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Determination and Enhancement of Anaerobic Dehalogenation Degradation of Chlorinated Organics in Aqueous Systems

Battelle Columbus Div., OH

Prepared for

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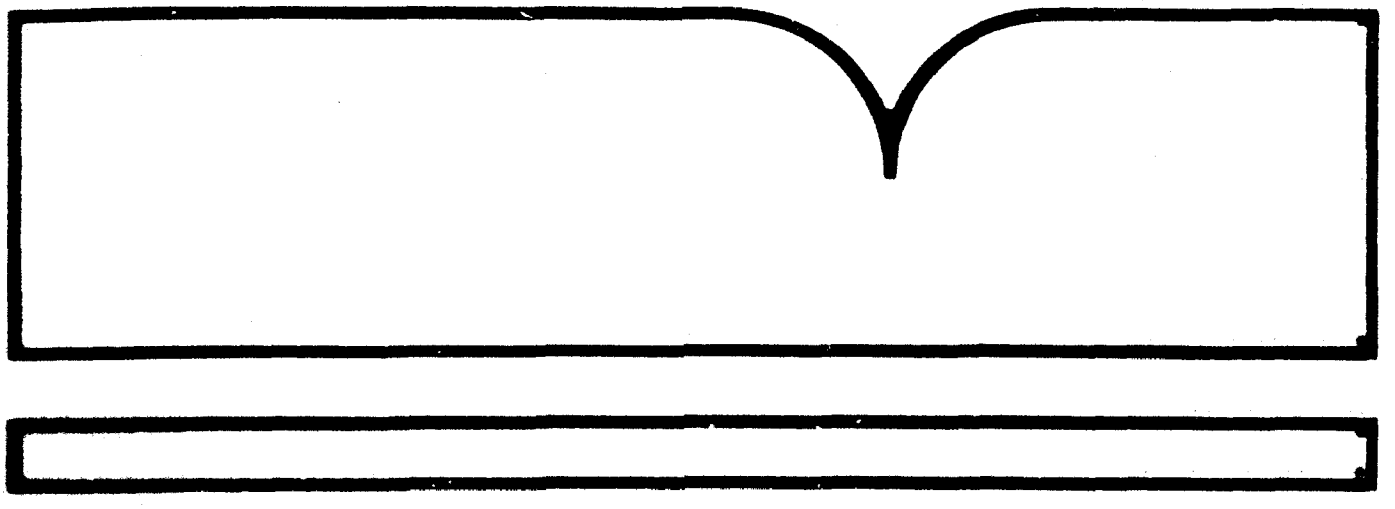

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NOTICE

This study has been funded wholly or in part by the United States Environmental Protection Agency under cooperative agreement (CR811120-02-4) to (Battelle Columbus Division). It has been subject to the Agency's review, and it has been approved for publication as an EPA document. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

Today's rapidly developing and changing technologies and industrial products and practices frequently carry with them the increased generation of materials that, if improperly dealt with, can threaten both public health and the environment. The U.S. Environmental Protection Agency is charged by Congress with protecting the Nation's land, air, and water systems. Under a mandate of national environmental laws, the agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. These laws direct the EPA to perform research to define our environmental problems, measure the impacts, and search for solutions.

The Risk Reduction Engineering Laboratory is responsible for planning, implementation, and management of research, development, and demonstration programs to provide an authoritative, defensible engineering basis in support of the policies, programs, and regulations of the EPA with respect to drinking water, wastewater, pesticides, toxic substances, solid and hazardous wastes, and Superfund-related activities. This publication is one of the products of that research and provides a vital communication link between the researcher and the user community.

The development of processes to remove toxic chemicals from [illegible] is an area of concern to the Water Engineering Research Laboratory. Microbially mediated aerobic degradation has been studied in order to provide technology for cleaning water. Investigation of anaerobic degradation began more recently and is of great interest. Anaerobic degradation may allow the degradation of [illegible] not degraded aerobically and will allow degradation of [illegible] in anaerobic environments. This paper discusses work done in the area of anaerobic dehalogenation of chlorinated aromatics. The goal of this work and similar work is to eventually provide efficient and economical methods to detoxify and degrade halogenated [illegible] that are present in anaerobic aqueous environments.

