous. 1986. Erste Allgemeine Verwaltungvorschrift zum Bundes-Immissionsschutzgesetz (Technische Anleitung zur Reinhaltung der Luft - TA Luft) vom 27-2-1986. Gemeinsames Ministerialblatt 37:95-144.

Anonymous. 1991. Nationale Milieuverkenning 2 1991-2010, Samson H.D. Tjeenk Willink, Alphen aan

den Rijn, The Netherlands (In Dutch).
Baggi, G., M.M. Boga, D. Catelani, E. Galli, and V. Treccani. 1983. Styrene catabolism by a strain of *Pseudomonas fluorescens*. System. Appl. Microbiol. **4**:141-147.
Beyreitz, G., R. Hübner and M. Saake. 1989. Biotechnologische behandelung Lösemittelhaltige Abluft. WLB Wasser Luft und Boden **9**:53-57.
de Bont, J.A.M., and W. Harder. 1978. Metabolism of ethylene by *Mycobacterium* E20. FEMS Microbiol. Lett. **3**:89-93.
de Bont, J.A.M., M.M. Attwood, S.B. Primrose, and W. Harder. 1979. Epoxidation of short chain alkenes in *Mycobacterium* E20: the involvement of a specific mono-oxygenase. FEMS Microbiol. Lett. **6**:183-188.
de Bont, J.A.M. 1976. Oxidation of ethylene by soil bacteria. Antonie van Leeuwenhoek **42**:59-71.
de Bont, J.A.M., S.B. Primrose, M.D. Collins, and D. Jones. 1980. Chemical studies on some bacteria which utilize gaseous unsaturated hydrocarbons. J. Gen. Microbiol. **117**:97-102.
de Bont, J.A.M., C.G. van Ginkel, J. Tramper, and K.C.A.M. Luyben. 1983. Ethylene oxide production by immobilized *Mycobacterium* Py1 in a gas/solid bioreactor. Enzyme Microb. Technol. **5**:55-60.
de Bont, J.A.M., J.P. van Dijken, and C.G. van Ginkel. 1982. The metabolism of 1,2-propanediol by the propylene oxide utilizing bacterium *Nocardia* A60. Biochim. Biophys. Acta **714**:465-470.
Brauer, H. 1984. Biologische Abluftreinigung. Chem. Ing. Tech. **56**:279-286.
Britton, L.N. 1984. Microbial degradation of aliphatic hydrocarbons, p. 89-129. *In* D.T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, New York.
de Bruin, W.P., M.J.J. Kotterman, M.A. Posthumus, G. Schraa, and A.J.B. Zehnder. 1992. Complete biological reductive transformation of tetrachloroethene to ethane. Appl. Environ. Microbiol. **58**:1996-2000.
Brunner, W., D. Staub, and T. Leisinger. 1980. Bacterial degradation of dichloromethane. Appl. Environ. Microbiol. **40**:950-958.
Bruyn, J. 1954. An intermediate in the oxidation of hexadecene-1 by *Candida lipolytica*. Koninkl. Akad. Wetenschap. Proc. Ser. C. **57**:41-45.
Cerniglia, C.E., W.T. Blevins, and J.J. Perry. 1976. Microbial oxidation and assimilation of propylene. Appl. Environ. Microbiol. **32**:764-768.
Cook, A.M., R. Scholtz, and T. Leisinger. 1988. Mikrobielle Abbau von halogenierten aliphatischen Verbindungen. GWF Wasser Abwasser **129**:61-69.
Davis, J.W., and C.L. Carpenter. 1990. Aerobic biodegradation of vinyl chloride in groundwater samples. Appl. Environ. Microbiol. **56**:3878-3880.
Diks, R.M.M., and S.P.P. Ottengraf. 1991. Process engineering aspects of biological waste gas purification. p. 353-367. *In* H. Verachtert and W. Verstraete (ed.), Environmental Biotechnology 1991. Royal Flemish Society of Engineers.
Diks, R.M.M. 1992. The removal of dichloromethane from waste gases in a biological trickling filter. Thesis Technical University of Eindhoven.
Ewers, J., D. Freier-Schröder, and H.-J. Knackmuss. 1990. Selection of trichloroethene (TCE) degrading bacteria that resist inactivation by TCE. Arch. Microbiol. **154**:410-413.
Fogel, M.M., A.R. Taddeo, and S. Fogel. 1986. Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. Appl. Environ. Microbiol. **51**:720-724.
Fox, B.G., J.G. Borneman, L.P. Wackett, and J.D. Lipscomb. 1990. Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: Mechanistic and environmental implications. Biochemistry **29**:6419-6427.
Freedman, D.L., and J.M. Gossett. 1991. Biodegradation of dichloromethane and its utilization as a growth substrate under methanogenic conditions. Appl. Environ. Microbiol. **57**:2847-2857.
Fujii, T., T. Ogawa, and H. Fukuda. 1985. A screening system for microbes which produce olefin hydrocarbons. Agric. Biol. Chem. **49**:651-657.
Furuhashi, K., M. Shintani, and M. Takagi. 1986. Effects of solvents on the production of epoxides by *Nocardia corallina* B-276. Appl. Microbiol. Biotechnol. **23**:218-223.
Gälli, R., and T. Leisinger. 1985. Specialized bacterial strains for the removal of dichloromethane from industrial waste. Conserv. Recycl. **8**:91-100.

Gälli, R., and T. Leisinger. 1988. Plasmid analysis and cloning of the dichloromethane-utilization genes of *Methylobacterium* sp. DM4. J. Gen. Microbiol. **134**:943-952.

Gallo, M., J.C. Bertrand, B. Roche, and E. Azoulay. 1973. Alkane oxidation in *Candida tropicalis*. Biochim. Biophys. Acta **296**:624-638.

Geigert, J., S.L. Neidleman, D.J. Dalietos, and S.K. DeWitt. 1983. Haloperoxidases: enzymatic synthesis of α,β-halohydrins from gaseous alkenes. Appl. Environ. Microbiol. **45**:366-374.

Geigert, J., T.D. Lee, D.J. Dalietos, D.S., Hirano, and S.L. Neidleman. 1986. Epoxidation of alkenes by chloroperoxidase catalysis. Biochem. Biophys. Res. Comm. **136**:778-782.

Gilewicz, M., G. Monpert, M. Acquaviva, G. Mille, and J.-C. Bertrand. 1991. Anaerobic oxidation of 1-*n*-heptadecene by a marine denitrifying bacterium. Appl. Microbiol. Biotechnol. **36**:252-256.

van Ginkel, C.G., and J.A.M. de Bont. 1986. Isolation and characterization of alkene-utilizing *Xanthobacter* spp. Arch. Microbiol. **145**:403-407.

van Ginkel, C.G., H.G.J. Welten, and J.A.M. de Bont. 1987a. Oxidation of gaseous and volatile hydrocarbons by selected alkene-utilizing bacteria. Appl. Environ. Microbiol. **53**:2903-2907.

van Ginkel, C.G., H.G.J. Welten, S. Hartmans, and J.A.M. de Bont. 1987b. Metabolism of *trans*-2-butene and butane in *Nocardia* TB1. J. Gen. Microbiol. **133**:1713-1720.

van Ginkel, C.G., E. de Jong, J.W.R. Tilanus, and J.A.M. de Bont. 1987c. Microbial oxidation of isoprene, a biogenic foliage volatile and of 1,3-butadiene, an anthropogenic gas. FEMS Microbiol. Ecol. **45**:275-279.

Gossett, J.M. 1987. Measurement of Henry's law constant for C_1 and C_2 chlorinated hydrocarbons. Environ. Sci. Technol. **21**:202-208.

Grady, C.P.L. 1983. Modeling of biological fixed films - a state-of-the-art review. *In* Y.C Wu and E.D. Smith (ed). Fixed-film biological processes for wastewater treatment. Noyes data corporation, Park Ridge, NJ.

Grbić-Galić, D., N. Churchman-Eisel, and I. Mraković. 1990. Microbial transformation of styrene by anaerobic consortia. J. Appl. Bacteriol. **69**:247-260.

Habets-Crützen, A.Q.H., S.J.N. Carlier, J.A.M. de Bont, D. Wistuba, V. Schurig, S. Hartmans, and J. Tramper. 1985. Stereospecific formation of 1,2-epoxypropane, 1,2-epoxybutane and 1-chloro-2,3-epoxypropane by alkene-utilizing bacteria. Enzyme Microb. Technol. **7**:17-21.

Hartmans, S., J.A.M. de Bont, J. Tramper, K.Ch.A.M. Luyben. 1985. Bacterial degradation of vinyl chloride. Biotechnol. Lett. **7**:383-388.

Hartmans, S., and J.A.M. de Bont. 1986. Acetol monooxygenase from *Mycobacterium* Py1 cleaves acetol into acetate and formaldehyde. FEMS Microbiol. Lett. **36**:155-158.

Hartmans, S., J.P. Smits, M.J. van der Werf, F. Volkering and J.A.M. de Bont. 1989. Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. Appl. Environ. Microbiol. **55**:2850-2855.

Hartmans, S., M.J. van der Werf, and J.A.M. de Bont. 1990. Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. Appl. Environ. Microbiol. **56**:1347-1351.

Hartmans, S. 1990. Biofiltration of nauseous gases, p. 1160-1165. *In* C. Christiansen, L. Munck, and J. Villadsen (ed.), Proceedings 5th European Congress on Biotechnology. Munksgaard, Copenhagen.

Hartmans, S. 1991. Biological waste-gas treatment. Paper presented at 4th International IGT symposium on Gas, Oil and Environmental Biotechnology, Colorado Springs, December 1991.

Hartmans, S., M.W. Jansen, M.J. van der Werf, and J.A.M. de Bont. 1991. Bacterial metabolism of 3-chloroacrylic acid. J. Gen. Microbiol. **137**:2025-2032.

Hartmans, S., E.J.T.M. Leenen, and G.T.H. Voskuilen. 1992. Membrane bioreactor with porous hydrophobic membranes for waste-gas treatment, p. 103-106. *In* A.J. Dragt and J. van Ham (ed.), Biotechniques for Air Pollution Abatement and Odour Control Policies, Elsevier Science Publishers, Amsterdam.

Heck, G., G. Müller und M. Ulrich. 1988. Reinigung lösungsmittelhaltiger Abluft: alternative mögelichkeiten. Chem. Ing. Tech. **60**:286-297.

Heyer, J. 1976. Mikrobiëlle Verwertung von Athylen. Z. Allg. Mikrobiol. **16**:633-637.

Higgins, I.J., R.C. Hammond, F.S. Sariaslani, D. Best, M.M. Davies, S.E Tryhorn, S.E. and F. Taylor. 1979. Biotransformation of hydrocarbons and related compounds by whole organism suspensions of methane-grown *Methylosinus trichosporium* OB3b. Biochem. Biophys. Res. Comm. **89**:671-677.

Holliger, H.C. 1992. Reductive dehalogenation by anaerobic bacteria. Thesis Wageningen Agricultural University.

Hou, C.T., R. Patel, A.I. Laskin, N. Barnabe, and I. Barist. 1983. Epoxidation of short-chain alkenes by resting-cell suspensions of propane-grown bacteria. Appl. Environ. Microbiol. **46**:171-177.

van den Hout, K.D. 1990. Industriële emissies in Nederland: derde inventarisatieronde 1985 t/m 1987. Publikatiesreeks Emissieregisttratie Nr. 1.

Hyman, M.R. and Wood, P.M. 1984. Ethylene oxidation by *Nitrosomonas europaea*. Arch. Microbiol. **137**:155-158.

Hyman, M.R., I.B. Murton, and D.J. Arp. 1988. Interaction of ammonia monooxygenase from *Nitrosomonas europaea* with alkanes, alkenes and alkynes. Appl. Environ. Microbiol. **54**:3187-3190.

Iida, M., and H. Iizuka. 1971. Enzymatic conversion of 1-decene to decyl alcohol by *Candida rugosa* JF 101. Z. Alg. Mikrobiol. **11**:301-305.

Iizuka, H., M. Iida, Y. Unami, and Y. Hoshino. 1968. *n*-Decane dehydrogenation by a cell-free extract of *Candida rugosa*. Z. Alg. Mikrobiol. **8**:145-149.

Ishikura, T and J.W. Foster. 1961. Incorporation of molecular oxygen during microbial utilization of olefins. Nature **192**:892-893.

Janssen, D.B., A. Scheper, and B. Witholt. 1984. Biodegradation of 2-chloroethanol and 1,2-dichloroethane by pure bacterial cultures, p. 169-178. *In* E.H. Houwink and R.R. van der Meer (ed.), Innovations in biotechnology. Progress in industrial microbiology, vol 20. Elsevier Science Publishers, Amsterdam.

Janssen, D.B., A. Scheper, L. Dijkhuizen, and B. Witholt. 1985. Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. Appl. Environ. Microbiol. **49**:673-677.

Janssen, D.B., R. Oldenhuis, and A.J. van den Wijngaard. 1989a. Hydrolytic and oxidative degradation of chlorinated aliphatic compunds by aerobic microorganisms, p. 105-128. *In* D. Kamely, A. Chakrabarty, and G. S. Omenn (ed.), Advances in applied biotechnology series, Vol. 4, Biotechnology and biodegradation. Gulf Publishing Company, Houston.

Janssen, D.B., F. Pries, J. van der Ploeg, B. Kazemier, P. Terpstra and B. Witholt. 1989b. Cloning of 1,2-dichloroethane degradation genes of *Xanthobacter autotrophicus* GJ10 and expression and sequencing of the *dhlA* gene. J. Bacteriol. **171**:6791-6799.

Kellens, M.J., H.L. Goderis, and P.P. Tobback. 1986. Biohydrogenation of unsaturated fatty acids by a mixed culture of rumen microorganisms. Biotechnol. Bioeng. **28**:1268-1276.

Keuning S., D.B. Janssen, and B. Witholt. 1985. Purification and characterization of hydrolytic haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. J. Bacteriol. **163**:635-639.

Kimmerle, K., C.M. Bell, W. Gudernatsch and H. Chmiel. 1988. Solvent recovery from air. J. Membrane Sci. **36**:477-488.

Klečka, G.M.. 1982. Fate and effects of methylene chloride in activated sludge. Appl. Environ. Microbiol. **44**:701-707.

Klug, M.J. and A.J. Markovetz. 1971. Utilization of hydrocarbons by micro-organisms. Adv. Microbiol. Physiol. **5**:1-39.

Kohler-Staub, D., and T. Leisinger. 1985. Dichloromethane dehalogenase of *Hyphomicrobium* sp. strain DM2. J. Bacteriol. **162**:676-681.

Kohler-Staub, D., S. Hartmans, R. Gälli, F. Suter, and T. Leisinger. 1986. Evidence for identical dichloromethane dehalogenases in different methylotrophic bacteria. J. Gen. Microbiol. **132**:2837-2843.

Kok, H.J.G. 1992. Bioscrubbing of air contaminated with high concentrations of hydrocarbons. p. 77-82. *In* A.J. Dragt and J. van Ham (ed.), Biotechniques for Air Pollution Abatement and Odour Control Policies, Elsevier Science Publishers, Amsterdam.

La Roche, S.D., and T. Leisinger. 1990. Sequence analysis and expression of the bacterial dichloromethane dehalogenase structural gene, a member of the glutathione *S*-transferase supergene family. J. Bacteriol. **172**:164-171.

La Roche, S.D., and T. Leisinger. 1991. Identification of *dcmR*, the regulatory gene governing expression of dichloromethane dehalogenase in *Methylobacterium* sp. strain DM4. J. Bacteriol. **173**:6714-6721.

van der Linden, A.C. 1963. Epoxidation of α-olefins by heptane-grown *Pseudomonas* cells. Biochim. Biophys. Acta **77**:157-159.

Leson, G., and A.M. Winer. 1991. Biofiltration: an innovative air pollution control technology for VOC emissions. J. Air Waste Manage. Assoc. **41**:1045-1054.

May, S.W. 1979. Enzymatic epoxidation reactions. Enzyme Microb. Technol. **1**:15-22.

Müller, R., and F. Lingens. 1988. Der mikrobielle Abbau von chlorierten Kohlenwasserstoffen. GWF Wasser Abwasser **129**:55-60.

Omori, T., Y. Jigami, and Y. Minoda. 1975. Isolation, identification and substrate assimilation specifity of some aromatic hydrocarbon-utilizing bacteria. Agric. Biol. Chem. **39**:1775-1779.

Ottengraf, S.P.P., J.J.P. Meesters, A.H.C. van den Oever, and H.R. Rozema. 1986. Biological elimination of volatile xenobiotic compounds in biofilters. Bioprocess Eng. **1**:61-69.

Ottengraf, S.P.P. 1986. Exhaust gas purification. *In* H.J. Rehm, and G. Reed (ed.), Biotechnology vol. 8. Microbial degradations. Chemie Verlag, Weinheim, Germany.

Ottengraf, S.P.P. 1987. Biological systems for waste gas elimination. Trends in Biotechnol. **5**:132-136.

Paul, H., C. Philipsen, F.J. Gerner and H. Strathmann. 1988. Removal of organic vapors from air by selective membrane permeation. J. Membrane Sci. **36**:363-372.

Phelps, T.J., K. Malachowsky, R.M. Schram, and D.C. White. 1991. Aerobic mineralization of vinyl chloride by a bacterium of the order *Actinomycetales*. Appl. Environ. Microbiol. **57**:1252-1254.

Rittmann, B.E., and P.L. McCarty. 1980. Utilization of dichloromethane by suspended and fixed-film bacteria. Appl. Environ. Microbiol. **39**:1225-1226.

Sallis, P.J., S.J. Armfield, A.T. Bull, and D.J. Hardman. 1990. Isolation and characterization of a haloalkane halidohydrolase from *Rhodococcus erythropolis* Y2. J. Gen. Microbiol. **136**:115-120.

Schink, B. 1985. Degradation of unsaturated hydrocarbons by methanogenic enrichment cultures. FEMS Microbiol. Ecol. **31**:69-77.

Schoepfer G.J. 1966. Stereospecific conversion of oleic acid to 10-hydroxystearic acid. J. Biol. Chem. **241**:5441-5447.

Scholtz, R., T. Leisinger, F. Suter, and A.M. Cook. 1987. Characterization of 1-chlorohexane halidohydrolase, a dehalogenase of wide substrate range from an *Arthrobacter* sp.. J. Bacteriol. **169**:5016-5021.

Scholtz, R., F. Messi, T. Leisinger, and A.M. Cook. 1988a. Three dehalogenases and physiological restraints in the biodegradation of haloalkanes by *Arthrobacter* sp. strain HA1. Appl. Environ. Microbiol. **54**:3034-3038.

Scholtz, R., L.P. Wackett, C. Egli, A.M. Cook, and T. Leisinger. 1988b. Dichloromethane dehalogenase with improved catalytic activity isolated from a fast-growing dichloromethane-utilizing bacterium. J. Bacteriol. **170**:5698-5704.

Shirai, K., and K. Hisatsuka. 1979. Production of β-phenetyl alcohol from styrene by *Pseudomonas* 305-STR-1-4. Agric. Biol. Chem. **43**:1399-1406.

Sielicki, M., D.D. Focht, and J.P. Martin. 1978. Microbial transformations of ^{14}C styrene in soil and enrichment cultures. Appl. Environ. Microbiol. **35**:124-128.

Stirling, D.I. and H. Dalton. 1979. The fortuitous oxidation and cometabolism of various carbon compounds by whole-cell suspensions of *Methylococcus capsulatus* (Bath). FEMS Microbiol. Lett. **5**:315-318.

Stucki, G., W. Brunner, D. Staub, and T. Leisinger. 1981a. Microbial degradation of chlorinated C_1 and C_2 hydrocarbons, p. 131-137. *In* T. Leisinger, A.M. Cook, R. Hütter, and J. Nuesch (ed.), Microbial degradation of xenobiotics and recalcitrant compounds. Academic Press, Inc., London.

Stucki, G., R. Gälli, H.-R. Ebershold, and T. Leisinger. 1981b. Dehalogenation of dichloromethane by cell extracts of *Hyphomicrobium* DM2. Arch. Microbiol. **130**:366-371.

Suylen, G.M.H., P.J. Large, J.P. van Dijken, and J.G. Kuenen. 1987. Mehtyl mercaptan oxidase, a key enzyme in the metabolism of mehtylated sulphur compounds by *Hyphomicrobium* EG. J. Gen. Microbiol. **133**:2989-2997.

Takeuchi, K., K. Koike, and S. Ito. 1990. Production of *cis*-unsaturated hydrocarbons by a strain of *Rhodococcus* in repeated batch culture with a phase-inversion, hollow-fiber system. J. Biotechnol. **14**:179-186.

Tardiff, G., C.W. Greer, D. Labbé, and P.C.K. Lau. 1991. Involvement of a large plasmid in the degradation of 1,2-dichloroethane by *Xanthobacter autotrophicus*. Appl. Environ. Microbiol. **57**:1853-1857.

Tausz, J. and P. Donath. 1930. Ueber die Oxydation des Wasserstoffs und Kohlenwasserstoffe mittels Bakterien. Hoppe-Seyler's Z. Physiol. Chem. **190**:141-168.

Traunecker, J., A. Preuß, and G. Diekert. 1991. Isolation and characterization of a methyl chloride utilizing, strictly anaerobic bacterium. Arch. Microbiol. **156**:416-421.

Tsien, H.-C., G.A. Brusseau, R.S. Hanson, and L.P. Wackett. 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **55**:3155-3161.

Tyler, S.C. 1991. The global methane budget, p.7-38. *In* J.E. Rogers, and W.B. Whitman (ed.), Microbial production and consumption of greenhouse gases: methane, nitrogen oxides, and halomethanes. American Society for Microbiology, Washington, D.C.

Vannelli, T., M. Logan, D.M. Arciero, and A.B. Hooper. 1990. Degradation of halogenated aliphatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Appl. Environ. Microbiol. **56**:1169-1171.

Vogel, T.M., C.S. Criddle, and P.L. McCarty. 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. **21**:722-736.

Wackett, L.P., G.A. Brusseau, S.R. Householder, and R.S. Hanson. 1989. Survey of microbial oxygenases: trichloroethylene degradation by propane-oxidizing bacteria. Appl. Environ. Microbiol. **55**:2960-2964.

Wallen, L.L., R.G. Benedict, and R.W. Jackson. 1962. The microbiological production of 10-hydroxystearic acid from oleic acid. Arch. Biochem. Biophys. **99**:249-253.

Watkinson, R.J. and H.J. Somerville. 1976. The microbial utilization of butadiene. *In* Proceedings International Biodegradation Symposium (3rd). Applied Science, Essex pp. 35-42.

Weightman, A.J., A.L. Weightman, and J.H. Slater. 1982. Stereospecificity of 2-monochloropriopionate dehalogenation by the two dehalogenases of *Pseudomonas putida* PP3: evidence for two different dehalogenation mechanisms. J. Gen. Microbiol. **128**:1755-1762.

Weijers, C.A.G.M., A. de Haan, and J.A.M. de Bont 1988a. Microbial production and metabolism of epoxides. Microbiol. Sci. **5**:156-159.

Weijers, C.A.G.M., C.G. van Ginkel, and J.A.M. de Bont. 1988b. Enantiomeric composition of lower epoxyalkanes produced by methane-, alkane-, and alkene-utilizing bacteria. Enzyme Microb. Technol. **10**:214-218.

van den Wijngaard, A.J., R.D. Wind, and D.B. Janssen. 1991. Kinetics of microbial degradation of 1,2-dichloroethane and 2-chloroethanol. p. 671-672. *In* H. Verachtert and W. Verstraete (ed.), Environmental Biotechnology 1991. Royal Flemish Society of Engineers.

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

Wolf, K., A. Yazdani, and P. Yates. 1991. Chlorinated solvents: will the alternatives be safer? J. Air Waste Manage. Assoc. **41**:1055-1061.

Yokota, T., H. Fuse, T. Omori, and Y. Minoda. 1986. Microbial dehalogenation of haloalkanes mediated by oxygenase or halidohydrolase. Agric. Biol. Chem. **50**:453-460.

Chapter 2

METHYL CHLORIDE: NATURALLY OCCURRING TOXICANT AND C-1 GROWTH SUBSTRATE

S. Hartmans, A. Schmuckle, A.M. Cook and Th. Leisinger.

A strain of *Hyphomicrobium* that could utilize methyl chloride as the sole carbon and energy source for growth was isolated from industrial sewage. The methylotroph utilized methyl chloride quantitatively with stoichiometric release of chloride ion. A specific growth rate (μ) of 0.09 h^{-1} was observed with about 1% (v/v) methyl chloride in the gas phase.

Journal of General Microbiology (1986) **132**:1139-1142.

Methyl chloride is a naturally occurring compound (see Harper, 1985; Singh *et al.*, 1983; White, 1982), which has been classified by the US Environmental Protection Agency as a priority pollutant (Keith and Telliard, 1979). It is a very water-soluble gas at normal temperature and pressure (Weast, 1984) and is used industrially as a methylating agent for silicones and lead (Edwards *et al.*, 1982). Methyl chloride is known to be detoxified in the liver (Kornbrust and Bus, 1983), to be oxidized by a methanotroph (Stirling and Dalton, 1979) and by isolated methane monooxygenase (Patel *et al.*, 1982), and to be enzymically hydrolysed (Keuning *et al.*, 1985), but it does not serve as a carbon source for growth (Stirling and Dalton, 1979; Janssen *et al.*, 1985) and further information on its biological fate is lacking (Watson *et al.*, 1980).

We have now isolated a bacterium that utilizes methyl chloride as a sole and growth-limiting source of carbon and energy.

MATERIALS AND METHODS

Materials. The inoculum was sludge from two industrial sewage plants (Schweizerhalle, Switzerland and Grenzbach, Germany). Methyl chloride (>99.5%, v/v) was purchased from Matheson; it was chromatographically pure and its identity was confirmed by mass spectrometry. Methane (>99.9%, v/v) and ethyl chloride (99.5%, v/v) were from Matheson. Other chemicals were from Fluka and were of the highest purity available. Liquid cultures were grown in screw-cap Erlenmeyer flasks closed with Mininert valves (Precision Sampling), which allowed ready sampling of the gas and liquid phases.

Growth. Cultures were grown in 100 mM-potassium phosphate buffer, pH 7.2, containing 25 mM-$(NH_4)_2SO_4$, 0.25 mM-$MgSO_4$ and trace elements (Cook and Hütter, 1981) to which the carbon source was added. Gases could be sterilized by filtration (0.2 μm pore diameter membrane filter) but we were able to draw gas from cylinder outlets by syringe and avoid contamination on injection of gas into the flask through the septum. Cultures were grown at 30°C on an orbital shaker and the volume of air above the culture was tenfold larger than the culture volume. Solid media were prepared by the addition of 1% (w/v) agar (Oxoid no. 1). Methyl chloride-minimal medium plates were incubated in desiccators with substrate (1%, v/v) in the gas phase. The substrate spectrum was examined in closed flasks, because the organism obviously grew with traces of carbon sources in the air.

To obtain growth yields, cultures containing different concentrations of substrates were allowed to grow into the stationary phase. Protein was measured and substrate exhaustion was confirmed. The yield was the slope of the line "protein concentration" vs "initial substrate concentration", and the correlation coefficient r was >0.985 (7 points). Cells for use in non-growing suspensions or in the preparation of cell extracts were harvested in the late exponential phase, washed and stored frozen; cells were disrupted in a French press (Stucki *et al.*, 1981).

Enrichment and isolation of organisms. The inoculum was used directly to inoculate a 30 ml culture in a closed 300 ml Erlenmeyer flask containing 90 μmol methyl chloride, with, in addition, 250 μmol methane as a putative major carbon source. The culture grew with disappearance of methyl chloride, within 7 d and was transferred to identical fresh medium. After three successful transfers, the properties of the system were examined and pure cultures were isolated (see Results and Discussion). The organism has been submitted to the Deutsche Sammlung von Mikroorganismen (Göttingen, FRG) and has received the accession number DSM 3646.

Analytical methods. Growth was quantified as protein (Cook and Hütter, 1981). Growth rates were calculated from measurements of OD_{546}. Dechlorination by whole cells and cell extracts were measured as release of Cl^-, determined colorimetrically (Bergmann and Sanik, 1957) and amperometrically (Hartmans *et al.*, 1985). Methyl chloride and other gases were determined by gas chromatography (Stucki *et al.*, 1981). Dissolved O_2 was measured polarimetrically (S3 Monitor; Yellow Springs Instruments).

RESULTS AND DISCUSSION

The enrichment culture grew in the presence of methyl chloride and there was total disappearance of methyl chloride as well as release of Cl^-. Since methyl chloride was chemically stable under the conditions of the enrichment culture, and non-methylotrophs (strains A and 99; Cook and Hütter, 1981) did not cause decay of methyl chloride, methyl chloride was not decomposing due to chemical methylation of bacteria. The culture did not grow with methane as the sole carbon source but growth in methyl chloride-minimal medium was observed. A pure culture, designated MC1, was obtained by alternately growing on methyl chloride-minimal agar and in selective liquid culture in methyl chloride-minimal medium. Purity (uniformity of colony morphology) was then confirmed on plates with methanol-minimal medium. Growth with methyl chloride as a source of carbon would appear to be unusual. Three organisms contain enzymes that dechlorinate the compound, but it does not serve as growth substrate (Stirling and Dalton, 1979; Patel *et al.*, 1982; Janssen *et al.*, 1985; Keuning *et al.*, 1985), and a *Hyphomicrobium* sp. that utilizes methylene chloride does not use methyl chloride (Stucki *et al.*, 1981).

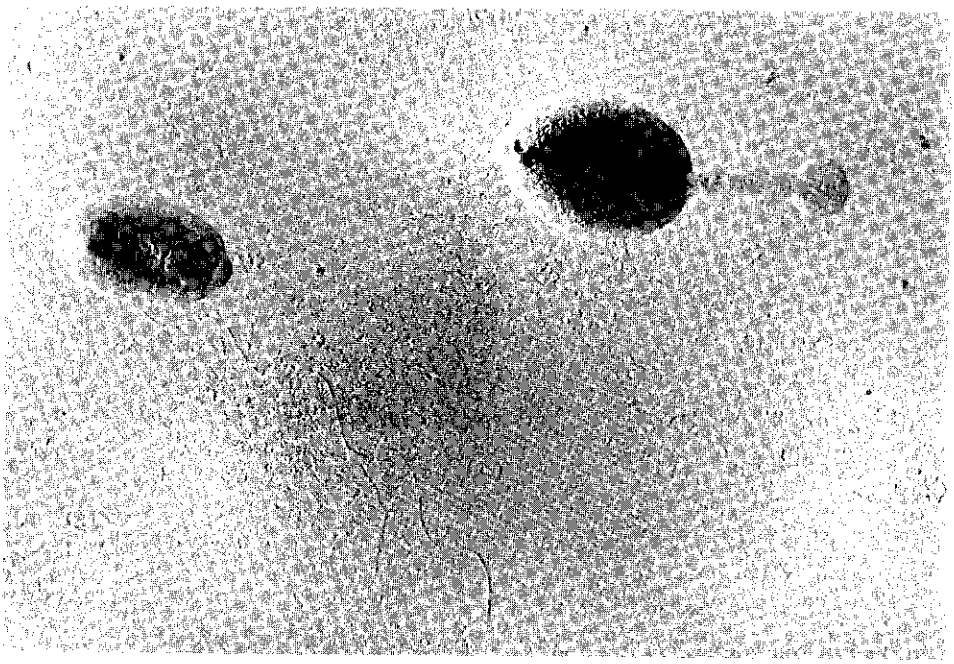

FIG. 1. Transmission electron micrograph of *Hyphomicrobium* sp. strain MC1. The organism was grown on methyl chloride minimal medium. Samples on carbon-coated Formvar films were air-dried and unidirectionally shadowed with 3 nm platinum/carbon at an angle of 45°. The bar represents 1 μm.

Strain MC1 had a very narrow substrate spectrum. Methyl chloride, methanol, formate and ethanol were good subtrates for growth whereas traces of growth were observed with acetate, butyrate and 3-hydroxybutyrate. Methane, methylene chloride, methylamine, ethyl chloride, 1,2-dichloroethane, pyruvate, glycerol, propionate, succinate and citrate did not support growth and the organism did not grow anaerobically in methanol-minimal medium containing nitrate.

Phase contrast and electron microscopy (Fig. 1) revealed that strain MC1 was pleiomorphic and Gram-negative. Plump motile rods with single sub-polar flagella were observed. Non-motile monoprosthecate rods with buds on the ends of the prostheca were also observed. These characteristics, together with methylotrophy, enable assignment of the strain to the genus *Hyphomicrobium* (Harder and Attwood, 1978).

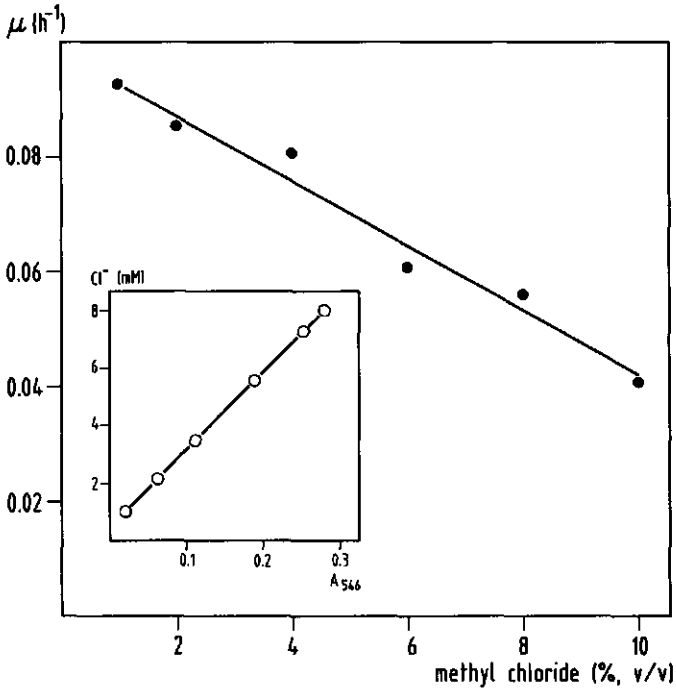

FIG. 2. Specific growth rate of *Hyphomicrobium* sp. strain MC1 as a function of substrate (methyl chloride) concentration. Each culture (150 ml) was inoculated with cells that were growing exponentially in homologous medium. Growth rates were estimated by following the increase in OD_{546} for at least three doublings. Substrate concentrations were kept within 0.2% of the values shown. Inset is the release of Cl^- during growth with about 2% (v/v) methyl chloride added to the gas phase.

The pure strain of *Hyphomicrobium* quantitatively utilized 90 μmol methyl chloride and released a mean of 88 ± 3 (SD) μmol Cl^-, which was identified by two independent methods. The yield of strain MC1 with methyl chloride, 5 g protein (mol C)$^{-1}$, lay between the yield with methanol [7 g protein (mol C)$^{-1}$; a normal value (Anthony, 1982)] and 2 g protein (mol C)$^{-1}$ observed with formate. The lower value with methyl chloride than with methanol implies that the product of dechlorination is formaldehyde and not methanol.

Substrate utilization (measured as release of Cl^-) was concomitant with growth (Fig. 2, inset). The specific growth rate, however, was dependent on the substrate concentration. A maximum growth rate of about 0.09 h^{-1} was observed at about 3 mM-methyl chloride (1%, v/v; Fig. 2); with 10% (v/v) substrate in the gas phase the rate was 0.04 h^{-1}. Growth rates of 0.12 and 0.04 h^{-1} were observed with methanol and formate, respectively.

The mechanism of dechlorination has not been elucidated. Washed cell suspensions of methyl chloride-grown cells, but not of methanol-grown cells, dechlorinated methyl chloride in an oxygen-dependent reaction, but there was no consumption of methane. We presume that oxidation of methyl chloride to formaldehyde by methane monooxygenase is not involved (cf. Stirling and Dalton, 1979). Hydrolytic dehalogenation (Keuning *et al.*, 1985) was not observed, and methanol, which was rapidly oxidized by methanol-grown cells, was only slowly oxidized by methyl chloride-grown cells. We suspect that methanol is not an intermediate in the degradation of methyl chloride and that there may be a methyl chloride monooxygenase analogous in reaction to methane monooxygenase.

Acknowledgement

We are grateful to Dr. D. Studer for preparing the electron micrographs and to Dr. E. Heinzle for obtaining the mass spectrum.

This investigation was supported by a grant from the Swiss Federal Institute of Technology, Zürich.

REFERENCES

Anthony, C. 1982. The Biochemistry of the Methylotrophs. p. 245-268. Academic Press, London.

Bergman, J.G., and J. Sanik. 1957. Determination of trace amounts of chlorine in naphtha. Anal. Chem. **29**:242-243.

Cook, A.M., and R. Hütter. 1981. *s*-Triazines as nitrogen sources for bacteria. J. Agric. Food Chem. **29**:1135-1143.

Edwards, R.R., I. Campbell, and G.S. Milne. 1982. The impact of chloromethanes on the environment: part 2, methyl chloride and methylene chloride. Chem. Industr. **1982**:619-622.

Harder, W., and M.M. Attwood. 1978. Biology, physiology and biochemistry of hyphomicrobia. Adv. Microbial Physiol. **17**:303-359.

Harper, D.B. 1985. Halomethane from halide ion - a highly efficient fungal conversion of environmental significance. Nature. **315**:55-57.

Hartmans, S., J.A.M. de Bont, J. Tramper, and K.C.A.M. Luyben. 1985. Bacterial degradation of vinyl chloride. Biotechnol. Lett. **7**:383-388.

Janssen, D.B., A. Scheper, L. Dijkhuizen, and B. Witholt, B. 1985. Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. Appl. Environ. Microbiol. **49**:673-677.

Keith, L.H., and W.A. Telliard. 1979. Priority pollutants: I - a perspective review. Environ. Sci. Technol. 13:416-423.

Keuning, S. D.B. Janssen, and B. Witholt. 1985. Purification and characterization of hydrolytic haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. J. Bacteriol. 163:635-639.

Kornbrust, D.J. and J.S. Bus. 1983. The role of glutathione and cytochrome P-450 in the metabolism of methyl chloride. Toxicol. Appl. Pharmacol. 67:246-256.

Patel, R.N., C.T. Hou, A.I. Laskin, and A. Felix. 1982. Microbial oxidations of hydrocarbons: properties of a soluble methane monooxygenase from a facultative methane-utilizing organism, *Methylobacterium* sp. strain CRL-26. Appl. Environ. Microbiol. 44:1130-1137.

Singh, H.B., L.J. Salas, and R.E. Stiltes. 1983. Methyl halides in and over the eastern Pacific (40°N – 32°S). J. Geophys. Res. 88:3684-3690.

Stirling, D.I., and H. Dalton 1979. The fortuitous oxidation and cometabolism of various carbon compounds by whole cell suspensions of *Methylococcus capsulatus* (Bath). FEMS Microbiol. Lett. 5:315-318.

Stucki, G., R. Gälli, H.-R. Ebersold, and T. Leisinger. 1981. Dehalogenation of dichloromethane by cell extracts of *Hyphomicrobium* DM2. Arch. Microbiol. 130:366-371.

Watson, A.J., J.E. Lovelock, and D.H. Stedman. 1980. The problem of atmospheric methyl chloride, p. 365-372. *In* M. Nicolet, and A.C. Aiken (ed.), Proceedings of the NATO Advanced Study Institute on Atmospheric Ozone: Its variation and Human Influences, Federal Aviation Administration. Washington, DC.

Weast, R.C. 1984. Handbook of Chemistry and Physics, p. C372, 65th edn. CRC Press, Boca Raton.

White, R.H. 1982. Biosynthesis of methyl chloride in the fungus *Phellinus pomaceus*. Arch. Microbiol. 132:100-102.

Chapter 3

AEROBIC VINYL CHLORIDE METABOLISM IN *MYCOBACTERIUM AURUM* L1

S. Hartmans and J.A.M. de Bont

Mycobacterium aurum L1, capable of growth on vinyl chloride as sole carbon and energy source, was previously isolated from soil contaminated with vinyl chloride. The initial step in vinyl chloride metabolism in strain L1 is catalyzed by alkene monooxygenase, transforming vinyl chloride to the reactive epoxide chlorooxirane. The enzyme responsible for chlorooxirane degradation appeared to be very unstable and thus hampered the characterization of the second step in vinyl chloride metabolism. Dichloroethenes are also oxidized by vinyl chloride-grown cells of strain L1, but they are not utilized as growth substrates. Three additional bacterial strains which utilize vinyl chloride as a sole carbon and energy source were isolated from environments with no known vinyl chloride contamination. The three new isolates were similar to strain L1 and were also identified as *Mycobacterium aurum*.

Applied and Environmental Microbiology (1992) **58**:1220-1226.

Vinyl chloride is carcinogenic in experimental animals and humans (Creech and Johnson, 1974; Maltoni and Lefemine, 1974). Consequently, the U.S. Environmental Protection Agency has classified vinyl chloride as a priority pollutant.

The compound is produced on a very large scale by the chemicals industry, mainly for use in the production of the polymer polyvinyl chloride (PVC). Associated with these large scale processes are inevitable losses to the environment. Vinyl chloride is a gas at ambient conditions (boiling point, 14°C at 1 atm), and consequently a large percentage of the industrial losses are to the atmosphere. However, due to its relatively short half-life of 20 h in the troposphere, vinyl chloride does not accumulate in the atmosphere (Guicherit and Schulting, 1985).