E. Timothy Oppelt, Acting Director
Risk Reduction Engineering Laboratory

ABSTRACT

Anaerobic degradation has the potential of providing efficient processes for destroying or detoxifying many environmental pollutants. Much interest is currently focused on the area of anaerobic degradation of halogenated organic compounds because of the toxicity of many of these compounds. This report summarizes initial efforts to isolate microorganisms capable of anaerobic dehalogenation; to examine the nutritional requirements of dehalogenating enrichments and a dehalogenating consortium; and to study the genetics of dehalogenation. The ultimate goal is to understand better the genetics of anaerobic dehalogenation and to use this information to develop engineered microorganisms with improved anaerobic dehalogenation capabilities.

Enrichments were made using secondary anaerobic digestor sludge (from Columbus, OH) as an inoculum. Anaerobic enrichments containing either 3- or 4-chlorobenzoate were established with a variety of terminal electron acceptors. Degradation of 4-chlorobenzoate was not observed. Degradation of 3-chlorobenzoate (3CB), as measured by High Pressure Liquid Chromatography (HPLC), was observed after 10-23 weeks, first in the nitrate enrichment and later in the methanogenic enrichments.

A 3CB degrading consortium, supplied by Dr. J. M. Tiedje, was examined to determine the effect of media composition on the consortium's population. These experiments were an effort to increase the proportion of the population which was capable of dehalogenation to aid in isolating the dehalogenator in pure culture. It was determined that when the consortium was grown in basal medium with 800 μM 3CB and 15 mM sodium nitrate, the microorganisms capable of benzoate degradation and methanogenesis were lost but dechlorination continued.

Genetic studies were begun using a pure culture of strain DCB-1 (obtained from Dr. J. M. Tiedje), the dehalogenating microorganism from

Tiedje's 3CB degrading consortium. Strain DCB-1 was examined for plasmid DNA using two different methods, alkaline lysis and salt-cleared lysis, but no plasmids were found. Therefore, it was presumed that the dehalogenase activity was chromosomally encoded. Genomic DNA was extracted and purified using a modified version of procedures previously used with anaerobes. A partial library was generated by cloning large DNA fragments into the cosmid pHC79 and small fragments into the plasmid pUC8. To aid in isolating recombinants with dehalogenase activity a rapid dehalogenase assay, based on the reaction which occurs between iodine and starch, was developed.

In summary, enrichments capable of degrading 3CB were isolated. It was shown that the population of a 3CB degrading consortium could be altered, by manipulating the growth medium, so that the fraction of the population capable of dehalogenation increased relative to the original consortium. Chromosomal DNA from strain DCB-1 was purified and found to be suitable for cloning manipulations. Finally, work began on the construction of a strain DCB-1 genomic library.

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16. ABSTRACT

This report summarizes initial efforts to isolate microorganisms capable of anaerobic dehalogenation; to examine the nutritional requirements of dehalogenating enrichments and a dehalogenating consortium; and to study the genetics of dehalogenation. The ultimate goal is to understand better the genetics of anaerobic dehalogenation and to use this information to develop capabilities. The important results from this work are summarized below.

Anaerobic enrichments were established in which 3-chlorobenzoate (3CB) but not 4-chlorobenzoate was degraded. Studies using a 3CB degrading consortium showed that specific manipulations of the growth medium could eliminate some members of the consortium while maintaining the organisms capable of dehalogenation. Such manipulations are useful in efforts to isolate organisms in pure culture. Genetic studies were begun using a pure culture of an anaerobic dehalogenator. No plasmids were found in this strain; therefore, it was presumed that the dehalogenase activity was chromosomally encoded. Genomic DNA was extracted and purified. A partial library was generated by cloning DNA fragments into the cosmid pHC79 and into the plasmid pUC8. A rapid dehalogenase assay was developed for the purpose of screening recombinants for dehalogenase activity.