The presence of vinyl chloride in groundwater is attributed mainly to the biological reduction of polychlorinated ethenes (Vogel and McCarty, 1985). Complete dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE) to ethene has been observed under methanogenic conditions, but the rate-limiting step is the conversion of vinyl chloride to ethene (Freedman and Gossett, 1989). More recently, reductive dechlorination of PCE to ethene via vinyl chloride in the absence of methanogenesis has also been reported (DiStefano *et al.*, 1991). As a result of these reductive dechlorination reactions, vinyl chloride concentrations of more than 1 mg/l have been detected in groundwater contaminated with PCE and TCE (Brauch *et al.*, 1987; Kästner, 1991; Milde *et al.*,1988). Therefore, further aerobic transformation of vinyl chloride could be of interest in the bioremediation of groundwater.

Aerobic vinyl chloride transformation has been observed with a wide range of microorganisms exhibiting monooxygenase activity. Methane- (Fogel *et al.*, 1986); Tsien *et al.*, 1989), propane- (Wackett *et al.*, 1989), isoprene- (Ewers *et al.*, 1990), and ammonia- (Vannelli *et al.*, 1990) utilizing bacteria have been shown to oxidize vinyl chloride, but no oxidation products were identified. By using purified soluble methane monooxygenase from *Methylosinus trichosporium* OB3b, the oxidation product of vinyl chloride was identified as chlorooxirane (Fox *et al.*, 1990).

Aerobic mineralization of vinyl chloride by groundwater (Davis and Carpenter, 1990) and by a Gram-positive, propane-grown bacterium (Phelps *et al.*, 1991) has been reported recently. To our knowledge, however, *Mycobacterium* strain L1 (Hartmans *et al.*, 1985) is the only bacterial strain described so far which utilizes vinyl chloride aerobically as a sole source of carbon and energy. *Mycobacterium* strain L1 was isolated from soil that had been contaminated with vinyl chloride-containing water for a number of years.

The present report describes the isolation of three additional vinyl chloride-degrading mycobacteria. Furthermore, growth of *Mycobacterium* strain L1 on vinyl chloride and the initial step in vinyl chloride metabolism were studied by using chemostat cultures.

MATERIALS AND METHODS

Isolation of vinyl chloride-degrading strains. Mycobacterium strain L1 was previously isolated from soil that had been contaminated for several years with water containing vinyl chloride (unpublished results, de Bont and van der Linden). Strain L1 has been deposited at the German Collection of Microorganisms and Cell Cultures (DSM), Braunschweig, Germany, under accession number DSM 6695. The new strains were isolated by the following procedure. Soil (20 g) or water (20 ml) was mixed with 10 ml mineral salts medium (MSM) which has been previously described (Hartmans *et al.*, 1985) and put in serum bottles (about 130 ml) which were sealed with rubber septa. After addition of 5 ml vinyl chloride the serum bottles were incubated statically in the dark at 30°C. After about 1 month, the content was diluted fivefold with MSM. A 30 ml quantity of this diluted suspension was once again incubated under the same conditions. After a total of 2 months, 1 ml of these enrichment cultures was added to 30 ml MSM in serum bottles of 130 ml. A 2 ml quantity of vinyl chloride was added, and cultures were incubated with gentle shaking at 30°C. The vinyl chloride concentration was determined weekly by analyzing headspace samples. Dilutions from cultures showing vinyl chloride degradation were plated on MSM agar plates which were incubated in a desiccator to which vinyl chloride (1% v/v) was added. After 2 and 4 weeks, the plates were inspected, and colonies which appeared to grow on vinyl chloride were streaked to purity by alternately plating them on glucose-yeast extract agar plates and on MSM agar plates which were incubated in a desiccator with vinyl chloride.

Maintenance and cultivation of strains. Vinyl chloride-degrading strains were grown on MSM agar plates at 30°C in a desiccator containing about 1% (v/v) vinyl chloride for about 1 month. Plates were sealed with paper adhesive tape. This usually prevented contamination of the plates with fungi during the prolonged incubation in the desiccator and also reduced the evaporation of water during subsequent storage outside the desiccator. Subculturing was routinely performed every 2 to 3 months. The MSM used in the enrichment cultures was the same as that described previously (Hartmans *et al.*, 1985). In subsequent experiments, the following composition of MSM was used: per litre of deionized water, 3.88 g of K_2HPO_4, 2.13 g of $NaH_2PO_4.2H_2O$, 2.0 g of $(NH_4)_2SO_4$, 0.1 g of $MgCl_2.6H_2O$, 10 mg of EDTA, 2 mg of $ZnSO_4.7H_2O$, 1 mg of $CaCl_2.2H_2O$, 5 mg of $FeSO_4.7H_2O$, 0.2 mg of $Na_2MoO_4.2H_2O$, 0.2 mg of $CuSO_4.5H_2O$, 0.4 mg of $CoCl_2.6H_2O$ and 1 mg of $MnCl_2.2H_2O$. In chemostat cultures, a buffer with a lower strength (containing 1.55 g of K_2HPO_4 and 0.85 g of $NaH_2PO_4.2H_2O$ per litre) was used.

Growth experiments. Gaseous substrates were added at concentrations of 5% (v/v). With CO_2 as the carbon source hydrogen gas (15% v/v) was also added. Liquid substrates were added at concentrations of 0.1% (v/v) and solid substrates at a concentration of 0.1% (w/v). Growth experiments with polychlorinated ethenes were performed by adding 50 μmol of substrate to Erlenmeyer flasks with a total volume of 300 ml and containing 100 ml of MSM and sealed with Teflon Mininert seals. Chemostat cultures were run at 30°C and pH 7 in a 3-litre Applikon fermentor with a working volume of 2 litres. The impeller speed was 750 rpm and the dilution rate was 0.02 h^{-1} with 2% (v/v) vinyl chloride or ethene at an aeration rate of 200 ml/min. Growth rates were usually determined by monitoring substrate depletion curves (Hartmans and Tramper, 1991). This method gave the same values as the growth rates that had been determined by monitoring the optical density at 660 nm and chloride liberation rates (Hartmans and Tramper, 1991).

Oxidation experiments with whole cells. Degradation experiments with whole cells were performed at 30°C with vinyl chloride-grown cells of strain L1 freshly harvested from the chemostat and washed with 50 mM phosphate buffer (pH 7.0). The oxidation of polychlorinated ethenes (5 μl) was performed in 300 ml flasks fitted with Teflon Mininert valves containing cells in 25 or 50 ml of 50 mM potassium phosphate buffer (pH 7.0). The oxidation of vinyl chloride by other alkene-utilizing bacteria was performed with cells which had been stored at -20°C and cultivated as described previously (Habets-Crützen *et al.*, 1984; van Ginkel *et al.*, 1986).

Preparation of cell extracts. Cells were harvested from the chemostat or from batch cultures in the exponential growth phase by centrifugation at 16,000 *g* for 10 min at 4°C, resuspended in about 200 ml of 50 mM potassium phosphate buffer (pH 7.0), and centrifuged once again. The pellet was subsequently resuspended in approximately 6 ml of the same buffer containing 2 mM dithiothreitol. The concentrated washed-cell suspension was placed on ice and disrupted by ultrasonication with a Branson B-12 sonifier with a power input of 10 W eight times for 15 s each time. Whole cells and cell

debris were removed by centrifugation at 27,000 *g* for 20 min at 4°C. The supernatant was used directly as the cell extract in the enzyme activity assays.

Enzyme assays. All assays were performed at 30°C. Spectrophotometric assays were performed on a Perkin-Elmer 550A spectrophotometer. Activities are expressed in nanomoles of product formed or of substrate consumed per minute per milligram of protein.

Alkene monooxygenase. Alkene monooxygenase was assayed by analyzing epoxypropane formation. Assays were done in 35-ml serum bottles sealed with rubber septa. The reaction mixture contained 2 μmol of NADH, cell extract and 50 mM potassium phosphate buffer (pH 7.3) in a total volume of 1 ml. The serum bottles were incubated at 30°C in a shaking water bath. After 2 min in the water bath, the reaction was started by the addition of 1 ml propene via the rubber septum. Epoxypropane formation was determined by analyzing headspace samples of 200 μl every 2 to 3 min during a total incubation time of about 20 min. The epoxypropane formation rate was constant during this time period.

Epoxyethane dehydrogenase. Epoxyethane dehydrogenase was assayed by determining epoxyethane consumption. The assay as by de Bont and Harder (1978) was slightly modified. The following was added to a serum bottle of 35 ml: 450 μl of 100 mM Tris hydrochloride (pH 8.5), 50 μl of 10 mM NAD^+, 50 μl of 10 mM coenzyme A, 50 μl of 2 mM FAD and water to give a total volume of 1 ml after the addition of extract. The bottle was sealed with a rubber septum and flushed with nitrogen for 2 to 3 min to remove most of the oxygen, and 1 ml of 1% (v/v) epoxyethane in nitrogen gas was added. The bottles were subsequently incubated in a shaking water bath, and after 2 min the reaction was started by the addition of cell extract (0.2 to 0.4 ml) through the septum with a syringe. The epoxyethane consumption rate was determined by analyzing headspace samples either until all epoxyethane was consumed or for a maximum of 50 min. The epoxyethane consumption rate was constant during this time period.

Isocitrate lyase and *isocitrate dehydrogenase* were assayed as described previously (Hartmans *et al.*, 1991a).

Identification of chlorooxirane. The following experiment was performed, starting with a steady-state culture of *Mycobacterium aurum* L1 operated at a dilution rate of 0.016 h^{-1} and an airflow of 85 ml/min containing vinyl chloride (7,500 ppmv). The dilution rate was reduced to zero, and 0.5 litre MSM was added to decrease the volume of the gas phase and thereby the residence time of the air passing through the fermentor. At the same time, the inlet vinyl chloride concentration was increased to 11,000 ppmv. After 8 h at these conditions, the vinyl chloride conversion was 97.4%. The airflow into the fermentor was subsequently increased to 200 ml per min with 6,750 ppmv of vinyl chloride, resulting in a conversion of 68%. The air leaving the fermentor was passed through 5 ml of ethanediol containing 4-(4-nitrobenzyl)pyridine for 30 min to trap any chlorooxirane formed. The air-vinyl chloride flow subsequently was replaced by 100 ml pure air per min for 43 min. After these 43 min, the original air-vinyl chloride mixture (200 ml/min; 6,750 ppmv) was passed through the fermentor again, and the gas phase leaving the fermentor was passed through 5 ml of ethanediol containing 4-(4-nitrobenzyl)pyridine for 30 min. The vinyl chloride conversion gradually decreased to 31% over 30 min. The ethanediol solutions were immediately analyzed for the chlorooxirane adduct of 4-(4-nitrobenzyl)-pyridine as described by Barbin *et al.* (1975).

Analytical methods. Biomass dry weights were routinely determined by measuring the absorbance of an appropriate dilution in MSM at 660 nm with a Perkin-Elmer 550A spectrophotometer. Dry weight versus optical density was assumed to be linear below an absorbance of 0.5, which corresponded with 130 mg (dry weight) per litre (determined with glucose-grown cells after 24 hours at 105°C).

Concentraions of vinyl chloride, ethene, epoxyalkanes and chlorinated ethenes were determined by analyzing 100-μl headspace samples on a Packard 430 gas chromatograph fitted with a stainless-steel Porapak R column (100-120 mesh, 110 cm × 1/8 inch inner diameter) and a flame ionization detector. The oven temperature was 180°C (200°C for the analysis of polychlorinated ethenes), and the carrier gas N_2, at a flow rate of 20 ml/min. Total glutathione was quantified as described by Tietze (1969). Protein was quantified by the Bradford (1976) method with bovine serum albumin as the standard.

Chemicals. Vinyl chloride (chloroethene) with a purity of 99.95%, propene, ethene and epoxyethane were from HoekLoos, Schiedam, The Netherlands. Vinyl bromide, 1,1-, *cis*- and *trans*-1,2-dichloroethene were from Janssen Chimica, Beerse, Belgium. 4-(4-Nitrobenzyl)pyridine, DL-isocitrate

and coenzyme A were from Sigma, St. Louis, MO. Dithiothreitol, NAD^+, NADH, $NADP^+$ and ATP were from Boehringer, Mannheim, Germany. Other chemicals were from Merck, Darmstadt, Germany.

RESULTS

Isolation and characterization of vinyl chloride-degrading strains

In the present investigation, a series of inocula from different sources without any known history of vinyl chloride contamination were used in enrichment cultures with vinyl chloride as the carbon source. Three vinyl chloride-degrading strains designated VC2, VC3, and VC4 were isolated from the 20 different inocula used. They were isolated from soil which had been treated with 1,3-dichloropropene for 20 years (VC2), from the sludge of an aerobic wastewater treatment plant mainly treating domestic wastewater (VC3) and from the River Rhine sampled at Wageningen (VC4), respectively. The new isolates all formed yellow colonies on agar plates, as did the previously isolated *Mycobacterium* strain L1. All four strains were tentatively identified as *M. aurum* at the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands. The colour and colony morphologies of the new isolates differed somewhat, indicating that they were different strains.

None of the ethene- or propene-utilizing strains of the genera *Mycobacterium* and *Xanthobacter* previously isolated in our laboratory (Habets-Crützen *et al.*, 1984; van Ginkel and de Bont, 1986) could grow with vinyl chloride as the sole carbon source. Alkene-grown cells did, however, oxidize vinyl chloride at initial rates similar to those of vinyl chloride-grown cells of strain L1. Ethene-grown cells of *Mycobacterium* strain E3 and propene-grown cells of *Xanthobacter* strain Py2 oxidized vinyl chloride with initial specific activities of 37 and 33 nmol min^{-1} mg $protein^{-1}$, respectively.

The four vinyl chloride-utilizing strains exhibited the same pattern of growth substrate utilization. Ethene, ethanol, acetate, 1-propanol, propionate, pyruvate and glycerol were all utilized as sole source of carbon and energy. All strains grew autotrophically on H_2. Methane, methanol, ethane, ethanediol, glycolate, glycine, propene, 2-propanol, 1,2-propanediol, chloroethane, chloroethanol and chloroacetate (0.05%, w/v) did not support growth.

Vinyl chloride degradation kinetics

As no obvious differences among the four strains were observed, subsequent experiments were performed with strain L1. Growth rates with vinyl chloride were determined a number of times, and they varied between 0.03 and 0.06 h^{-1}. A growth rate of about 0.04 h^{-1} was determined most frequently. The observed growth rates were influenced by the history of the inoculum. Prolonged subculturing in liquid batch cultures with vinyl chloride as the carbon source resulted in an increase of the growth rate with vinyl chloride, whereas subculturing on glucose-yeast extract agar plates resulted in a decreased growth rate with vinyl chloride. Both effects

were reversible.

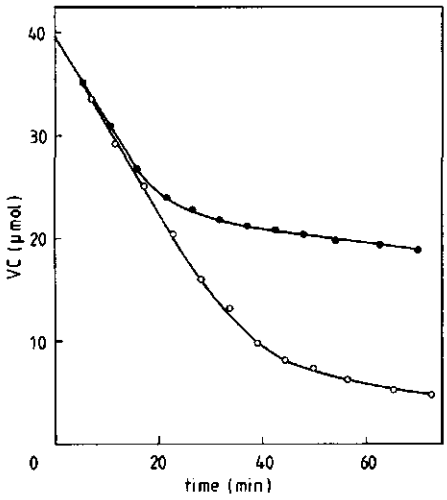

FIG. 1. Vinyl chloride degradation by washed cells of *M. aurum* L1. (●) Total volume of liquid phase (3 ml) containing 16 mg of freshly harvested vinyl chloride-grown cells in phosphate buffer; (○) same composition plus 16 mg of heat-inactivated cells.

Strain L1 was grown in a vinyl chloride-limited chemostat at a number of dilution rates between 0.012 and 0.032 h^{-1} for several months. After an apparent steady state was obtained, the vinyl chloride concentration in the air from the chemostat was determined several times during a time period which varied from 1 to 5 days. From these data and from the maximum specific growth rate of 0.040 h^{-1} an apparent K_S of 3.2 μM was calculated. In calculating the K_S the gas-phase vinyl chloride concentration in the chemostat was assumed to be in equilibrium with the liquid-phase concentration.

Vinyl chloride metabolism

Freshly harvested, vinyl chloride-grown, washed cells of strain L1 oxidized vinyl chloride at a rate of 55 nmol min^{-1} mg of dry $weight^{-1}$. Very rapid inactivation of vinyl chloride degradation was, however, observed (Fig. 1). Incubation of cells at the same densities for 1 h at 30°C before vinyl chloride was added gave an almost identical vinyl chloride degradation curve (results not shown). Inactivation could be delayed by adding boiled cells (Fig. 1). No vinyl chloride degradation was observed when only boiled cells were present (results not shown). We concluded from this experiment that the inactivation was caused by a toxic metabolite of vinyl chloride metabolism which could accumulate extracellularly.

The most obvious toxic product that could be formed from vinyl chloride is chlorooxirane, the product of vinyl chloride epoxidation, or its rearrangement

product, chloroacetaldehyde. The involvement of chlorooxirane in vinyl chloride metabolism would require the presence of monooxygenase activity in vinyl chloride-grown cells. It was previously shown that vinyl chloride- and ethene-grown cells of strain L1 oxidized propene to 1,2-epoxypropane (Weijers *et al.*, 1988), indicating that alkene monooxygenase was present in cells grown on both substrates. Alkene monooxygenase could also be assayed in crude extracts of ethene- or vinyl chloride-grown cells (Table 1). As alkene monooxygenase is a multicomponent enzyme (Hartmans *et al.*, 1991b), the observed specific activities vary with the protein content of the assay (Table 1). Vinyl chloride oxidation by dialyzed crude extracts was dependent on the presence of both oxygen and NADH. No monooxygenase activity was detected in cells grown on acetate or succinate.

TABLE 1. Enzyme activities in cell extracts of *Mycobacterium aurum* L1 grown on various carbon sources.

	Activity (nmol min^{-1} (mg protein)$^{-1}$) in extracts from cells grown on:			
Enzyme	Vinyl chloride	Ethene	Acetate	Succinate
Alkene monooxygenase	2.35 (7.8)[a] 2.02 (5.2) 1.13 (2.6)	1.60 (12.2) 1.32 (8.1) 0.96 (4.1)	ND	ND
Epoxyethane dehydrogenase	2.22 (5.2) 0.99 (2.6)	– (8.1) 7.4 (4.1)	ND	ND
Isocitrate lyase	111	89	87	3
Isocitrate dehydrogenase	245	272	550	346

[a] Values in parentheses indicate the amount of protein (mg) in the assay. Vinyl chloride- and ethene-grown cells were from chemostat cultures (D = 0.02 h^{-1}); acetate and succinate cells were batch grown. ND, not detectable; –, reaction too fast to allow a reliable activity determination.

On several occasions, after an interruption in the vinyl chloride supply to the continuous culture, a significant drop in vinyl chloride conversion was observed. This could be the result of monooxygenase inactivation due to a temporary accumulation of chlorooxirane upon restoration of the vinyl chloride supply to the culture.

To verify the presumed formation of chlorooxirane, the air leaving the fermentor was analyzed by passing it through a solution containing 4-(4-nitrobenzyl)pyridine in ethanediol as described by Barbin *et al.* (1975). This was done with air from the

fermentor prior to the vinyl chloride supply being interrupted for 43 min and also with the air from the fermentor directly after the vinyl chloride supply was restored. The broken line in Fig. 2 indicates the theoretical outlet concentrations that would be observed if no vinyl chloride were transformed after the vinyl chloride supply was restored. The characteristic spectrum of the chlorooxirane adduct (Barbin *et al.*, 1975) was detected only in the solution through which the air from the fermentor had been passed after the interruption in the vinyl chloride supply (Fig. 3). This confirms that chlorooxirane is the oxidation product of vinyl chloride. It also indicates that the enzyme responsible for the degradation of the chlorooxirane is unstable or that the activity of this enzyme is very sensitive to changes in the intracellular environment (e.g., cofactor levels) due to the absence of an exogenous carbon and energy source.

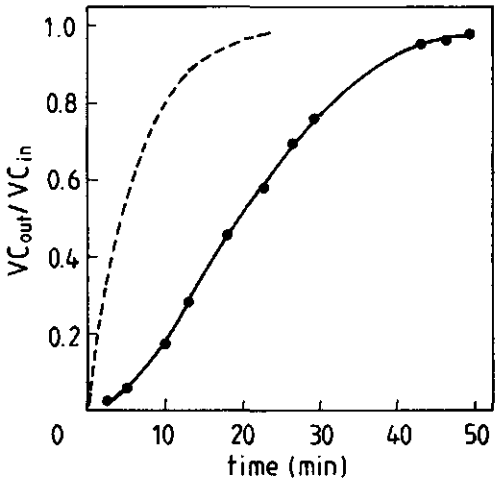

FIG. 2. Vinyl chloride outlet concentrations upon restoring the vinyl chloride supply to a culture of *M. aurum* L1 which had been without vinyl chloride for 43 min (—) and the theoretical outlet concentrations that would be observed if no vinyl chloride were transformed after the vinyl chloride supply was restored (- -).

Mammalian metabolism of chlorooxirane involves the formation of *S*-formyl methyl glutathione (Duverger *et al.*, 1981), either directly or via chloroacetaldehyde. Extracts of batch-grown cultures of strain L1, however, only contained about 1 μmol total glutathione per gram protein after growth on vinyl chloride, ethene or acetate.

The growth yield of *M. aurum* L1 with ethene and vinyl chloride as substrate was determined using closed batch cultures. The observed yields were 0.77 and 0.22 g of biomass per g of substrate utilized, respectively. Calculation of the growth yields on a molar basis results in 21.7 and 13.8 grams of biomass formed per mol of ethene or vinyl chloride utilized, indicating that the oxidation of vinyl chloride yields

less ATP than the oxidation of ethene.

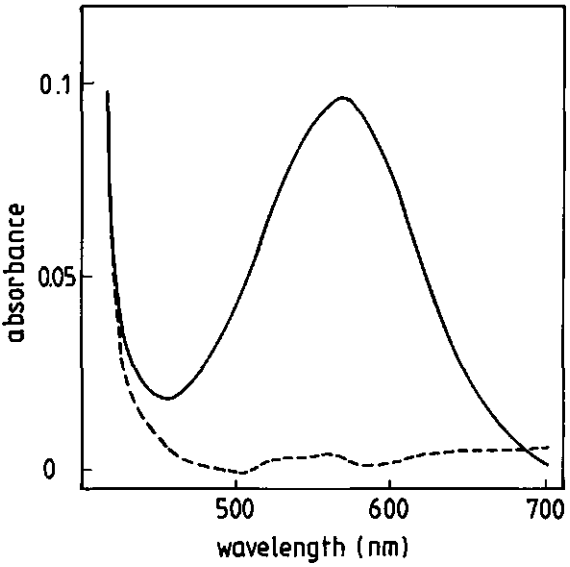

FIG. 3. Absorption spectra of 4-(4-nitrobenzyl)pyridine adducts in ethanediol solutions through which the air from the fermentor was passed prior to the interruption in the vinyl chloride supply (- -) and directly after restoration of the vinyl chloride supply (——).

Oxidation of polychlorinated ethenes by *M. aurum* L1

TABLE 2. Whole-cell oxidation rates of chlorinated alkenes by vinyl chloride-grown *M. aurum* L1 cells.

Substrate	Initial oxidation rate nmol min^{-1} (mg dry weight)$^{-1}$
Vinyl chloride (chloroethene) (350)[a]	55
1,1-Dichloroethene (165)	10
cis-1,2-Dichloroethene (860)	30
trans-1,2-Dichloroethene (425)	25
Trichloroethene (320)	<1

[a]Values in parentheses indicate the initial substrate concentration (μM), calculated by using the partition coefficients determined by Gossett (1987).

Table 2 shows the initial oxidation rates of various chlorinated ethenes by vinyl chloride-grown cells of *M. aurum* L1. Ethene oxidation resulted in epoxyethane

accumulation and with *trans*-1,2-dichloroethene as the substrate, an unidentified compound, probably the epoxide (Janssen *et al.*, 1988), accumulated. Vinyl bromide and the compounds listed in Table 2 were also tested as growth substrates with strain L1. Growth was determined as substrate depletion. With ethene, vinyl chloride and vinyl bromide more than 50% of the substrate was consumed within 6 days. With the polychlorinated ethenes as growth substrates no significant substrate degradation or carbon dioxide formation could be observed, even after 3 weeks.

DISCUSSION

Isolation of vinyl chloride utilizers

In contrast to the previously isolated strain L1, the three new strains were isolated from environments not known to be contaminated with vinyl chloride. Prolonged contamination with vinyl chloride is therefore apparently not a prerequisite for the evolution of the vinyl chloride degradative pathway in bacteria.

It is remarkable that all four isolates were strains of *M. aurum*, although colony morphologies and pigmentations indicated that the four strains were not identical. A similar situation has, however, been observed when ethene is used as the carbon source in enrichment cultures. Until now, all strains isolated from enrichment cultures with ethene as sole carbon and energy source were identified as mycobacteria (Hartmans *et al.*, 1989a). A *Xanthobacter* sp. isolated from an enrichment culture with propene as the sole carbon and energy source was also capable of growth with ethene (van Ginkel and de Bont, 1986), but the growth rate was much lower than the growth rates of the ethene-utilizing mycobacteria. None of the ethene- or propene-utilizing bacteria tested could grow with vinyl chloride.

Until now, there were no reports concerning the isolation of pure cultures of vinyl chloride-utilizing microorganisms in the literature. This may be due in part to the low concentrations of vinyl chloride used in the published studies concerning aerobic degradation of vinyl chloride. Davis and Carpenter (1990), for example, used 0.1 and 1 μg of vinyl chloride per litre. This concentration is very low compared with the apparent K_S of strain L1 for vinyl chloride of 200 μg/litre. We have observed the induction of vinyl chloride degradation by glucose-grown cells of strain L1 at a concentration of 10 μg/litre but have not tested lower concentrations. Phelps *et al.* (1991) used 1 mg of vinyl chloride per litre in the presence of propane (5% v/v), but their isolate was apparently not capable of growth on vinyl chloride as the sole source of carbon and energy. The recovery of labeled CO_2 (Davis and Carpenter, 1990; Phelps *et al.*, 1991), assuming that vinyl chloride is also epoxidated in these cases, could indicate that these cultures had enzymes which very efficiently transform chlorooxirane, but it more likely indicates that these cultures were able to metabolize the products formed from the alkylation of glutathione (Plugge and Safe, 1977) or coenzyme A (Simon *et al.*, 1985) by chlorooxirane or chloroacetaldehyde, the rearrangement product of chlorooxirane.

The observed variation in the growth rate of strain L1 with vinyl chloride, which depended on the maintenance conditions of the culture, probably indicates that the regulation of vinyl chloride metabolism in *M. aurum* L1 is still genetically unstable. Indeed, we have observed that subculturing strain L1 on agar slants with yeast extract-glucose for more than 2 years resulted in the loss of the capacity of the total population to grow on vinyl chloride.

Vinyl chloride metabolism

Alkene monooxygenase activity was present in crude extracts of vinyl chloride-grown *M. aurum* L1. Combined with the observation that vinyl chloride degradation can be competitively inhibited by the addition of ethene or propene, which are both oxidized to the corresponding epoxides, this is strong evidence that the initial step in vinyl chloride metabolism is indeed catalyzed by alkene monooxygenase. The presumed product of vinyl chloride oxidation, chlorooxirane, is a very reactive and unstable compound (rearranging to chloroacetaldehyde with a half-life of 1.6 min in Tris-HCl buffer pH 7.4 at 37°C (Barbin *et al.* (1975]), indicating that during growth with vinyl chloride the epoxide must be metabolized very effectively to prevent the accumulation of toxic levels within the cell. However, on the basis of the inactivation of washed cells degrading vinyl chloride (Fig. 1), this very effective enzyme would also appear to be very unstable, rapidly losing its activity in the absence of an inducer. This hypothesis was confirmed by the experiment in which the vinyl chloride supply to a chemostat culture was interrupted for only 43 min. After the vinyl chloride supply was restored, chlorooxirane was detected in the air from the chemostat. No chlorooxirane could be detected prior to the interruption (Fig. 3). Apparently, this time period was already long enough to allow (some) loss of activity of the chlorooxirane transforming enzyme, consequently resulting in the accumulation of the inhibitory epoxide. In studying the inhibitory effects of the less reactive epoxide 1,2-epoxypropane, it was previously demonstrated that the inhibitory effect on the monooxygenases examined was much stronger than that on other physiological functions of the cell (Habets-Crützen and de Bont, 1985). Although not examined in the present investigation, this is probably also the case with alkene monooxygenase inhibition by chlorooxirane.

The apparent instability of the chlorooxirane-degrading enzyme, in combination with the reactivity and instability of the epoxide itself, makes further elucidation of the vinyl chloride degradative pathway very difficult. The observed presence of epoxyethane dehydrogenase activity in extracts of vinyl chloride-grown cells does not necessarily indicate the involvement of this enzyme in vinyl chloride metabolism. In the ethene-utilizing *Mycobacterium* strain E3, the monooxygenase and the epoxide dehydrogenase are both induced by epoxyalkanes (unpublished results). A number of other possible enzymatic transformations of epoxides can, however, be ruled out. Hydrolysis or isomerization (Hartmans *et al.*, 1989b) of the epoxide would result in glycolaldehyde and chloroacetaldehyde, respectively. As strain L1 does not

grow on ethanediol, glycolate, or chloroethanol, this would seem unlikely to occur. Only very low levels of glutathione could be detected in strain L1 grown on various substrates, indicating that glutathione-dependent transformation of chlorooxirane is also unlikely.

Oxidation of other chlorinated ethenes

Washed cells of vinyl chloride-grown *M. aurum* L1 oxidized dichloroethenes at rates which were in the same range as vinyl chloride oxidation (Table 2). Trichloroethene (TCE) was not oxidized at a detectable rate at the concentration tested. However, an isoprene-utilizing strain, which also exhibits alkene monooxygenase activity, was recently shown to oxidize chlorinated ethenes, including TCE (Ewers *et al.*, 1990). With the exception of 1,1-dichloroethene, which was oxidized at the same rate, the oxidation rates of vinyl chloride (25%) and the 1,2-dichloroethenes (2 to 10%) were somewhat lower than the rates we observed with strain L1 (Table 2). The TCE oxidation rate was rather low, 0.15 nmol min^{-1} mg $protein^{-1}$ when the TCE concentration was 6 μM (Ewers *et al.*, 1990).

Conclusions

Vinyl chloride metabolism in *M. aurum* L1 proceeds via oxidation of vinyl chloride to chlorooxirane by alkene monooxygenase (Fig. 4).

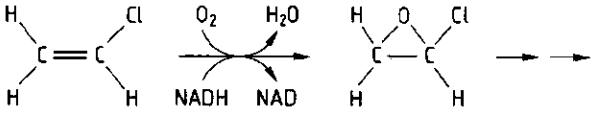

FIG. 4. Initial step in vinyl chloride metabolism of *M. aurum* L1, catalyzed by alkene monooxygenase.

The apparent instability of the enzyme responsible for the further metabolism of chlorooxirane in combination with the toxic characteristics of this compound can result in inhibition of the monooxygenase-oxidizing vinyl chloride. This aspect of vinyl chloride degradation by *M. aurum* L1 does not favour the application of this strain in the removal of vinyl chloride from waste gases, as the concentration can fluctuate to a great extent, but it need not be problematic in the bioremediation of groundwater. Furthermore, strain L1, and alkene-utilizing strains in general, may prove to be of interest in view of the cometabolic degradation of various other chlorinated ethenes.

Acknowledgements

We thank Dr. D.G. Groothuis (RIVM, Zeist) for identifying the vinyl chloride utilizers, Dr. G. Lebbink (IB, Haren) for supplying the 1,3-dichloropropene-treated soil, and Paul van Eijck and Ronald Niermans for performing some of the experimental work. The investigations were financed in part by the Dutch Ministry of Housing, Physical Planning and Environment.

REFERENCES

Barbin, A., H. Brésil, A. Croisy, P. Jacquignon, C. Malaveille, R. Montesano, and H. Bartsch. 1975. Liver-microsome-mediated formation of alkylating agents from vinyl bromide and vinyl chloride. Biochem. Biophys. Res. Comm. **67**:596-603.

de Bont, J.A.M., and W. Harder. 1978. Metabolism of ethylene by *Mycobacterium* E20. FEMS Microbiol. Lett. **3**:89-93.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.

Brauch, H.-J., W. Kühn, and P. Werner. 1987. Vinylchlorid in kontaminierten Grundwässern - vinyl chloride in contaminated groundwater. Vom Wasser **68**:23-32.

Creech J.L., and M.N. Johnson. 1974. Angiosarcoma of liver in the manufacture of polyvinyl chloride. J. Occup. Med. **16**:150-151.

Davis, J.W., and C.L. Carpenter. 1990. Aerobic biodegradation of vinyl chloride in groundwater samples. Appl. Environ. Microbiol. **56**:3878-3880.

DiStefano, T.D., J.M. Gossett, and S.H. Zinder. 1991. Reductive dechlorination of high concentrations of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. Appl. Environ. Microbiol. **57**:2287-2292.

Duverger, M., M. Lambotte, E. Malvoisin, C. de Meester, F. Poncelet, and M. Mercier. 1981. Metabolic activation and mutagenicity of 4 vinylic monomers (vinyl chloride, styrene, acrylonitrile, butadiene). Tox. Eur. Res. **3**:131-140.

Ewers, J., D. Freier-Schröder, and H.-J. Knackmuss. 1990. Selection of trichloroethene (TCE) degrading bacteria that resist inactivation by TCE. Arch. Microbiol. **154**:410-413.

Fogel, M.M., A.R. Taddeo, and S. Fogel. 1986. Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. Appl. Environ. Microbiol. **51**:720-724.

Fox, B.G., J.G. Borneman, L.P. Wackett, and J.D. Lipscomb. 1990. Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. Biochemistry **29**:6419-6427.

Freedman, D.L., and J.M. Gossett. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Appl. Environ. Microbiol. **55**:2144-2151.

van Ginkel, C.G., H.G.J. Welten, and J.A.M. de Bont. 1986. Epoxidation of alkenes by alkene-grown *Xanthobacter* spp. Appl. Microbiol. Biotechnol. **24**:334-337.

van Ginkel, C.G., and J.A.M. de Bont. 1986. Isolation and characterization of alkene-utilizing *Xanthobacter* spp. Arch. Microbiol. **145**:403-407.

Gossett, J.M. 1987. Measurement of Henry's law constant for C_1 and C_2 chlorinated hydrocarbons. Environ. Sci. Technol. **21**:202-208.

Guicherit, R., and F.L. Schulting. 1985. The occurrence of organic chemicals in the atmosphere of the Netherlands. Sci. Total Environ. **43**:193-219.

Habets-Crützen, A.Q.H., L.E.S. Brink, C.G. van Ginkel, J.A.M. de Bont, and J. Tramper. 1984. Production of epoxides from gaseous alkenes by resting-cell suspensions and immobilized cells of alkene-utilizing bacteria. Appl. Microbiol. Biotechnol. **20**:245-250.

Habets-Crützen, A.Q.H., and J.A.M. de Bont. 1985. Inactivation of alkene oxidation by epoxides in alkene- and alkane-grown bacteria. Appl. Microbiol. Biotechnol. **22**:428-433.

Hartmans, S., J.A.M. de Bont, J. Tramper, and K.C.A.M. Luyben. 1985. Bacterial degradation of vinyl chloride. Biotechnol. Lett. **7**:383-388.

Hartmans, S., J.A.M. de Bont, and W. Harder. 1989a. Microbial metabolism of short-chain unsaturated hydrocarbons. FEMS Microbiol. Rev. **63**:235-264.

Hartmans, S., J.P. Smits, M.J. van der Werf, F. Volkering, and J.A.M. de Bont. 1989b. Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. Appl. Environ. Microbiol. **55**:2850-2855.

Hartmans, S., and J. Tramper. 1991. Dichloromethane removal from waste gases with a trickle-bed bioreactor. Bioprocess Eng. **6**:83-92.

Hartmans, S., M.W. Jansen, M.J. van der Werf, and J.A.M. de Bont. 1991a. Bacterial metabolism of

3-chloroacrylic acid. J. Gen. Microbiol. **137**:2025-2032.

Hartmans, S., F.J. Weber, B.P.M. Somhorst, and J.A.M. de Bont. 1991b. Alkene monooxygenase from *Mycobacterium*: a multicomponent enzyme. J. Gen. Microbiol. **137**:2555-2560.

Janssen, D.B., G. Grobben, R. Hoekstra, R. Oldenhuis, and B. Witholt. 1988. Degradation of *trans*-1,2-dichloroethene by mixed and pure cultures of methanotrophic bacteria. Appl. Microbiol. Biotechnol. **29**:392-399.

Kästner, M. 1991. Reductive dechlorination of tri- and tetrachloro-ethylenes depends on transition from aerobic to anaerobic conditions. Appl. Environ. Microbiol. **57**:2039-2046.

Maltoni, C., and G. Lefemine. 1974. Carcinogenicity bioassays of vinyl chloride. I. Research plan and early results. Environ. Res. **7**:387-405.

Milde, G., M. Nerger, and R. Mergler. 1988. Biological degradation of volatile chlorinated hydrocarbons in groundwater. Water Sci. Tech. **20**:67-73.

Phelps, T.J., K. Malachowsky, R.M. Schram, and D.C. White. 1991. Aerobic mineralization of vinyl chloride by a bacterium of the order *Actinomycetales*. Appl. Environ. Microbiol. **57**:1252-1254.

Plugge, H., and S. Safe. 1977. Vinyl chloride metabolism: a review. Chemosphere **6**:309-325.

Simon, P., H.M. Bolt, and J.G. Filser. 1985. Covalent interaction of reactive metabolites with cytosolic coenzyme A as mechanism of haloethylene-induced acetonemia. Biochem. Pharmacol. **34**:1981-1986.

Tietze, F. 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Anal. Biochem. **27**:502-522.

Tsien, H.-C., G.A. Brusseau, R.S. Hanson, and L.P. Wackett. 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **55**:3155-3161.

Vannelli, T., M. Logan, D.M. Arciero, and A.B. Hooper. 1990. Degradation of halogenated aliphatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Appl. Environ. Microbiol. **56**:1169-1171.

Vogel, T.M., and P.L. McCarty. 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. **49**:1080-1083.

Wackett, L.P., G.A. Brusseau, S.R. Householder, and R.S. Hanson. 1989. Survey of microbial oxygenases: trichloroethylene degradation by propane-oxidizing bacteria. Appl. Environ. Microbiol. **55**:2960-2964.

Weijers, C.A.G.M., C.G. van Ginkel, and J.A.M. de Bont. 1988. Enantiomeric composition of lower epoxyalkanes produced by methane-, alkane-, and alkene-utilizing bacteria. Enzyme Microb. Technol. **10**:214-218.

Chapter 4

ALKENE MONOOXYGENASE FROM *MYCOBACTERIUM*: A MULTICOMPONENT ENZYME

S. Hartmans, F.J. Weber, B.P.M. Somhorst and J.A.M. de Bont

A NADH- or NADPH-dependent alkene monooxygenase (AMO) activity has been detected in cell-free extracts of the ethene-utilizing *Mycobacterium* E3 and *Mycobacterium aurum* L1. The activity was not linear with protein concentration in the assay suggesting AMO is a multicomponent enzyme. The inhibition pattern of AMO activity was very similar to the inhibition patterns published for the three-component soluble methane monooxygenases. Fractionation of crude extracts revealed that combination of two fractions was required to restore AMO activity. The first fraction was inhibited by acetylene, indicating it contained an oxygenase component. The second fraction contained reductase activity which was absent from non-induced cells. This reductase activity is probably the NADH-acceptor reductase of AMO.

Journal of General Microbiology (1991) 137:2555-2560.

Ethene-utilizing mycobacteria have been studied mainly in relation to the possibility of using them to produce optically active epoxides (Habets-Crützen *et al.*, 1985; Weijers *et al.*, 1988; Hartmans *et al.*, 1989). Most studies have been done with whole cells and as yet little is known about the epoxidation reaction at the enzyme level. De Bont and Harder (1978) demonstrated that the initial step in ethene metabolism in *Mycobacterium* E20 was the oxidation of ethene to epoxyethane by alkene monooxygenase (AMO). Low activities of AMO were also detected in crude extracts of *Mycobacterium* E20 (de Bont *et al.*, 1979).

Oxidation of short-chain alkenes by methane monooxygenases also results in epoxide formation. Several methane monooxygenases have been studied in the past decade (Anthony, 1986). The most detailed studies have been performed by H. Dalton and co-workers (see Anthony, 1986, for a review) with the soluble methane monooxygenase (MMO) of the type I methanotroph *Methylococcus capsulatus* (Bath). The MMO of *M. capsulatus* (Bath) has been resolved into three components that are all required for monooxygenase activity. Component A exhibits oxygenase activity in the presence of components C (reductase) and B (regulatory protein). In the absence of protein B, components A and C exhibit "NADH oxidase" activity, reducing oxygen to water at the expense of NADH (Anthony, 1986). From the facultative methane-utilizing *Methylobacterium* sp. strain CRL-26 a similar soluble MMO has been purified which did not require a regulatory type B component (Patel and Savas, 1987). Recently, it was shown that *Methylosinus trichosporium* OB3b, a type II methanotroph, also contains a three-component soluble MMO, very similar to the *M. capsulatus* (Bath) enzyme (Fox and Lipscomb, 1988; Fox *et al.*, 1989). Oxidation of alkenes to the corresponding epoxides by *Pseudomonas oleovorans* is also catalyzed by a three-component monooxygenase, although this enzyme has no similarity to the soluble methane monooxygenases (May, 1979).

The methane- and alkane-utilizing bacteria oxidize a wide range of hydrocarbons (Colby *et al.*, 1977; May, 1979; Stirling and Dalton, 1979; Higgins *et al.*, 1983; Witholt *et al.*, 1990) due to the broad substrate specificity of the monooxygenases present. The alkene-utilizing bacteria generally only oxidize alkenes to the corresponding epoxides (van Ginkel *et al.*, 1987). Based on this substrate specificity of AMO activity in whole cells the enzyme is expected to differ from reported monooxygenases.

This report describes the initial characterization of AMO from the ethene-utilizing *Mycobacterium* E3 (Habets-Crützen *et al.*, 1984).

MATERIALS AND METHODS

Strains and cultivation. Mycobacterium E3 was isolated with ethene (Habets-Crützen *et al.*, 1984) and *Mycobacterium aurum* L1 with vinyl chloride as carbon source (Hartmans *et al.*, 1986). Production of biomass was by growth on ethene in a fed-batch manner in a 2 or 10 litre Applikon fermentor at pH 7 and 30°C. Ethene was supplied continuously as a 2% (v/v) mixture in air at a rate of 0.2 vvm. About half of the fermentation liquid, containing approximately 3 g biomass (wet weight) per litre, was harvested daily. After harvesting, the volume which had been withdrawn from the fermentor was

replaced with new mineral salts medium. If biomass was not harvested daily, mineral medium was supplied continuously at a rate of 0.02 h^{-1}. The mineral salts medium contains per litre deionized water: 1.55 K_2HPO_4, 0.85 g $NaH_2PO_4.2H_2O$, 2.0 g $(NH_4)_2SO_4$, 0.1 g $MgCl_2.6H_2O$, 10 mg EDTA, 2 mg $ZnSO_4.7H_2O$, 1 mg $CaCl_2.2H_2O$, 5 mg $FeSO_4.7H_2O$, 0.2 mg $Na_2MoO_4.2H_2O$, 0.2 mg $CuSO_4.5H_2O$, 0.4 mg $CoCl_2.6H_2O$ and 1 mg $MnCl_2.2H_2O$. Maintenance of strains, culture harvesting and storage of harvested cells were as previously described (Hartmans and de Bont, 1986).

Preparation of cell extracts. Crude cell extracts were prepared as described previously (Hartmans and de Bont, 1986) except that 10% (v/v) glycerol (87%) was added before sonication. All chromatographic steps were performed at 4°C. Dialysis of crude extracts was done overnight at 4°C against 200 volumes of 50 mM potassium phosphate buffer, pH 7.3, with 8.7% glycerol (Buffer A).

Fractionation on a DEAE-Sepharose CL-6B column (25 cm × 2.5 cm). Elution was with a linear gradient of 0 to 1 M NaCl in 1 litre of buffer A at a flow rate of 0.8 ml min^{-1}. Routinely about 0.5 g of protein was applied to the column. The protein elution pattern was very reproducible, facilitating the localization of fractions X and Y. Concentration of the pooled fractions (fraction X eluted at a NaCl concentration of about 220 mM and fraction Y eluted at about 300 mM NaCl) from the DEAE-Sepharose column was done by ultrafiltration with an Amicon 8050 concentrator with Filtron membranes. The concentrated protein solution was subsequently diluted (1:10) with buffer A and once again concentrated to remove most of the NaCl.

Enzyme assays. All assays were done at 30°C. Spectrophotometric assays were done on a Perkin-Elmer 550A spectrophotometer. Activities are expressed in nmol product formed min^{-1} (mg protein)$^{-1}$.

Alkene monooxygenase. This was assayed by analyzing epoxypropane formation. Assays were done in serum bottles (35 ml) sealed with a rubber septum. The reaction mixture contained 2 μmol NADH, cell extract and buffer A in a total volume of 1 ml. The serum bottles were incubated at 30°C in a reciprocating water-bath. After 2 min in the water-bath the reaction was started by the addition of 1 ml propene via the rubber septum. Epoxypropane formation was determined by taking head-space samples every 2 to 3 min during a total incubation time of about 20 min. Epoxypropane formation was linear during this time period. Inhibitors were tested by adding them to the assay mixture (incubated at 30°C) 5 min before the reaction was started by the addition of propene. Extracts used in determining the pH optimum of AMO were prepared in demineralized water containing 0.85% (w/v) NaCl and 8.7% glycerol. Immediately after preparation, the extract it was diluted 2-fold with the appropriate buffer (0.1 M potassium phosphate for pH 7.0 to 8.0, and 0.1 M Bis-Tris for pH 5.7 to 7.0) and used to determine AMO activity.

NAD(P)H-acceptor reductase. These activities were assayed as described by Colby and Dalton (1979) using potassium ferricyanide as artificial acceptor in buffer A.

Component X. This was assayed either by adding acetylene-inactivated crude extract or fraction Y to the assay. For both assays it was verified that component X was the rate-limiting component in the total assay mix.

Component Y. This was assayed by adding fraction X to the assay in amounts sufficient to ensure that component Y was the rate-limiting component in the total assay mix.

Inactivation with acetylene. Inactivation of extracts was done by incubating extracts under the same conditions as were used for the AMO activity assay except that propene was omitted and 5% (v/v) acetylene was added to the gas phase. After incubation for 30 min at 30°C the gas phase was flushed with air to remove all acetylene.

Slab gel electrophoresis. Flat polyacrylamide gels (140 × 80 × 3 mm) were prepared containing 7.7% (w/v) polyacrylamide and 2.7% (w/v) cross-linker in 100 mM phosphate buffer pH 7.3. The vertical slab gels were run under non-denaturing conditions immersed in sodium phosphate buffer (50 mM pH 7.3) in a GE-2/4 gel electrophoresis apparatus from Pharmacia. Protein samples applied onto the gel contained 10% (w/v) sucrose and 0.02% bromophenol blue. Electrophoresis was started at 200 V until the proteins entered the gel (15 to 20 min) and was subsequently continued at 60 V until the dye reached the bottom of the gel. NADH-reductase activity staining was done immediately after electrophoresis by incubating the gel in 30 ml of buffer A with 1 mM NADH and 0.6 mM 4-nitro blue tetrazolium chloride in the dark at 30°C for 30 to 60 min.

Analytical methods. Protein was determined by the Bradford (1976) method using bovine serum

albumin as standard. Epoxypropane was determined by analyzing 200 μl headspace samples on a Packard 430 gas chromatograph fitted with a stainless steel Porapak R column (100-120 mesh, 110 cm × 1/8 inch i.d.) and a flame-ionization detector. The oven temperature was 180°C and the carrier gas N_2 at 20 ml min^{-1}.

Chemicals. Ethene, propene and carbon monoxide were from Hoek Loos (Schiedam, The Netherlands). Epoxypropane and glycerol (87%) were from Merck. Acetylene was prepared from $CaCl_2$ (Aldrich) as described by Burris (1974). NADH, NADPH and 4-nitro blue tetrazolium chloride were from Boehringer. *o*-Phenantroline was from Janssen and 8-hydroxyquinoline was from Sigma. DEAE-Sepharose C-6B was from Pharmacia and membrane filters with a nominal cutoff of 10 kDa (Omega NMWL 10K) were from Filtron Corporation.

RESULTS AND DISCUSSION

Determination of AMO activity

AMO activity was assayed by determining epoxypropane formation from propene by headspace analysis. The rate of epoxypropane degradation by crude extracts is insignificant and using headspace analysis the formation of 2 nmol epoxypropane could be detected. Cell-free extracts were routinely prepared in the presence of glycerol as this significantly enhanced the stability of AMO activity. Extracts of ethene-grown, but not glucose-grown cells of *Mycobacterium* strains E3 and L1 contained AMO activity. In both strains AMO activity was strictly dependent on NAD(P)H and oxygen. Extracts of *Mycobacterium* E3 contained slightly higher specific AMO activities than those from strain L1 and were consequently used in most experiments. The pH optimum for AMO activity was at pH 7.3 to 7.4, with activities of about 10% of the maximum at pH 6.0 and pH 8.0. Ultracentrifugation of crude extract at 150,000 × *g* for 90 min resulted in complete recovery of activity in the supernatant indicating that AMO is a soluble enzyme. Elution of crude extracts over a G-10 Sephadex column resulted in significant loss of AMO activity, which could not be restored by the addition of the salt peak. Dialysis by dilution and subsequent concentration using a membrane with a 10 kDa cutoff was, however, possible without significant loss of AMO activity.

AMO, as with other soluble monooxygenases capable of oxidizing short-chain alkenes (Colby and Dalton, 1976; Fox and Lipscomb, 1988), probably consists of more than one component since activity as a function of the protein concentration in the assay mix gave a non-linear relationship (Fig. 1). From Fig. 1 specific activities up to 1.1 nmol epoxypropane min^{-1} (mg protein)$^{-1}$ can be calculated. This is significantly lower than the epoxypropane formation rate of 16 nmol min^{-1} (mg protein)$^{-1}$ reported for whole cells of *Mycobacterium* E3 (Habets-Crützen *et al.*, 1984). Preparation of extracts without glycerol gave initial specific activities which were about twofold higher.

The specific activities of AMO are also very low when compared with the specific activities of about 80 nmol epoxypropane min^{-1} (mg protein)$^{-1}$ reported for MMO from *Methylococcus capsulatus* (Bath) (Colby *et al.*, 1977) and *Methylosinus trichosporium* OB3b (Fox and Lipscomb, 1988).

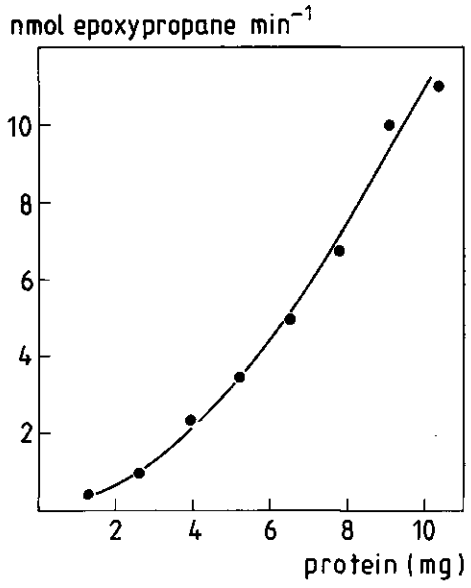

FIG. 1. Relationship between alkene monooxygenase activity and the amount of protein in the assay.

Inhibitors of AMO activity

Using crude extracts several potential inhibitors of AMO activity were tested (Table 1). *o*-Phenantroline, 8-hydroxyquinoline and potassium cyanide all significantly inhibited AMO activity. Other chelating agents tested did not result in significant inhibition, suggesting that any metal ion involved in alkene oxidation by AMO is well-shielded from attack by most metal-binding compounds. AMO activity, inhibited for 40% with 2 mM *o*-phenantroline, was restored completely by the addition of 2 mM Fe^{2+}, but not by adding Cu^{2+}, Zn^{2+}, Mn^{2+} or Mg^{2+}, indicating iron plays an essential role in AMO activity.

Carbon monoxide did not inhibit AMO, indicating that a P450 type of cytochrome is not involved. The strong inhibitory effect observed with acetylene has also been reported for several other monooxygenases (Hyman and Arp, 1988; Hyman and Wood, 1985; Prior and Dalton, 1985). Inhibition by acetylene is dependent on the presence of both oxygen and NADH, and is a result of the transformation of acetylene by the monooxygenase to a reactive species which subsequently inactivates the active centre of the enzyme (Prior and Dalton, 1985).

The observed inhibition pattern for AMO of *Mycobacterium* E3 is very similar to the inhibition patterns reported for the soluble methane monooxygenases of *Methylococcus capsulatus* (Bath) (Stirling and Dalton, 1977) and *Methylosinus trichosporium* OB3b (Scott *et al.*, 1981). Further characterization of the oxygenase component of the soluble MMO of *M. capsulatus* (Bath) (component A) revealed

the presence of an unusual non-haem iron centre (Green and Dalton, 1988). The iron in component A could be removed by dialysis against 8-hydroxyquinoline, resulting in an inactive protein. Reconstitution of the iron centre by incubating with iron-EDTA also resulted in reactivation of protein A (Green and Dalton, 1988).

TABLE 1. Inhibitors of alkene monooxygenase activity.

Inhibitor	Concentration	Inhibition (%)*
KCN	1 mM	0
KCN	5 mM	33
KCN	10 mM	>90
o-Phenantroline	1 mM	23
o-Phenantroline	5 mM	[b]100
8-Hydroxyquinoline	2 mM	53
Thiourea	10 mM	0
Allylthiourea	10 mM	0
NaN_3	10 mM	0
2,2'-Dipyridyl	10 mM	0
Salicylic acid	10 mM	0
EDTA	10 mM	0
Carbon monoxide	20 %	<5
Acetylene	5 %	>85

* Activity was determined by adding propene after 5 min preincubation at 30°C with inhibitor.
[b] Activity levelled off to zero during assay.

Separation of AMO components

AMO in crude extracts prepared without glycerol lost 50 % of its activity after 5 h at 4°C. The crude enzyme preparation could be stabilized by adding glycerol. With 2.6, 4.4, and 8.7% glycerol added to crude extracts, AMO activity half-life 4°C was extended to 9, 18 and 40 h respectively.

In all subsequent experiments 8.7% glycerol was added to extracts and buffers. The addition of β-mercaptoethanol, PMSF, DTT, EDTA, NADH, $FeSO_4$, $Fe(NH_4)_2(SO_4)_2.6H_2O$ as well as storage under anaerobic conditions did not affect the stability of AMO activity.

Separation of the individual components of AMO was attempted on a DEAE Sepharose CL-6B column. Fractions pooled progressively to give 50 ml pools were concentrated to assay AMO activity. No activity could be detected in any of these concentrated 50 ml pools. Combination of all concentrated fractions, however, resulted in the recovery of some AMO activity. Apparently, the phenomena which

resulted in the irreversible inactivation of AMO activity after elution over a G-10 Sephadex column played a less-significant role in DEAE column chromatography. The rate-limiting component of AMO activity was located by combining concentrated fractions from the DEAE Sepharose CL-6B column with crude extract and assaying for increased AMO activities. The rate-limiting component, designated component X, was located in the pooled fractions eluting between 545 and 615 ml (Fig. 2). These pooled fractions did not exhibit any AMO activity without addition of crude extract. Using crude extract which had been stored for some time at −20°C the increase in AMO activity upon addition of the fraction containing component X was even more significant: the activity of thawed crude extract containing 4.5 mg protein increased from 0.8 nmol epoxypropane min^{-1} to 10 nmol min^{-1} when 7.2 mg protein of fraction X was added.

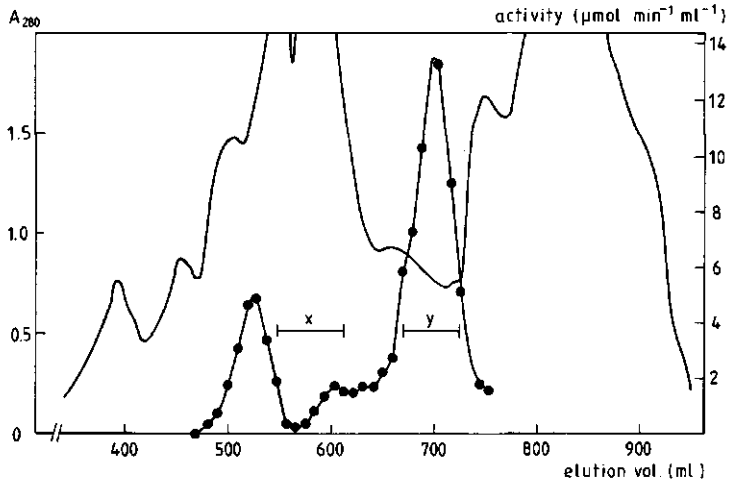

FIG. 2. DEAE-Sepharose CL-6B fractionation of crude extract. Fractions of 8 ml were collected. The horizontal lines indicate the elution volumes used to prepare fractions X and Y. NAD(P)H-acceptor reductase activities were assayed with $K_3Fe(CN)_6$ as artificial acceptor (●).

A preparation containing this presumably most labile component X of AMO was used to locate a second fraction which upon combination with fraction X gave AMO activity. This second fraction was designated fraction Y.

At this stage it was verified that both components X and Y were only present in cells grown with ethene as growth substrate. Combination of fractions X (2.2 mg protein) or Y (1.2 mg protein) with crude extracts (7.8 mg protein) of glucose-grown cells did not result in detectable levels of AMO activity, indicating that components X and Y were indeed not present in extracts of glucose-grown cells. Furthermore, fractions containing components X and Y were also prepared from extracts of ethene-grown *Mycobacterium aurum* L1 cells. All combinations of fractions X and Y of the two strains resulted in activity, indicating a certain degree of similarity

between the AMOs of the two strains.

Characterization of fractions X and Y.

In an initial characterization of fractions X and Y it was determined which of these fractions contained NADH-acceptor reductase activity by following the reduction of the artificial acceptor $K_3Fe(CN)_6$ (Colby and Dalton, 1979). Significant reductase activity was present in fraction Y. In a subsequent separation of fractions X and Y this assay was used to locate the reductase component Y more precisely (Fig. 2). Several peaks exhibiting reductase activity were located. Fractions from the peak with the highest reductase activity (670 to 730 ml) gave AMO activity upon combination with fraction X obtained by concentrating the protein peak which eluted between 545 and 615 ml. The location of fractions X and Y used in the activity determinations shown in Table 2 are depicted by the horizontal lines in Fig. 2.

TABLE 2. Specific activities of alkene monooxygenase fractions

Protein in assay (mg)				Activity	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]	
cfe	cfe/Ac	X	Y	(nmol min^{-1})	X	Y
12.5	0.0	0.0	0.0	3.10	0.25	–
0.0	0.0	1.1	1.9	0.46	0.42	–
12.5	0.0	6.0	0.0	5.0	–	0.41
0.0	0.0	7.6	0.62	1.23	–	1.99
0.0	9.0	1.1	0.0	0.45	0.41	–

cfe, cell-free extract (stored at 4°C during preparation of X and Y); cfe/Ac, cell-free extract inactivated with acetylene; X and Y, preparations fractionated with DEAE column as depicted in Fig. 2.

As component X was assumed to be rate-limiting in crude extracts, the activity of crude extract can be assumed to be determined by the amount of component X present. The specific activity of fraction X in the presence of excess component Y was 0.41 nmol min^{-1} (mg protein)$^{-1}$, which is only slightly higher than the specific activity of crude extract (0.25 nmol min^{-1} (mg protein)$^{-1}$). The apparent purification of the reductase component Y appeared to be slightly higher, with the specific activity increasing from 0.41 to 1.99 nmol min^{-1} (mg protein)$^{-1}$ (Table 2). These low increases in specific activities could indicate that a third component is required for AMO activity, as has been established for the soluble MMOs (Anthony, 1986; Fox *et al.*, 1989). If such a third component is required it is apparently present in limiting amounts in either fraction X or Y (or both). Attempts

to further purify components X and Y should resolve this matter in the future.

Addition of fraction X to crude extracts which had been inactivated by incubation with acetylene restored AMO activity. As can be seen in Table 2 this gave the same specific activity [0.45 nmol min^{-1} (mg protein)$^{-1}$] based on fraction X protein, as the assay with excess component Y [0.46 nmol min^{-1} (mg protein)$^{-1}$]. Addition of fraction Y to acetylene-inactivated extracts did not result in restoration of AMO activity. Apparently, inactivation with acetylene results in inactivation only of component X. Inactivation of component X with acetylene is accomplished only in the presence of active AMO. Incubation of fractions X and Y separately with acetylene in the presence of NADH and oxygen for 30 min and subsequent removal of acetylene before combining both fractions gave active AMO. Combination of fractions X and Y before incubation with acetylene resulted in inactivation of AMO activity. These results indicated that both preparations are essential to produce the AMO-inactivating product of acetylene and that fraction X appears to contain the AMO component which can be inactivated by acetylene. Prior and Dalton (1985) demonstrated that only one protein was labelled in soluble and particulate fractions of crude extracts of *Methylococcus capsulatus* (Bath) after incubation with ^{14}C labelled acetylene. For the soluble MMO preparation the labelled polypeptide corresponded to the α-subunit of component A, the oxygenase component of the soluble MMO. By analogy with the soluble MMO of *Methylococcus capsulatus* (Bath), fraction X could very well contain the oxygenase component of AMO.

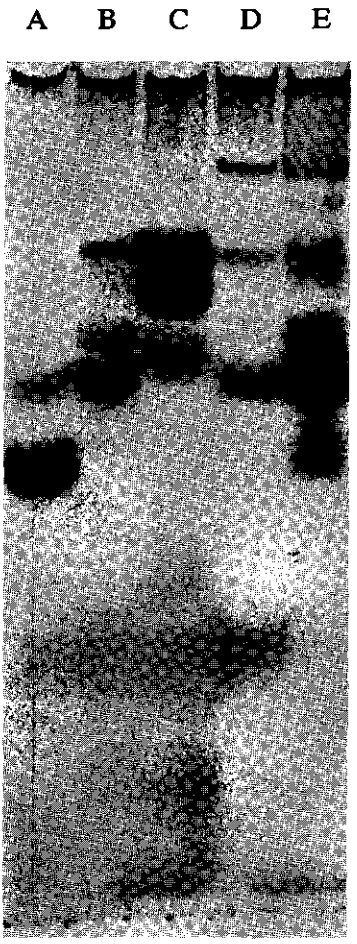

FIG. 3. Non-denaturing gel electrophoresis showing NADH reductase activity stained with 4-nitro blue tetrazolium chloride. Lane A, crude extract of ethene-grown cells; lane B, crude extract of glucose-grown cells; lane C, first peak with reductase activity from DEAE column (at 530 ml); lane D, fraction X; lane E, fraction Y.

The specific activity of fraction Y when assayed with potassium ferricyanide was 16.6 μmol min^{-1} (mg protein)$^{-1}$ compared to 230 μmol min^{-1} (mg protein)$^{-1}$ reported for the purified component C of *Methylococcus capsulatus* (Bath) (Colby and Dalton, 1979). Using gel electrophoresis under non-denaturing conditions fraction Y was compared with several other samples after activity staining with 4-nitro blue tetrazolium chloride. As can be seen in Fig. 3 a band with reductase activity at the same migration distance as the major reductase component of fraction Y was present only in crude extracts of ethene-grown cells. Fraction X and extracts of glucose-grown cells did not exhibit any detectable reductase activity at this migration distance. This indicates that the major band in the lane with fraction Y probably is the reductase component of AMO.

It is therefore concluded that in both of the mycobacteria studied a multicomponent AMO is induced after growth on ethene. In extracts of *Mycobacterium* E3 two fractions (designated X and Y) were located after separation on an anionic exchanger; upon combination these two fractions gave AMO activity. Based on inhibition experiments with acetylene it was concluded that fraction X contains an oxygenase component. Fraction Y contained significant reductase activity which is induced after growth with ethene. From the present data it is not clear if, in analogy with the soluble MMO, a third component is required for AMO activity. Attempts to further purify components X and Y should resolve this matter in the future.

REFERENCES

Anthony, C. 1986. Bacterial oxidation of methane and methanol. Adv. Microbial Physiol. **27**:113-210.

de Bont, J.A.M., and W. Harder. 1978. Metabolism of ethylene by *Mycobacterium* E20. FEMS Microbiol. Lett. **3**:89-93.

de Bont, J.A.M., M.M. Attwood, S.B. Primrose, and W. Harder. 1979. Epoxidation of short chain alkenes in *Mycobacterium* E20: the involvement of a specific mono-oxygenase. FEMS Microbiol. Lett. **6**:183-188.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.

Burris, R.H. 1974. Methodology, p. 23. *In* A. Quispel (ed.), The biology of nitrogen fixation. North Holland Publishing Co., Amsterdam.

Colby, J., D.I. Stirling, and H. Dalton. 1977. The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath). Biochem. J. **165**:395-402.

Colby, J., and H. Dalton. 1979. Characterization of the second prosthetic group of the flavoenzyme NADH-acceptor reductase (component C) of the methane mono-oxygenase from *Methylococcus capsulatus* (Bath). Biochem. J. **177**:903-908.

Fox, B.G., and J.D. Lipscomb. 1988. Purification of a high specific activity methane monooxygenase hydroxylase component from a type II methanotroph. Biochem. Biophys. Res. Comm. **154**:165-170.

Fox, B.G., W.A. Froland, J.E. Dege, and J.D. Lipscomb. 1989. Methane monooxygenase from *Methylosinus trichosporium* OB3b. J. Biol. Chem. **264**:10023-10033.

van Ginkel, C.G., H.G.J. Welten, and J.A.M. de Bont. 1987. Oxidation of gaseous and volatile hydrocarbons by selected alkene-utilizing bacteria. Appl. Environ. Microbiol. **53**:2903-2907.

Green, J., and H. Dalton. 1988. The biosynthesis and assembly of protein A of soluble methane monooxygenase of *Methylococcus capsulatus* (Bath). J. Biol. Chem. **263**:17561-17565.

Habets-Crützen, A.Q.H., L.E.S. Brink, C.G. van Ginkel, J.A.M. de Bont, and J. Tramper. 1984.

Production of epoxides from gaseous alkenes by resting-cell suspensions and immobilized cells of alkene-utilizing bacteria. Appl. Microbiol. Biotechnol. **20**:245-250.

Habets-Crützen, A.Q.H., S.J.N. Carlier, J.A.M. de Bont, D. Wistuba, V. Schurig, S. Hartmans, and J. Tramper. 1985. Stereospecific formation of 1,2-epoxypropane, 1,2-epoxybutane and 1-chloro-2,3-epoxypropane by alkene-utilizing bacteria. Enzyme Microb. Technol. **7**:17-21.

Hartmans, S., J.A.M. de Bont, J. Tramper, and K.C.A.M. Luyben. 1985. Bacterial degradation of vinyl chloride. Biotechnol. Lett. **7**:383-388.

Hartmans, S., and J.A.M. de Bont. 1986. Acetol monooxygenase from *Mycobacterium* Py1 cleaves acetol into acetate and formaldehyde. FEMS Microbiol. Lett. **36**:155-158.

Hartmans, S., J.A.M. de Bont, and W. Harder. 1989. Microbial metabolism of short-chain unsaturated hydrocarbons. FEMS Microbiol. Rev. **63**:235-264.

Higgins, I.J., D.J. Best, and R.C. Hammond. 1983. New findings in methane-utilizing bacteria highlight their importance in the biosphere and their commercial potential. Nature **286**:561-564.

Hyman, M.R., and D.J. Arp. 1988. Acetylene inhibition of metalloenzymes. Anal. Biochem. **173**:207-220.

Hyman, M.R., and P.M. Wood. 1985. Suicidal inactivation and labelling of ammonia mono-oxygenase by acetylene. Biochem. J. **227**:719-725.

May, S.W. 1979. Enzymatic epoxidation reactions. Enzyme Microb. Technol. **1**:15-22.

Patel, R.N., and J.C. Savas. 1987. Purification and properties of the hydroxylase component of methane monooxygenase. J. Bacteriol. **169**:2313-2317.

Prior, S.D., and H. Dalton. 1985. Acetylene as a suicide substrate and active site probe for methane monooxygenase from *Methylococcus capsulatus* (Bath). FEMS Microbiol. Lett. **29**:105-109.

Scott, D., J. Brannan, and I.J. Higgins. 1981. The effect of growth conditions on intracytoplasmic membranes and methane mono-oxygenase activities in *Methylosinus trichosporium* OB3b. J. Gen. Microbiol. **125**:63-72.

Stirling, D.I., and H. Dalton. 1977. Effect of metal-binding agents and other compounds on methane oxidation by two strains of *Methylococcus capsulatus*. Arch. Microbiol. **114**:71-76.

Weijers, C.A.G.M., C.G. van Ginkel, and J.A.M. de Bont. 1988. Enantiomeric composition of lower epoxyalkanes produced by methane-, alkane- and alkene-utilizing bacteria. Enzyme Microb. Technol. **10**:214-218.

Witholt, B., M.-J. de Smet, J. Kingma, J.B. van Beilen, M. Kok, R.G. Lageveen, R.G. and G. Eggink. 1990. Bioconverison of aliphatic compounds by *Pseudomonas oleovorans* in multiphase bioreactors: background and economic potential. Trends Biotechnol. **8**:46-52.

Chapter 5

USE OF VINYL CHLORIDE IN THE ISOLATION OF MUTANTS OF *MYCOBACTERIUM* E3 DEFECTIVE IN GROWTH ON ETHENE

S. Hartmans, F.J. Weber, G.T.H. Voskuilen and J.A.M. de Bont

Mutants of *Mycobacterium* E3 unable to grow on ethene were isolated with a frequency of 10 to 20% using vinyl chloride as a mutagen-precursor. The method is based on the transformation of vinyl chloride to the very mutagenic chlorooxirane by alkene monooxygenase of wild-type cells. One ethene-negative mutant still capable of growth with epoxyethane was characterized as lacking the putative reductase component of the multicomponent alkene monooxygenase.

Abbreviations: AMO, alkene monooxygenase; MMO, methane monooxygenase: MSM, mineral salts medium; YEG, yeast extract-glucose; MSG, mineral salts glucose.

The initial step in short-chain alkene metabolism in alkene-utilizing microorganisms is the oxidation of alkenes to epoxyalkanes, catalyzed by alkene monooxygenase (AMO) (Hartmans *et al.*, 1989). AMO from the ethene-utilizing *Mycobacterium* E3 requires at least two fractions for activity (Hartmans *et al.*, 1991). One of these fractions is inhibited by acetylene and probably contains the oxygenase component (X) whereas the other fraction contains reductase activity, and therefore probably contains the reductase component (Y).

Several other monooxygenases which epoxidate alkenes have already been well characterized and were all multicomponent enzymes (see May, 1979; Anthony, 1986).

Inhibition of AMO from *Mycobacterium* E3 by chelating compounds was very similar to the inhibition reported for the soluble methane monooxygenases (Hartmans *et al.*, 1991). The specific activity of AMO in crude extracts of strain E3 was, however, much lower than the activities reported for the soluble methane monooxygenases (MMO) of *Methylococcus capsulatus* (Bath) (Colby *et al.*, 1977) and *Methylosinus trichosporium* OB3b (Fox *et al.*, 1989). MMO from *M. capsulatus* (Bath) has been purified and characterized in detail by the group of H. Dalton (see Anthony, 1986, for an overview). The *M. trichosporium* OB3b MMO has recently also been purified and characterized (Fox *et al.*, 1989). This work revealed that these soluble methane monooxygenases were very similar and consist of three components. The genes encoding the subunits of the oxygenase component and the reductase component of the soluble MMO from *M. capsulatus* (Bath) have been cloned and sequenced using oligonucleotide probes specific to the *N*-terminal amino acid sequence of the individual purified proteins (Mullens and Dalton, 1987; Stainthorpe *et al.*, 1989; 1990). Subsequently, heterologous probes derived from *M. capsulatus* (Bath) were used to isolate the MMO genes of *M. trichosporium* OB3b (Cardy *et al.*, 1991). Classical genetics with methanotrophs has, however, been less successful, in part because of the problems encountered in isolating the necessary MMO-deficient mutants (McPheat *et al.*, 1987).

The multicomponent alkane hydroxylase (May, 1979) of the alkane-utilizing *Pseudomonas putida* has also been studied quite extensively. Much of the progress in the area of the genetics and regulation of the alkane hydroxylase was made possible by using hydroxylase-deficient mutants.

To achieve a better understanding of the alkene monooxygenase, the isolation of AMO-deficient mutants of ethene-utilizing bacteria would clearly be very helpful. Such mutants could be used in an assay system (*in vitro* complementation) to facilitate the purification of the individual components of the enzyme and could also be used in studies concerning the regulation of AMO activity.

The objective of the present investigation was to isolate AMO-deficient mutants of *Mycobacterium* E3. This paper describes a new mutant isolation method using vinyl chloride as a mutagen-precursor which is transformed *in situ* into active mutagen only in cells with monooxygenase activity.

MATERIALS AND METHODS

Chemicals. The gases ethene, epoxyethane, propene and vinyl chloride were from Hoek Loos (Schiedam, NL). Other chemicals were from the sources described previously (Hartmans *et al.*, 1991).

Organism and growth conditions. Mycobacterium E3 was previously isolated with ethene as growth substrate (Habets-Crützen *et al.*, 1984). The mineral salts medium, growth conditions, culture harvesting and storage of harvested cells were as previously described (Hartmans *et al.*, 1991). Solid media were prepared by adding 1.2% (w/v) agar (Oxoid No.1) to the mineral salts medium (MSM). Per litre MSM 0.25 g yeast extract and 1 g glucose was added for yeast extract-glucose (YEG) plates and 1 g glucose or sodium acetate was added for glucose and acetate plates respectively.

Preparation of cell extracts. Crude cell extracts, acetylene inactivated extracts and preparations containing component X or Y were prepared as described previously (Hartmans *et al.*, 1991).

Isolation of AMO-deficient mutants. **Method I:** E3 cells from yeast extract-glucose agar slants were suspended in sterile water and diluted to about 1000 cells/ml. 0.1 ml of such a suspension was plated on YEG plates. YEG plates were placed in a desiccator and vinyl chloride (2.5% v/v) and ethene (0.25 % v/v) were added to the gas phase. All plates were incubated at 30°C. After 10 days the plates were removed from the desiccator and individual colonies were transferred to YEG parent plates. After good growth had occurred on the parent plates these were replica plated on MSM plates (twice) and glucose plates. The first series of MSM plates was placed in a desiccator with 2% (v/v) ethene and the second series was placed in a desiccator to which 2 mM (based on the volume of the solidified liquid phase of the agar plates) epoxyethane was added. The epoxyethane concentration in the desiccator was monitored every two days by headspace analysis and epoxyethane was added, if due to hydrolysis or consumption, the concentration had fallen below 0.5 mM. **Method II:** 0.1 ml of a suspension of ethene-grown cells of strain E3 containing approximately 500 cells/ml was spread on MSM plates and incubated in a desiccator containing vinyl chloride (1% v/v), ethene (0.02% v/v) and epoxyethane (2 mM based on the total liquid volume of the agar plates). The epoxyethane concentration in the desiccator was controlled as described above. After three weeks material from the outer perimeter of the larger colonies was transferred to YEG parent plates. Phenotypic characterization of the putative mutants was as described under method I.

Determination of reversion frequencies. 100 µl aliquots of suitable decimal dilutions were plated on YEG plates and MSM plates which were incubated with ethene in the gas phase (2% v/v). After three weeks of incubation at 30°C the number of colonies formed were compared.

Enzyme assays. Alkene monooxygenase was assayed as described previously (Hartmans *et al.*, 1991) by gas chromatographically determining epoxypropane formation rates. Activity staining of NADH-reductase after non-denaturing gel electrophoresis was performed as before (Hartmans *et al.*, 1991).

Analytical methods. Ethene, vinyl chloride and epoxyethane were determined by analyzing 100 µl headspace samples on a gas chromatograph with a Porapak R column (Hartmans *et al.*, 1991).

RESULTS

Isolation of AMO-deficient mutants

Incubation of *Mycobacterium* E3 on yeast extract-glucose agar plates in the presence of vinyl chloride (2.5% v/v) and ethene (0.25% v/v) resulted in large (approximately 40%) and small colonies. Most of the large colonies had lost the ability to grow on ethene, whereas all but one of the small colonies tested still grew with ethene. Unfortunately, with one exception, all the colonies that had lost the capacity to grow with ethene had also lost the capacity to grow with epoxyethane (Table 1). The one colony that had lost the capacity to grow with ethene but which could still utilize epoxyethane as growth substrate was designated mutant AMO$^-$-1.

In a second experiment two series of 10 plates with an average of 127 ± 20 and

63 ± 9 colonies respectively were incubated with vinyl chloride and ethene. After 10 days an average of 60 and 32 colonies had appeared on the YEG-vinyl chloride plates corresponding with viability percentages of 47% and 51% respectively. 28% of the colonies on the YEG-vinyl chloride plates were judged as large and 72% were judged as small colonies. As in the first experiment, again almost 80% of the large colonies were mutants unable to utilize ethene. In this experiment there were also several mutants (7%) amongst the small colonies (Table 1). Unfortunately the percentage of ethene-negative, epoxyethane positive, mutants was once again very low (1.4%). The three mutants recovered from the second experiment were designated AMO$^-$-2, AMO$^-$-3 and AMO$^-$-4, respectively.

TABLE 1. Selection of alkene monooxygenase deficient mutants.

	Growth substrate	Small colonies	Large colonies
Experiment 1	glucose	156	108
	ethene	155	20
	epoxyethane	ND	21
Experiment 2	glucose	87	263
	ethene	81	54
	epoxyethane	82	56

Although ethene minus phenotypes were isolated at a high frequency with the above method almost all of these mutants had also lost the capacity to grow on epoxyethane. We therefore used a second method to isolate ethene minus mutants still capable of utilizing epoxyethane by employing epoxyethane instead of glucose as growth substrate during the isolation procedure (method II).

Using this method we did not observe a very distinct difference between large and small colonies as was observed with glucose as growth substrate. Nevertheless, we tested 80 colonies from the plates that had been incubated for 4 weeks with the mixture of vinyl chloride, ethene and epoxyethane for growth on ethene and epoxyethane. This gave 5 colonies which no longer grew on ethene or epoxyethane and 11 colonies which did not grow on ethene, but still utilized epoxyethane as growth substrate. These 11 mutants of the desired phenotype were not further characterized.

Characterization of mutants

The stability of the four alkene monooxygenase-deficient mutants isolated with method I was determined by plating dilutions on YEG plates and on mineral salts medium plates which were incubated with ethene. After three weeks colony counts were performed and reversion rates calculated.

TABLE 2. Reversion rates of alkene monooxygenase deficient mutants.

Mutant	Reversion rate
AMO$^-$-1	2×10^{-7}
AMO$^-$-2	5×10^{-8}
AMO$^-$-3	5×10^{-8}
AMO$^-$-4	1×10^{-6}

Complementation experiments were performed in an attempt to study the nature of the AMO$^-$ mutations. Extracts of the mutants were prepared from cells grown with epoxyethane and combined with preparations derived from extracts of the wild-type strain by anion exchange chromatography, or by inactivation with acetylene. AMO activity in extracts of mutant AMO$^-$-1 could be restored by adding the anion exchange fraction containing the reductase component Y of AMO (Hartmans *et al.*, 1991) and also by adding extracts inactivated with acetylene. Addition of the anion exchange fraction containing the oxygenase component X did not result in activity. Complementation experiments with the other mutants only gave very low levels of AMO activity, or no activity at all.

Crude extracts of epoxyethane-grown mutant AMO$^-$-1 were also analyzed with non-denaturing slab gel electrophoresis followed by activity staining for reductase activity. No reductase activity was observed at the migration distance were the partially purified reductase preparation (component Y) or extracts of epoxyethane-grown E3 cells normally exhibited activity (Hartmans *et al.*, 1991). Based on these observations it is concluded that epoxyethane-grown cells of mutant AMO$^-$-1 do not synthesize a functional reductase component of AMO.

DISCUSSION

Vinyl chloride is oxidized by crude cell-free extracts of *Mycobacterium* E3 in an oxygen- and NADH-dependent reaction. Addition of ethene to extracts degrading vinyl chloride resulted in a lower vinyl chloride degradation rate indicating that both substrates are oxidized by the same enzyme. Based on these experiments we assumed that vinyl chloride is epoxidated to chlorooxirane (chloroepoxyethane) by AMO analogous to the oxidation of vinyl chloride by MMO (Fox *et al.*, 1990) and liver microsomes (Barbin *et al.*, 1975). Chlorooxirane is very unstable and rearranges to chloroacetaldehyde in Tris-HCl buffer pH 7.4 at 37°C with a half-life of 1.6 min (Barbin *et al.*, 1975). Both products are mutagenic intermediates in mammalian vinyl chloride metabolism (Malaveille *et al.*, 1975).

We reasoned that cells capable of oxidizing vinyl chloride will produce chlorooxirane provided an active monooxygenase and sufficient reducing equivalents

are present. The *in vivo* production of the reactive chlorooxirane should result in the inhibition of physiological functions and consequently in retardation of growth. Cells without monooxygenase activity will not produce chlorooxirane from vinyl chloride and will not be affected by the presence of this compound. Consequently vinyl chloride might be used to isolate AMO-deficient mutants of *Mycobacterium* E3 by selecting the large colonies grown in the presence of vinyl chloride. Ethene was included in the incubation atmosphere to assure induction of AMO as it was assumed it would be the inducer of AMO. Vinyl chloride was added in a tenfold higher concentration than ethene to ensure that AMO would predominantly transform vinyl chloride rather than ethene. Substrate competition experiments had previously shown that the affinity of ethene-grown cells for vinyl chloride and ethene was, based on gas phase concentrations, about the same.

To minimize the effects of chlorooxirane (or its rearrangement product chloroacetaldehyde) produced by colonies possessing AMO activity on colonies not oxidizing vinyl chloride present on the same plate only very few cells (50 to 100) were applied onto the YEG-vinyl chloride plates. With this procedure extremely high overall mutation frequencies ranging from 10 to 20% per original viable cell were obtained. This frequency was even higher (80%) when only the larger colonies are considered. This high mutation rate is not surprising when it is realized that viable wild-type cells constantly produce mutagenic compounds from vinyl chloride until a mutation resulting in loss of AMO activity occurs.