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SECTION 1

INTRODUCTION

Many halogenated organic compounds can pose a serious environmental problem because of their toxicity and the tendency for accumulation in sediments and soils affecting both flora and fauna (Edwards, 1973; Chapman, 1978; Schneider, 1979). Microbial degradation has the potential to remove these halogenated compounds either in situ or in conjunction with above ground waste treatment. Aerobic degradation has been extensively studied. Some organic compounds do not appear to be degraded aerobically (DiGeronimo et al., 1979; Bouwer, et al., 1981) and others are degraded in a manner in which highly toxic intermediates are formed (DiGeronimo, et al., 1979; Horvath and Alexander, 1970; Evans, et al., 1971a; b; Gaunts and Evans, 1971; Ahmed and Focht, 1973; Reineke and Knackmuss, 1980; Hartman, et al., 1979). Relatively little is known about anaerobic degradation of these compounds. However, recent investigations indicate that anaerobic degradation of a number of halogenated organic compounds such as halogenated benzoates (Horowitz, et al., 1982; Suflita, et al., 1982; Horowitz, et al., 1983), halogenated phenols (Ide, et al., 1972; Murthy, et al., 1979; Boyd, et al., 1983; Boyd and Shelton, 1984) and halogenated short chain aliphatic compounds (Bouwer, et al., 1981; Bouwer and McCarty, 1983 a; b) does occur in the environment. These studies have shown that some compounds which are not degraded aerobically are readily degraded anaerobically. In contrast to aerobic degradation, the first step of anaerobic degradation is dehalogenation (Suflita, et al., 1982; Horowitz, et al., 1983) leading immediately to the formation of a less toxic, more biodegradable compound.

The overall objective of the program is the development of engineered microorganisms to cause the destruction of hazardous organic compounds (e.g., chlorinated organics) under anaerobic conditions. An understanding

of microbially mediated anaerobic dehalogenation and the exploitation of this process may result in the significant reduction of toxic and hazardous wastes in the United States. Therefore, the success of this program would greatly assist the EPA in detoxifying many recalcitrant compounds which in the past have not been biodegradable or accessible to other chemical and physical destruction techniques.

If the dehalogenase activity is encoded by a gene or genes carried by the plasmid, then plasmid genes specifying the anaerobic degradation or biotransformation of chlorinated organics can be introduced into suitable hosts using genetic engineering techniques. Strains so modified could then be examined to study and enhance the degradation of organic chemical contaminants present in hazardous waste sites.

If dehalogenase activity is a chromosomally encoded activity, then a genomic library will have to be constructed for each dehalogenating microorganism. At present, since there are no suitable probes for the dehalogenase enzyme gene, or the enzyme itself, the gene will have to be isolated using a screening method that detects the activity of the enzyme.

In order to study the genetics and biochemistry of dehalogenation and to begin a cloning effort, a pure culture of a dehalogenating organism was needed. Dr. J. M. Tiedje and associates developed the first anaerobic dehalogenating consortium and isolated the first anaerobic dehalogenating microorganism in pure culture (Shelton and Tiedje, 1984 a; b). When this study began, neither Tiedje's consortium nor pure culture of DCB-1 were available for study. It was not until later in the program that the consortium and then DCB-1 became available. This study, therefore, was divided into three phases. The first phase of this study was an effort to detect the presence of anaerobic dehalogenators in Columbus sewage and to isolate a pure culture of a dehalogenating microorganism. In the second phase of this study, we obtained the 3-chlorobenzoate (3CB) degrading consortium of Dr. Tiedje. This consortium was used as a model system for the isolation and identification of the organisms responsible for dehalogenation. In the third phase of this study, a pure culture of DCB-1, the organism responsible for dehalogenation in Tiedje's consortium, was made available to us and genetic studies on this dehalogenating bacteria were begun.

The work described in this report is an initial effort to examine anaerobic dehalogenation by applying some of the expertise gained in studying aerobic degradation processes.

SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

The ultimate goal of the work with anaerobic dehalogenating bacteria is to develop microorganisms capable of degrading hazardous organic compounds (eg., chlorinated organics) to environmentally safe forms under anaerobic conditions. This work will also provide information on the mechanism of anaerobic dehalogenation. To achieve this goal, we recommend completing the generation of the DCB-1 library and the cloning of the dehalogenase gene(s).

When Columbus sewage was used as an inoculum for the various enrichments discussed in this report, dehalogenation of 3-chlorobenzoate (3CB) was detected. The Battelle laboratory was the second laboratory to report this activity. Anaerobic dehalogenation is an activity found in multiple sewage samples; it is not an isolated activity (Shelton and Tiedje, 1984 a;b, our work). Dehalogenation of 4-chlorobenzoate (4CB) was not detected in the primary enrichment from Columbus sewage; this result is in agreement with the work of others (Horowitz, et al., 1982).

A hypothesis that H_2 at a concentration of 10% would aid in the establishment of a 3CB degrading consortium was shown not to be true for the conditions we used. Experiments with the dehalogenating consortium of Tiedje showed that varying the growth conditions (change in the terminal electron acceptor) could enhance the growth of the dehalogenating bacteria relative to other bacteria originally present in the consortium.

Pure cultures of DCB-1, the microorganism responsible for the dehalogenation of 3CB in the Tiedje consortium, were examined for the presence of plasmid DNA by a variety of methods (Hansen and Olsen, 1978 and Birnboim and Doly, 1979); no plasmids were observed. The absence of plasmid DNA indicated that a total genomic library of DCB-1 needed to be generated in order to clone the gene or genes responsible for anaerobic dehalogenation. Our efforts have shown that genomic-DNA isolated from DCB-1 can be cloned in and banked in *E. coli*.

SECTION 3

MATERIALS AND METHODS

ANAEROBIC METHODS AND MEDIA

Media and Strains

The anaerobic techniques used for the handling of the inocula, preparing the media, and handling of enrichments and cultures were essentially those of Hungate (1950) as modified by Bryant (1972) and Balch and Wolfe (1976). For the work with the enrichments and the consortium, the basal medium contained rumen fluid, B-vitamins, minerals, $NaHCO_3$, Na_2S reducing solution, resazurin redox indicator, and a 90% N_2-10% CO_2 gas phase (final pH 7.0) (Table 1). $NaHCO_3$ solution was prepared by dissolving 5.0 g in distilled water to make 100 ml, filter-sterilizing the solution, aseptically bubbling it with an 80 percent N_2:20% CO_2 gas mixture for 30 min. and then dispensing the solution under the same gas mixture in appropriate amounts into sterile 18 x 150 mm anaerobic culture tubes fitted with standard black butyl rubber stoppers (No. 1). Cysteine-sulfide solution contained 1.25% each of cysteine/HCl and $Na_2S.9H_2O$ and was prepared as previously described (Bryant and Robinson, 1961). Gas mixtures were obtained by using a gas proportioner with FM112-02G flow tubes (Aalborg Instruments and Controls, Inc., Monsey, NJ) which in turn was connected to the gasing apparatus described by Bryant (1972). Yeast extract (0.1%, w/v), or other growth supplements (i.e., volatile and branched-chain fatty acids [Table 2]) can be used as substitutes for rumen fluid. The terminal electron acceptor was CO_2 for methanogenic media, while 20 mM Na_2SO_4, 15 mM KNO_3 or 20 mM sodium fumarate was added for sulfate, nitrate or fumarate enrichments, respectively. Fermentative enrichments were prepared by including the carbohydrates similar to those used in the Complex

Carbohydrate medium (CM) of Leedle and Hespell (1980). The basal medium was used, but in addition, the medium contained 800 μM 3CB, Neopeptone (0.1%, Difco), Tryptone (0.1%, Difco), and 0.05% (vol/vol) each of cellulose (Sigmacell, type 100), cellobiose, glucose, maltose, pectin (citrus fruit), soluble starch (potato), xylan (larchwood) and glycerol.