Unfortunately almost all ethene negative mutants (>98%) were also unable to grow on epoxyethane. This indicates that the expression of the two enzymes involved, AMO and epoxyethane dehydrogenase (de Bont and Harder, 1978), is under coordinated control and that regulatory mutants are produced much more easily than mutants producing inactive AMO.

Although method I resulted in the isolation of mutants no longer capable of growth on ethene with a high frequency, this method was rather inefficient for the isolation of the ethene minus epoxyethane utilizing phenotype (less than 1% of the large colonies).

A clear division between large and small colonies was not observed with the second method using epoxyethane as growth substrate instead of YEG. Nevertheless the method was extremely successful since 11 of the 80 colonies tested had the desired phenotype.

The isolation of methane monooxygenase-deficient mutants of two methanotrophs (Nicolaidis and Sargent, 1987; McPheat *et al.*, 1987) involved a similar procedure as described here. However, dichloromethane was used instead of vinyl chloride. These mutants were obtained by plating large numbers of cells on methanol plates which were incubated in the presence of dichloromethane. Dichloromethane is transformed by methane monooxygenase to carbon monoxide which inhibits growth of the methanotrophs. Mutants without methane monooxygenase were dichloromethane resistant and formed larger colonies, apparently because no carbon

monoxide was formed. Dichloromethane can induce frameshift mutations in *Salmonella typhimurium* without biochemical activation (Jongen *et al.*, 1978) but dichloromethane and its oxidation product carbon monoxide are considerably less mutagenic than the oxidation products of vinyl chloride. This is exemplified by the mutation frequency using the dichloromethane procedure with *Methylosinus trichosporium* OB3b which, although Nicolaidis and Sargent (1987) did not report any mutation frequencies, was probably less than 10^{-9}. As vinyl chloride is also transformed by methane monooxygenase (Fox *et al.*, 1990) the use of vinyl chloride instead of dichloromethane should be much more effective in isolating methane monooxygenase-deficient mutants.

The mutational specificity of chlorooxirane has been investigated in E. coli by analyzing the reversion of *trp A* mutants (Barbin *et al.*, 1985). It was shown that chlorooxirane induces base-pair substitution mutations, predominantly GC → AT transitions, but not frameshift mutations. In this respect chlorooxirane is comparable with the often used mutagen ethyl methanesulphonate (EMS) which also specifically induces GC → AT transitions. Treatment of *E. coli trp A* mutants with chlorooxirane resulted in a higher reversion frequency (on a molar basis) compared with treatment with EMS (0.5 mM chlorooxirane for 6 min, 35% survival, reversion frequency = 187×10^{-9} vs. 80 mM EMS for 1 hour, 72% survival, reversion frequency = 120×10^{-9}) (Barbin *et al.*, 1985). These results demonstrate the reactivity and strong mutagenic properties of chlorooxirane.

Although chlorooxirane mainly induces base-pair substitutions (Barbin *et al.*, 1985) the four AMO-deficient mutants isolated were relatively stable (Table 2). The reversion frequencies may in fact be even lower than shown in Table 2 as strain E3 can grow to some extent on impurities in agar resulting in micro-colonies which should have a higher rate of reversion than single cells.

From the complementation experiments of mutant AMO$^-$-1 with partially purified preparations of AMO it appears that this mutant lacks the reductase component Y of AMO (Hartmans *et al.*, 1991). At present we are further characterizing the isolated mutants and we have purified the reductase component of AMO using mutant AMO$^-$-1 in a complementation assay (Weber *et al.*, 1992).

REFERENCES

Anthony, C. 1986. Bacterial oxidation of methane and methanol. Adv. Microbial Physiol. **27**:113-210.

Barbin, A., H. Brésil, A. Croisy, P. Jacquignon, C. Malaveille, R. Montesano, and H. Bartsch. 1975. Liver-microsome-mediated formation of alkylating agents from vinyl bromide and vinyl chloride. Biochem. Biophys. Res. Comm. **67**:596-603.

Barbin, A., F. Besson M.-H. Perrard, J.-C. Béréziat, J, Kaldor, G. Michel, and H. Bartsch. 1985. Induction of specific base-pair substitutions in *E. coli trp A* mutants by chloroethylene oxide, a carcinogenic vinyl chloride metabolite. Mut. Res. **152**:147-156.

de Bont, J.A.M., and W. Harder. 1978. Metabolism of ethylene by *Mycobacterium* E20. FEMS Microbiol. Lett. 3:89-93.

Cardy, D.L.N., V. Laidler, G.P.C. Salmond, and J.C. Murrell. 1991. The methane monooxygenase gene cluster of *Methylosinus trichosporium*: cloning and sequencing of the *mmoC* gene. Arch Microbiol **156**:in press.

Colby, J., D.I. Stirling, and H. Dalton. 1977. The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath). Biochem. J. **165**:395-402.

Fox, B.G., W.A. Froland, J.E. Dege, and J.D. Lipscomb. 1989. Methane monooxygenase from *Methylosinus trichosporium* OB3b. J. Biol. Chem. **264**:10023-10033.

Fox, B.G., J.G. Borneman, L.P. Wackett, and J.D. Lipscomb. 1990. Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. Biochemistry **29**:6419-6427.

Habets-Crützen, A.Q.H., L.E.S. Brink, C.G. van Ginkel, J.A.M. de Bont, and J. Tramper. 1984. Production of epoxides from gaseous alkenes by resting-cell suspensions and immobilized cells of alkene-utilizing bacteria. Appl. Microbiol. Biotechnol. **20**:245-250.

Hartmans, S., J.A.M. de Bont, and W. Harder. 1989. Microbial metabolism of short-chain unsaturated hydrocarbons. FEMS Microbiol. Rev. **63**:235-264.

Hartmans, S., F.J. Weber, B.P.M. Somhorst, and J.A.M. de Bont. 1991. Alkene monooxygenase from *Mycobacterium*: a multicomponent enzyme. J. Gen. Microbiol. **137**:2555-2560.

Jongen, W.M.F., G.G. Alink, and J.H. Koeman. 1978. Mutagenic effect of dichloromethane on *Salmonella typhimurium*. Mut. Res. **56**:245-248.

Malaveille, C., H. Bartsch, A. Barbin, A.M. Camus, R. Montesano, A. Croisy, and P. Jacquignon. 1975. Mutagenicity of vinyl chloride, chloroethylene oxide, chloroacetaldehyde and chloroethanol. Biochem. Biophys. Res. Comm. **63**:363-370.

May, S.W. 1979. Enzymatic epoxidation reactions. Enzyme Microb. Technol. **1**:15-22.

McPheat, W.L., N.H. Mann, and H. Dalton. 1987. Isolation of mutants of the obligate methanotroph *Methylomonas albus* defective in growth on methane. Arch. Microbiol. **148**:40-43.

Mullens, I.A., and H. Dalton. 1987. Cloning of the gamma-subunit methane monooxygenase from *Methylococcus capsulatus*. Bio/Technol. **5**:490-493.

Nicolaidis, A.A., and A.W. Sargent. 1987. Isolation of methane monooxygenase-deficient mutants from *Methylosinus trichosporium* OB3b using dichloromethane. FEMS Microbiol. Lett. **41**:47-52.

Stainthorpe, A.C., J.C. Murrell, G.P.C. Salmond, H. Dalton, and V. Lees. 1989. Molecular analysis of methane monooxygenase from *Methylococcus capsulatus* (Bath). Arch. Microbiol. **152**:154-159.

Stainthorpe, A.C., V. Lees, G.P.C. Salmond, H. Dalton, and J.C. Murrell. 1990. The methane monooxygenase gene cluster of *Methylococcus capsulatus* (Bath). Gene **91**:27-34.

Weber, F.J., W.J.H. van Berkel, S. Hartmans, and J.A.M. de Bont. 1992. Purification and properties of the NADH-reductase component of alkene monooxygenase from *Mycobacterium* strain E3. J. Bacteriol. **174**:3275-3281.

Chapter 6

BACTERIAL METABOLISM OF 3-CHLOROACRYLIC ACID

S. Hartmans, M.W. Jansen, M.J. van der Werf and J.A.M. de Bont

Two bacterial strains were isolated with 3-chloroacrylic acid (CAA) as sole source of carbon and energy. Strain CAA1, a *Pseudomonas cepacia* sp., was capable of growth with only the *cis*-isomer of CAA. Strain CAA2, a coryneform bacterium, utilized both isomers of CAA as sole source of carbon and energy. Strain CAA1 contained *cis*-CAA hydratase and strain CAA2 contained two hydratases, one with *cis*-3-CAA hydratase activity and one with *trans*-CAA hydratase activity. The product of the hydratase activities with CAA was malonate semialdehyde. In both strains malonate semialdehyde was subsequently decarboxylated by a cofactor-independent decarboxylase yielding acetaldehyde and CO_2.

Journal of General Microbiology (1991) **137**: 2025-2032.

The biodegradability of halogenated hydrocarbons receives considerable attention in view of the persistence of some of these compounds in the environment. Degradation of a number of chlorinated hydrocarbons by pure cultures has, however, been demonstrated. In several cases the dechlorination step has also been characterized. Specific dechlorination reactions of chlorinated hydrocarbons that have been studied in detail are the hydrolytic dehalogenases acting on chlorinated alkanes (Kohler-Staub and Leisinger, 1985; Keuning *et al.*, 1985; Scholtz *et al.*, 1987) or 2-chloroalkanoic acids (Motosugi *et al.*, 1982; Smith *et al.*, 1990). The oxidative dechlorination of chlorinated alkanes has also been reported (Yokota *et al.*, 1986). The dechlorination reactions of unsaturated chlorinated hydrocarbons have been less well studied. Within this category of compounds most attention has focussed on the chlorinated ethenes due to their broad use and widespread occurrence in the environment. Transformation of all chlorinated ethenes has been observed under anaerobic conditions (Fathepure and Boyd, 1988; Freedman and Gossett, 1989; Bagley and Gossett, 1990). Aerobic transformation has been observed only for mono-, di-, and trichloroethenes. Transformation (co-oxidation) of these compounds has been demonstrated with various types of microorganisms (e.g. toluene-, methane-, alkane- and ammonium-utilizers) (Wackett *et al.*, 1989). Microorganisms utilizing chlorinated ethenes as sole carbon and energy source have been isolated only with monochloroethene (vinyl chloride) (Hartmans *et al.*, 1985). The initial step in the aerobic transformation of chlorinated ethenes is generally assumed to be an epoxidation of the carbon-carbon double bond (Hartmans *et al.*, 1989). The further metabolism of the reactive and unstable chloroepoxides is not known, but extensive dechlorination is often observed.

Aerobic degradation of 3-chloroallyl alcohol, which also contains a chloroethenyl group, has been studied by Belser and Castro (1971). It is formed from the soil disinfectant 1,3-dichloropropene as a result of chemical hydrolysis. The 3-chloroallyl alcohol-degrading *Pseudomonas* sp. isolated by Belser and Castro (1971) degrades 3-chloroallyl alcohol via 3-chloroacrylic acid (CAA). Incubation of whole cells with CAA resulted in the formation of 3-oxo-propanoic acid (malonate semialdehyde). The dechlorination of CAA was, however, not investigated in any further detail.

We now report on the dechlorination of CAA in more detail. The CAA degradative pathway and the dechlorination reaction in particular were studied in two bacterial strains isolated with CAA (Hartmans *et al.*, 1988).

MATERIALS AND METHODS

Chemicals. *cis*-3-Chloroacrylic acid and *trans*-3-chloroacrylic acid were from Ventron (Karlsruhe, Germany), 3-chloropropionic acid, *cis*- and *trans*-1,2-dichloroethene, 1,3-dichloropropene, fumaric acid, acrylic acid, crotonic acid, cinnamic acid, methacrylic acid and MOPS were from Janssen Chimica (Beerse, Belgium). *trans*-2-Pentenoic acid and maleic acid were from Aldrich Chemie (Brussels, Belgium), 1-chloropropene was obtained from ICN Biomedicals (Plainview, New York) and vinyl chloride was from Hoek Loos (Schiedam, The Netherlands). 2-Chloroacrylic acid was from Heraeus (Karlsruhe, Germany). 3-Chloroallyl alcohol was a gift from the Institute for Pesticide Research (Wageningen, The Netherlands). L- and *meso*-diaminopimelic acid were from L. Light and Co,

(Colnbrook, UK), DL-isocitrate and Coenzyme A from Sigma. NAD^+ and $NADP^+$ were from Boehringer. Other chemicals were from Merck.

Isolation and cultivation. Enrichment cultures were set up using a mixture of soil and water samples as inoculum in mineral salts medium with one of the following compounds as sole carbon source. 1,3-Dichloropropene and chloroallyl alcohol were added at a concentration of 1 mM in Erlenmeyer flasks sealed with Mininert valves to prevent evaporation. *cis*- or *trans*-CAA were added at a concentration of 2.5 mM. After incubation for 2 weeks at 30°C 1 ml from the enrichment cultures was transferred to fresh medium with the same carbon source. After growth was observed in these cultures, cells were streaked to purity on glucose/yeast extract agar plates. Non-sterile controls without inoculum were also made and incubated under identical conditions to monitor any chemical hydrolysis of the chlorinated substrates. 1,3-Dichloropropene was assayed by taking headspace samples which were analyzed on a gas chromatograph. Chemical hydrolysis or biodegradation of the two other compounds was monitored by determining Cl^- formation rates in both the enrichment cultures and the non-sterile controls. From the depletion curve observed for 1,3-dichloropropene in the control flasks a half-life of 92 h was calculated.

The mineral salts medium contains per litre deionized water: 3.88 g K_2HPO_4, 2.13 g $NaH_2PO_4.2H_2O$, 2.0 g $(NH_4)_2SO_4$, 0.1 g $MgCl_2.6H_2O$, 10 mg EDTA, 2 mg $ZnSO_4.7H_2O$, 1 mg $CaCl_2.2H_2O$, 5 mg $FeSO_4.7H_2O$, 0.2 mg $Na_2MoO_4.2H_2O$, 0.2 mg $CuSO_4.5H_2O$, 0.4 mg $CoCl_2.6H_2O$ and 1 mg $MnCl_2.2H_2O$.

Cultures were grown in the mineral salts medium with 0.1% (w/v) carbon source, unless indicated otherwise. When CAA was used as a carbon source it was sterilized separately by filtration and added at a concentration of 5 mM. Culture conditions, maintenance of strains, culture harvesting and storage of harvested cells were as previously described (Hartmans and de Bont, 1986).

Preparation of cell extracts. Crude cell extracts and dialyzed extracts were prepared as described previously (Hartmans and de Bont, 1986).

Separation of hydratases. Cell extract (150 mg protein) from glucose-grown (0.25% (w/v)) strain CAA2 cells, which had been induced with 2 mM *cis*-CAA in the late exponential growth phase, was fractionated on a DEAE-Sepharose CL-6B column (25 cm × 2.5 cm). Elution was with 50 mM potassium phosphate buffer, pH 7.0, with a linear gradient of 100 to 500 mM NaCl at a flow rate of 50 ml h^{-1}. Fractions of 8.3 ml were collected.

Fractions containing hydratase activity were concentrated and washed with phosphate buffer (50 mM, pH 7.0), in order to reduce the Cl^- concentration, by ultrafiltration across an Amicon YM 10 membrane in an Amicon 8050 concentrator. These hydratase preparations could be stored at -20°C without any apparent loss of activity and were used for K_M, pH optimum and substrate specificity determinations.

Isolation of 2,4-dinitrophenylhydrazones. The partially purified hydratase preparations were incubated with the appropriate CAA isomer until reaction was complete. After filtration over an Amicon YM 10 filter (to remove protein) the filtrate was reacted with 2,4-dinitrophenylhydrazine and analyzed by mass spectrometry as described by van den Tweel and de Bont (1985).

Enzyme assays. All assays were performed at 30°C. Spectrophotometric assays were performed on a Perkin-Elmer 550A spectrophotometer. Activities are expressed in nmol product formed min^{-1} (mg protein)$^{-1}$. One unit (U) is the amount of enzyme which catalyzes the transformation of one μmol of substrate min^{-1}.

(i) 3-Chloroacrylic acid hydratase. The reaction mixture (total volume 2 ml) contained 100 μmol potassium phosphate, pH 7.0, 10 μmol phenylhydrazine hydrochloride and cell extract. The reaction was initiated by the addition of 10 μmol *cis*- or *trans*-CAA and phenylhydrazone formation was recorded at 315 nm. By comparing phenylhydrazone formation rates with Cl^- liberation rates (amperometrically) under the same conditions, a molar absorption coefficient for the phenylhydrazone of 2.2×10^3 l mol^{-1} cm^{-1} was calculated with both isomers of CAA. At 300 nm an absorption coefficient of 4.7×10^3 l mol^{-1} cm^{-1} was calculated. Van den Tweel and de Bont (1985) reported an absorption coefficient for the phenylhydrazone of malonate semialdehyde of 4×10^3 l mol^{-1} cm^{-1} at 300 nm.

(ii) Fumarase (EC 4.2.1.2). Activity was assayed at 240 nm (fumarate formation) in a reaction mixture (total volume 2 ml) containing 100 μmol potassium phosphate, pH 7.0, and 5 μmol L-malate

and enzyme. An absorption coefficient of 2.3×10^3 l mol^{-1} cm^{-1} was used in calculating fumarase activities.

(iii) Malonate semialdehyde decarboxylase (EC 4.1.1.-). Activity was determined by analyzing CO_2 or acetaldehyde formation in 30 ml serum bottles containing 50 μmol potassium phosphate, pH 7.0, 5 μmol *cis*-CAA, 0.2 U partially purified *cis*-CAA hydratase devoid of malonate semialdehyde decarboxylase activity and cell extract in a total volume of 1 ml.

(iv) Malonate decarboxylase (EC 4.1.1.-). Activity was determined by analyzing CO_2 formation in 30 ml serum bottles containing 50 μmol potassium phosphate, pH 7.0, 5 μmol malonate and cell extract in a total volume of 1 ml.

(v) Acetaldehyde dehydrogenase (EC 1.2.1.3). The reaction mixture (total volume 2 ml) contained 100 μmol Tris/HCl, pH 8.0, 2 μmol NAD^+ and cell extract. The reaction was started by the addition of 10 μmol acetaldehyde and NADH formation was recorded at 340 nm.

(vi) Isocitrate lyase (EC 4.1.3.1). The reaction mixture (total volume 2 ml) contained cell extract, 100 μmol Tris/HCl, pH 8.0, 100 μmol $MgCl_2$, and 10 μmol phenylhydrazine hydrochloride. The reaction was started by the addition of 20 μmol isocitrate and hydrazone formation was recorded at 324 nm. Activities were calculated using an absorption coefficient for the phenylhydrazone of 1.7×10^4 l mol^{-1} cm^{-1}.

(vii) Isocitrate dehydrogenase (EC 1.1.1.42). The reaction mixture (total volume 2 ml) contained cell extract, 100 μmol Tris/HCl pH 8.0, 2 μmol $NADP^+$ and 2 μmol $MgSO_4$. The reaction was started by adding 8 μmol isocitrate and NADPH formation was recorded at 340 nm.

Analytical methods. Protein was determined by the Lowry method using bovine serum albumin as standard. CO_2 concentrations were determined by analyzing headspace samples on a gas chromatograph (Hartmans *et al.*, 1985). Acetaldehyde and vinyl chloride (at 180°C) and 1,3-dichloropropene (at 210°C) were assayed on a Packard 430 gas chromatograph fitted with a Porapak R column (Hartmans *et al.*, 1985). Cl^- was determined colorimetrically (Bergmann and Sanik, 1957) or amperometrically (Hartmans *et al.*, 1985). The substrate specificity of the hydratases with the chlorinated substrates was determined by incubating enzyme and 5 mM substrate in 100 mM MOPS/KOH buffer and assaying Cl^- formation by the colorimetric method. The substrate specificity of the hydratases with the nonchlorinated substrates was determined spectrophotometrically at 220 nm by incubating 0.5 mM substrate in 50 mM potassium phosphate buffer, pH 7, with 0.05 U ml^{-1} of partially purified hydratase. Chromatography of whole-cell hydrolysates was performed as described by Becker *et al.* (1964). *Arthrobacter globiformis* (ATCC 8010), *Arthrobacter simplex* (NCIB 8929) and *Brevibacterium linens* (ATCC 9175) were used as reference strains containing lysine, L-DAP and *meso*-DAP respectively. Phenylhydrazones were extracted with ethyl acetate, which was subsequently removed by evaporation, and assayed by reverse phase HPLC using a C-18 column (200 by 3 mm; Chrompack, Middelburg, The Netherlands) and detected at 280 nm by means of a Perkin-Elmer variable wavelength detector. The eluent was acrylonitrile in Milli-Q water (60:40, v/v) at a flow rate of 0.6 ml min^{-1}.

RESULTS AND DISCUSSION

Isolation and characterization of new isolates

To isolate microorganisms containing CAA-dechlorinating activity, enrichment cultures were set up with 1,3-dichloropropene, chloroallyl alcohol or one of the CAA isomers as the sole carbon source. With 1,3-dichloropropene and chloroallyl alcohol no differences were observed either in substrate depletion or Cl^- formation between enrichment cultures and the controls without inoculum. Although biodegradation of 1,3-dichloropropene has been observed (van Dijk, 1980), to our knowledge, no pure cultures have been isolated. In contrast, enrichment cultures with either isomer of CAA as sole carbon source resulted in rapid microbial growth. Growth was also observed in the non-sterile control containing *trans*-CAA. Strains

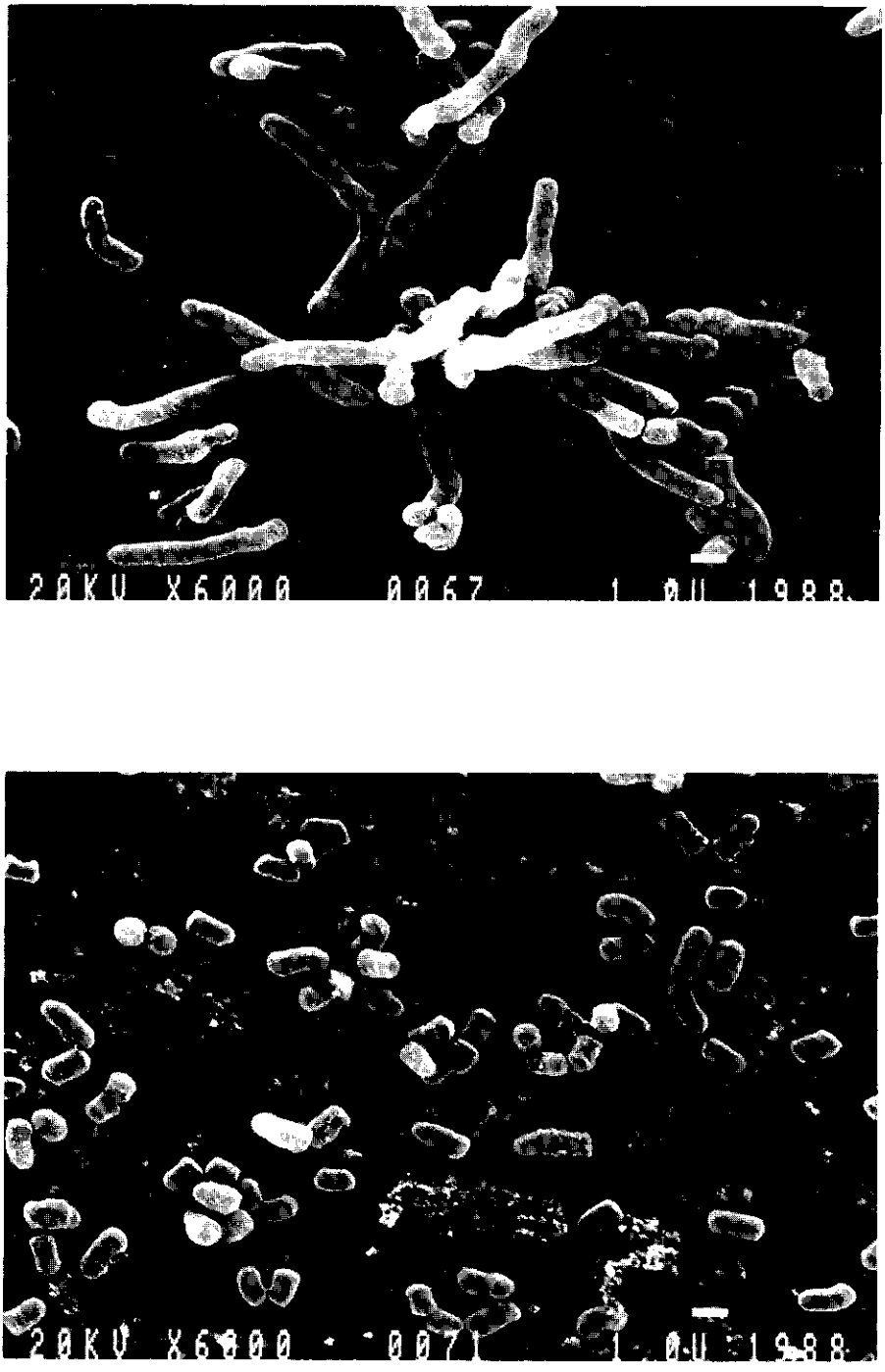

FIG. 1. Photomicrographs of strain CAA2 after growth for 8 h (top) and 96 h (bottom) on mineral salts medium with 0.1% (w/v) glucose. Bars, 1 μm.

CAA1 and CAA2 were isolated from the enrichment cultures with *cis*-CAA and *trans*-CAA respectively.

Strain CAA1 was a Gram-negative, straight and motile rod. It was catalase and oxidase positive and possessed β-galactosidase activity. Denitrification, indole formation, urease activity and gelatin hydrolysis were negative. Glucose, arabinose, mannose, mannitol, *N*-acetylglucoside, gluconic acid, capric acid, adipic acid, malic acid and phenylacetic acid were all utilized as growth substrates. Maltose was not utilized. Based on these observations strain CAA1 was identified as a strain of *Pseudomonas cepacia*. The organism also utilized acetate, propionate, fumarate and malonate as growth substrates. *trans*-CAA, acrylic acid and methanol were not utilized. The doubling time of strain CAA1 with *cis*-CAA was 2.6 h and with malonate, a possible intermediate of *cis*-CAA metabolism, 2.2 h.

Strain CAA2 was creamy to light-orange in colour. The isolate was Gram-positive and not acid fast. Catalase and oxidase tests were positive. The morphology of strain CAA2 depended on the age of the culture. In exponentially growing cultures strain CAA2 occurred as irregular rods (Fig. 1, top). In the stationary growth phase strain CAA2 formed coccoid cells, often in the V-arrangement typical of coryneform bacteria (Fig. 1, bottom). Thin-layer chromatography of strain CAA2 hydrolysates showed *meso*-diaminopimelic acid to be present. Based on the presence of *meso*-DAP and the microscopic morphology, strain CAA2 was tentatively designated as a *Brevibacterium* sp. (Jones and Keddie, 1986). The isolate grew with both CAA isomers, as well as with acetate, propionate and fumarate. Malonate, acrylic acid and methanol were not utilized as sole carbon source. The doubling time of strain CAA2 with *cis*-CAA was 3 h. The doubling time with *trans*-CAA was difficult to determine as concentrations higher than 2-4 mM *trans*-CAA clearly inhibited growth.

Neither strain grew with 1,3-dichloropropene or chloroallyl alcohol at a concentration of 1 mM.

3-Chloroacrylic acid dechlorinating activities

The initial step in CAA metabolism was studied using cell extracts. Cell extracts of *cis*-CAA-grown cells of both strains exhibited rapid Cl^- formation upon addition of *cis*-CAA. Dialysis of crude extracts did not affect the specific rate at which Cl^- was liberated from *cis*-CAA. Cell extracts of *cis*-CAA-grown strain CAA1 did not dechlorinate *trans*-CAA, whereas cell extracts of strain CAA2 grown with *cis*-CAA dechlorinated both isomers of CAA. Extracts of *trans*-CAA-grown strain CAA2 also dechlorinated both isomers of CAA. The cofactor independent Cl^- formation observed in cell-free extracts after *cis*-CAA addition could be explained by assuming an enzymic hydration of the double bond of *cis*-CAA. Hydration of the double bond would yield 3-chloro-2-hydroxypropanoic acid or the unstable 3-chloro-3-hydroxypropanoic acid, which rapidly chemically decomposes to malonate semialdehyde (3-oxopropanoic acid) and HCl. This would be in accordance with the

previously reported malonate semialdehyde formation by whole cells incubated with *cis*-CAA (Belser and Castro, 1971). A similar type of reaction has been reported for the hydration by fumarase of monofluorofumarate yielding α-fluorohydrin (α-fluoromalate) which subsequently decomposed to oxaloacetate. Hydration of monochlorofumarate with fumarase, however, yielded the chemically stable β-chloro-*threo*-L-malate (Marletta *et al.*, 1982).

Larger amounts of cells were required to study the dechlorinating activities in more detail and to identify the product of the reaction. As CAA is relatively expensive, strain CAA2 was grown on glucose with 2 mM *cis*-CAA added in the late exponential growth phase (2 h before harvesting) to produce biomass with dechlorinating activities. Cells cultivated in this manner contained specific dechlorinating activities similar to the levels present in cells grown with *cis*-CAA as sole carbon and energy source. With extracts from cells cultivated in this manner the dechlorinating activities of strain CAA2 were investigated in more detail.

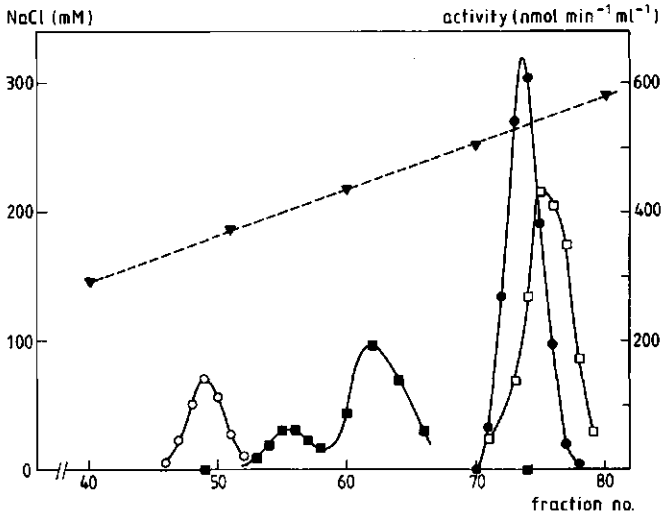

FIG. 2. Separation of *cis*-CAA hydratase (O), fumarase (■), *trans*-CAA hydratase (●) and malonic semialdehyde decarboxylase (□) activities with a DEAE-Sepharose CL-6B column (25 cm × 2.5 cm). Elution was with 50 mM potassium phosphate buffer, pH 7.0, with a linear gradient of 100 to 500 mM NaCl (▾) at a flow rate of 50 ml h^{-1}. Fraction volume was 8.3 ml.

The activities were partially purified using anion exchange column chromatography. Fractionation of extracts on a DEAE-Sepharose CL-6B column resulted in the separation of the *cis*- and *trans*-CAA dechlorinating activities (Fig. 2), indicating that the two isomers are dechlorinated by two distinct enzymes. Fumarase activity was also located. No fumarase activity coincided with either of

the two CAA-dechlorinating activities. The fractions containing the respective dechlorinating activities were pooled and dialyzed to remove excess Cl^-. These partially purified preparations were used to identify the product of the dechlorinating reaction and to determine the pH-optimum, the K_M for CAA and the substrate specificities. The specific activities of the partially purified preparations of the *cis*-CAA and *trans*-CAA dechlorinating activities were, respectively, 1300 and 2600 nmol min^{-1} mg $protein^{-1}$.

Product identification

The product of the CAA dechlorinating activity was assumed to be malonic semialdehyde. To confirm this hypothesis 2,4-dinitrophenylhydrazones were prepared after incubating the partially purified *trans*-CAA dechlorinating activity with its substrate. Van den Tweel and de Bont (1985) reported that the mass spectra of the malonate semialdehyde and acetaldehyde 2,4-dinitrophenylhydrazones are identical due to the decarboxylation of malonate semialdehyde during 2,4-dinitrophenylhydrazone preparation. The mass spectrum of the 2,4-dinitrophenylhydrazone of the product formed by the *trans*-CAA dechlorinating activity was indeed identical to that of the 2,4-dinitrophenylhydrazone of authentic acetaldehyde and to the spectrum of the 2,4-dinitrophenylhydrazone of acetaldehyde published by Kanner and Bartha (1982). Further identification of the product was undertaken using HPLC, as the identification of acetaldehyde only gave indirect evidence for the formation of malonate semialdehyde from CAA.

Analysis of the unsubstituted phenylhydrazones formed during the standard CAA hydratase assay using phenylhydrazine, rather than 2,4-dinitrophenylhydrazine, was possible with reverse-phase HPLC. Both dechlorinating activities gave phenylhydrazones with the same retention time (4.1 min) upon HPLC analysis and the same UV spectrum. Incubation of cell extracts of 3-butyn-1-ol-grown *Pseudomonas* BB1 (van den Tweel and de Bont, 1985) with propynoic acid and phenylhydrazine yields the phenylhydrazone of malonate semialdehyde. This phenylhydrazone was, based on HPLC and UV analysis, identical to the phenylhydrazones that were formed from CAA. The phenylhydrazone of acetaldehyde also had the same UV spectrum (dissolved in ethylacetate) but was, probably due to the very low solubility in the HPLC eluent, not detectable with HPLC. The phenylhydrazone of pyruvate had a markedly different UV spectrum and also a different retention time (2.7 min) upon HPLC analysis.

Based on these results it is concluded that strain CAA2 contained two distinct enzymes that dechlorinate the respective isomers of CAA, both yielding malonate semialdehyde as product. Belser and Castro (1971) had already demonstrated that whole cells could transform CAA to malonate semialdehyde, but they proposed a difficult-to-envisage "hydroxylation" of CAA as the dechlorinating step. Although the dechlorinating enzymic activities could perhaps be characterized as dehalogenases (halidohydrolases), characteristics such as the pH optimum and substrate specificity

indicate that these enzymes share more similarity with hydratases like fumarase. Therefore, it is assumed that the CAA hydratases should indeed be classified as hydratases (EC 4.2.1.-) rather than as dehalogenases.

The pH optimum of *cis*-CAA hydratase was rather broad and lay between pH 7.3 and 8.0, whereas the pH-optimum of *trans*-CAA hydratase was more pronounced at pH 7.3. The K_M for *cis*-CAA was 2.5 mM and the K_M for *trans*-CAA was 0.2 mM. In both cases slight substrate inhibition was observed at substrate concentrations above 5 mM. The relatively broad pH optimum observed for *cis*-CAA hydratase is possibly a reflection of the substrate concentration of 2.5 mM which was used in the activity assay. A similar observation has been made for fumarase activity where the pH optimum (pH 6.9) was much less pronounced when the fumarate concentration in the activity assay approached the K_M value (Alberty *et al.*, 1954). The pH optima of the two hydratases are therefore very similar to the pH-optimum of fumarase. In contrast, the pH optima of dehalogenases are much higher. For haloalkane dehalogenases pH optima of 8.2 and 9.5 have been determined (Keuning *et al.*, 1985; Scholtz *et al.*, 1987) and for 2-haloacid dehalogenases pH optima of 9.5 are common (Smith *et al.*, 1990).

The substrate specificity of the two hydratases was very high. Several chlorinated compounds were tested as potential substrates for both hydratases. No enzymic liberation of Cl^- (detection level <3% of the activity measured with the CAA isomer) was, however, detected with 1,3-dichloropropene, 3-chloroallyl alcohol, 2-chloroacrylic acid, 1-chloropropene, vinyl chloride, *cis*-1,2-dichloroethene, *trans*-1,2-dichloroethene or 3-chloropropionic acid.

Special attention was given to vinyl chloride as we are interested in methods to remove this carcinogenic compound from industrial wastes (Hartmans *et al.*, 1985). Hypothetically, hydration of vinyl chloride would result in the formation of either 1-chloroethanol or 2-chloroethanol. 1-Chloroethanol would decompose to acetaldehyde and HCl, but 2-chloroethanol is stable under the assay conditions. By adding 1,2-dichloroethane-grown cells of *Xanthobacter autotrophicus* GJ10 (Janssen *et al.*, 1985) to the incubation mixture of CAA hydratase with vinyl chloride, any 2-chloroethanol that would be formed would be oxidized and dehalogenated resulting in Cl^- formation. However, also in the presence of *X. autotrophicus* GJ10 cells no Cl^- formation was detected, indicating that vinyl chloride is not hydrated by the CAA hydratases of strain CAA2. Apparently, the presence of a carboxyl group adjacent to the chloroethenyl group is a prerequisite for enzymic hydration of the carbon-carbon double bond by the two CAA hydratases of strain CAA2.

Besides chlorinated compounds several non-chlorinated unsaturated carboxylic acids were tested as substrates for the partially purified hydratases. Hydration of acrylic acid, propynoic acid, crotonic acid, *trans*-2-pentenoic acid, cinnamic acid, methacrylic acid and maleic acid was, however, not observed with either of the two hydratase preparations (detection level lower than 1% of the activity observed with the appropriate CAA isomer as substrate).

The high substrate specificity of the CAA hydratases also indicates that these enzymes apparently share more similarity with a hydratase like fumarase, which has a relatively high substrate specificity (Teipel *et al.*, 1968), than with the dehalogenases, which generally transform a wide range of substrates (Smith *et al.*, 1990). The observed high substrate specificity gives rise to speculations concerning the physiological function of these enzymes. If CAA is indeed the only substrate transformed, this could indicate that these enzymes have evolved as a result of the large scale application in agriculture of the nematocide 1,3-dichloropropene. In that case, however, the CAA-utilizers would also be expected that grow on chloroallyl alcohol, the product formed upon chemical hydrolysis of 1,3-dichloropropene.

A better understanding of the evolutionary origin of the CAA hydratases will, however, require characterization of the nucleotide sequences of the corresponding genes. In this respect the recent data showing extensive sequence homology between fumarase and L-aspartate ammonia-lyase (Takagi *et al.*, 1986) is very interesting. Possibly the CAA-hydratases share a common evolutionary origin with these enzymes.

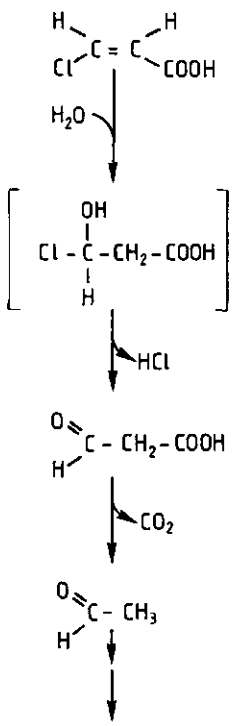

FIG. 3. Proposed pathway for *cis*-CAA degradation in *Pseudomonas cepacia* strain CAA1 and the coryneform strain CAA2.

3-Chloroacrylic degradation pathway

Having established that the product of CAA dechlorination was malonic semialdehyde, the degradative pathway of this not-very-common metabolic intermediate was studied. Two enzymic activities oxidizing malonic semialdehyde have been described previously. A NAD^+-dependent dehydrogenase (EC 1.2.1.15) which oxidizes malonic semialdehyde to malonate (Nakamura and Bernheim, 1961) and a NAD^+- and CoA-dependent malonate semialdehyde oxidative decarboxylase (EC 1.2.1.18) forming acetyl-CoA (Yamada and Jakoby, 1960). Interestingly, *cis*-CAA-dependent formation of CO_2 was detected in dialyzed extracts without adding NAD^+ or CoA. Incubation of *trans*-CAA with the partially purified preparation of *trans*-CAA hydratase also resulted in CO_2 formation. No CO_2 formation was observed when *cis*-CAA was incubated with the partially purified *cis*-CAA hydratase.

This indicated that the partially purified *trans*-CAA hydratase preparation contained a cofactor-independent malonate semialdehyde decarboxylase, presumably yielding acetaldehyde and CO_2, which was not present in the partially purified *cis*-CAA hydratase. Subsequent experiments with the partially purified *trans*-CAA hydratase did indeed reveal simultaneous formation of acetaldehyde and CO_2 from *trans*-CAA. Combination of the *trans*-CAA and *cis*-CAA hydratase preparations also resulted in acetaldehyde and CO_2 formation from *cis*-CAA. When phenylhydrazine was added to the incubation mixture, acetaldehyde and CO_2 formation were no longer observed, but the Cl^- formation rate was unaffected.

As malonate semialdehyde was not available from a commercial source the partially purified *cis*-CAA hydratase preparation, devoid of malonate semialdehyde decarboxylase, was used to produce malonate semialdehyde from *cis*-CAA *in situ* to assay malonate semialdehyde decarboxylase activity. With this assay we were able to locate the malonate semialdehyde decarboxylase activity peak in the fractions from the DEAE-Sepharose column. As anticipated, malonate semialdehyde decarboxylase-activity more or less coincided with *trans*-CAA hydratase activity (Fig. 2).

TABLE 1. Enzyme activities in *Pseudomonas cepacia* CAA1
(Activities expressed in nmol min^{-1} mg $protein^{-1}$)

	Growth substrate:		
	cis-CAA	malonate	succinate
cis-CAA dehalogenase	240	<5	<5
MSA decarboxylase	530	40	30
Malonate decarboxylase	<20	470	<20
Acetaldehyde dehydrogenase	40	35	35
Isocitrate lyase	825	695	5
Isocitrate dehydrogenase	585	680	550

With the observed enzyme activities a degradative pathway for *cis*- and *trans*-CAA in *cis*-CAA-grown strain CAA2 can be envisaged which proceeds via malonate semialdehyde to acetaldehyde. Subsequently, enzyme activities in crude extracts of *cis*-CAA-grown cells of both strains and in extracts of cells grown with control substrates were compared. As can be seen in Tables 1 and 2, CAA hydratase and malonate semialdehyde decarboxylase activities were present only after growth with *cis*-CAA. NAD^+-dependent acetaldehyde dehydrogenase activities in *cis*-CAA grown cells of both strains were rather low. No PMS-dependent acetaldehyde dehydrogenase activity could be detected in extracts of ethanol-grown cells of strain CAA2. In spite of the observed low acetaldehyde dehydrogenase activities we

propose for both strains the *cis*-CAA degradation pathway summarized in Fig. 3. Induction of isocitrate lyase activity during growth with *cis*-CAA is also in agreement with the above pathway.

TABLE 2. Enzyme activities in strain CAA2
(Activities expressed in nmol min^{-1} mg $protein^{-1}$)

	Growth substrate:		
	cis-CAA	ethanol	succinate
cis-CAA dehalogenase	450	<10	<10
trans-CAA dehalogenase	1200	<10	12
MSA decarboxylase	1160	55	60
Acetaldehyde dehydrogenase	25	200	5
Isocitrate lyase	1020	1010	15
Isocitrate dehydrogenase	560	645	855

The presence of malonate decarboxylase activity in cell extracts of malonate-grown strain CAA1, and the absence of this activity in *cis*-CAA grown cells, is a further confirmation that, in this isolate, malonate is not an intermediate of *cis*-CAA metabolism.

Having established the pathway for *cis*-CAA degradation an explanation for the inhibition of strain CAA2 by the growth substrate *trans*-CAA was still required. Initial *trans*-CAA concentrations higher than 2-4 mM gave inconsistent growth and often resulted in a transient accumulation of acetaldehyde. This accumulation of the toxic aldehyde possibly explained the poor growth of strain CAA2 with *trans*-CAA. No acetaldehyde formation was observed with *cis*-CAA as carbon source at similar concentrations, probably because *cis*-CAA hydratase activity is lower.

Acknowledgements

We thank Alex van Neerven for photography and Dr D.B. Janssen for providing *Xanthobacter autotrophicus* GJ10.

REFERENCES

Alberty, R.A., V. Massey, C. Frieden, and A.R. Fuhlbrigge. 1954. Studies of the enzyme fumarase. III. The dependence of kinetic constants at 25°C upon the concentration and pH of phosphate buffers. J. Am. Chem. Soc. 76:2485-2493.

Bagley, D.M., and J.M. Gossett. 1990. Tetrachloroethene transformation to trichloroethene and *cis*-1,2-dichloroethene by sulfate-reducing enrichment cultures. Appl. Environ. Microbiol. 56:2511-2516.

Becker, B., M.P. Lechevalier, R.E. Gordon, and H.A. Lechevalier. 1964. Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12:421-423.

Belser, N.O., and C.E. Castro. 1971. Biodehalogenation - The metabolism of the nematocides *cis*- and *trans*-3-chloroallyl alcohol by a bacterium isolated from soil. J. Agricul. Food Chem. **19**:23-26.

Bergmann, J.G., and J. Sanik. 1957. Determination of trace amounts of chlorine in naphtha. Anal. Chem. **29**:241-243.

van Dijk, H. 1980. Dissipation rates in soil of 1,2-dichloropropane and 1,3- and 2,3-dichloropropenes. Pest. Sci. **11**:625-632.

Fathepure, B.Z., and S.A. Boyd. 1988. Dependence of tetrachloroethylene dechlorination on methanogenic substrate consumption by *Methanosarcina* sp. strain DCM. Appl. Environ. Microbiol. **54**:2976-2980.

Freedman, D.L., and J.M. Gossett. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Appl. Environ. Microbiol. **55**:2144-2151.

Hartmans, S., J.A.M. de Bont, J. Tramper, and K.C.A.M. Luyben. 1985. Bacterial degradation of vinyl chloride. Biotechnol. Lett. **7**:383-388.

Hartmans, S., and J.A.M. de Bont. 1986. Acetol monooxygenase from *Mycobacterium* Py1 cleaves acetol into acetate and formaldehyde. FEMS Microbiol. Lett. **36**:155-158.

Hartmans, S., M.W. Jansen, and J.A.M. de Bont. 1988. 3-Chloroacrylic acid metabolism in bacteria, p. 205. *In* C. Ratledge, A. Szentirmai, G. Barabás, and F. Kevei (ed.), Microbial Physiology and the Manufacturing Industry. OMIKK, Budapest.

Hartmans, S., J.A.M. de Bont, and W. Harder. 1989. Microbial metabolism of short-chain unsaturated hydrocarbons. FEMS Microbiol. Rev. **63**:235-264.

Janssen, D.B., A. Scheper, L. Dijkhuizen, and B. Witholt. 1985. Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. Appl. Environ. Microbiol. **49**:673-677.

Jones, D., and R.M. Keddie. 1986. Genus *Brevibacterium*, p. 1301-1313. *In* P.H.A. Sneath, N.S. Mair, M.E. Sharpe, and J.G. Holt (ed). Bergey's Manual of Systematic Bacteriology, vol 2. Williams and Wilkins, Baltimore.

Kanner, D., and R. Bartha. 1982. Metabolism of acetylene by *Nocardia rhodochrous*. J. Bacteriol. **150**:989-992.

Keuning, S., D.B. Janssen, and B. Witholt. 1985. Purification and characterization of hydrolytic dehalogenase from *Xanthobacter autotrophicus* GJ10. J. Bacteriol. **163**:635-639.

Kohler-Staub, D., and T. Leisinger. 1985. Dichloromethane dehalogenase of *Hyphomicrobium* sp. strain DM2. J. Bacteriol. **162**:676-681.

Marletta, M.A., Y.F. Cheung, and C. Walsh. 1982. Stereochemical studies on the hydration of monofluorofumarate and 2,3-difluorofumarate by fumarase. Biochemistry **21**:2637-2644.

Motosugi, K., N. Esaki, and K. Soda, K. 1982. Purification and properties of a new enzyme, DL-2-haloacid dehalogenase, from *Pseudomonas* sp. J. Bacteriol. **150**:522-527.

Nakamura, K. and F. Bernheim. 1961. Studies on malonic semialdehyde dehydrogenase from *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **50**:147-152.

Scholtz, R., T. Leisinger, F. Suter, and A.M. Cook. 1987. Characterization of 1-chlorohexane halidohydrolase, a dehalogenase of wide substrate range from an *Arthrobacter* sp. J. Bacteriol. **169**:5016-5021.

Smith, J.M., K. Harrison, and J. Colby. 1990. Purification and characterization of D-2-haloacid dehalogenase from *Pseudomonas putida* strain AJ1/23. J. Gen. Microbiol. **136**:881-886.

Takagi, J.S., M. Tokushige, Y. Shimura, and M. Kanehisa. 1986. L-aspartate ammonia-lyase and fumarate hydratase share extensive sequence homology. Biochem. Biophys. Res. Comm. **138**:568-572.

Teipel, J.W., G.M. Hass, and R.L. Hill. 1968. The substrate specificity of fumarase. J. Biol. Chem. **243**:5684-5694.

van den Tweel, W.J.J., and J.A.M. de Bont. 1985. Metabolism of 3-butyn-1-ol by *Pseudomonas* BB1. J. Gen. Microbiol. **131**:3155-3162.

Wackett, L.P., G.A. Brusseau, S.R. Householder, and R.S. Hanson. 1989. Survey of microbial oxygenases: Trichloroethylene degradation by propane-oxidizing bacteria. Appl. Environ. Microbiol. **55**:2960-2964.

Yamada, E.W., and W.B. Jakoby. 1960. Aldehyde oxidation - V. Direct conversion of malonic semialdehyde to acetyl-coenzyme A. J. Biol. Chem. 235:589-594.

Yokota, T., H. Fuse, T. Omori, and Y. Minoda. 1986. Microbial dehalogenation of haloalkanes mediated by oxygenase or halidohydrolase. Agricul. Biol. Chem. 50:453-460.

Chapter 7

BACTERIAL DEGRADATION OF STYRENE INVOLVING A NOVEL FLAVIN ADENINE DINUCLEOTIDE-DEPENDENT STYRENE MONOOXYGENASE

S. Hartmans, M.J. van der Werf and J.A.M. de Bont.

By using styrene as the sole source of carbon and energy in concentrations of 10 to 500 μM, 14 strains of aerobic bacteria and two strains of fungi were isolated from various soil and water samples. In cell extracts of 11 of the bacterial isolates, a novel flavin adenine dinucleotide-requiring styrene monooxygenase activity that oxidized styrene to styrene oxide (phenyl oxirane) was detected. In one bacterial strain (S5), styrene metabolism was studied in more detail. In addition to styrene monooxygenase, cell extracts from strain S5 contained styrene oxide isomerase and phenylacetaldehyde dehydrogenase activities. A pathway for styrene degradation via styrene oxide and phenylacetaldehyde to phenylacetic acid is proposed.

Applied and Environmental Microbiology (1990) 56:1347-1351.

Styrene is used in large quantities by the chemical industry, mainly as a starting material for synthetic polymers such as polystyrene and styrene-butadiene rubber. It is also used as a solvent in the polymer processing industry and consequently is present in many industrial effluents. Airborne emissions of styrene often cause problems, even at low concentrations (less than 1 vpm), due to the malodorous properties of the compound. Removal of styrene from industrial waste gases could be accomplished by using styrene-degrading bacteria as biocatalysts. In view of this potential application, it is important to know the metabolic fate of the styrene that is transformed by the biocatalysts in order to prevent the possible accumulation of more toxic styrene degradation products, e.g., styrene oxide.

Mammalian metabolism of styrene has been studied quite extensively in view of the intensive industrial use of styrene and its possible toxic and carcinogenic properties (Vainio *et al.*, 1982). The first step in the major pathway of mammalian styrene metabolism is the oxidation to styrene oxide.

Knowledge concerning the microbial metabolism of styrene is very scarce. The first attempt to isolate styrene-degrading microorganisms from more than 100 soil samples was unsuccessful (Omori *et al.*, 1975). Subsequently, Sielicki *et al.* (1978) described a styrene-utilizing mixed culture, and the isolation of pure cultures degrading styrene was first reported by Shirai and Hisatsuka (1979b) who isolated 31 strains. One strain, designated a *Pseudomonas* sp., was studied in more detail. On the basis of the detection of small amounts of styrene oxide when cells were incubated in the presence of styrene, it was proposed that in this *Pseudomonas* strain, styrene is degraded via styrene oxide (Shirai and Hisatsuka 1979a).

Biotransformation of styrene to styrene oxide by whole cells has been described previously (Higgins *et al.*, 1979; Furuhashi *et al.*, 1986). By using *Methylosinus trichosporium* OB3b cells, styrene oxide was the only product detected when this methanotroph was incubated with styrene (Higgins *et al.*, 1979). The oxidation is probably a result of the broad specificity of the methane monooxygenase present in these cells. *Nocardia corallina* B-276, which contains a monooxygenase acting on a wide range of 1-alkenes, also forms styrene oxide from styrene (Furuhashi *et al.*, 1986). Oxidation of styrene to styrene oxide by cell-free extracts has been reported for *Methylococcus capsulatus* (Bath) (Colby *et al.*, 1977) and the propane-utilizing *Brevibacterium* sp. CRL 56 (Hou *et al.*, 1983). Styrene oxidation by cell-free extracts of styrene-grown microorganisms has, to our knowledge, not been reported in the literature.

Recently, we described a *Xanthobacter* species isolated on styrene that contained a novel styrene oxide isomerase activity which isomerized styrene oxide to phenylacetaldehyde (Hartmans *et al.*, 1989). Styrene transformation by whole cells of this strain was oxygen dependent, but we were not successful in resolving the nature of the oxidation product. We therefore set out to isolate other styrene-degrading organisms. As we anticipated that styrene would be toxic at higher concentrations, three different isolation methods were used in which relatively low

concentrations of styrene were employed. All three methods resulted in various new isolates. In addition to two fungal isolates, 14 bacterial isolates, which appeared to be morphologically different, were selected. In the new bacterial isolates, we investigated the transformation of styrene. In most strains, we detected a novel flavin adenine dinucleotide (FAD)-requiring styrene monooxygenase (SMO) activity which formed styrene oxide. The SMO and styrene metabolism in one strain were studied in more detail, and a degradative pathway for styrene is proposed.

MATERIALS AND METHODS

Isolation of styrene utilizers. Styrene-degrading microorganisms were enriched either by adding 5 μl of styrene directly to an Erlenmeyer flask containing 50 ml of mineral salts medium with inoculum or by adding 25 μl of styrene to a test tube with 5 ml dibutyl phthalate which was placed in a similar Erlenmeyer flask with 50 ml of mineral salts medium and inoculum. The mineral salts medium has been described previously (Hartmans *et al.*, 1989), and various local soil and water samples were used as inoculum. The Erlenmeyer flasks (300 ml) were fitted with Teflon Mininert valves (Precision Sampling) to prevent styrene evaporation. Flasks were incubated at 30°C on a shaker. After growth was observed, 0.1 ml of serial dilutions was plated onto agar plates with mineral salts medium. Plates were incubated in a desiccator containing a flask with 2% (v/v) styrene in dibutyl phthalate. The third method used to isolate styrene-degrading microorganisms consisted of directly plating dilutions of samples without prior enrichment onto agar plates with mineral salts medium and incubating these plates in a desiccator in which styrene was supplied via the gas phase as described above. Colonies that developed on the agar plates with styrene as the carbon source were isolated and checked for purity by plating on yeast extract-glucose agar plates. The fungi were isolated from enrichment cultures containing 0.5 mM styrene in mineral salts medium which had been adjusted to pH 4.5 by the addition of hydrochloric acid.

Growth conditions. Strains were subcultured once a month and grown at 30°C on mineral medium agar slopes in a desiccator with an Erlenmeyer containing 2% (v/v) styrene in dibutyl phthalate. After 1 week, the agar slants were removed from the desiccator and stored at room temperature. Growth experiments were performed using the mineral salts medium described previously (Hartmans *et al.*, 1989), with the carbon sources (Table 1) added aseptically at a concentration of 0.01% (w/v) after sterilization of the mineral salts medium. Cultures were incubated at 30°C on a shaker (Hartmans *et al.*, 1989). Culture doubling times with styrene as growth substrate were determined by monitoring the absorbance increase at 660 nm with a Vitatron UPS photometer of cultures growing in a fermentor with a working volume of 1 litre at 30°C. Styrene was supplied via the gas phase by passing 10% (20 ml/min) of the total air flow (200 ml/min) into the fermentor through a bubble column containing styrene which was kept at 20°C. Styrene-grown cells for the preparation of cell extracts of the strains S1, S3, S4, S5, S6, S8, S9, and S14 were grown as batch cultures in the fermentor under the same conditions as described above. Styrene-grown cells for the preparation of cell extracts of strains S7, S10, S11, and S12 were grown in 5-liter Erlenmeyer flasks as previously described for *Xanthobacter* strain 124X (Hartmans *et al.*, 1989).

Cells were harvested, concentrated, and washed with potassium phosphate buffer by centrifugation (Hartmans and de Bont, 1986) and were used directly to prepare cell extracts. Cell extracts were prepared by ultrasonication, and dialysis of extracts was performed with a Sephadex G-25 column with 50 mM potassium phosphate buffer, pH 7.0 (Hartmans and de Bont, 1986).

Experiments with whole cells. Oxygen uptake experiments with washed cells were performed with dilute suspensions of freshly harvested, washed cells (Hartmans *et al.*, 1989). The incubation of strain S5 cells with styrene was performed as described previously for *Xanthobacter* strain 124X (Hartmans *et al.*, 1989).

Enzyme assays. All assays were performed at 30°C by using extracts from freshly harvested cells. Activities are expressed in nanomoles of product formed (NADH or NADPH) or substrate consumed (styrene or O_2) min^{-1} mg of $protein^{-1}$.

Styrene monooxygenase (SMO) activity was measured by determining styrene consumption in the headspace of 30-ml vials fitted with Teflon Mininert valves preventing styrene evaporation. The reaction mixture consisted of cell extract (usually 5 to 10 of mg protein per 0.5 ml), 0.2 ml of a solution containing 5 mM NADH or NADPH, and 0.1 mM FAD in water and potassium phosphate buffer (50 mM, pH 7.0) to a total volume of 2.0 ml. The vial was placed in a shaking water bath, and after 2 min the reaction was started by the addition of 0.1 ml of phosphate buffer saturated with styrene. The temperature optimum of SMO was determined with the standard assay at different temperatures. The pH optimum was determined at 30°C with 50 mM potassium phosphate buffers at the desired pH.

Styrene oxide isomerase, 2-phenylethanol dehydrogenase [$NAD(P)^+$-dependent], and phenylacetaldehyde dehydrogenase [$NAD(P)^+$-dependent] were assayed spectrophotometrically as described previously (Hartmans *et al.*, 1989). Phenazine methosulfate (PMS)-dependent 2-phenylethanol dehydrogenase and phenylacetaldehyde dehydrogenase were assayed by determining oxygen consumption rates as previously described (Hartmans *et al.*, 1989).

Determination of partition coefficient. The partition coefficient of styrene between air and mineral salts medium (Henry coefficient) was determined by measurement of the styrene content in the gas phase in a series of Erlenmeyer flasks fitted with Teflon Mininert valves. The Erlenmeyers all contained the same amount of styrene and varying ratios of air-mineral salts medium. After equilibration at 30°C, samples of the gas phase were analyzed, and the partition coefficient was calculated for all pairs of Erlenmeyers by using the peak area determined by the integrator of the gas chromatograph in combination with the volumes of the gas and liquid phases. The dibutyl phthalate-mineral salts medium partition coefficient was determined in a similar way by varying the amount of dibutyl phthalate added to Erlenmeyers containing a fixed amount of mineral salts medium and styrene.

Analytical methods. Protein was determined by the method of Lowry *et al.*, (1951) by using bovine serum albumin as the standard. Spectrophotometric assays were performed on a Perkin-Elmer 550A spectrophotometer. Oxygen uptake experiments were carried out by using a biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio). Styrene was determined by gas chromatographic analysis of headspace samples (Hartmans *et al.*, 1989). Reverse-phase HPLC analysis of styrene oxide, 2-phenylethanol, phenylacetaldehyde and phenylacetic acid was performed as previously described (Hartmans *et al.*, 1989).

Chemicals. Aromatic compounds were obtained from Janssen Chimica, Beerse, Belgium, except for styrene oxide, benzene and mandelic acid which were from E. Merck AG, Darmstadt, Germany. PMS and styrene glycol were from EGA Chemie, Steinheim, Germany. Biochemicals were from Boehringer, Mannheim, Germany, and Sephadex G-25 was from Pharmacia, Uppsala, Sweden. All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

Isolation of styrene-degrading bacteria

The three different methods described in the Materials and Methods section were all successfully used to isolate styrene-degrading microorganisms from various soil and water samples. In earlier experiments (Omori *et al.*, 1975), 101 soil samples were tested but no styrene-degrading microorganisms with styrene as carbon source at a concentration of 2% were isolated. This concentration is well above the solubility of styrene in water of 1.5 mM at 25°C (Banerjee *et al.*, 1980), and it is therefore not surprising that no organisms were isolated under these conditions. Recently, however, *Pseudomonas putida* IH-2000 was isolated in the presence of 30% (v/v) toluene (Inoue and Horikoshi, 1989). This solvent-resistant strain also grew on a complex medium in the presence of high concentrations of styrene, thus illustrating that high styrene concentrations are not always growth inhibiting.

From our results, it appeared that it is possible to isolate styrene-degrading organisms very readily, provided low substrate concentrations are used. Styrene-degrading microorganisms are apparently very common in nature. This is not surprising when it is realized that styrene is also produced in natural ecosystems (Shirai and Hisatsuka, 1979b). Styrene concentrations in the aqueous phase of the closed cultures were calculated using an air-mineral salts medium partition coefficient (Henry coefficient) of 0.2 and a dibutyl phthalate-mineral salts medium partition coefficient of 2,000. Enrichment cultures set up with dibutyl phthalate as a substrate reservoir (resulting in a styrene concentration in the aqueous phase of about 22 μM) as well as enrichment cultures with 0.01% (v/v) styrene added directly to the mineral salts medium (resulting in a styrene concentration of about 0.5 mM in the aqueous phase after equilibration of styrene between the air and aqueous phase), resulted in styrene-dependent growth with all inocula used. It was also possible to routinely obtain colonies of styrene-utilizing organisms by directly plating soil and water samples on mineral salts medium plates which were incubated in an atmosphere in equilibrium with a 2% (v/v) styrene in dibutyl phthalate, resulting in a concentration of about 0.09 mM styrene in the aqueous phase of the agar plates.

Although dibutyl phthalate itself can support microbial growth when added directly to the growth medium, transfer of dibutyl phthalate via the gas phase, as would be the case in our enrichment flasks, is apparently too slow to allow substantial microbial growth. The vapour pressure for dibutyl phthalate is about 0.01 Pa at 25°C (Howard *et al.*, 1985) whereas the vapour pressure for styrene at the concentration used in the enrichment cultures 11 Pa (110 vpm), illustrating why styrene transfer was much higher than dibutyl phthalate transfer. The solubility of dibutyl phthalate in water at 20°C is about 10 μM (Howard *et al.*, 1985), which is about half of the equilibrium concentration of styrene in the aqueous phase under the enrichment conditions. Apparently, the mass transfer resistance in the gas phase is rate limiting for dibutyl phthalate transfer to the aqueous phase. An organic solvent as a reservoir for toxic compounds in enrichment experiments offers the advantageous possibility of achieving relatively high biomass concentrations at low substrate concentrations in the water phase without having to continuously monitor and adjust the substrate concentration.

By using the three different methods, many different isolates were obtained. Fourteen bacterial isolates that appeared to be morphologically different after growth on agar slants were selected for further study. They were designated strains S1 to S14. Organisms with a macroscopic morphology similar to that of strain S1 were present in all enrichment cultures. The strains S6, S8, S9, and S12 were Gram negative and motile. All other bacterial isolates were Gram positive. Further identification of the isolated bacteria was not attempted. Two styrene-degrading fungi were also isolated, but not further studied.

Growth experiments

The doubling time of strain S1 with styrene as the carbon source was 4.6 h. Strains S2, S7, S10, and S11 formed clumps during growth in liquid cultures, thus preventing a reliable determination of growth rates. Strains S2, S3, S4, S5, S6, S10 and S13 were isolated from inocula spread directly onto agar plates and incubated with styrene. The doubling times with styrene as the growth substrate of the strains isolated directly from agar plates (S3 and S4, 5.6 h; S5, 3.4 h; S6, 4.4 h; and S13, 9 h) did not differ significantly from the values obtained for the strains which were isolated from the enrichment cultures (S8, 2.5 h; S9, 3.9 h; and S14, 7.5 h). We did not determine the doubling time of strain S12 as we did not succed in growing it in the fermentor. All the new isolates grew faster with styrene as growth substrate than did the previously described *Xanthobacter* strain 124X (Hartmans *et al.*, 1989), which has a doubling time of 19 h.

To further characterize the new isolates, growth experiments were performed with various aromatic compounds as the sole source of carbon and energy (Table 1). Apart from styrene, all strains utilized styrene oxide and 2-phenylethanol as a growth substrate. Strain S3 was the only strain that could utilize all aromatic compounds tested as the growth substrate. This strain also utilized the polycyclic aromatic compounds anthracene, phenanthrene, and pyrene as sole sources of carbon and energy.

TABLE 1. Ability of carbon sources to support growth of styrene-utilizing strains.

	Growth by strain[a]:														
Substrate	124X	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14
Styrene	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Styrene oxide	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±
2-Phenylethanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1-Phenylethanol	+	+	+	+	+	−	−	−	−	−	−	±	−	+	−
Acetophenone	−	+	±	+	+	+	−	−	−	−	−	+	−	+	−
Styrene glycol	−	±	−	+	−	−	−	−	−	−	+	±	+	+	−
Ethylbenzene	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−
Toluene	+	−	−	+	+	+	−	−	−	−	+	±	−	−	−
Benzene	−	−	−	+	−	−	−	±	−	±	+	−	−	−	−

[a] + good growth, ± slight growth, − no growth.

Oxidation of potential intermediates of styrene degradation

Respiration experiments using suspensions of styrene-grown cells were performed to get an indication of possible intermediates of styrene metabolism. The addition of styrene oxide and phenylacetaldehyde resulted in an increase in the rate of oxygen consumption with all strains. Phenylacetic acid was oxidized by all strains except for strain S3. This strain, however, was the only one which oxidized acetophenone and ethylbenzene. Styrene glycol was not oxidized by any of the strains. The above results indicate that, with the exception of perhaps strain S3, which may attack styrene on the aromatic moiety of the molecule, all strains could degrade styrene via styrene oxide, phenylacetaldehyde, and phenylacetic acid.

With all strains tested, styrene degradation by suspensions of styrene-grown cells was inhibited under an atmosphere of nitrogen gas but could be restored after the addition of oxygen. This indicated that styrene is initially attacked by an oxygenase-type enzyme, possibly resulting in styrene oxide formation. To provide evidence that styrene is oxidized to styrene oxide, experiments using cell extracts were performed.

Styrene degradation in cell extracts

Styrene degradation in crude extracts was tested in all strains except in strains S2 and S13. In the presence of NADH, styrene degradation was detected in all crude extracts tested with the exception of extracts from strains S3, S14 and *Xanthobacter* strain 124X. In some cases, activity was also present in the absence of exogenously added NADH, possibly because sufficient NAD or NADH was already present in these extracts. With crude extracts from strain S14, styrene degradation was observed only if NADH was replaced by NADPH. With extracts from strains S1, S5, S8, S9, S10, S12, and S14, it was shown that styrene degradation by cell extracts was also dependent on the presence of molecular oxygen. To further assess the dependency of the reaction on the presence of NADH or NADPH, extracts of a number of strains were dialyzed. By using these dialyzed extracts, styrene consumption was no longer detected. Apparently, besides both NADH (or NADPH) and molecular oxygen, another low-molecular-weight component is required for enzymic activity.

By adding FAD to the assay mixture, styrene degradation activity of dialyzed extracts was restored. In this manner, it was possible to detect NADH-, O_2- and FAD-dependent styrene oxygenase activity in dialyzed extracts prepared from styrene-grown cells of strains S1, S4, S5, S6, S8, S9, S10, S11 and S12. Styrene oxygenase in strain S14 differed from the activity in the other strains in that, besides FAD, it had an absolute requirement for NADPH instead of NADH. NADPH could not replace NADH in the styrene oxygenase assay of extracts derived from the other strains.

Styrene degradation in strain S5

It was decided to examine styrene degradation in more detail in strain S5. This

nonmotile, Gram-positive organism formed pink colonies on agar plates. Incubation of a suspension of washed, styrene-grown cells with styrene resulted in the transient accumulation of phenylacetic acid (Fig. 1). We also attempted to detect product formation in crude extracts. To determine the optimal conditions for styrene degradation in crude extracts, the pH and temperature optima of the assay for styrene oxidation were determined (Fig. 2). The optima were close to the conditions already used in the assay for SMO activity. Incubation of dialyzed cell extracts with styrene in the presence of NADH, FAD, and molecular oxygen revealed styrene-dependent accumulation of three aromatic compounds. On the basis of retention time and UV-spectrum upon HPLC analysis of the reaction mixture, phenylacetaldehyde, 2-phenylethanol and phenylacetic acid were identified. No styrene oxide (phenyloxirane) or any hydroxy styrenes, which would result from chemical rearrangement of arene oxides formed as a result of epoxidation of the aromatic ring, could be detected. That styrene oxide was the initial oxidation product of styrene is, however, in agreement with the formation of the detected compounds. Crude extracts of styrene-grown cells contained a high styrene oxide isomerase activity (Hartmans *et al.*, 1989), which transforms any styrene oxide produced to phenylacetaldehyde. Crude extracts also contained NAD^+-dependent 2-phenylethanol dehydrogenase and NAD^+-dependent phenylacetaldehyde dehydrogenase (Table 2). These three enzymic activities would result in the transformation of any styrene oxide formed from styrene into the three aromatic compounds detected. Besides an NAD^+-dependent phenylacetaldehyde dehydrogenase, $NADP^+$- and PMS-dependent phenylacetaldehyde dehydrogenase activities were also present in cell extracts from styrene-grown cells (Table 2).

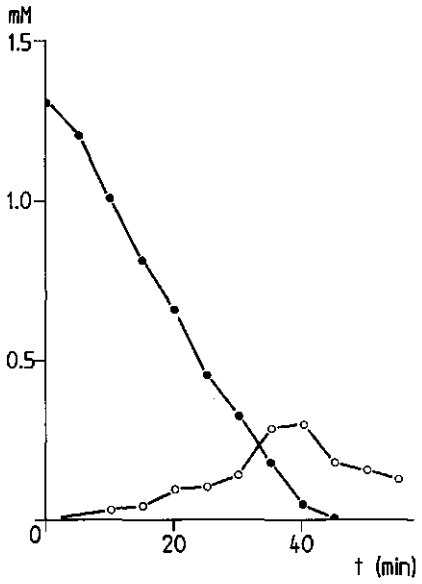

FIG. 1. Transient accumulation of phenylacetic acid (O) from styrene (●) by washed cells of strain S5 (1 mg protein per ml).

The above results suggest a degradative pathway of styrene involving an initial epoxidation to styrene oxide, which is subsequently isomerized to phenylacetaldehyde and oxidized to phenylacetic acid. The proposed pathway for

styrene degradation in strain S5 is shown in Fig. 3 and is based on simultaneous adaptation experiments, the transient accumulation of phenylacetic acid (Fig. 1), and the presence of the required enzymic activities (Table 2). The involvement of phenylacetic acid in bacterial styrene metabolism has been proposed previously (Baggi *et al.*, 1983; Sielicki *et al.*, 1975), but evidence concerning the involvement

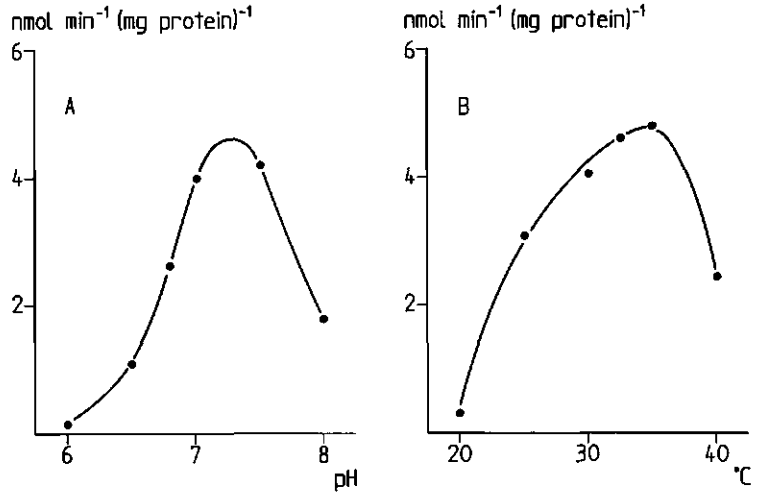

FIG. 2. Specific acitivities of styrene monooxygenase in crude extracts of strain S5 as a function of pH (A) and temperature (B).

TABLE 2. Enzyme activities in cell-free extracts of styrene-grown strain S5.

Enzyme	Specific activity (nmol min^{-1} mg protein)$^{-1}$
Styrene monooxygenase	7
Styrene oxide isomerase	395
Phenylacetaldehyde dehydrogenase	
PMS-dependent	132
NAD^+-dependent	162
$NADP^+$-dependent	25
2-Phenylethanol dehydrogenase	
PMS-dependent	0
NAD^+-dependent	0.5
$NADP^+$-dependent	0.3

of styrene oxide as an intermediate in bacterial styrene metabolism is not available. Shirai and Hisatsuka (1979a) detected styrene oxide in the culture broth of a

Pseudomonas species degrading styrene and isolated 2-phenylethanol from whole-cell incubations with both styrene and styrene oxide. These authors suggested that styrene was transformed by a monooxygenase to styrene oxide, which was then reduced to 2-phenylethanol. Considering our results, it would seem possible that the reduction of styrene oxide to 2-phenylethanol as observed by Shirai and Hisatsuka (1979a) proceeds in two steps involving phenylacetaldehyde as an intermediate.

The further metabolism of phenylacetic acid was not investigated, although several potential intermediates of phenylacetic acid metabolism were tested as growth substrates. Mandelic acid and 4-hydroxyphenylacetic acid did not support growth of strain S5, whereas 2- and 3-hydroxyphenylacetic acid did.

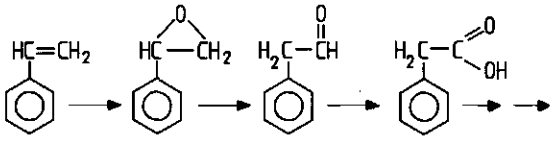

FIG. 3. Proposed pathway of styrene metabolism in strain S5.

The oxidation of styrene to styrene oxide has been demonstrated previously by using crude cell extracts from the propane-utilizing *Brevibacterium* sp. strain CRL 56 (Hou *et al.*, 1983) and the methylotroph *Methylococcus capsulatus* (Bath) (Colby *et al.*, 1977). The SMO activity present in styrene-degrading bacteria differs from these monooxygenases and alkene monooxygenase (de Bont and Harder, 1978) in its dependence on FAD for enzymic activity. The methane, alkane, and alkene monooxygenase activities can be easily determined by analyzing the rate of 1,2-epoxypropane formation from propene. Whole cells of strain S5 containing SMO activity did not oxidize propene, further indicating that SMO differs from the bacterial monooxygenases described by others. The requirement of FAD for SMO activity indicates the involvement of a flavoprotein in styrene oxidation. Most known bacterial flavin-dependent monooxygenases only perform hydroxylation reactions on the ring of substituted aromatic compounds (Müller, 1985). To our knowledge, there are no flavin-dependent monooxygenases that epoxidate alkenes (Walsh and Jack Chen, 1988). SMO could thus appear to be a flavoprotein with a novel catalytic activity.

The previously described *Xanthobacter* strain 124X (Hartmans *et al.*, 1989) and strain S3 were the only strains in which no SMO activity was detected. Styrene metabolism in these strains possibly proceeds via an initial oxidation of the aromatic nucleus. An oxidation of this type has previously been proposed for a styrene-degrading *Nocardia* species in which styrene was apparently degraded via a dihydrodihydroxy derivative with the side chain still intact (Andreoni *et al.*, 1978).

Although styrene oxide is probably an intermediate in the styrene degradation pathway of most organisms, we could not detect the accumulation of this toxic compound in growing cultures or in suspensions of washed cells incubated with

styrene. Apparently, styrene oxide isomerase activities are sufficiently high to prevent such an accumulation. On the basis of these observations, it would seem that application of styrene-degrading bacteria in processes for the biological treatment of waste gases containing styrene will not result in the accumulation of the more toxic styrene oxide.

REFERENCES

Andreoni, V., G. Baggi, E. Galli, and V. Treccani. 1978. The microbial degradation of styrene. Soc. Gen. Microbiol. Quart. **6**:18-19.

Baggi, G., M.M. Boga, D. Catelani, E. Galli, and V. Treccani. 1983. Styrene catabolism by a strain of *Pseudomonas fluorescens*. System. Appl. Microbiol. **4**:141-147.

Banerjee, S., S.H. Yalkowsky, and S.C. Valvani. 1980. Water solubility and octanol/water partition coefficients of organics. Limitations of the solubility-partition coefficient correlation. Environ. Sci. Technol. **14**:1227-1229.

de Bont, J.A.M., and W. Harder. 1978. Metabolism of ethylene by *Mycobacterium* E20. FEMS Microbiol. Lett. **3**:89-93.

Colby, J., D.I. Stirling, and H. Dalton. 1977. The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath). Biochem. J. **165**:395-402.

Furuhashi, K., M. Shintani, and M. Takagi. 1986. Effects of solvents on the production of epoxides by *Nocardia corallina* B-276. Appl. Microbiol. Biotechnol. **23**:218-223.

Hartmans, S., and J.A.M. de Bont. 1986. Acetol monooxygenase from *Mycobacterium* Py1 cleaves acetol into acetate and formaldehyde. FEMS Microbiol. Lett. **36**:155-158.

Hartmans, S., J.P. Smits, M.J. van der Werf, F. Volkering, and J.A.M. de Bont. 1989. Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. Appl. Environ. Microbiol. **55**:2850-2855.

Higgins, I.J., R.C. Hammond, F.S. Sariaslani, D. Best, M.M. Davies, S.E. Tryhorn, and F. Taylor. 1979. Biotransformation of hydrocarbons and related compounds by whole organism suspensions of methane-grown *Methylosinus trichosporium* OB3b. Biochem. Biophys. Res. Comm. **89**:671-677.

Hou, C.T., R. Patel, A.I. Laskin, N. Barnabe, and I. Barist. 1983. Epoxidation of short-chain alkenes by resting-cell suspensions of propane-grown bacteria. Appl. Environ. Microbiol. **46**:171-177.

Howard, P.H., S. Banerjee, and K.H. Robbilard. 1985. Measurement of water solubilities, octanol/water partition coefficients and vapor pressures of commercial phthalate esters. Environ. Toxicol. Chem. **4**:653-661.

Inoue, A., and K. Horikoshi. 1989. A *Pseudomonas* thrives in high concentrations of toluene. Nature, **338**:264-266.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.

Müller, F. 1985. Flavin-dependent hydroxylases. Biochem. Soc. Trans. **13**:443-447.

Omori, T., Y. Jigami, and Y. Minoda. 1975. Isolation, identification and substrate assimilation specificity of some aromatic hydrocarbon utilizing bacteria. Agr. Biol. Chem. **39**:1775-1779.

Shirai, K., and K. Hisatsuka. 1979a. Production of β-phenetyl alcohol from styrene by *Pseudomonas* 305-STR-1-4. Agric. Biol. Chem. **43**:1399-1406.

Shirai, K., and K. Hisatsuka. 1979b. Isolation and identification of styrene assimilating bacteria. Agric. Biol. Chem. **43**:1595-1596.

Sielicki, M., D.D. Focht, and J.P. Martin. 1978. Microbial transformations of styrene and [^{14}C]styrene in soil and enrichment cultures. Appl. Environ. Microbiol. **35**:124-128.

Vainio, H., F. Tursi, and G. Belvedere. 1982. What are the significant metabolites of styrene? *In* E. Hietanen, M., Laitinen, and O. Hänninen (ed.), Cytochrome P-450, Biochemistry, Biophysics and Environmental Implications. Elsevier Biomedical Press, Amsterdam.

Walsh, C.T., and Y.-C. Jack Chen. 1988. Enzymatische Baeyer-Villiger-Oxidationen durch flavinabhängige Monooxygenasen. Angewan. Chem. **100**:342-352.

Chapter 8

CHARACTERIZATION OF A *MYCOBACTERIUM* SP. AND A *XANTHOBACTER* SP. FOR THE REMOVAL OF VINYL CHLORIDE AND 1,2-DICHLOROETHANE FROM WASTE GASES

S. Hartmans, A. Kaptein, J. Tramper and J.A.M. de Bont

Mycobacterium aurum L1 and *Xanthobacter autotrophicus* GJ10 were characterized with respect to their potential use in the biological treatment of waste gases containing vinyl chloride and 1,2-dichloroethane. Vinyl chloride at a concentration of 125 g/m^3 in the gas phase was not toxic but 1,2-dichloroethane at 22 g/m^3 already resulted in a decreased growth rate. Kinetic properties of washed cells were determined and chemostat cultures were run to optimize the medium for vinyl chloride removal. Using a mixed culture of the two strains, simultaneous removal and mineralization of vinyl chloride and 1,2-dichloroethane was demonstrated. The affinity constants of growing cells found for the two substrates are, however, significantly higher than the maximal allowable concentrations of vinyl chloride and 1,2-dichloroethane in waste gases.

Applied Microbiology and Biotechnology (1992) 37:796-801

Vinyl chloride (VC) is mainly produced by dehydrochlorination of 1,2-dichloroethane (DE). Inevitable losses of VC and DE to the environment occur during this process and also in the subsequent polymerization of VC to polyvinyl chloride (PVC).

VC is carcinogenic in humans and DE is a suspected carcinogen. Legislation on the maximal allowable concentrations of these pollutants in industrial waste gases is consequently strict. For instance in the Federal Republic of Germany, according to the TA-Luft, the maximal allowable concentrations of VC or DE in waste gas may not exceed 5 mg/m^3 (Anonymous, 1986).

Biological treatment of waste gases containing low concentrations of pollutants has been advocated as a cost-effective method (Ottengraf, 1986). We have previously demonstrated effective removal of dichloromethane from waste gases using a trickle-bed bioreactor (Hartmans and Tramper, 1991).

The VC-utilizing *Mycobacterium aurum* L1 (Hartmans *et al.*, 1985; Hartmans and de Bont, 1992) and the DE-utilizing *Xanthobacter autotrophicus* GJ10 (Janssen *et al.*, 1985) have been previously described. In the present investigation we have evaluated the possibility of using these organisms in the biological treatment of waste gas containing VC and DE. Growth conditions, substrate and product toxicity, kinetic properties and mass-transfer aspects have been studied using both batch and chemostat cultures.