TABLE 1. COMPOSITION OF BASAL MEDIUM

Component	Percent*
Clarified rumen fluid	5.0
Mineral solution+	5.0
Trace mineral solution§	0.1
Vitamin solution$^{\phi}$	0.5
Resazurin (0.1% wt/vol)	0.1
$NaHCO_3$ solution	7.0
Cysteine-sulfide solution	2.0
Gas phase (N_2:CO_2)	80:20

*All concentrations were in percent (vol/vol) unless otherwise indicated.

+Mineral solution contained (g/liter): KH_2PO_4, 10.0; $MgCl_2.6H_2O$, 6.6; NH_4Cl, 8.0; $CaCl_2.2H_2O$, 1.0

§Trace mineral solution is the trace element solution listed in Aragno and Schlege 1. 1981. (p. 874) with the addition of (g/liter:$FeCl_2.4H_2O$, 1.5; Na_2SeO_3, 0.1.).

$^{\phi}$Vitamin solution contained (mg/liter): 20 mg each of biotin and folic acid; 10 mg of pyridoxal-hydrochloride; 60 mg lipoic acid; and 50 mg each of riboflavin, thiamine hydrochloride, calcium-D-pantothenate, cyanocobalamin, p-aminobenzoic acid and nicotinic acid.

TABLE 2. VOLATILE FATTY ACID MIX*

Component	Volume (ml)
Acetic Acid	17
Propionic Acid	6
Butyric Acid	4
Isobutyric Acid	1
Valeric Acid	1
Isovaleric Acid	1
DL-Alpha-Methyl Butyrate	1

*Adjusted pH to 7.5 with 10 N NaOH. Brought to final volume of 200 ml with distilled water. Store sealed in refrigerator.

Stock cultures of DCB-1 were maintained in basal medium (Shelton & Tiedje, 1984b) containing 10-20% (v/v) clarified rumen fluid and 0.2% (w/v) pyruvate. Rumen fluid was obtained from fistulated cows at the Ohio Agricultural Research Development Center. The crude product was filtered through cheese cloth and centrifuged at 9820 x g, autoclaved and stored at 4°C. Prior to use, the liquid was further clarified by centrifugation. In order to increase cell yield and to establish a larger inoculum, DCB-1 was transferred to basal medium containing 0.2% pyruvate, 0.1% yeast extract, and 10 mM $Na_2S_2O_3$. For DNA extraction, DCB-1 was grown in one liter round bottom flasks in basal medium consisting of 20% clarified rumen fluid, and 0.2% pyruvate. All cultures were grown in an atmosphere of 80% N_2:20% CO_2.

The *E. coli* strains and vectors used in this work are listed in Table 3. *E. coli* strains were grown on LB plates, LB broth (Miller, 1972) or nutrient broth (Difco) with the appropriate antibiotic, if necessary, for selecting recombinants. (Ampicillin was used at a final concentration of 30-40 μg/ml and tetracycline was used at a final concentration of 15 μg/ml).

TABLE 3. MICROORGANISMS AND VECTORS

Strain	Genotype	Source
DCB-1		J. Tiedje
E. coli		
AC80	thr leu met hsdR-hsd+	L. Bopp
JM83	ara Δ (lac-pro) strA thi (80 dlacI^{q}ZΔ M15)	
MM294	endA1 thi-1 hsdR17 supE44	B. Bachmann
DH5α	endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 80dlacZΔM15	BRL*
Vectors		
pBR322	Ap^{R} Tc^{R}§	BRL
pHC79	Ap^{R} Tc^{R} cos	BRL
pUC8	Ap^{R} lacZα	BRL

*Bethesda Research Laboratories

§ampicillin resistance, Ap^{R}; tetracycline resistance, Tc^{R}

Tests for redox growth range and dehalogenation capability of the E. coli recombinants carrying DCB-1 DNA inserts were performed in basal medium supplemented with 20% clarified rumen, 0.2% pyruvate, plus the necessary antibiotic to maintain selective pressure for the plasmid. In addition, AC80 required 0.05% casamino acids in the medium to satisfy its auxotrophic requirements. The bacteria were incubated at 37°C.

Most Probable Numbers (MPN) Analysis

The MPN analysis procedure was used in experiments studying the 3CB degrading consortium. Ten-fold serial dilutions were prepared with anaerobic dilution solution (Table 4). One ml of each dilution was inoculated into triplicate tubes containing nine ml of the desired medium.