MATERIALS AND METHODS

Maintenance and cultivation of strains. Mycobacterium aurum L1 (DSM 6695) was maintained on mineral salts medium (MSM) agar plates at 30°C, which were incubated for about 1 month in a desiccator containing about 1% (v/v) VC in the gas phase. Plates were sealed with paper adhesive tape. This usually prevented contamination of the plates with fungi during the prolonged incubation in the desiccator and also reduced evaporation of water during subsequent storage outside the desiccator. Subculturing was routinely performed every 2 - 3 months. MSM contained per litre deionized water 3.88 g K_2HPO_4, 2.13 g $NaH_2PO_4.2H_2O$, 2.0 g $(NH_4)_2SO_4$, 0.1 g $MgCl_2.6H_2O$, 10 mg EDTA, 2 mg $ZnSO_4.7H_2O$, 1 mg $CaCl_2.2H_2O$, 5 mg $FeSO_4.7H_2O$, 0.2 mg $Na_2MoO_4.2H_2O$, 0.2 mg $CuSO_4.5H_2O$, 0.4 mg $CoCl_2.6H_2O$ and 1 mg $MnCl_2.2H_2O$. In chemostat cultures a lower buffer strength was used with 1.55 g K_2HPO_4 and 0.85 g $NaH_2PO_4.2H_2O$ per litre. *Xanthobacter autotrophicus* GJ10 (ATCC 43050) was maintained on yeast-extract glucose agar slants (Habets-Crützen *et al.*, 1984).

Growth experiments. Growth experiments with VC and DE were performed at 30°C in Erlenmeyer flasks with a total volume of 300 ml containing 100 ml MSM and sealed with Teflon Mininert valves. If only VC was used as substrate, rubber septa could also be used. Growth rates were determined by monitoring substrate depletion curves (Hartmans and Tramper, 1991).

Continuous cultures were run at 30°C in a 3-litre Applikon fermentor with a working volume of 2 litres. The pH was kept at 7.0 using 1 M NaOH and the impeller speed was 750 rpm. Dilution rates were determined by measuring the rate with which liquid from the fermentor accumulated in the waste vessel. VC-in-air mixtures were supplied to the fermentor using Brooks 5850 TR Mass-Flow Controllers (Rosemount, Schiedam, The Netherlands). DE-in-air mixtures were prepared by mixing air saturated with DE with pure air. DE-saturated air was prepared by passing air at a controlled rate through a bubble column containing DE. The temperature of the bubble column was kept constant at 20°C.

DE degradation by washed whole cells. Experiments with whole cells were performed with biomass harvested from the chemostat operated at a dilution rate of 0.02 h^{-1}. Cells were harvested by centrifugation, washed with 50 mM phosphate buffer pH 7.0 and stored at -20°C until required. DE

degradation rates were determined by analyzing headspace samples from 300 ml flasks fitted with Teflon Mininert valves containing cells in 50 ml phosphate buffer, pH 7.0. The flasks were incubated at 30°C in a shaking water bath.

Analytical methods. VC and DE were determined by analyzing 100 μl headspace samples on a Packard 430 gas chromatograph fitted with a stainless-steel Porapak R column (100-120 mesh, 110 cm × 1/8" i.d.) and a flame ionization detector. The oven temperature was 180°C for analysis of VC and 210°C for analysis of DE. The carrier gas was N_2 at 20 ml/min. VC-in-air calibration standards were prepared by adding a precise volume of VC to serum bottles sealed with rubber septa and wrapped in aluminum foil. DE-in-air calibration standards were prepared by adding an exact amount of DE to flasks partly filled with MSM (with NaN_3 to prevent bacterial growth) and fitted with Teflon Mininert valves (Precision Sampling). With the partition coefficient (0.056 at 30°C) and the volumes of the two phases in such a flask, the DE concentration in the gas phase can be calculated. Chloride-ion concentrations were determined with a Marius micro chlor-o-counter (Marius, Utrecht, The Netherlands). Protein was determined as described by Habets-Crützen *et al.*, (1984). Dry weights were determined after drying at 105°C.

Determination of partition coefficients. Gas/liquid partition coefficients were determined in a similar way as the EPICS method (Equilibrium Partitioning In Closed Systems) described by Gossett (1987). Briefly, this involved the measurement of the VC or DE content in the gas phase of a series of Erlenmeyer flasks fitted with Teflon Mininert valves. The flasks all contained the same amount of VC or DE and varying ratios of air/MSM. After equilibration at 30°C, samples of the gas phase were analyzed and the partition coefficient was calculated for all pairs of Erlenmeyers flasks using the peak-area determined by the integrator of the gas chromatograph in combination with the volumes of the gaseous and aqueous phases. For VC and DE values of 1.25 and 0.056, respectively, were determined at 30°C for the dimensionless partition coefficient ([kg m^{-3} air]/[kg m^{-3} liquid]).

Chemicals. Vinyl chloride (chloroethene) with a purity of 99.95% was obtained from HoekLoos, Schiedam, The Netherlands and 1,2 dichloroethane >99.8% was from Janssen Chimica, Beerse, Belgium. Other chemicals were from Merck, Darmstadt, Germany.

RESULTS

Substrate and product toxicity

The concentrations of DE and VC in the liquid phase during start-up of a bioreactor are in equilibrium with the concentrations in the waste gas and may reach inhibitory levels.

We therefore tested the effect of different concentrations of VC and DE on the growth of strains L1 and GJ10. VC up to 125 g/m^3 in the gas phase did not influence the growth rates of strain L1 on VC and strain GJ10 on DE respectively. In contrast, the growth rate of strain L1 growing on VC was reduced by about 50% with 22 g/m^3 DE in the gas phase and no growth was observed with 45 g/m^3 DE in the gas phase. Strain GJ10 grew well at an initial DE concentration of 22 g/m^3 in the gas phase (4 mM). Higher concentrations were not tested.

Biodegradation of chlorinated hydrocarbons is accompanied by the formation of hydrochloric acid. Subsequent neutralization with sodium hydroxide results in the accumulation of sodium chloride. Sodium chloride up to a concentration of 100 mM did not influence the growth rate of strain L1 on VC, while the growth rate of strain GJ10 on DE was reduced from 0.066 to 0.03 h^{-1} in the presence of 100 mM sodium chloride.

Kinetic parameters

The growth rate of strain L1 with VC increased upon prolonged subculturing on VC. Depending on the history of the culture the growth rate varied between 0.03 and 0.06 h^{-1}. Cells taken from the chemostat which had been run for more than 2 months under VC limitation at dilution rates varying between 0.013 and 0.025 h^{-1} exhibited a growth rate of 0.04 h^{-1}. The Michaelis-Menten constant (K_M) of 3.2 μM for VC of washed cells of strain L1 was determined previously from initial degradation velocities at different VC concentrations (Hartmans *et al.*, 1985).

Using washed cells of strain GJ10 a K_M of 0.65 mM was determined for DE from initial DE-degradation rates at DE concentrations ranging from 0.2 to 5 mM. At initial DE concentrations between 1 and 5 mM the Lineweaver-Burk plot deviated from the straight line, suggesting substrate inhibition. The apparent maximal velocity (V_{max}) was 167 nmol min^{-1} mg $protein^{-1}$ but the highest degradation rate actually measured was only 85 nmol min^{-1} mg $protein^{-1}$.

An estimate of the substrate concentration constant (K_S) of strain GJ10 growing on DE was made from a DE depletion curve in a closed culture. The method is based on the assumption that the substrate to biomass conversion ratio is independent of the growth rate. DE-depletion was followed by analyzing headspace samples at regular intervals. Towards the end of the experiment samples were taken every hour. From these data the substrate consumption rate ($\Delta S/\Delta t$) was calculated at different concentrations of DE. Growth rates were calculated by dividing ($\Delta S/\Delta t$) by the amount of substrate consumed at that point. No correction was made for the inoculum, which was less than 1% of the amount of biomass formed after all the DE had been consumed. Plotting the calculated growth rates against the corresponding DE concentrations in a Lineweaver-Burk plot gave Fig. 1.

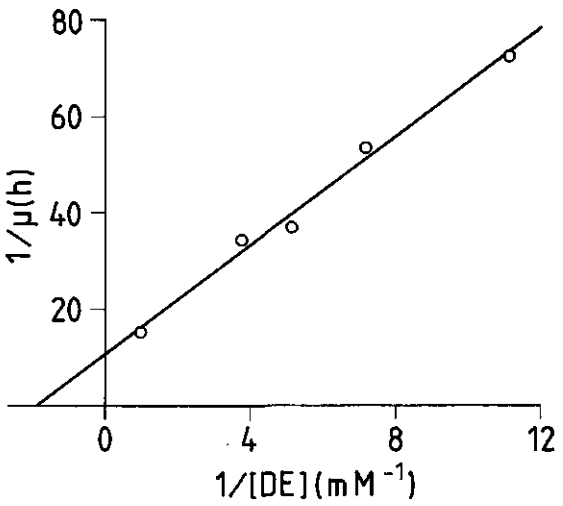

FIG. 1. Lineweaver-Burk plot of growth rates at various DE concentrations for *Xanthobacter autotrophicus* GJ10 growing on DE in a closed culture.

This plot gives a K_S of 0.53 mM and an apparent μ_{max} of 0.093 h^{-1}. Plotting the same data in an Eadie-Hofstee plot gave a K_S of 0.63 mM and an apparent μ_{max} of 0.106 h^{-1}.

Chemostat cultures of *Mycobacterium aurum* L1

To assure a reliable long-term operation of a bioreactor for waste-gas treatment the biocatalytic activity should be maintained by growth *in situ* to compensate for death, inactivation or wash-out. Chemostat cultures can be used to determine the relationship between substrate concentration and growth rate. For a chemostat culture operated at a dilution rate of 0.012 h^{-1}, we previously reported 93% VC-removal from air containing 1% VC (v/v), which passed through the fermentor at a rate of 1.2 h^{-1} (0.02 vvm) (Hartmans *et al.*, 1985). Subsequent experiments, however, indicated that VC removal was not mass-transfer-limited but that some component of the mineral salts medium was growth-limiting.

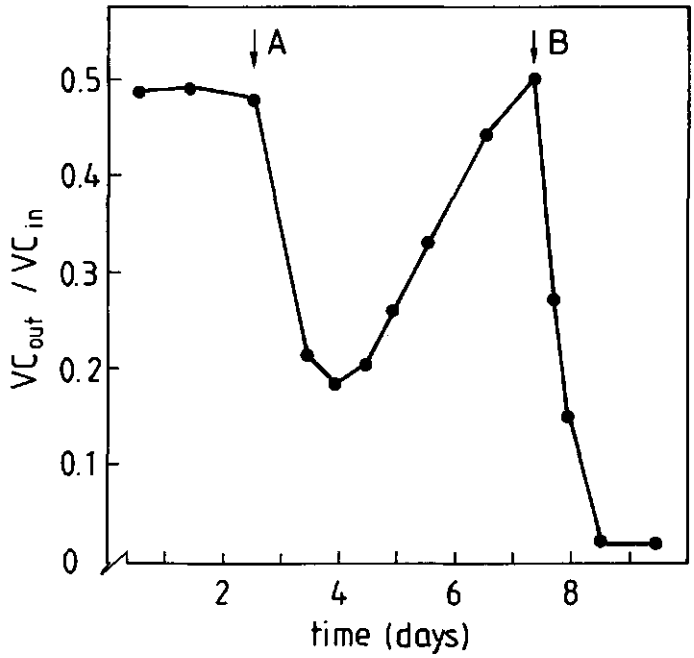

FIG. 2. Effect of $FeSO_4.7H_2O$ additions on VC removal by *M. aurum* L1 in a continuous culture of 2 l at a dilution rate of 0.02 h^{-1} aerated with air containing 45 g/m^3 VC at a rate of 40 ml min^{-1}. At (A) 5 mg $FeSO_4.7H_2O$ was added to the culture and at (B) 9 mg $FeSO_4.7H_2O$ was added to the culture and the medium reservoir was supplemented with an additional 5 mg/l $FeSO_4.7H_2O$.

Comparison of the previously used mineral salts medium (Hartmans *et al.*, 1985), which contains 0.2 ml/l of the Vishniac and Santer trace element solution, with a

number of media described in the literature revealed that iron would most likely be the limiting component. It was also observed that the trace element solution described by Vishniac and Santer (1957) contained a lot of zinc. This is, however, apparently based on a misprint. The zinc concentration should actually be tenfold lower (Robertson and Kuenen, 1983). Figure 2 shows that the addition of extra iron to a chemostat culture results in much better VC removal.

Based on these observations a new medium was formulated with 5 mg/l $FeSO_4.7H_2O$ and 2 mg/l $ZnSO_4.7H_2O$, compared to 1 and 4.4 mg/l, respectively, in the previously used medium.

Using the improved mineral salts medium, much lower VC concentrations were obtained with the chemostat cultures. Figure 3 shows the reciprocal outlet concentration of VC as a function of the reciprocal dilution rate for a number of steady states with strain L1. The apparent K_S derived from Fig. 3 was identical to the K_M of washed cells for VC at 100 ppm (250 mg/m^3) in the gas phase.

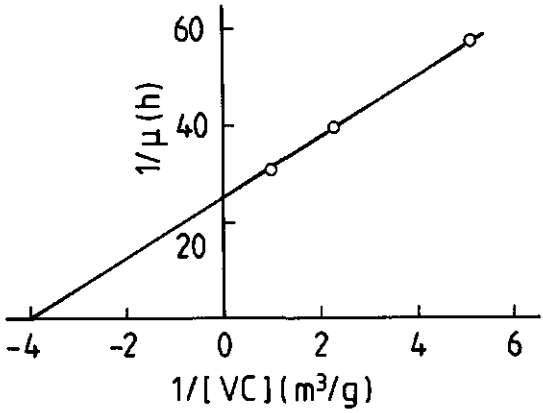

FIG. 3. Lineweaver-Burk plot of VC concentration in outlet air versus dilution rate at steady states of *M. aurum* L1 chemostat cultures.

Inactivation of vinyl chloride degradation

Another aspect of VC degradation requiring further investigation was the previously observed inactivation of washed cells degrading VC (Hartmans *et al.*, 1985). Using a much higher biomass density (5.3 mg/ml) than was used previously, a much more rapid inactivation of VC degradation was observed. Only 7% of the initial activity remained after 60 min, probably as a result of the formation of the epoxide of VC (chlorooxirane) or its rearrangement product chloroacetaldehyde (Hartmans and de Bont, 1992). These compounds are both very reactive alkylating agents (Malaveille *et al.*, 1975).

Moreover, on several occasions inactivation of the VC-degrading population in the continuous culture was observed after an interruption in the VC supply to the culture. Figure 4 shows the observed VC conversion upon restoring the VC supply

to a continuous culture of *M. aurum* L1 and *X. autotrophicus* GJ10 that had been without VC for 9 hours (indicated by bar B in Fig. 5). The broken line indicates the theoretical outlet concentrations that would be observed if no VC was transformed after the VC supply was restored. Shorter interruptions in the supply of VC did not usually result in complete inhibition of VC transformation but a temporarily reduced VC conversion was always observed. Figure 5 shows the time required for the culture to regain the original VC elimination efficiency. DE removal was only slightly affected by the interruption in the VC supply. The lower DE conversion on days 6-8, compared to the conversion on day 1, is due to the increase in the dilution rate on day 1 (indicated by arrow A in Fig. 5).

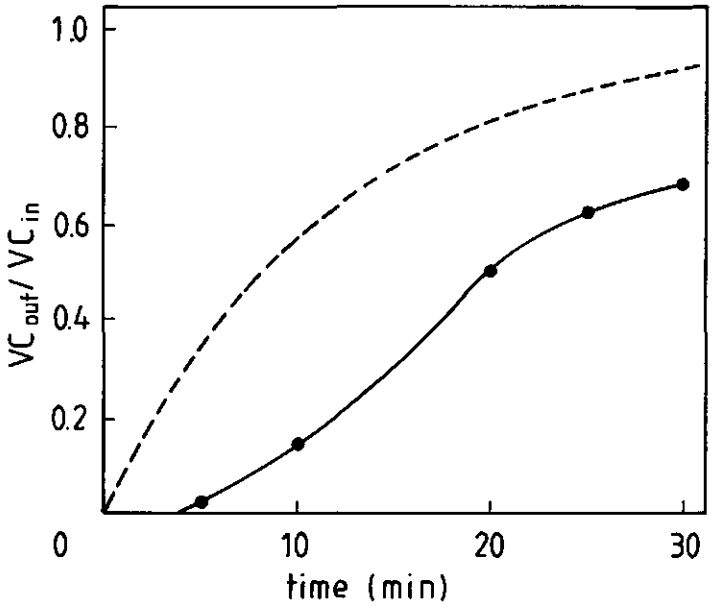

FIG. 4. VC outlet concentration upon restoring the VC supply to a continuous coculture of *M. aurum* L1 and *X. autotrophicus* GJ10 after 9 h without VC. The broken line indicates the theoretical outlet concentration that would be observed if no VC was transformed after restoring the VC supply.

Simultaneous removal of VC and DE

Several steady states of chemostat cultures containing both strains L1 and GJ10 and supplied with a mixture of VC and DE were obtained. Table 1 shows the data for two such steady states. The presence of strain GJ10 and DE did not influence VC removal, as could be expected from the batch experiments.

DISCUSSION

Although DE already affects the growth rate of strain L1 significantly at a concentration of 4 mM, the corresponding concentration in the gas phase of 22

g/m^3 is much higher than the levels that will routinely be encountered in waste gases containing DE. Therefore it is not expected that substrate toxicity will cause any problems in the biological removal of DE and VC from waste gases.

Sodium chloride at a concentration of 100 mM already had a significant effect on the growth rate of strain GJ10 with DE. Therefore an efficient bioreactor for DE removal should incorporate biomass retention allowing wash-out of chloride ions. Application of a trickle-bed bioreactor as we have previously demonstrated for dichloromethane removal from waste gas (Hartmans and Tramper, 1991) seems logical. Diks and Ottengraf (1991), however, reported that strain GJ10 did not form a stable biofilm in the trickle-bed bioreactor that they studied.

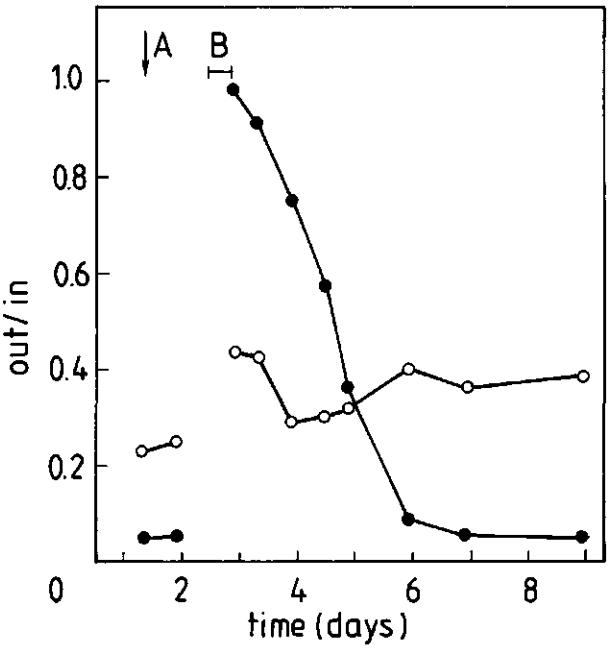

FIG. 5. Effect of an interruption in the VC supply of 9 hours (indicated by bar B) on VC (●) and DE (○) conversion of a continuous culture with strains L1 and GJ10. Aeration was at a rate of 11.6 h^{-1} with air containing 4.65 g/m^3 VC and 2 g/m^3 DE. At point A the dilution rate was changed from 0.013 to 0.018 h^{-1}.

The most important parameter with respect to the biodegradation of contaminants to very low concentrations is the substrate affinity constant. In our view a stable system for waste gas treatment requires *in situ* regeneration of the biocatalyst i.e. *in situ* growth should be possible. The K_S for a particular growth substrate gives an indication of the substrate concentration range required for good growth. K_S values are usually determined using chemostat cultures by determining the concentration of the growth-rate-limiting substrate at different dilution rates.

For growth on VC with the medium that we used previously (Hartmans *et al.*,

1985), we observed that VC concentrations in the air from the chemostat increased with an increase in the amount of VC supplied to the fermentor, suggesting that VC was not the growth-limiting substrate. As illustrated in Fig. 2, iron proved to be the limiting component in the growth medium. The positive effect of adding iron to the mineral salts medium could be a reflection of the requirement of iron by the enzyme involved in the initial transformation of VC (alkene monooxygenase), but could also indicate that *M. aurum* L1 is relatively inefficient at scavenging iron from its environment. The latter would seem to be the case based on the data for *Mycobacterium smegmatis*, which contains 5-8 μmol iron per gram dry weight when grown under iron-sufficient conditions (McCready and Ratledge, 1978). The previously used medium contains 3.5 μM iron and the culture only contained 0.3 g/l biomass (dry weight) under the iron limiting conditions (Fig. 2). Therefore a value of almost 12 μmol iron per gram dry weight would have been attainable if strain L1 would have taken up all the iron present in the medium.

Using the improved medium strain L1 was grown VC-limited in the chemostat at various growth rates. The Lineweaver-Burk plot using gas-phase VC concentrations from chemostat steady states (Fig. 3) gave an apparent K_S of 250 mg/m^3 VC in the gas phase and a μ_{max} of 0.04 h^{-1}. However, this method of estimating the K_S requires some discussion.

As VC is transferred from the gas phase to the liquid phase a driving force must be present, i.e., the liquid-phase concentration is not in equilibrium with the gas-phase concentration, but is lower. The magnitude of this difference is, however, difficult to estimate, as exemplified by the following.

In the classical approach to mass transfer in fermentors, mainly applied to oxygen transfer, the gas phase is assumed to be ideally mixed and mass transfer is described with the following equation in which the outlet gas concentration is used for C_G.

$$\text{Specific Mass Transfer Rate} = k_La \times (C_G/m - C_L)$$

where k_La = volumetric mass transfer coefficient, C_G = gas-phase concentration, m = partition coefficient and C_L = liquid-phase concentration. For oxygen m is high (34) and only a small percentage of the oxygen in air is transferred. Consequently the outlet oxygen concentration can be used for C_G as this will not differ much from the actual average C_G. This assumption is, however, not applicable for gas/liquid transfer of components with a (much) lower partition coefficient. In the latter case conversions of more than 99% can be observed and if an ideally mixed gas phase is assumed, extremely high values for k_La would be required to explain the observed mass transfer rates. For example for the situation in Table 1, for the outlet VC concentration of 225 mg/m^3, a k_La of 0.079 s^{-1} would be required assuming that the gas phase is ideally mixed and the liquid-phase concentration is zero. This is about five times higher than the estimated k_La for VC of 0.017 s^{-1} calculated from experimentally determined k_La values for oxygen and ethene

assuming a reciprocal relationship between values and diffusion coefficients in water (unpublished results). Furthermore, the assumption that the VC concentration in the liquid phase is zero is not realistic, indicating that the value of 0.079 s^{-1} for k_LA is a minimal value for the situation in which the gas phase is assumed to be ideally mixed. Therefore, the gas phase cannot be assumed to be ideally mixed. The other extreme, would be to assume that the gas flow through the fermentor is completely plug flow. If this is assumed for the same data in Table 1 the average gas-phase concentration would be the logarithmic average (1.466 g/m^3) of the inlet and outlet concentrations. If the liquid-phase concentration is assumed to be in equilibrium with the outlet VC concentration a k_LA value of 0.014 s^{-1} would be required, which is quite close to the value of 0.017 s^{-1} calculated from the other k_LA data. If plug flow and a liquid-phase concentration of zero would be assumed, a k_LA of only 0.012 s^{-1} would be required to result in the observed outlet concentration.

TABLE 1. Gas phase concentrations at steady-state conditions of chemostat cultures with *M. aurum* L1 and *X. autotrophicus* GJ10 growing on vinyl chloride and 1,2-dichloroethane.

D (h^{-1})	ϕ_v (h^{-1})	VC_{in} (g/m^3)	VC_{out} (g/m^3)	DE_{in} (g/m^3)	DE_{out} (g/m^3)	Chloride Recovery (%)
0.0127	11.5	4.67	0.225	1.98	0.455	101
0.0184	11.5	4.63	0.238	2.14	0.87	97

D, Dilution rate; ϕ_v, Specific air flow; VC and DE, concentrations in the gas phase.

These calculations demonstrate that the gas phase in our fermentor is far from ideally mixed. The actual liquid concentrations are of course lower than the values calculated from the outlet concentrations in the gas phase, but it is difficult to estimate how large the differences are. It is therefore rather fortuitous that the Lineweaver-Burk plot using the outlet gas-phase concentrations gives an apparent K_S of 3.2 mM which is equal to the value determined for the K_M of washed cells. The actual value will probably be slightly lower.

The value of 0.53 mM for the K_S of strain GJ10 for DE that we estimated from a batch growth experiment is reasonably close to the value of 0.26 mM recently reported by van den Wijngaard *et al.*, (1991). These authors used the more conventional method employing chemostat cultures to determine this value. The μ_{max} reported by van den Wijngaard *et al.*, (1991) for growth of GJ10 is slightly higher at 0.104 h^{-1} than our value of 0.094 h^{-1}. This difference could be due to the presence of vitamins in the growth medium of van den Wijngaard *et al.*, (1991).

Determination of the μ_{max} for growth with DE is, however, rather difficult to perform as the substrate is already toxic at concentrations of about 20 times the K_S.

Using the kinetic parameters K_S = 0.53 mM and μ_{max} = 0.094 h^{-1}, DE concentrations for the chemostat cultures can be calculated. For the two dilution rates in Table 1 the corresponding DE outlet concentrations, assuming equilibrium between the gaseous and aqueous phase, would be 0.46 and 0.72 g/m^3, respectively. These values are very close to the experimentally determined values (Table 1).

Van den Wijngaard *et al.,* (1991) recently described two new DE-degrading strains containing the same dehalogenase as strain GJ10. These new strains, however, contain respectively about 2- and 15-fold higher levels of the dehalogenase. The K_M of the purified dehalogenase was reported to be 0.63 mM, which is very close to the value of 0.65 mM that we determined for washed cells of strain GJ10. These new strains had apparent K_S values of 0.23 mM and 0.03 mM, respectively (van den Wijngaard *et al.,* 1991).

The method we have used to determine the K_S of strain GJ10 for DE with closed batch cultures would seem generally applicable for bacteria with a high K_S in relation to the detection limit of the substrate being studied. Especially for substrates that can be analyzed by simple headspace analysis the method is very suitable. A good water solubility (low partition coefficient) is, however, required to assure that the gas and liquid phases are in equilibrium. Furthermore, it is advisable to use initial substrate concentrations that are not too high to avoid high biomass densities resulting in relatively high substrate consumption rates at the end of the growth experiment. This method is not suitable for the determination of the K_S for VC as this compound has a much higher partition coefficient than DE.

The reduced VC transformation observed after an interruption in the VC supply to the chemostat culture (Figs. 4 and 5) is probably the result of a temporary accumulation of chlorooxirane directly after the VC supply is restored due to insufficient activity of the enzyme transforming this toxic intermediate. Indeed after an interruption in the VC supply of less than 1 h chlorooxirane could be detected in the air from the fermentor, whereas under normal conditions no chlorooxirane was detectable (Hartmans and de Bont, 1992). This could indicate that the enzyme required for the further metabolism of chlorooxirane is not synthesized in sufficient amounts in the absence of its inducer (VC or chlorooxirane) and is very unstable. The temporary accumulation of chlorooxirane had almost no effect on the DE conversion (Fig. 5). This is in line with earlier studies on the inactivation of bacteria by epoxides (Habets-Crützen and de Bont, 1985). In these studies 1,2-epoxypropane was shown to inactivate alkene monooxygenase at much lower concentrations than were required for inhibition of other physiological functions of the cell.

In conclusion, there are at least two problems that prevent the successful application of the strains studied here to remove VC and DE from waste gases to the required low levels of 5 mg/m^3. For the VC-degrading strain the observed instability is an obvious problem and for both strains the gas-phase concentrations

corresponding with substrate affinity constants are much higher than 5 mg/m^3. Even the K_S value of the DE-utilizing strain recently described (van den Wijngaard *et al.*, 1991), which is tenfold lower than the K_S of strain GJ10, still corresponds with a gas-phase concentration of 160 mg/m^3.

Acknowledgement

We thank Dr. D.B. Janssen from the University of Groningen for kindly providing *X. autotrophicus* GJ10, Anne Hof for k_LA determinations and Wim Wiegant for discussions.

These investigations were supported in part by the Dutch Ministry of Housing, Physical Planning and Environment.

REFERENCES

Anonymous 1986. Erste Allgemeine Verwaltungvorschrift zum Bundes-Immissionsschutzgesetz (Technische Anleitung zur Reinhaltung der Luft - TA Luft) vom 27-2-1986. Gemeinsames Ministerialblatt **37**:95-144.

Diks, R.M.M., and S.P.P. Ottengraf. 1991. Process engineering aspects of biological waste gas purification, p. 353-367. *In* H. Verachtert, and W. Verstraete (ed.), Environmental Biotechnology 1991. Royal Flemish Society of Engineers.

Gossett, J.M. 1987. Measurement of Henry's law constant for C_1 and C_2 chlorinated hydrocarbons. Environ. Sci. Technol. **21**:202-208.

Habets-Crützen, A.Q.H., L.E.S. Brink, C.G. van Ginkel, J.A.M. de Bont, and J. Tramper. 1984. Production of epoxides from gaseous alkenes by resting-cell suspensions and immobilized cells of alkene-utilizing bacteria. Appl. Microbiol. Biotechnol. **20**:245-250.

Habets-Crützen, A.Q.H., and J.A.M. de Bont 1985. Inactivation of alkene oxidation by epoxides in alkene- and alkane-grown bacteria. Appl. Microbiol. Biotechnol. **22**:428-433.

Hartmans, S., J.A.M. de Bont, J. Tramper, and K.Ch.A.M. Luyben. 1985. Bacterial degradation of vinyl chloride. Biotechnol. Lett. **7**:383-388.

Hartmans, S., and J.A.M. de Bont. 1992. Aerobic vinyl chloride metabolism in *Mycobacterium aurum* L1. Appl. Environ. Microbiol. **58**:1220-1226.

Hartmans, S., and J. Tramper. 1991. Dichloromethane removal from waste gases with a trickle-bed bioreactor. Bioprocess. Eng. **6**:83-92.

Janssen, D.B., A. Scheper, L. Dijkhuizen, and B. Witholt. 1985. Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. Appl. Environ. Microbiol. **49**:673-677.

Malaveille, C., H. Bartsch, A. Barbin, A.M. Camus, R. Montesano, A. Croisy, and P. Jacquignon. 1975. Mutagenicity of vinyl chloride, chloroethyleneoxide, chloroacetaldehyde and chloroethanol. Biochem. Biophys. Res. Comm. **63**:363-370.

McCready, K.A. and C. Ratledge. 1978. Amounts of haem and related compounds in *Mycobacterium smegmatis* grown in various concentrations of iron. Biochem. Soc. Trans. **6**:421-423.

Ottengraf, S.P.P. 1986. Exhaust gas purification, p. 425-452. *In* W. Schönborn (ed.), Biotechnology vol 8, Microbial Degradations. Chemie Verlag, Weinheim, Germany.

Robertson, L.A., and J.G. Kuenen. 1983. *Thiospaera pantotropha* gen. nov. sp. nov., a facultatively anaerobic, facultatively autotrophic sulphur bacterium. J. Gen. Microbiol. **129**:2847-2855.

van den Wijngaard, A.J., R.D. Wind, and D.B. Janssen. 1991. Kinetics of microbial degradation of 1,2-dichloroethane and 2-chloroethanol, p. 671-672. *In* H. Verachtert, and W. Verstraete (ed.), Environmental Biotechnology 1991. Royal Flemish Society of Engineers.

Vishniac, W., and M. Santer. 1957. The *Thiobacilli.* Bacteriol. Rev. **21**:195-213.

Chapter 9

DICHLOROMETHANE REMOVAL FROM WASTE GASES WITH A TRICKLE-BED BIOREACTOR

S. Hartmans and J. Tramper

A 66 l trickle-bed bioreactor was constructed to assess the possibilities of eliminating dichloromethane from industrial waste gases. The trickle-bed bioreactor was filled with a randomly-stacked polypropylene packing material over which a liquid phase was circulated. The pH of the circulating liquid was externally controlled at a value of 7 and the temperature was maintained at 25°C. The packing material was very quickly covered by a dichloromethane-degrading biofilm which thrived on the dichloromethane supplied via the gas phase. The biological system was very stable and not sensitive to fluctuations in the dichloromethane supply. Removal of dichloromethane from synthetic waste gas was possible down to concentrations well below the maximal allowable concentration of 150 mg/m^3 required by West-German law for gaseous emissions. At higher dichloromethane concentrations specific dichloromethane degradation rates of 200 $g\ h^{-1}\ m^{-3}$ were possible. At very low inlet concentrations, dichloromethane elimination was completely mass-transfer limited.

The gas-phase mixing could be described by a series of 10 to 7 identical ideally-mixed tanks for superficial gas velocities ranging from 150 to 450 m/h. Dichloromethane elimination with the trickle-bed bioreactor was modelled using an overall mass-transfer coefficient that was dependent on the gas and liquid velocities. Mass-transfer resistance within the biofilm was also accounted for. Using the model, elimination efficiencies were predicted which were very close to the experimentally observed values.

Bioprocess Engineering (1991) 6:83-92.

Dichloromethane (methylene chloride) is produced in large amounts by the chemical industry. It is mainly applied as a solvent in metal degreasing, in paint removers and in the pharmaceutical industry. Although some dichloromethane containing wastes are incinerated, it can be assumed that the greater part of the dichloromethane produced is eventually lost to the environment. Due to its low boiling point (40.1°C) and high vapour pressure (47 kPa at 20°C) significant amounts of dichloromethane reach the environment via gaseous emissions. In 1980 dichloromethane and tetrachloroethylene were estimated to be the predominant chlorinated hydrocarbons emitted into the ambient air in the Netherlands, with dichloromethane emissions totaling 5800 tons per year (Guicherit and Schulting, 1985). Besides being present in gaseous emissions, dichloromethane can also be detected in many aqueous industrial effluents.

Recently the regulations concerning the maximal allowable concentrations of pollutants in industrial waste gases have been reviewed in the FRG (Technische Anleitung Luft, 1986). The concentration of dichloromethane in industrial waste-gas emissions in West Germany must now be lower than 150 mg/m^3. It is expected that in the near future these new regulations will also be applied in other European countries. Therefore, a considerable interest exists in the development of techniques for the elimination of dichloromethane and other chemicals from waste gases.

For many years dichloromethane was considered to be non-biodegradable (Klečka, 1982) and was described as being ubiquitous in aqueous environments (Rittmann and McCarty, 1980). Degradation of dichloromethane by biofilms (Rittmann and McCarty, 1980) and mineralization by activated sludge (Klečka, 1982) were, however, subsequently demonstrated.

Nowadays dichloromethane degrading bacteria are isolated quite readily from activated sludge and most water and soil samples. Strains belonging to the genera *Hyphomicrobium, Pseudomonas* and *Methylobacterium* have been described (Brunner *et al.*, 1980; Stucki *et al.*, 1981; Gälli and Leisinger, 1985).

Bacterial metabolism of dichloromethane has been shown to be brought about by a glutathione-dependent dichloromethane dehalogenase yielding formaldehyde and hydrochloric acid (Stucki *et al.*, 1981). The dehalogenase from *Hyphomicrobium* DM2 has been purified and characterized (Kohler-Staub and Leisinger, 1985). Further work revealed that several dichloromethane-degrading strains belonging to different genera all contained the same dehalogenase (Kohler-Staub *et al.*, 1986). Recently, however, a fast-growing dichloromethane-degrading bacterium designated DM11 was isolated that contained a novel dehalogenase with improved catalytic activity (Scholtz *et al.*, 1988).

Once the existence of dichloromethane-degrading bacteria had been established, the potential of using these microorganisms to treat dichloromethane containing wastewater was studied by several groups. The most detailed study was performed by Gälli (1987), who described a fluidized-bed bioreactor containing sand particles with an attached dichloromethane-degrading biofilm. Using a synthetic waste water

containing 10 kg/m^3 dichloromethane, specific degradation rates of 1.6 kg m^{-3} h^{-1} could be achieved. A disadvantage of the fluidized-bed bioreactor described by Gälli, is the necessity of a separate mass-transfer device to supply the oxygen required for biodegradation.

Biofilters have received much attention during the last decade for the biological treatment of waste gases due to their low operational costs and proven reliability (Ottengraf, 1986). A limitation of the biofilter (or compost filter) is, however, that the degradation of compounds such as chlorinated hydrocarbons will result in acidification of the packing material and subsequent loss of biological activity.

In view of the above limitations we have investigated the possibility of using a trickle-bed bioreactor to treat waste gases containing dichloromethane. A 66 l reactor, very similar to the high-rate biological filters used in aerobic wastewater treatment, was constructed. Contrary to the situation for wastewater treatment, the aqueous phase is circulated and the pollutant is removed from the gas phase flowing through the reactor. Special attention is given to the mass transfer aspects associated with the removal of dichloromethane from the gas phase.

MATERIALS AND METHODS

Microorganisms. The dichloromethane-degrading strains *Hyphomicrobium* DM2 and *Methylobacterium* DM4 have been described previously (Stucki *et al.*, 1981; Gälli and Leisinger, 1985) and were a kind gift of Dr R. Gälli and Prof Th. Leisinger. *Hyphomicrobium* DM20 was isolated from an enrichment culture with dichloromethane (2 mM) as carbon source and a mixture of local soil and water samples as inoculum. Strain DM21 was isolated from the trickle-bed bioreactor.

Growth conditions and media. The mineral salts medium was made up of 10 ml of stock solution A and 25 ml of stock solution B per litre demineralized water. Stock solution A contained per litre demineralized water: 200 g $(NH_4)_2SO_4$, 10 g $MgCl_2.6H_2O$, 1 g EDTA, 0.2 g $ZnSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.5 g $FeSO_4.7H_2O$, 0.02 g $Na_2MoO_4.2H_2O$, 0.02 g $CuSO_4.5H_2O$, 0.04 g $CoCl_2.6H_2O$ and 0.1 g $MnCl_2.2H_2O$. Stock solution B contained per litre demineralized water: 155 g K_2HPO_4 and 85 g $NaH_2PO_4.2H_2O$.

Determination of growth rates and dichloromethane degradation rates. Growth rates were determined at 30°C. Biomass increase was determined with a nephelometer (Evans Electroselenium Ltd., UK). Amperometric measurement of chloride liberation rates as a result of dichloromethane degradation was also used to determine growth rates (Hartmans *et al.*, 1985). A third method to determine growth rates, especially useful at low dichloromethane concentrations, was with the following equation:

$$\ln(S_t - S) = \ln(S_t - S_0) + \mu t$$

with S = substrate concentration at time t, S_0 = substrate concentration at time zero and S_t = the theoretical initial substrate concentration which includes the substrate that would have been necessary to form the biomass present in the inoculum. This equation is valid as long as $S >> K_S$ implying μ equals μ_{max}. Furthermore, the yield and maintenance coefficients are assumed constants. S,t data points were determined from substrate depletion curves of closed cultures. Values for S_t were estimated with the least squares criterion. Calculation of all growth rates was done from a series of data points collected during at least two culture doubling times.

Determination of the specific dichloromethane degradation activity of the circulating liquid from the trickle-bed bioreactor was performed by measuring the dichloromethane consumption rate of this suspension (2 ml in a 30 ml vial incubated in a reciprocally-shaking water bath at 30°C) by periodically analyzing headspace samples after the addition of dichloromethane (100 μg). The effect of temperature on the relative dichloromethane-degradation rates by *Hyphomicrobium* DM20 was

determined in a similar fashion with dichloromethane-grown cells.

Determination of partition coefficient. The gas/liquid partition coefficient of dichloromethane was determined in a similar way as the EPICS method (Equilibrium Partitioning In Closed Systems) described by Gossett (1987). Briefly, this involved the measurement of the dichloromethane content in the gas phase of a series of Erlenmeyer flasks fitted with teflon Mininert valves. The Erlenmeyers all contained the same amount of dichloromethane and varying ratios of air/mineral salts medium. After equilibration at 30°C, samples of the gas phase were analyzed and the partition coefficient was calculated for all pairs of Erlenmeyers using the peak-area determined by the integrator of the gas chromatograph in combination with the volumes of the gas and liquid phases.

Trickle-bed bioreactor. A schematic diagram of the trickle-bed bioreactor is shown in Fig. 1. The actual reactor consisted of a vertical perspex pipe with an internal diameter of 0.29 m and a height of 1.34 m. The polypropylene packing material (Filterpak, Mass Transfer International, Heversham, UK) was randomly stacked to a height of 0.9 or 1 m. Filterpak consists of 27 mm segments of corrugated pipe with a diameter of 50 mm with two vertical partitioning segments at right angles to one another on the inside. The specific surface area of the packing material is 118 m^{-1} (manufacturer's specifications). The packing material was supported by a perforated plate of stainless steel (diameter of 0.285 m with holes of 8 mm diameter at a centre to centre distance of 19 mm) which was placed at 0.14 m from the bottom of the reactor. A similar stainless steel plate at the top of the packing supported an aluminium perforated plate (diameter 0.25 m, with 3 mm holes at a centre to centre distance of 6 mm). This construction allowed a sufficient radial distribution of the liquid which was supplied via eight openings (i.d. 6 mm) situated 0.1 m above the top sieve plates. The liquid was collected at the bottom of the reactor at a steady-state level of about 4 cm and ran into the mixing vessel which was placed 0.8 m below the bottom of the reactor. The liquid applied to the top of the bioreactor was pumped up from the mixing vessel by a 0.5 Hp centrifugal pump. The flow rate could be adjusted by a valve and was measured with a rotameter (0.1 - 1 m^3/h) during the second run. The combined volumes of the aqueous phases in the reactor, mixing vessel and piping was 17.6 l at a liquid circulation rate of 1 m^3/h. The liquid in the mixing vessel was gently mixed by a turbine stirrer. The pH was controlled with a pH-controller (Type 505A, LH-Fermentation, Slough, UK) coupled to a peristaltic pump which added demineralized water containing NaOH (4 g/l) and K_2HPO_4 (1 g/l). The pH of the system was kept at pH 7 ± 0.5 unless indicated otherwise. The temperature of the mixing vessel could be controlled by a coil heat exchanger through which water from a thermostated water bath was circulated. Unless indicated otherwise the temperature of the mixing vessel was kept at 25 ± 2°C.

A synthetic dichloromethane-containing waste gas was made by mixing air saturated with dichloromethane with pure air in a mixing zone. This mixture was then introduced at the bottom of the bioreactor directly below the bottom sieve plate. The air saturated with dichloromethane was prepared by passing air at a controlled rate (5850 TR Brooks Massflow Controller, Veenendaal, The Netherlands) through a bubble column containing dichloromethane. The temperature of the bubble column was kept at 20°C, allowing evaporation rates of 1 to 15 g dichloromethane per hour, depending on the air-flow rate through the column. The evaporation rate of dichloromethane from the bubble column was calibrated by gravitametrically determining dichloromethane evaporation at an air flow entering the bubble column of 22.3 ml/min. This corresponded with an evaporation rate of 3.84 g/h. This value is very close to the value of 4.12 which can be calculated assuming a partial pressure of dichloromethane of 47.2 kPa at 20°C. It was furthermore shown that the inlet dichloromethane concentrations were linear with the air flow through the bubble column in the range of 5 to 75 ml/min. The air flow indicated by the mass-flow controller, in combination with the value of 3.84 g/h at an air flow of 22.3 ml/min, was routinely used to calculate evaporation rates of dichloromethane. The inlet and outlet dichloromethane concentrations were determined at points A and B (Fig. 1) by flushing serum bottles with air from the sampling ports for several minutes with a diaphragm pump. Immediately after sampling, the dichloromethane content of the gas phase in these bottles was analyzed gas chromatographically. The main flow of air passed through a rotameter with a low accuracy, thus only allowing an indication of the magnitude of the air flow. Reliable determinations of the actual air flow (m^3/h) entering the bioreactor were calculated from dichloromethane-evaporation rates in combination with the inlet concentrations of dichloromethane.

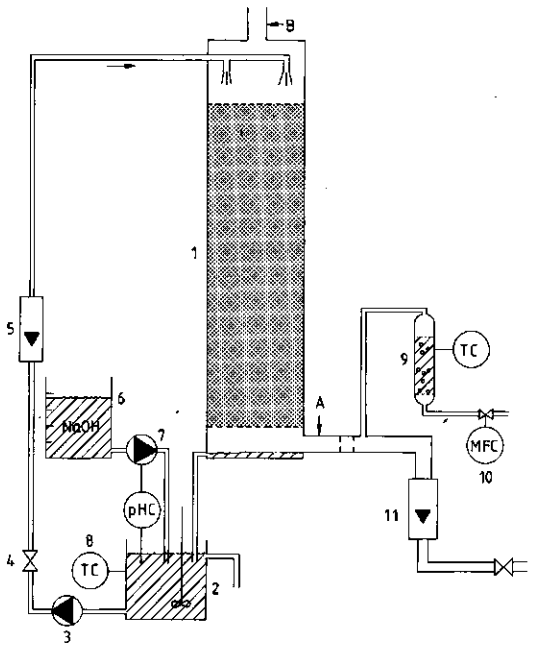

FIG. 1. Schematic diagram of the trickle-bed bioreactor. (1) Packing material in reactor, (2) Mixing vessel, (3) Liquid circulation pump, (4) Valve, (5) Liquid rotameter, (6) NaOH reservoir, (7) Pump linked to pH-controller, (8) Temperature control, (9) Bubble column with thermojacket, (10) Mass flow controller, (11) Air rotameter, A and B sampling ports.

The reactor was inoculated with 2 l of *Hyphomicrobium* DM20 grown in batch culture with 0.2% (v/v) methanol in mineral salts medium. To supply the biomass growing in the bioreactor with minerals, stock solution A was added to the mixing vessel daily. One ml of stock solution A was added for each litre of $NaOH/K_2HPO_4$ solution consumed during the preceding 24 hours.

Analytical methods. Dichloromethane concentrations were determined by analysis of 100 μl gas-phase samples on a Packard 430 gas chromatograph fitted with a stainless steel Porapak R column (100-120 mesh, 110 cm × 1/8" i.d.) and a flame ionization detector. The oven temperature was 210°C and the carrier gas N_2 at 20 ml/min. Dichloromethane-in-air calibration standards were prepared by adding an exact amount of dichloromethane to Erlenmeyer flasks partly filled with mineral salts medium (with a small amount of NaN_3 to prevent bacterial growth) and fitted with Teflon Mininert valves (Precision Sampling). With the values of the partition coefficient (30°C) and the volumes of the two phases in such an Erlenmeyer, the dichloromethane concentration in the gas phase can be calculated.

Residence time distribution curves were determined using propene (20 to 50 ml) as a pulse tracer injected at sampling point A (Fig. 1). The propene content of the air leaving the bioreactor could be continuously analyzed with a portable Photo Ionization Detector with a sampling pump (PID 580 OVM, Thermoelectron, Hopkinton, Mass.). The mean residence time in the sampling tubing was subtracted from the recorded residence times before these were used to calculate the moments of the residence-time distribution curves with a computer. The model used to describe the mixing of the gas flow was a cascade of N identical, ideally-mixed tanks.

Chemicals. All solid and liquid chemicals were from E. Merck, Darmstadt, Germany. Propene was

from Hoekloos, Schiedam, The Netherlands.

RESULTS AND DISCUSSION

Comparison of strains degrading dichloromethane

A prerequisite for an efficient transfer of dichloromethane from a waste gas to an aqueous phase containing dichloromethane-degrading bacteria is a low dichloromethane concentration in the water phase. Therefore, three dichloromethane-degrading bacterial strains were compared with respect to their substrate affinity. This was done by comparing the growth rates with dichloromethane as growth substrate at different concentrations (Table 1).

TABLE 1. Specific growth rates of dichloromethane utilizers (h^{-1}).

Dichloromethane concentration range (mM)	DM2	DM4	DM20
0.015 - 0.001[a)]	0.107 ± 0.005	0.133 ± 0.010	0.103 ± 0.005
0.7 - 0.05[a)]	0.103 ± 0.010	0.091 ± 0.004	0.114 ± 0.009
1 - 0.1[b)]	0.081 ± 0.003	0.092 ± 0.004	0.105 ± 0.012
5 - 3[b)]	0.069 ± 0.002	0.067 ± 0.005	0.084 ± 0.004
5 - 1[c)]	0.08	0.077	0.10
10 - 5[d)]	0.07	0.09	–

Methods used to determine growth rates: [a)] dichloromethane depletion curves, [b)] Cl^- - formation, [c)] biomass formation, [d)] Gälli (1986).

The new isolate, *Hyphomicrobium* DM20, had a slightly higher growth rate than the two previously described strains (DM2 and DM4). Recently however, after we had completed our experiments, Scholz *et al.* (1988) have described a dichloromethane-degrading bacterium (strain DM11) with an even higher specific growth rate of 0.22 h^{-1}.

The specific growth rate of the three dichloromethane-degrading strains tested was still maximal at dichloromethane concentrations of 1-15 μM in the liquid phase. This was somewhat unexpected in view of the K_M of 30 μM which had previously been determined for the purified dichloromethane dehalogenase from *Hyphomicrobium* DM2 (Kohler-Staub and Leisinger, 1985) and the value of 20 μM for the K_M of the dehalogenase from *Methylobacterium* DM4 (Scholz *et al.*, 1988). The K_M values of the dehalogenases from these two strains would indeed be expected to be the same as it was previously shown that the dehalogenases from *Hyphomicrobium* DM2 and *Methylobacterium* DM4 are identical (Kohler-Staub *et al.*, 1986). Both strains grow faster on methanol than on dichloromethane (Sholtz *et al.*, 1988) and during incubation of *Hyphomicrobium* DM2 cells with dichloromethane no formaldehyde formation could be detected (Stucki *et al.*, 1981). These observations indicate that dehalogenation is the rate-limiting step during growth on

dichloromethane and would imply that the K_M and K_S of whole cells have about the same value. As we could not detect any decrease in growth rate at dichloromethane concentrations below the K_M values reported in the literature for the purified enzymes, the K_S of the growing cells and hence the K_M of *in vivo* enzymic activity is apparently much lower than the K_M of the purified enzymes. The method of headspace analysis that we applied was unfortunately not sensitive enough to determine the K_M for dichloromethane of whole cells. Subsequently we have assumed a K_M of 1 μM for whole cells, but the actual value is expected to be lower. This corresponds with a dichloromethane concentration of 10.6 mg/m^3 in air at 30°C, which is well below the concentration of 150 mg/m^3, which probably will be the maximal allowable concentration in industrial gaseous emissions in the future. Based on these data we concluded that effective treatment of waste gases contaminated with dichloromethane would be feasible using a bioreactor with a high gas/liquid mass-transfer capacity. The trickle-bed bioreactor was chosen because it provides a high gas/liquid interface at a relatively-low power consumption.

In an earlier experiment (unpublished results), strains DM2 and DM4 were both inoculated into a chemostat which was run at a dilution rate of 0.04 h^{-1} with dichloromethane as growth-limiting substrate. The chemostat was run for more than 30 days without one of the two strains becoming dominant in the liquid phase. Wall growth was, however, also observed. In the biofilm which formed on the glass wall of the fermentor, the *Hyphomicrobium* DM2 appeared to be the predominating organism. As dichloromethane degradation is accompanied with HCl formation, which results in NaCl accumulation when neutralized with NaOH, we have also tested the salt tolerance of the three strains by determining the growth rates with 200 mM NaCl added to the growth medium. This resulted in substantially lower specific growth rates of 0.022, 0.029 and 0.042 h^{-1} for strains DM2, DM4, and DM20, respectively.

Dichloromethane elimination with the trickle-bed bioreactor

Based on the observed growth rates at elevated NaCl concentrations and the fact that the *Hyphomicrobium* species formed a biofilm in the chemostat experiment, we inoculated the trickle-bed bioreactor with *Hyphomicrobium* DM20, assuming that this strain would probably also form a biofilm. The trickle-bed bioreactor was run twice. The first time with a packing of 0.9 m and a liquid circulation rate of about 0.3 to 0.5 m^3/h. Formation of a biofilm on the polypropylene packing was already observed several days after inoculation. The reactor was run for several weeks with dichloromethane supplied at a rate of 3.84 g/h and a superficial gas velocity of about 40 m/h, resulting in an inlet dichloromethane concentration of almost 1.5 g/m^3. Within a week a dichloromethane conversion of 85% was already observed. At this stage it was also verified if the dichloromethane removed from the air passing through the reactor was indeed dechlorinated. This was done by

gravitametrically monitoring the NaOH addition rate. In these experiments the K_2HPO_4 was omitted from the 0.1 M NaOH solution which was pumped into the mixing vessel to neutralize HCl produced as a result of dichloromethane degradation. In this way the observed dichloromethane removal (calculated from the % conversion) could indeed be accounted for by NaOH consumption.

Interruptions of several days in the dichloromethane supply had hardly any effect on the system. Once the dichloromethane supply was restored, the original dichloromethane-elimination efficiency was regained very rapidly. This is an important observation in view of the often discontinuous character of industrial waste-gas emissions. A pH drop to a value of 3.5, which occurred as a result of the NaOH reservoir running empty, caused dichloromethane degradation to cease. However, after readjustment of the pH to 7 the original dichloromethane-degradation capacity was regained within several days. The bioreactor forms a very stable system and only requires a minimum of control.

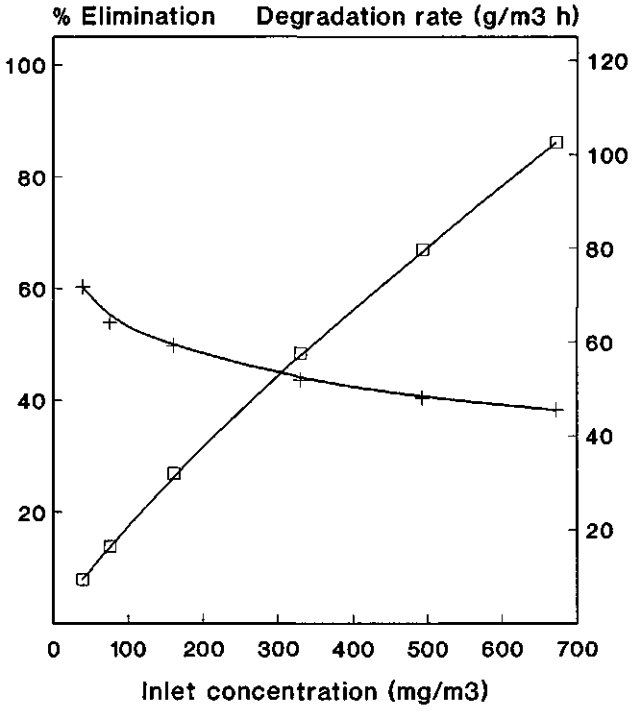

FIG. 2. Dichloromethane elimination efficiencies (+) and specific degradation rates (□) with the 0.9 m trickle-bed bioreactor at a superficial gas velocity of 360 m/h.

No pressure difference could be measured between the inlet and outlet gas phase, so that only a minimal power consumption is required to pass the waste gas through

the reactor making the energy required to circulate the liquid phase the main energy operating costs.

The reactor was run for a period of three months with dichloromethane loading rates varying from 1.9 to 4.8 g/h, corresponding to inlet dichloromethane concentrations of 0.7 to 1.8 g/m^3. Under these conditions dichloromethane conversions of 80 to 95% were recorded.

Figure 2 shows a typical curve of the efficiency of the 0.9 m trickle-bed bioreactor at various dichloromethane inlet concentrations for a relatively high gas velocity averaging 360 m/h. Specific degradation rates which are also shown ($g\ m^{-3}\ h^{-1}$) were calculated by multiplying the percentage of dichloromethane conversion with the dichloromethane evaporation rate (g/h) and dividing this value by the volume of the packed bed (0.059 m^3 for a bed height of 0.9 m). At lower gas velocities and higher inlet concentrations of dichloromethane, resulting in a much higher driving force for mass transfer, specific degradation rates as high as 200 $g\ m^{-3}\ h^{-1}$ were achieved. To allow sufficiently high dichloromethane evaporation rates the temperature of the bubble column containing dichloromethane was increased to 25°C during these experiments.

At the end of this period of three months, experiments were performed in which the effect of temperature on the dichloromethane elimination efficiency of the bioreactor was determined. The temperature of the liquid phase was varied for a short time (0.5 to 2 hours) for two situations. One was at a relatively low superficial gas velocity ($v_{g,s}$) of 77 m/h and high inlet concentration of dichloromethane (1.89 g/m^3) and the second was at a high $v_{g,s}$ of 370 m/h and a very low inlet concentration of dichloromethane (79 mg/m^3). After about 15 minutes at the new temperature a constant outlet concentration of dichloromethane was observed. As shown in Fig. 3 the effect of the temperature on these two situations is markedly different. As a comparison, the temperature effect on the degradation rate of dichloromethane by free cells is also shown. As can be seen in Fig. 3 the temperature hardly affects the dichloromethane elimination efficiency under conditions where dichloromethane degradation in the bioreactor is obviously completely mass-transfer limited. Apparently the temperature dependency of the diffusion coefficient, which results in a higher mass-transfer resistance at lower temperatures, is compensated by the increased solubility of dichloromethane at these lower temperatures (a lower partition coefficient) resulting in an increased driving force for mass transfer. The curve in Fig. 3 for the situation with the higher inlet concentration of dichloromethane indicates a gradual change from a reaction-limited situation at the lower temperatures (15 to 20°C) to a partially mass-transfer-limited situation in the higher temperature range (25 to 30°C).

After these experiments the bioreactor was dismantled, cleaned and repacked to height of 1 m, resulting in an effective packed-bed volume of 0.066 m^3. At this stage a rotameter was installed which allowed measurement of the liquid flow rate. The start-up conditions were identical to those for the 0.9 m bioreactor, except that now

the liquid-circulation rate could be measured and was set at 1 m^3/h. After four weeks a gradual change in colour of the biofilm was observed. Especially in areas with relatively high liquid velocities, e.g., places where the packing material makes contact with the wall of the bioreactor, the biofilm started turning pink. Six weeks after the reactor had been inoculated the pink dichloromethane-degrading bacterium appeared to be the predominant dichloromethane-degrading microorganism. This pink methylotrophic organism was apparently better adapted to the conditions prevailing in the trickle-bed bioreactor than the *Hyphomicrobium* DM20 which was used as inoculum. After ten weeks, during which the biological system proved to be very stable, the bioreactor was supplied with a constant flow of dichloromethane of 3.84 g/h at an average air flow rate of about 40 m/h for two consecutive weeks. This was the starting situation from which in the course of another two weeks a series of measurements were done under varying conditions.

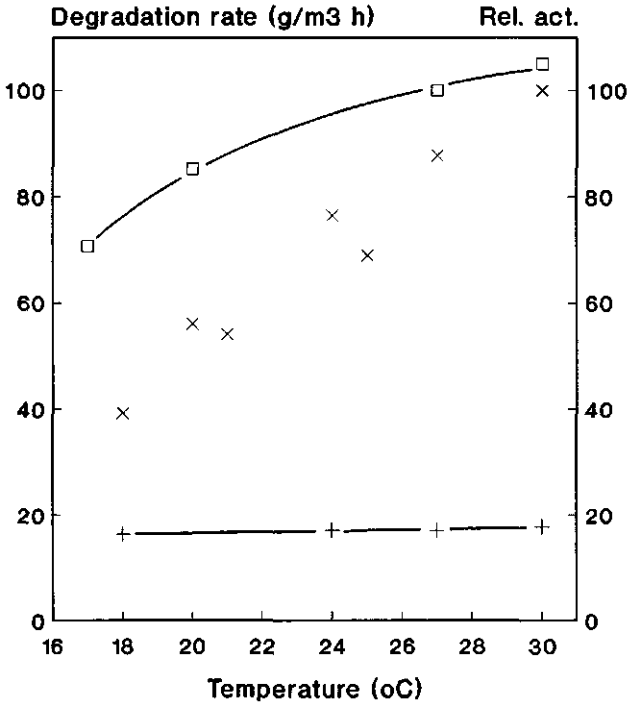

FIG. 3. Temperature effect on specific dichloromethane degradation rates with the 0.9 m trickle-bed bioreactor. (□) Inlet concentration 1.89 g/m^3 and superficial gas velocity 77 m/h; (×) Relative activity of free cells; (+) Inlet concentration 79 mg/m^3 and superficial gas velocity 370 m/h.

These varying conditions were generally applied for a short period only (0.5 to 2 h), long enough to allow stabilization and measurement of both the outlet and the inlet

concentrations of dichloromethane. Between each series of measurements, the dichloromethane supply was reset to 3.84 g/h and the air flow was readjusted to about 40 m/h.

Mass-transfer aspects of dichloromethane elimination

The following experiments were performed to illustrate the mass-transfer resistances associated with the transfer of dichloromethane from the gas phase to the biofilm. Figure 4 shows an enhancement of the elimination efficiency as a result of an increased liquid velocity indicating the existence of mass-transfer resistance in the liquid phase.

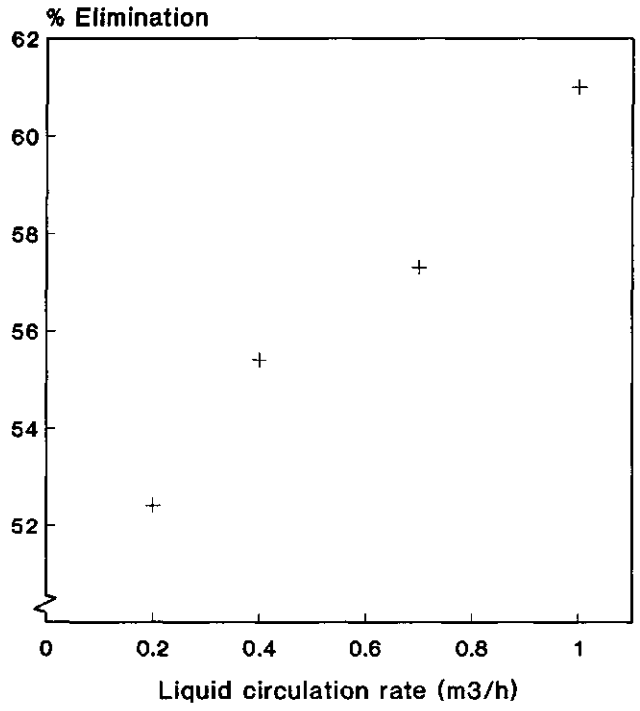

FIG. 4. Effect of liquid circulation rate on dichloromethane elimination with the 1 m trickle-bed bioreactor at an inlet concentration of 347 mg/m^3 and a superficial gas velocity 335 m/h.

It was verified that the decrease in elimination efficiency at lower liquid circulation rates was not caused by a decrease in the pH of the liquid leaving the reactor. No significant differences in pH could be measured.

Besides the biomass in the biofilm covering the packing material there was also suspended biomass present in the circulating liquid. The circulating liquid was shown to have a specific dichloromethane degradation rate of 36 g m^{-3} h^{-1}. This

correspond with a specific degradation capacity, based on the bioreactor volume, of 9.5 g m^{-3} h^{-1}. In Tables 2 and 3 the results of a series of experiments are described with varying inlet concentrations of dichloromethane at an average air flow of 233 m/h. These experiments were all performed on the same day.

TABLE 2. Dichloromethane-elimination efficiency including suspended biomass at an average gas velocity of 233 m/h.

Dichloromethane evaporation rate (g/h)	Dichloromethane concentration (mg/m^3) Inlet	Outlet	Conversion %	Specific degradation rate (g m^{-3} h^{-1})	Calculated minimal $k_{o,l}$ $\times 10^5$ m/s
0.96	66	8.2	87.2	12.68	13.48
1.92	117	20.1	82.8	24.09	12.89
2.88	184	37.7	79.6	34.73	11.08
3.84	248	59.7	75.9	44.16	9.75
4.80	305	85.2	72.1	52.44	8.95
6.72	429	140.6	67.2	68.42	7.79
8.64	549	194	64.7	84.70	7.30
11.52	727	296	59.3	103.51	6.35

TABLE 3. Dichloromethane-elimination efficiency after removing excess suspended biomass at an average gas velocity of 233 m/h.

Dichloromethane evaporation rate (g/h)	Dichloromethane concentration (mg/m^3) Inlet	Outlet	Conversion %	Specific degradation rate (g m^{-3} h^{-1})	Calculated minimal $k_{o,l}$ $\times 10^5$ m/s
0.96	68	11.85	82.5	12.00	10.99
1.92	126	28.0	77.8	22.63	10.21
2.88	187	49	73.8	32.20	9.20
4.80	315	99	68.6	49.89	7.86
8.64	559	220	60.6	79.32	6.43
11.52	746	321	57.0	99.49	5.81

Between the experiments shown in Tables 2 and 3 the liquid phase was washed by adding 30 l of water, with about the same ionic composition as the circulating liquid, to the mixing vessel at a rate of about 2 litres per minute. This theoretically results in removal of about 82% ($1 - e^{-30/17.6}$) of the suspended biomass. As can be seen by comparing Tables 2 and 3, the suspended biomass does indeed affect the observed dichloromethane-elimination efficiencies, resulting in lower elimination

efficiencies after removal of a substantial portion of the suspended biomass. In Tables 2 and 3 we have also calculated the theoretical minimal values for the overall mass-transfer coefficients (based on the liquid phase) required to account for the observed mass-transfer rates. For the calculation of the minimal $k_{o,l}$ values, plug flow of the gas phase and a dichloromethane concentration of zero in the bulk liquid phase have been assumed. This situation would be encountered if all the transferred dichloromethane is immediately consumed in the circulating liquid phase. With these assumptions the logarithmic average concentration of dichloromethane in the gas phase $((c_{in} - c_{out}) / \ln (c_{in}/c_{out}))$ was assumed to be the mean driving force for mass transfer. This value for the driving force was used in combination with the observed specific mass-transfer rate and the assumed specific gas/liquid interface of 118 m^2/m^3 to calculate the minimal $k_{o,l}$ values. At very low inlet concentrations of dichloromethane, combined with relatively high activities of suspended biomass, the dichloromethane concentration in the water phase approaches zero and consequently the calculated minimal $k_{o,l}$ approaches the actual value. The decrease in the calculated minimal $k_{o,l}$ value required at higher dichloromethane transfer rates is of course a reflection of the increase of the dichloromethane concentration in the bulk liquid phase, which is necessary as a driving force to transport dichloromethane into the biofilm. This thus illustrates that at higher dichloromethane degradation rates a significant mass-transfer resistance is present within the biofilm.

TABLE 4. Dichloromethane-elimination efficiency at different gas velocities.

Gas velocity (m/h)	Dichloromethane concentration (mg/m^3) Inlet	Outlet	Conversion %	Specific degradation rate ($g\ m^{-3}\ h^{-1}$)	Calculated minimal $k_{o,l}$ ($\times 10^5$ m/s)
145	160	14.4	91.0	21.18	10.31
249	140	31.6	77.4	27.02	10.91
336	156	53.7	65.6	34.35	10.54
475	147	67.6	54.0	37.70	10.85
156	299	44.8	85.0	39.56	8.69
242	288	90.1	68.7	47.97	8.29
339	309	132.8	57.0	59.69	8.41
461	303	159	47.5	66.33	8.74

If for two situations with different specific degradation rates the calculated minimal $k_{o,l}$ values are the same, this implies that the actual $k_{o,l}$ values for these two situations differ, with a higher actual $k_{o,l}$ for the situation with the higher mass transfer rate. This situation was observed when we increased the gas velocities while

keeping the inlet concentrations almost the same. The specific degradation rates increased with an increase in the gas velocity, whereas the calculated minimal $k_{o,l}$ values were almost constant for the two series (Table 4). This indicates that the actual $k_{o,l}$ increases with increasing gas velocity and implicates the existence of gas-phase resistance for dichloromethane transfer, assuming that the gas velocity does not affect the liquid mixing.

Modelling dichloromethane elimination with the trickle-bed bioreactor

To achieve a better understanding of dichloromethane elimination, simulation of the process of mass transfer and dichloromethane degradation in the trickle-bed bioreactor was attempted. The approach has been to use our computer program BIOSIM (de Gooijer *et al.*, 1989), with which the optimal volumes of a series of ideally stirred-tank reactors (STR) can be calculated for a desired conversion by immobilized biocatalysts. The program calculates the internal and external effectiveness factors for the immobilized biocatalysts obeying Michaelis-Menten kinetics resulting in an overall effectiveness factor for each one of the series of stirred tanks. The overall conversion must be varied iteratively until the calculated total volume of the series of stirred tanks equals the actual volume of the trickle-bed bioreactor being simulated.

The number of STR's used in the simulations was calculated from the residence-time-distribution curves of the gas phase. The residence-time distribution of the gas phase was determined at four gas velocities and with varying liquid-circulation rates. The liquid velocity had no significant effect on the gas-phase mixing characteristics. With increasing gas velocity the plug-flow character of the gas phase decreased slightly. Using a series of N identical, ideally-mixed tanks as a model for gas-phase mixing the following values for N were calculated for the superficial gas velocities indicated: 10.6 (128 m/h), 9.3 (235 m/h), 7.9 (357 m/h) and 7.1 (447 m/h). At these gas velocities an increase in the gas velocity apparently results in an increased turbulence and consequently a better axial mixing of the gas phase. At gas velocities below 100 m/h the number of identical, ideally-mixed tanks required to describe the gas-phase mixing decreased with decreasing gas velocity indicating that transport as a result of axial diffusion becomes relatively more important under these conditions.

In the BIOSIM program an overall external mass-transfer coefficient was used to calculate the external effectiveness factor. In the case of the trickle-bed bioreactor where the substrate for the biocatalyst is supplied via the gas phase, the total external mass-transfer resistance can be assumed to consist of the following 3 components: (i) a gas/liquid, gas-side resistance ($1/k_g$) resulting in a concentration gradient between the bulk of the gas phase and the gas/liquid interface, (ii) a gas/liquid, liquid-side resistance ($1/k_l$), resulting in a concentration gradient between the gas/liquid interface and the bulk liquid and (iii) a biofilm/liquid, liquid-side resistance ($1/k_s$), possibly resulting in a concentration gradient between the biofilm/liquid interface and the bulk liquid phase. As the contribution of the

latter component ($1/k_s$) to the total external mass transfer resistance was difficult to estimate, unless a very discrete interface is indeed assumed to exist, it was neglected. Consequently, the total external mass-transfer coefficient ($k_{o,l}$) used in the simulations was calculated with equation (1):

$$m/k_{o,l} = 1/k_g + m/k_l \qquad (1)$$

In equation (1) m is the partition coefficient of dichloromethane, which has a value of 0.125 at 30°C. Values of k_g were calculated from equation (2) and k_l was calculated with equation (6).

$$Sh = (k_g \times d) \,/\, D_{DM,g} \qquad (2)$$

Equation (3) was used to estimate values for Sh (Ranz and Marshall, 1952)

$$Sh = 2 + 0.57 \times Re^{0.5} \times Sc^{0.33} \qquad (3)$$

with

$$Re = \rho_g \times v_g \times d/\mu_g \qquad (4)$$

and

$$Sc = \mu_g/(\rho_g \times D_{DM,g} \qquad (5)$$

For the estimation of the gas velocity (v_g) in equation (4) a porosity of 0.9 was used to calculate v_g from the superficial gas velocity ($v_g = v_{g,s}/0.9$). The manufacturer reports a porosity of 0.93 for Filterpak, but as we have applied a higher liquid flow rate (twice the advised upper limit) a slightly larger larger liquid hold-up (resulting in a slightly lower porosity) has been assumed. The following values for the various parameters were used in the above equations: d (the packing material was assumed to be comparable to spheres with a diameter of 0.04 m) = 0.04 m , $D_{DM,g}$ (diffusion coefficient of dichloromethane in air at 30°C) = 1.0×10^{-5} m^2/s (estimated with the Chapman-Enskog kinetic theory (Bird *et al.*, 1960), ρ_g (density of air) = 1.164 kg/m^3, μ_g (dynamic viscosity of air at 30°C) = 1.87×10^{-5} $N \times$ s/m^2.

The liquid-phase mass-transfer coefficient was estimated with the correlation of Morris and Jackson (Lyderson, 1983):

$$k_l = C \times (\rho_l/\mu_l)^{0.2} \times D_{DM,l}{}^{0.5} \times L^{0.7} \qquad (6)$$

The following values for the various parameters were used: C (liquid-film packing factor) was estimated to be 800 from data for comparable packing materials (Lyderson, 1983), ρ_l (liquid density) = 1000 kg/m^3, m_l (dynamic viscosity of water at 30°C) = 8×10^{-4} $N \times$ s/m^2, $D_{DM,l}$ (diffusion coefficient of dichloromethane in water at 30°C = 1.57×10^{-9} m^2/s (calculated with the Wilke-Chang equation

(Wilke and Chang, 1955), $L = v_s/a$ with v_s (superficial liquid flow rate) = 4.21×10^{-3} m/s and a (specific surface area) = 118 m^2/m^3. The k_l calculated with equation (6) was 4.0×10^{-4} m/s for a liquid circulation rate of 1000 l/h.

To simulate the specific surface area of the trickle-bed bioreactor (118 m^2/m^3) covered with a biofilm, the model used to simulate the trickle-bed bioreactor was assumed to be a reactor filled for 39.33% with gel beads having a diameter of 0.02 m containing dichloromethane-degrading bacteria homogeneously spread throughout the bead. The volumetric gas-flow rate and inlet concentrations of dichloromethane were transformed to a liquid-flow rate and liquid concentrations of dichloromethane using the partition coefficient of 0.125. The other parameters required for the simulation were: The maximal specific dichloromethane consumption rate (V_{max}), which was estimated to be 1.852×10^{-4} kg dichloromethane kg^{-1} biomass s^{-1} by assuming a growth yield of 0.15 kg biomass per kg dichloromethane consumed (estimated from protein yield data of Scholtz *et al.* (1988) and assuming biomass contains 50% protein) and a maximal growth rate of 0.1 h^{-1}. The biocatalyst gel load (X), or biofilm density, was assumed to be 50 kg biomass per m^3 gel beads which is an average value when compared with data from the literature, although biofilm densities as high as 105 kg/m^3 have also been reported (Hoehn and Ray, 1973). The Michaelis-Menten constant (K_M), was set at 1 μM (8.5×10^{-5} kg/m^3), based on the growth experiments, but could very well be lower. The diffusion coefficient of dichloromethane within the biofilm (D_B), was assumed to be 80% of the value of the diffusion coefficient in water resulting in a value of $D_B = 1.26 \times 10^{-9}$ m^2/s. A temperature of 30°C was assumed in all simulations and furthermore it was assumed that there was only dichloromethane consumption in the biofilm and not in the circulating liquid phase.

Comparison of simulations with experimental results

Figure 5 shows the dichloromethane elimination efficiencies predicted by the model for the gas velocities and inlet concentrations from Table 3. A $k_{o,l}$ of 1.85×10^{-4} m/s estimated with equation (1) was used (with $k_g = 2.75 \times 10^{-3}$ m/s and $k_l = 4.0 \times 10^{-4}$ m/s) and the gas-phase mixing was described by a series of 9 ideally-mixed tanks. The experimentally-determined elimination efficiencies after washing out excess biomass (data from Table 3) are also shown.

The model describes the experimental results surprisingly well, even though we have had to estimate the values of most parameters and have not incorporated a potentially existing mass-transfer resistance between the liquid phase and the biofilm ($1/k_s$). Incorporation of such a mass-transfer resistance in the model would result in lower elimination efficiencies, especially at relatively low inlet concentrations. This effect could, however, be compensated for to some extent by simultaneously incorporating a higher biomass concentration and a lower value for the K_M in the model, which as discussed above, could be possible.

As was previously reported, the conversion achieved with the optimal design

(used in BIOSIM) is very close to the design of a series of equal ideally-mixed tank reactors (Luyben and Tramper, 1982). For the simulations in Fig. 5 the differences in the calculated elimination efficiencies for the optimal and equal size design were indeed very small. The differences never exceeded 0.06%, thus justifying the use of the easier to calculate optimal design.

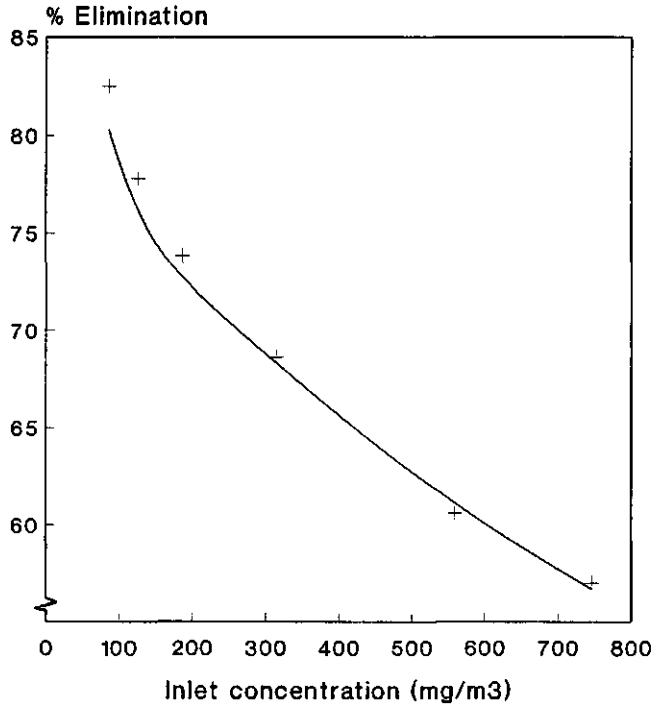

FIG. 5. Experimentally determined values (+) and simulated values (—) for dichloromethane elimination with the 1 m trickle-bed bioreactor at an average superficial gas velocity of 233 m/h.

In Table 5 the predicted elimination efficiencies at different gas velocities are compared with the experimentally determined data from Table 4. The gas velocity affects dichloromethane transfer in two ways. Increased gas velocities result in a better mixing of the gas phase which results in a decrease in the gas-phase resistance (increased k_g and thus $k_{o,l}$, Table 5), but also in a decreased mean driving force (less identical ideally-mixed tanks to describe the gas-phase mixing). The experimentally observed efficiencies are all slightly higher than the values predicted by the model. However, this underestimation is never more than 9% and can be attributed to the presence of significant amounts of active biomass in the liquid phase.

TABLE 5. Simulated dichloromethane-elimination efficiencies at different gas velocities.

Superficial gas velocity (m/h)	$k_{o,l} \times 10^4$ (m/s)	Number of identical ideally mixed reactors	Experimentally determined conversion (%)	Simulated conversion (%)
145	1.74	10	91.0	87.4
156	1.74	10	85.0	82.2
249	1.92	9	77.4	72.1
242	1.92	9	67.7	67.3
336	2.04	8	65.6	61.0
339	2.04	8	57.0	54.0
475	2.17	7	54.0	49.3
461	2.17	7	47.5	43.6

Using the model, the maximal oxygen uptake of the biofilm was also estimated. Assuming a K_M for oxygen of 1% of the air saturated concentration in water at 30°C (7.5 g/m^3), a maximal oxidation rate which equals the maximal dichloromethane oxidation rate (on molar basis), a diffusion coefficient for oxygen in the biofilm of 1.9×10^{-9} m^2/s (80% of the value in water used by Brink and Tramper (1986)) and neglecting mass-transfer resistance in the gas phase ($k_{o,l}$ equals k_l which was estimated with equation (6) to be 5.0×10^{-4} m/s) a maximal oxygen uptake rate of 2.93×10^{-7} kg m^{-2} s^{-1} was calculated. This would allow a maximal dichloromethane oxidation rate of 7.78×10^{-7} kg m^{-2} s^{-1} corresponding to a maximal specific dichloromethane-degradation capacity of the trickle-bed bioreactor of 331 g m^{-3} h^{-1}, more than 60% above the experimentally observed specific degradation rate. Feeding the trickle-bed bioreactor with dichloromethane concentrations that would allow higher degradation rates could result in the accumulation of the toxic formaldehyde, the product of dichloromethane dehalogenation. The dehalogenase activity is oxygen independent but degradation of formaldehyde requires oxygen. We were unable to experimentally verify the occurrence of oxygen limitation, due to the limited dichloromethane evaporation capacity of our bubble column. Accordingly, no formaldehyde could be detected in the liquid phase of the mixing vessel at the highest-observed specific degradation rate of 200 g m^{-3} h^{-1}.

The BIOSIM program describing mass transfer to and substrate consumption in a biofilm has also been used to verify experimental data from Harris and Hansford (1976) for oxygen-limited glucose consumption by a biofilm growing on a vertical plate over which a film of liquid was flowing. The average maximal glucose removal rate they observed was 2.2×10^{-6} kg COD m^{-2} s^{-1}, which corresponds to an oxygen consumption rate of 7.04×10^{-7} kg m^{-2} s^{-1}. To calculate the maximal

oxygen flux into the biofilm with the BIOSIM program the values of Harris and Hansford (1976) were used for the following parameters: V_{max} = 1.78 × 10^{-4} s^{-1}, K_M = 2.5 × 10^{-5} kg/m^3, biomass concentration in the biofilm (X) 90 kg/m^3, D_B 2.5 × 10^{-9} m^2/s, a bulk-liquid oxygen concentration of 8 × 10^{-3} kg/m^3 and a $k_{o,l}$ of 4 × 10^{-4} m/s. The resulting calculated maximal oxygen flux into the biofilm was 6.98 × 10^{-7} kg m^{-2} s^{-1}, which is very close to the value of 7.04 × 10^{-7} kg m^{-2} s^{-1} (calculated from the oxygen limited removal of glucose) observed by Harris and Hansford.

One aspect has to be considered in more detail. The $k_{o,l}$ used by Harris and Hansford was estimated from their experimental data, and assumed to describe the mass-transfer resistance in a stagnant water layer adjacent to the biofilm ($1/k_s$). Mass-transfer resistance on the air side of the water film was apparently not considered, although oxygen transfer from the air was necessary to prevent oxygen depletion of the water flowing over the biofilm. The k_l we have used in our model has been estimated from an empirical equation for the liquid-side mass-transfer coefficient of the gas-liquid interface of a liquid film flowing over the packing material. We have disregarded any mass-transfer resistance existing on the biofilm side of the liquid film. In our model the k_l is dependent on the liquid-flow rate (equation 2) whereas in the experiments of Harris and Hansford there was no apparent effect of the flow rate on mass-transfer, implying a constant k_s, and providing support for their assumption of a stagnant water layer between the biofilm and the bulk liquid.

Although the two approaches are fundamentally different, both models result in a satisfactory prediction of the observed experimental data. A better understanding of the relative mass-transfer resistances in the trickle-bed bioreactor would however, require more experimental data especially for situations without any dichloromethane consumption in the liquid phase. In our view, however, the present model is sufficiently accurate to use for the dimensioning of trickle-bed bioreactors for the elimination of dichloromethane from waste gases. Design of large-scale trickle-bed bioreactors can probably be done based on the reported pilot-scale experiments as long as the mixing characteristics and velocities of both the liquid and gas phases are of the same magnitude as used in the pilot-scale experiments.

Conclusions

From the pilot-scale experiments we have reported here, we conclude that elimination of dichloromethane from waste gases is technically possible with the relatively uncomplicated trickle-bed bioreactor. Especially for the treatment of large volumes of waste gases the trickle-bed bioreactor is better suited than the previously described fluidized-bed bioreactor (Gälli, 1987). Although three phase fluidized-bed bioreactors have a larger specific surface area for biofilm formation (up to 1000 m^2/m^3 (Etzensperger *et al.*, 1989), allowing higher volumetric degradation rates at the same dichloromethane flux into the biofilm, the energy costs required blow the

air through the reactor will be considerable. The elimination efficiencies achieved with the 1 m trickle-bed reactor are not very high but increasing the bed height will probably result in elimination efficiencies high enough to cope with most industrial waste-gas emissions. To lower the operating costs of a trickle-bed bioreactor the liquid flow rate may be reduced. This will result in lower energy costs, but due to the concomitant decrease in mass-transfer efficiency a larger reactor will be required to achieve the same dichloromethane elimination efficiency. Obviously there is an optimum where the sum of the operating and investment costs is minimal.

Acknowledgements

We thank Miss M.P. Jansen and Mr H. Oude Luttighuis for participating in the experimental work. We are grateful to Dr R. Gälli and Prof Th. Leisinger (ETH-Zürich) for providing *Hyphomicrobium* DM2 and *Methylobacterium* DM4 and thank Ir C.D. de Gooijer for advice on the computer simulations.

This work was supported by the Dutch Ministry of Housing, Physical Planning and Environment (project number 64.10.15.02).

REFERENCES

Bird, R.B., W.E Stewart, and E.N. Lightfoot. 1960. Transport phenomena. New York: John Wiley and Sons.

Brink, L.E.S., and J. Tramper. 1986. Modelling the effects of mass transfer of propene epoxidation of immobilized *Mycobacterium* cells: Pseudo-one-substrate conditions and negligible product inhibition. Enzyme Microb. Technol. 8:281-288.

Brunner, W.B., D. Staub, and T. Leisinger. 1980. Bacterial degradation of dichloromethane. Appl. Environ. Microbiol. 40:950-958.

Etzensperger, M., S. Thoma, S. Petrozzi, and I.J. Dunn. 1989. Phenol degradation in a three-phase biofilm fluidized sand bed reactor. Bioproc. Eng. 4:175-181.

Gälli, R., and T. Leisinger. 1985. Specialized strains for the removal of dichloromethane from industrial waste. Cons. Recycling 8:91-100.

Gälli, R. 1987. Biodegradation of dichloromethane in waste water using a fluidized bed bioreactor. Appl. Microbiol. Biotechnol. 27:206-213.

de Gooijer, C.D., H.J.J Hens, and J. Tramper. 1989. Optimum design for a series of continuous stirred tank reactors containing immobilized biocatalyst beads obeying intrinsic Michaelis-Menten kinetics. Bioproc. Eng. 4:153-158.

Gossett, J.M. 1987. Measurement of Henry's law constant for C_1 and C_2 chlorinated hydrocarbons. Environ. Sci. Technol. 21:202-208.

Guicherit, R., and F.L. Schulting. 1985. The occurrence of organic chemicals in the atmosphere of the Netherlands. Sci. Total Environ. 43:193-219.

Harris, N.P., and G.S. Hansford. 1976. A study of substrate removal in a microbial film reactor. Water Res. 10:935-943.

Hartmans, S., J.A.M. de Bont, J. Tramper, and K.Ch.A.M. Luyben. 1985. Bacterial degradation of vinyl chloride. Biotechnol. Lett. 7:383-388.

Hoehn, R.C., and A.D. Ray. 1973. Effects of thickness on bacterial film. Journal Water Pollution Control Federation 45:2302-2320.

Klečka, G.M. 1982. Fate and effects methylene chloride in activated sludge. Appl. Environ. Microbiol. 44:701-707.

Kohler-Staub, D., and T. Leisinger. 1985. Dichloromethane dehalogenase of *Hyphomicrobium* sp. strain DM2. J. Bacteriol. 162:676-681.

Kohler-Staub, D., S. Hartmans, R. Gälli, F. Suter, and T. Leisinger. 1986. Evidence for identical dichloromethane dehalogenases in different methylotrophic bacteria. J. Gen. Microbiol. 132:2837-2843.

Luyben, K.C.A.M., and J. Tramper. 1982. Optimal design for continuous stirred tank reactors in series using Michaelis-Menten kinetics. Biotech. Bioeng. 24:1217-1220.

Lyderson, A.L. 1983. Mass transfer in engineering practice. Chicester: John Wiley and Sons.

Ottengraf, S.P.P. 1986. Exhaust gas purification, p. 425-452. *In* W. Schönborn (ed.), Biotechnology, vol 8, Microbial degradations. Chemie Verlag, Weinheim, Germany.

Ranz, W.E., and W.R. Marshall. 1952. Evaporation from drops. Chem. Eng. Progr. 48:141-146,173-180.

Rittmann, B.E., and P.L. McCarty. 1980. Utilization of dichloromethane by suspended and fixed film bacteria. Appl. Environ. Microbiol. 39:1225-1226.

Stucki, G., R. Gälli, H.R., Ebershold, and T. Leisinger. 1981. Dehalogenation of dichloromethane by cell extracts of *Hyphomicrobium* DM2. Arch. Microbiol. 130:366-371.

Scholtz, R., L.P. Wackett, C. Egli, A.M. Cook, and T. Leisinger. 1988. Dichloromethane dehalogenase with improved catalytic activity isolated from a fast-growing dichloromethane-utilizing bacterium. J. Bacteriol. 170:5698-5704.

Wilke, C.R., and P. Chang. 1955. Correlation of diffusion coefficients in dilute solutions. Am. Inst. Chem. Eng. J. 1:264-270.

Chapter 10

GENERAL DISCUSSION

In this general discussion I will focus on the following aspects:

- The isolation of microorganisms with biodegradative potential
- Initial modes of attack on unsaturated and chlorinated hydrocarbons
- The potential of the trickle-bed bioreactor in waste-gas treatment

Isolation of microorganisms with biodegradative potential

To isolate microorganisms capable of degrading a specific compound various strategies can be used. The simplest method assumes that the desired microorganism is already present in Nature and that isolation is possible using simple enrichment techniques. The enrichment technique I have used involved incubation of an inoculum in a mineral salts medium of pH 7 at 30°C with a specific carbon source. The inoculum was usually a mixture of soil and water samples but in a number of cases distinct samples, often taken from polluted environments were used. Usually a series with varying concentrations of the carbon source was tested to assess if the compound of interest was toxic. Toxicity and biodegradation can be very easily monitored by determining carbon dioxide-formation rates.

Although not tested in detail, the effect of the carbon source concentration can be crucial. In Chapter 3 we assume that this is the reason why other researchers have not isolated vinyl chloride-utilizing microorganisms as they have used substrate concentrations that are too low to actually support growth. On the other hand our success in isolating styrene-utilizing microorganisms is probably due to the low concentrations of styrene we used in the enrichment cultures (Chapter 7). Effects of the substrate concentration on the isolation of microorganisms had already been reported in 1926 by den Dooren de Jong.

As I was also interested in finding microorganisms that would hydrate the double

bond of vinyl chloride, rather than oxidizing it to the reactive and carcinogenic epoxide, I set up enrichment cultures with vinyl chloride as carbon source under denitrifying conditions. These were, however, not successful. Isolation of bacteria on 3-chloroacrylic acid, e.g., vinyl chloride substituted with a carboxylic acid group, resulted in the desired 3-chloroacrylic acid hydrating activities, but these enzymes did not transform vinyl chloride.

If the desired phenotypes are not isolated readily more elaborate enrichment techniques may be used. One approach is to use more than one carbon source in the enrichment culture. Beside the compound of interest, a structurally related compound that has been shown to be biodegradable is added to increase the population of microorganisms that may also have the potential to utilize the compound of interest. This approach was used in isolating the methyl chloride degrading *Hyphomicrobium* MC1 (Chapter 2). In this case methane was added to the original enrichment cultures to increase the numbers of methane-utilizers, some of which might also have, or develop, the capacity to utilize methyl chloride. In this particular case this proved to be unnecessary as the isolated methyl chloride-utilizing strain MC1 did not utilize methane. Preferably, this type of experiment is performed in a continuous culture allowing a large population to grow on the substrate known to be biodegradable. By repeatedly adding new inocula, and perhaps periodically some mutagenic chemicals, the chance of finding the desired phenotype can be enhanced. In this way microorganisms are challenged to develop new pathways by altering their own genetic information due to mutations in structural and/or regulatory genes, or by recruitment of genetic information from other microorganisms.

New degradation pathways can also be constructed *in vitro*. This has been demonstrated for the degradation of a number of aromatic compounds (Timmis, 1989). The major problem in applying the vinyl chloride degrading *Mycobacterium aurum* L1 is the apparent instability of the epoxide transforming enzyme (Chapter 3). If styrene oxide isomerase (Hartmans *et al.*, 1989) would also transform the epoxide of vinyl chloride to chloroacetaldehyde it should theoretically be possible to construct a pathway involving alkene monooxygenase (Chapter 4), styrene oxide isomerase and the lower part of the 1,2-dichloroethane pathway as described by Janssen (1985) in *Xanthobacter autotrophicus* GJ10.

Initial modes of attack of unsaturated and chlorinated hydrocarbons

The strains isolated on vinyl chloride all oxidized vinyl chloride to the epoxide, analogous to the microorganisms previously isolated with short-chain alkenes. Hydration of the chlorinated carbon-carbon double bond was only observed with 3-chloroacrylic acid (Chapter 6) i.e. when the double bond was "activated" by a carboxylic group. Hydrolytic release of Cl^- has also been demonstrated with a new isolate degrading 2-chloroacrylic acid (pers. comm. van der Werf). Interestingly almost all styrene-degrading isolates degraded styrene by initial oxidation of the

unsaturated side-chain, rather than direct oxidation of the aromatic nucleus.

Although the alkene and styrene monooxygenases were not studied in great detail it is clear that these two enzymes are quite different. Alkene monooxygenase appears to be similar to the soluble methane monooxygenase but styrene monooxygenase is quite different from the monooxygenases studied so far that have the capacity to epoxidate the carbon-carbon double bond.

Of the dehalogenation reactions involved in the degradation of the chlorinated compounds discussed in this thesis only dehalogenation of 3-chloroacrylic acid was demonstrated in cell-free extracts (Chapter 6). The two hydratases, each specific for one of the isomers of 3-chloroacrylic acid were separated. These enzymes, like most hydratases, appeared to have a very high substrate specificity. Very similar results were published recently (Hylckama Vlieg and Janssen, 1992). These authors have purified and characterized two dehalogenases from a 3-chloroacrylic acid-utilizing coryneform bacterium, strain FG41. With the exception of the K_M of the *trans*-3-chloroacrylic acid dehalogenase of strain FG41, which was somewhat higher than the value we found for the strain CAA2 enzyme (Chapter 6) there were no differences between the enzymes of the two coryneforms. Hylckama Vlieg and Janssen (1992) also determined the N-terminal amino acid sequences of the two dehalogenases but found no homology between the two enzymes or homology with other known N-terminal sequences.

Dehalogenation of methyl chloride by *Hyphomicrobium* MC1 was shown to be oxygen-dependent, but a monooxygenase type of reaction requiring reducing equivalents can be ruled out on the basis of the growth yields (Chapter 2).

The enzymatics of Cl^- removal in the vinyl chloride degradation pathway of *Mycobacterium aurum* L1 (Chapter 3) are still a complete mystery. Formation of 2-chloroacetyl-CoA from chlorooxirane can, however, not be ruled out. Subsequent hydrolytic dechlorination of the coenzyme A ester as was recently described by Smith *et al.* (1991) for 3-chloro-2-methylpropionic acid would yield glycollyl-CoA. However, lack of growth of strain L1 on ethanediol or its oxidation products, which can also be envisaged to be degraded via glycollyl-CoA, do not support the involvement of glycollyl-CoA in vinyl chloride metabolism. Castro *et al.* (1992) very recently reported acetaldehyde formation from vinyl chloride by a *Pseudomonas* sp. grown with 3-chloropropanol. The mechanism of chlorine elimination is, however, not clear. Hydrolysis of the chlorine-carbon bond or hydration of the carbon-carbon double bond both yield unstable intermediates that rearrange to acetaldehyde. The The possibility that chlorooxirane is involved in the transformation of vinyl chloride to acetaldehyde can not be eliminated based on the results of Castro *et al.*, (1992).

Possibilities and limitations of the trickle-bed bioreactor

The performance of the trickle-bed bioreactor for the removal of dichloromethane from waste gases was shown to be quite good (Chapter 9). It should, however, be realized that a specific degradation rate of 50 grams of

dichloromethane m^{-3} h^{-1} only amounts to 7 grams of carbon m^{-3} h^{-1}. With other waste gas compositions, resulting in higher specific degradation rates of carbon, we have subsequently observed clogging of the trickle-bed bioreactor due to excessive growth (Weber, pers. comm.). Diks (1992) also observed this phenomenon and suggests that, depending on the packing material, a reasonable maximal elimination capacity allowing long-term stable operation of the trickle-bed bioreactor is in the range of 12 grams of carbon m^{-3} h^{-1}. Weber (pers. comm.) has also observed stable operation of the trickle-bed bioreactor at a specific carbon removal rate of 13 grams of carbon m^{-3} h^{-1} in a trickle-bed bioreactor treating waste gas containing toluene and butylacetate. The toluene concentration was, however, only reduced from about 80 mg m^{-3} in the inlet waste gas to 50-60 mg m^{-3} in the outlet, at a specific waste-gas flow of 200 h^{-1}. Butylacetate ($\pm$75 mg m^{-3} inlet concentration) was removed almost completely, once more illustrating the effect of the Henry coefficient on the efficiency with which contaminants can be removed from waste gases. These rates are actually lower than the rates of 25 - 35 grams carbon m^{-3} h^{-1} reported for biofilters (Beyreitz, 1989), suggesting that mineralization processes possibly operate more efficiently in a biofilter.

The simulations of dichloromethane removal with the trickle-bed bioreactor that were made in Chapter 9 are very different from the approach used by Diks (1992) to model the trickling filter he studied. Contrary to our conclusions, Diks (1992) reports that the removal of dichloromethane is not very much mass-transfer limited. However, Diks did not report many data in the range we studied, e.g. at dichloromethane inlet-concentrations well below 1 g m^{-3}.

To further validate the assumptions that we have made to simulate dichloromethane removal with the trickle-bed bioreactor using the BIOSIM program (Chapter 9) additional experiments should be performed with compounds with different Henry coefficients.

Conclusions

One conclusion that can be drawn from the results described in this thesis, and of course numerous other publications, is that microorganisms capable of growth on xenobiotic compounds can very often be isolated directly from Nature. Many xenobiotics which were assumed not to be persistent a number of years ago have since then been demonstrated to support growth of pure cultures. I expect that this trend will continue. Trichloroethene for example, is oxidized by a number of microorganisms exhibiting monooxygenase activity, but no pure cultures, capable of growth on trichloroethene as a sole source of carbon and energy, have been isolated yet. There is, however, no apparent reason why this should not be possible.

An aspect not discussed in this thesis until now is the spin-off that biodegradation research may have for an area such as biocatalysis. Sometimes the enzymatic activities discovered while studying the biodegradative pathways of xenobiotic compounds can be applied in the synthesis of specific chemicals (Ribbons *et al.*,

1989; Taylor, 1990).

Although in many cases growth of pure cultures on specific pollutants has been demonstrated this does not always implicate that it is possible to subsequently apply these cultures in existing bioreactors to treat waste gases (Chapter 8).

Therefore numerous challenges still exist for microbiologists as well as for engineers to further explore and develop the field of biological techniques for waste treatment.

REFERENCES

Beyreitz, G., R. Hübner, and M. Saake. 1989. Biotechnologische behandelung Lösemittelhaltige Abluft. WLB Wasser Luft und Boden **9**:53-57.

Diks, R.M.M. 1992. The removal of dichloromethane from waste gases in a biological trickling filter. Thesis Technical University of Eindhoven.

Castro, C.E., R.S. Wade, D.M. Riebeth, E.W. Bartnicki, and N.O. Belser. 1992. Biodehalogenation: Rapid metabolism of vinyl chloride by a soil *Pseudomonas* sp. Direct hydrolysis of a vinyl C-Cl bond. Environ. Toxicol. Chem. **11**:757-764.

den Dooren de Jong, L.E. 1926. Bijdrage tot de kennis van het mineralisatieproces. Thesis Technical University of Delft, Nijgh and van Dittmars, Rotterdam.

Hartmans, S., J.P. Smits, M.J. van der Werf, F. Volkering, and J.A.M. de Bont. 1989. Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. Appl. Environ. Microbiol. **55**:2850-2855.

Hylckama Vlieg, J.E.T., and D.B. Janssen. 1992. Bacterial degradation of 3-chloroacrylic acid and the characterization of *cis*- and *trans*-specific dehalogenases. Biodegradation. **2**:139-150.

Janssen, D.B., A. Scheper, L. Dijkhuizen, and B. Witholt. 1985. Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. Appl. Environ. Microbiol. **49**:673-677.

Ribbons, D.W., S.J.C. Taylor, C.T. Evans, S.D. Thomas, J.T. Rossiter, D.A. Widdowson, and D.J. Williams. 1989. Biodegradations yield novel intermediates for chemical synthesis, p. 213-245. *In* D. Kamely, A. Chakrabarty, and G. S. Omenn (ed.), Advances in applied biotechnology series. Vol. 4, Biotechnology and biodegradation. Gulf Publishing Company, Houston.

Smith, M.R., W.J.J van den Tweel, and J.A.M. de Bont, 1991. Degradation of 3-chloro-2-methylpropionic acid by *Xanthobacter* sp. CIMW 99. Appl. Microbiol. Biotechnol. **36**:246-251.

Taylor, S.C. 1990. S-2-chloropropanoic acid by biotransformation , p. 170-176. *In* L.G. Copping, R.E. Martin, J.A. Pickett, C. Bucke, and A.W. Bunch (ed.), Opportunities in Biotransformations. Elsevier Applied Science, London.

Timmis, K.N., F. Rojo, and J.L. Ramos. 1989. Design of new pathways for the catabolism of environmental pollutants, p. 61-82. *In* D. Kamely, A. Chakrabarty, and G. S. Omenn (ed.), Advances in applied biotechnology series. Vol 4, Biotechnology and biodegradation. Gulf Publishing Company, Houston.

SUMMARY

The original goal of the research described in this thesis was to develop a biological process for the removal of vinyl chloride from waste gases. The gaseous and carcinogenic vinyl chloride is used to produce the plastic polyvinyl chloride (PVC). During this production process waste gases containing vinyl chloride are generated. As a microorganism capable of growth on vinyl chloride as the sole carbon and energy source had been isolated it was envisaged that it might be possible to remove vinyl chloride from waste gases with a biological process.

Besides the original strain, *Mycobacterium* L1, three additional vinyl chloride-utilizing strains were isolated subsequently. All strains were tentatively identified as *Mycobacterium aurum*.

The first step in vinyl chloride metabolism in strain L1 was shown to be the oxidation of vinyl chloride to the corresponding epoxide, chlorooxirane, by alkene monooxygenase. Chlorooxirane is also the product of vinyl chloride oxidation in the human liver and is responsible for the carcinogenic properties of vinyl chloride. Alkene monooxygenase is also present in *Mycobacterium* E3 after growth on ethene. Extracts from strain E3 could be fractionated yielding two fractions which upon combination exhibited alkene monooxygenase activity, indicating that the enzyme consists of at least two components. One fraction was inhibited by acetylene, indicating it contained the oxygenase component of alkene monooxygenase, whereas the other fraction contained significant reductase activity. The corresponding fractions could also be obtained from extracts of vinyl chloride-grown cells of strain L1. Alkene monooxygenase appeared to be similar to the soluble three-component methane monooxygenases. These enzymes also oxidize alkenes to the corresponding epoxides.

The capacity of alkene monooxygenase to oxidize vinyl chloride to the mutagenic and toxic chlorooxirane was exploited to generate and select monooxygenase mutants of the ethene-utilizing strain E3. As long as cells exhibit monooxygenase activity they produce chlorooxirane from vinyl chloride and are hampered in their growth. However, when monooxygenase activity is lost, due to a mutation, these cells are no longer inhibited by the presence of vinyl chloride. Using this technique a mutant of strain E3 no longer capable of growth on ethene was isolated. Subsequently, it was shown that this mutant lacks the reductase component of alkene monooxygenase. Growth of the mutant on epoxyethane (oxirane) resulted in synthesis of the other alkene monooxygenase component(s). Extracts of such cells could be used to detect and subsequently purify the reductase component of alkene monooxygenase.

During growth of strain L1 on vinyl chloride in chemostat cultures it became evident that the original mineral salts medium was not optimal. The addition of extra iron to the medium resulted in an enhanced vinyl chloride consumption. Chemostat cultures were also used to determine to what levels vinyl chloride could be removed. This type of experiment was also done with the *Xanthobacter autotrophicus* GJ10 isolated by D.B. Janssen (University of Groningen) to determine

1,2-dichloroethane removal from air. 1,2-Dichloroethane is the precursor in the major production process of vinyl chloride. The concentrations of both compounds in the air that had passed through the cultures were significantly higher than the maximal allowable concentrations in waste gases according to the German legislation (TA-Luft). Therefore the affinity of both strains towards the respective substrates is too low to apply them in waste-gas treatment.

The enzyme transforming chlorooxirane appeared to be very unstable. After a short interruption in the supply of vinyl chloride to a culture of strain L1 inactivation took place upon restoring the vinyl chloride supply to the culture. This is probably caused by chlorooxirane accumulation due to an insufficient activity of the chlorooxirane transforming enzyme after such an interruption in the supply of vinyl chloride. The chlorooxirane subsequently inactivates cell components including alkene monooxygenase. Based on the observed inactivation and the relatively low affinity for vinyl chloride it was concluded that development of a process to remove vinyl chloride based on the application of *Mycobacterium aurum* L1 was not feasible.

As the formation of chlorooxirane is one of the major drawbacks of strain L1, microorganisms were isolated on compounds structurally related to vinyl chloride. Using this approach we hoped to isolate strains which add a water molecule to the double bond of vinyl chloride, resulting in formation 2-chloroethanol or acetaldehyde. 3-Chloroacrylic acid and styrene were used as vinyl chloride analogues in enrichment cultures. The enrichment cultures with 3-chloroacrylic acid did indeed result in the isolation of bacteria which hydrated the double bond of 3-chloroacrylic as the initial step in the degradation pathway of this compound. Unfortunately these enzymes did not exhibit any activity with vinyl chloride as substrate. With styrene as carbon source a number of microorganisms were isolated. Almost all of these isolates oxidized the unsaturated alifatic moiety of styrene yielding styrene oxide (phenyloxirane). The flavine adenine dinucleotide-dependent styrene monooxygenase has a high substrate specificity, only oxidizing phenyl substituted alkenes. In contrast to alkene monooxygenase the enzyme appears to require only one component for activity.

Besides vinyl chloride, biodegradation of methyl chloride, the simplest chlorinated hydrocarbon was studied. Methyl chloride-grown cells of the isolated *Hyphomicrobium* strain MC1 could dechlorinate methyl chloride only under aerobic conditions. Simple hydrolytic dehalogenation was therefore not taking place. No methane monooxygenase activity could be detected in methyl chloride-grown cells.

To be able to study a bioreactor for the removal of a chlorinated hydrocarbon from air, dichloromethane was selected as model contaminant. Dichloromethane is applied on a large scale as a solvent and consequently is present in numerous industrial waste gases. The strains isolated and characterized by the group of Th. Leisinger (ETH Zürich), as well as a new isolate, were shown to have a much better affinity towards dichloromethane than the value published for the purified

dehalogenase. Due to this high affinity these microorganisms can be applied to remove dichloromethane to concentrations well below the maximal allowable levels in waste gases.

A 0.066 m^3 trickle-bed bioreactor was studied for the elimination of dichloromethane from waste gases. The reactor was filled with a polypropylene packing material on which a biofilm developed. The air containing dichloromethane was forced through the reactor counter-current to a circulating aqueous phase. The aqueous phase is used to neutralize and remove the hydrochloric acid formed during dichloromethane degradation. The biological system was very stable and not sensitive to fluctuations in the dichloromethane supply.

Dichloromethane elimination with the trickle-bed bioreactor was determined at various gas and liquid flows and dichloromethane concentrations. It was possible to simulate the observed dichloromethane elimination efficiencies surprisingly well with a model incorporating Michaelis-Menten kinetics, diffusion in the biofilm and mass-transfer resistance in the gaseous as well as the liquid phase.

On the basis of the experimental results it was concluded that removal of dichloromethane is technically feasible with the trickle-bed reactor.

SAMENVATTING

Het oorspronkelijke doel van het onderzoek dat in dit proefschrift wordt beschreven was om na te gaan of vinylchloride door microbiologische afbraak uit industriële afvalgassen was te verwijderen. Het betrof hier met name afvalgassen van een bedrijf waar uitgaande van het gasvormige vinylchloride het polymeer polyvinylchloride (PVC) werd geproduceerd. Voorafgaand aan dit onderzoek was namelijk een microorganisme geisoleerd dat in staat was om met vinylchloride als enige koolstof- en energiebron te groeien.

Dit microorganisme, *Mycobacterium* L1, en een drietal daarna geisoleerde stammen die zeer veel op stam L1 leken, werden onderzocht op hun capaciteiten om op vinylchloride en andere verbindingen te groeien. Er waren vrijwel geen verschillen tussen de afzonderlijke stammen welke alle geidentificeerd zijn als *Mycobacterium aurum*.

De eerste stap van het vinylchloride metabolisme in stam L1 is bestudeerd en bleek gekatalyseerd te worden door het alkeen monooxygenase. Dit enzym oxydeert vinylchloride tot het overeenkomstige epoxide, chlooroxiraan. Dit is ook het produkt dat ontstaat bij vinylchloride afbraak in de menselijke lever en is verantwoordelijk voor de kankerverwekkende eigenschap van vinylchloride. Alkeen monooxygenase is ook aanwezig in de stam *Mycobacterium* E3 na groei op etheen. Dit enzym bleek uit meerdere componenten te bestaan en werd gescheiden in twee fracties die gezamelijk monooxygenase activiteit bezitten. De ene fractie was te inactiveren met acetyleen, hetgeen duidt op de aanwezigheid van de oxygenase component van het monooxygenase, terwijl de andere fractie een hoge reductase activiteit vertoonde. De overeenkomstige fracties konden ook in stam L1 worden aangetoond. Het alkeen monooxygenase bleek veel overeenkomst te vertonen met de oplosbare methaan monooxygenases, die ook in staat zijn om alkenen te epoxyderen, en die uit drie afzonderlijke componenten bestaan.

Door gebruik te maken van de eigenschap dat alkeen monooxygenase vinylchloride kan omzetten in het mutagene chlooroxiraan is een simpele methode ontwikkeld om monooxygenase mutanten te isoleren van microorganismen die vinylchloride kunnen oxyderen maar niet in staat zijn om op deze verbinding te groeien. Zolang cellen van een dergelijke stam monooxygenase activiteit bezitten wordt vinylchloride omgezet in het toxische en mutagene chlooroxiraan. Deze cellen worden hierdoor gehinderd in hun groei. Zodra echter een mutatie ontstaat, waardoor geen actief monooxygenase meer wordt aangemaakt, zullen deze mutanten niet meer gehinderd worden door de aanwezigheid van vinylchloride en sneller groeien dan het wild-type. Met deze techniek is een mutant van stam E3 geisoleerd die niet meer in staat was om op etheen te groeien en die bij nader onderzoek de reductase component van het alkeen monooxygenase complex bleek te missen. Bij groei van deze mutant op epoxyethaan (oxiraan) worden wel de andere component(en) van het alkeen monooxygenase complex aangemaakt zodat deze mutant gebruikt kan worden om de reductase component van het alkeen monooxygenase complex afzonderlijk te meten en dus te zuiveren.

Bij groei van stam L1 op vinylchloride in continu cultures bleek dat het gebruikte medium niet optimaal was. Het bleek te weinig ijzer te bevatten voor goede groei van stam L1. Met dit type cultures werd ook bepaald tot welke concentratie stam L1 vinylchloride uit lucht kan verwijderen. Tevens werd dit type experiment uitgevoerd met de door D.B. Janssen (RU Groningen) geisoleerde *Xanthobacter autotrophicus* GJ10 voor de verwijdering van 1,2-dichloorethaan uit lucht. 1,2-Dichloorethaan is de grondstof voor de chemische produktie van vinylchloride. Voor beide verbindingen was de concentratie in de lucht nadat deze door de continu culture was geleid echter aanzienlijk hoger dan de wettelijk maximaal toegestane concentraties volgens de Duitse TA-Luft. Beide stammen hebben dus een onvoldoende affiniteit voor de te verwijderen verbindingen.

Bovendien bleek het enzym dat chlooroxiraan, het eerste intermediair in the vinylchloride afbraakroute, omzet bijzonder instabiel te zijn. Na een korte onderbreking in de aanvoer van vinylchloride bleek stam L1 zichzelf te inactiveren nadat de vinylchloride aanvoer werd hersteld. Dit wordt waarschijnlijk veroorzaakt doordat het enzym dat chlooroxiraan omzet, na een dergelijke onderbreking, onvoldoende activiteit bezit om alle door het monooxygenase gevormde chlooroxiraan direct om te zetten. Hierdoor worden tal van celcomponenten, waaronder het alkeen monooxygenase, geinactiveerd door chlooroxiraan.

Concluderend kon worden vastgesteld dat het niet mogelijk is om, gebruik makend van stam L1, een betrouwbaar proces op te zetten voor de biologische verwijderijng van vinylchloride uit industriële afvalgassen.

Omdat één van de nadelen van stam L1 de afbraak via het giftige chlooroxiraan betrof, is getracht microorganismen te isoleren op verbindingen die qua structuur op vinylchloride lijken in de veronderstelling dat dan mogelijkerwijs stammen worden geisoleerd die als eerste stap in de afbraakroute vinylchloride een watermolecuul aan de dubbele binding adderen. Hiervoor werden 3-chlooracrylzuur en styreen gebruikt. Styreenafbraak was ook van belang bezien vanuit de stankoverlast die veroorzaakt wordt door bedrijven die met deze verbinding werken. Met 3-chlooracrylzuur werden inderdaad bacteriën gevonden die de twee isomeren van deze verbinding afbreken door in de eerste stap een watermolecuul aan de dubbele binding te adderen. Deze enzymen vertoonden echter geen activiteit met vinylchloride als substraat. Met styreen als substraat werd een aantal verschillende microorganismen gevonden. Vrijwel alle onderzochte stammen oxydeerden styreen op de alifatische zijketen tot het overeenkomstige epoxyde (fenyloxiraan). Het hiervoor verantwoordelijke flavine adenine dinucleotide afhankelijke styreenmonooxygenase heeft een hoge substraat specificiteit. Het oxydeert alleen fenyl gesubstitueerde alkenen. Verder lijkt dit monooxygenase uit slechts één component te bestaan.

Naast vinylchloride is ook de afbraak van methylchloride, de eenvoudigste gechloreerde koolwaterstof, onderzocht. Er is een *Hyphomicrobium* MC1 geisoleerd die na groei op methylchloride slechts onder aerobe condities deze verbinding kon

afbreken. Hydrolytische dehalogenering was dus uitgesloten. Er kon geen methaan monooxygenase activiteit gevonden worden in methylchloride gekweekte cellen.

Om uiteindelijk toch onderzoek aan een bioreactor voor de reiniging van afvalgassen te kunnen doen is de afbraak en verwijdering van dichloormethaan uit lucht onderzocht. Dichloormethaan wordt veel toegepast als oplosmiddel en komt daardoor vaak voor in industriële afvalgassen. Twee van de reeds beschikbare stammen, geisoleerd door de groep van Th. Leisinger uit Zürich, en een eigen isolaat bleken een veel betere affiniteit voor dichloormethaan te hebben dan gepubliceerd was voor het gezuiverde dehalogenase. Door deze hoge affiniteit voor dichloormethaan zijn deze bacteriën in staat om dichloormethaan tot voldoende lage concentraties af te breken voor toepassing in de afvalgasreiniging.

Voor de verwijdering van dichloormethaan uit lucht is de trickle-bed bioreactor onderzocht. Deze reactor bestaat uit een pakking van kunststof waarop een biofilm kan ontstaan. De dichloormethaan bevattende lucht werd door de reactor geleid in tegenstroom met een waterfase die over de pakking werd gerecirculeerd. Middels de waterfase kan het zoutzuur dat ontstaat tijdens de afbraak van dichloormethaan worden geneutraliseerd en afgevoerd.

Dichloormethaan-verwijdering met de trickle-bed bioreactor is bij verschillende lucht en vloeistof debieten en dichloormethaan concentraties onderzocht. De gevonden dichloormethaan-verwijdering bleek verassend goed te beschrijven met een model waarin Michaelis-Menten kinetiek, diffusie in de biofilm en stof transport weerstand in zowel de gas- als de waterfase geincorporeerd zijn.

Op basis van de uitgevoerde experimenten kon geconcludeerd worden dat de biologische verwijdering van dichloormethaan uit lucht middels een trickle-bed bioreactor technisch goed uitvoerbaar is.

Nawoord

Op de valreep (natuurlijk) dit nawoord waarin ik een aantal mensen wil bedanken die hebben bijgedragen aan de uiteindelijke totstandkoming van dit proefschrift.
Allereerst mijn promotoren Jan de Bont en Hans Tramper. Dankzij jullie vertrouwen en de geboden vrijheid heb ik mij met plezier kunnen uitleven in mijn pogingen de veelal weerbarstige "beestjes" hun geheimen te ontfutselen. Dat de meeste bevindingen uiteindelijk ook nog geopenbaard zijn is met name aan jullie te danken.
De studenten Paul van Eijck, Philippe Schyns, Ronald Niermans, Allard Kaptein, Wim van Ravens, Hans Oude Luttighuis, Matthijs Jansen, Gijsbert Voskuilen, Mariët van der Werf, René Stunnenberg, Marion Jansen en Anne Hof die in het kader van hun doctoraal onderzoek bijdragen hebben geleverd en die voor de broodnodige afwisseling hebben gezorgd.
Dianne Somhorst en Frans Weber, dankzij jullie vasthoudendheid hebben we het alkeen monooxygenase toch nog enigszins te pakken gekregen.
Mijn vele kamergenoten en medewerkers bij achtereenvolgens de Vakgroep Microbiologie, de Sectie Proceskunde en de Sectie Industriële Microbiologie. Jullie hebben zelden een opgeruimd bureau gezien.
Medewerkers van de diverse afdelingen van de Centrale Dienst, met name de mechanische werkplaats en de voormalige service afdeling, de tekenkamer en de afdelingen elektro en fotografie. Maar ook de dames van de kantine en het bibliotheekpersoneel.
Tot slot wil ik mijn ouders bedanken voor de mogelijkheden die zij mij hebben geboden en vooral Deborah voor haar geduld en vertrouwen.

Curriculum vitae

Sybe Hartmans werd geboren op 21 oktober 1957 te Korogwe, in het voormalige Tanganyika. De lagere school werd doorlopen in Nieuw-Zeeland. Het diploma Atheneum B werd in 1976 behaald aan "Het Wagenings Lyceum". In datzelfde jaar werd begonnen met de studie levensmiddelentechnologie aan de toenmalige Landbouwhogeschool te Wageningen. Afstudeervakken waren technische microbiologie, proceskunde en organische chemie. Het doctoraal examen landbouwwetenschappen werd behaald met lof in november 1983. Aansluitend hierop werd hij voor 3 jaar aangesteld als wetenschappelijk assistent bij de vakgroep Microbiologie van de Landbouwhogeschool te Wageningen. In 1985 werden 4 maanden doorgebracht aan het Mikrobiologisches Institut van de Eidgenössische Technische Hochschule te Zürich, Zwitserland. Van december 1986 tot december 1988 was hij als toegevoegd onderzoeker werkzaam bij de sectie Proceskunde, vakgroep Levensmiddelentechnologie van de Landbouwuniveristeit. Het onderzoek dat gedurende de periode december 1983 - december 1988 is verricht, is grotendeels in dit proefschrift beschreven.
Sedert 1 december 1988 is hij werkzaam als universitair docent bij de sectie Industriële Microbiologie, vakgroep Levensmiddelentechnologie van de Landbouwuniversiteit te Wageningen.

Bibliography

Habets-Crützen, A.Q.H., S.J.N. Carlier, J.A.M. de Bont, D. Wistuba, V. Schurig, S. Hartmans, and J. Tramper. 1985. Stereospecific formation of 1,2-epoxypropane, 1,2-epoxybutane and 1-chloro-2,3-epoxypropane by alkene-utilizing bacteria. Enzyme Microb. Technol. 7:17-21.

Hartmans, S., J.A.M. de Bont, J. Tramper, and K.Ch.A.M. Luyben. 1985. Bacterial degradation of vinyl chloride. Biotechnol. Lett. 7:383-388.

de Bont, J.A.M., M.J.A.W. Vorage, S. Hartmans, and W.J.J. van den Tweel. 1986. Microbial degradation of 1,3-dichlorobenzene. Appl. Environ. Microbiol. 52:677-680.

Hartmans, S., and J.A.M. de Bont. 1986. Acetol monooxygenase from *Mycobacterium* Py1 cleaves acetol into acetate and formaldehyde. FEMS Microbiol. Lett. 36:155-158.

Hartmans, S., A. Schmuckle, A.M. Cook, and T. Leisinger. 1986. Methyl chloride: naturally occurring toxicant and C-1 growth substrate. J. Gen. Microbiol. 132:1139-1142.

Kohler-Staub, D., S. Hartmans, R. Gälli, F. Suter, and T. Leisinger. 1986. Evidence for identical dichloromethane dehalogenases in different methylotrophic bacteria. J. Gen. Microbiol. 132:2837-2843.

van Ginkel, C.G., H.G.J. Welten, S. Hartmans, and J.A.M. de Bont. 1987. Metabolism of *trans*-2-butene and butane in *Nocardia* TB1. J. Gen. Microbiol. 133:1713-1720.

Archelas, A., S. Hartmans, and J. Tramper. 1988. Stereoselectieve epoxidation of 4-bromo-1-butene and 3-butene-1-ol with three alkene-utilizing bacteria. Biocatalysis 1:283-292.

Hartmans, S., J.A.M. de Bont, and W. Harder. 1989. Microbial metabolism of short-chain unsaturated hydrocarbons. FEMS Microbiol. Rev. 63:235-264.

Hartmans, S., J.P. Smits, M.J. van der Werf, F. Volkering, and J.A.M. de Bont. 1989. Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. Appl. Environ. Microbiol. 55:2850-2855.

Hartmans, S., M.J. van der Werf, and J.A.M. de Bont. 1990. Bacterial metabolism of styrene involving a novel FAD-dependent styrene monooxygenase. Appl. Environ. Microbiol. 56:1347-1351.

Hartmans S., and J. Tramper. 1991. Dichloromethane removal from waste gases with a trickle-bed bioreactor. Bioprocess Eng. 6:82-93.

Hartmans, S., M.W. Jansen, M.J. van der Werf, and J.A.M. de Bont. 1991. Bacterial metabolism of 3-chloroacrylic acid. J. Gen. Microbiol. 137:2025-2032.

Hartmans, S., F.J. Weber, B.P.M. Somhorst, and J.A.M. de Bont. 1991. Alkene monooxygenase from *Mycobacterium* E3: a multicomponent enzyme. J. Gen. Microbiol. 137:2555-2560.

Hartmans, S., and J.A.M. de Bont. 1992. The genus *Mycobacterium*-nonmedical p. 1214-1237. *In* A. Balows, H.G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The Prokaryotes, 2nd Ed. Springer Verlag, New York.

Hartmans, S., and J.A.M. de Bont. 1992. Aerobic vinyl chloride metabolism in *Mycobacterium aurum* L1. Appl. Environ. Microbiol. 58:1220-1226.

Weber, F.J., W.J.H. van Berkel, S. Hartmans, and J.A.M. de Bont. 1992. Purification and properties of the NADH-reductase component of alkene monooxygenase from *Mycobacterium* strain E3. J. Bacteriol. 174:3275-3281.

van der Werf, M.J., W.J.J. van den Tweel, and S. Hartmans. 1992. Screening for microorganisms producing D-malate from maleate. Appl. Environ. Microbiol. 58:2854-2860.

Hartmans, S., A. Kaptein, J. Tramper, and J.A.M. de Bont. 1992. Characterization of a *Mycobacterium* sp. and a *Xanthobacter* sp. for the removal of vinyl chloride and 1,2-dichloroethane from waste gases. Appl. Microbiol. Biotechnol. 37:796-801.