The MPN was estimated from the number of tubes in each triplicate set which were positive for a desired characteristic (Rodino, 1972).

TABLE 4. ANAEROBIC DILUTION SOLUTION*

Component	Percent
Mineral Solution	5.0
Trace Mineral Solution	0.1
Sodium Bicarbonate (5%)	2.5
Cysteine HCl/Sodium Sulfide (1.25% ea.)	2.0
Resazurin (0.1%)	0.1
Distilled H_2O	To 100 ml
Gas Phase (N_2/CO_2)	90:10
Final pH	7.2

*Prepare as described for basal medium and aliquoted in 4.5 ml amounts in septum-lipped tubes.

Source and Collection of Inoculum

The source of inoculum for the enrichments was secondary anaerobic digested sludge (Jackson Pike Plant, Columbus, Ohio). Inoculum was collected in a sterile 2 liter glass carboy containing a stir bar for mixing and sealed with a black rubber stopper fitted with a one-way gas valve to allow release of gas pressure.

Enrichments

Enrichments were prepared by adding sterile anaerobic solutions of 3CB or 4CB to the basal medium with 10% sewage sludge as inoculum. Some enrichments were set up in order to determine the effect of hydrogen on the development of actively degrading consortia by adding 10% H_2 to the atmosphere. Enrichments were incubated at 35°C. Degradation of the halogenated compound was followed over time by HPLC analysis. Once

degradation was observed, the enrichments were transferred to fresh media and passed several times to stabilize the activity.

Sample Collection, Storage and Analysis

Zero time samples of the gas phase and culture fluid were collected with a syringe. The gas sample was analyzed immediately by gas chromatography (GLC) for the presence of CH_4 and CO_2. The culture fluid was filtered (0.45 μm) or centrifuged to remove cellular material and stored frozen until it was analyzed. Initially, weekly samples of the gas phase and culture fluid were taken. Sampling intervals were altered when indicated as necessary by the data. Degradation of the parent compound and appearance of intermediates was followed using GLC or HPLC analysis.

Redox Studies

To determine the potential expression of a dehalogenase gene isolated from an obligate anaerobe and cloned into a facultative anaerobe, it was decided to evaluate the clones for dehalogenase activity over a wide range of redox conditions. Normally, DCB-1 is grown in a medium with an E_h of approximately -570 mV while the host E. coli strains JM83 and AC80 are grown under normal aerobic conditions. Each recipient was first grown under varying conditions of E_h to establish the limits of growth. JM83 and AC80 grew both aerobically and at E_h values as low as -570 mV. Additional tests with strain JM83 showed that growth occurred in the presence of sodium thioglycolate (<-100 mV), cysteine-HCl (-210 mV), dithiothreitol (-330 mV), and anaerobically with no extraneous reductant.

DEHALOGENASE SCREENING ASSAY

A dehalogenase screen was developed as a method to examine a large number of recombinants rapidly. Using a glass pipet, 1 drop each of the following reagents were added to the well of a white porcelain well plate: 0.2% KNO_2, 0.4% starch, 2% $ZnCl_2$ solution, and 1.9N HCl. A drop of liquid culture medium was then added to the well containing the reagents. A bluish purple color indicated a positive reaction for the presence of iodide. Iodide was present if the 3-iodobenzoate originally present had been dehalogenated. To confirm the validity of the spot test for dehalogenation,

DCB-1 was grown in the presence of 800 μM 3-iodobenzoate and analyzed for dehalogenation by both HPLC and the spot test described above over a 23 day period.

GENOMIC DNA ISOLATION AND PURIFICATION

In order to obtain sufficient quantities of purified genomic DNA, a modification of the Marmur (1961) procedure was used (J. Knutson, Michigan State University personal communication, 1985). Additional modifications were required and were made as follows: Proteinase K (Sigma) was used at a final concentration of 1 mg/ml to optimize cell breakage. RNase (Sigma) at a final concentration of 20 μg/ml was used to reduce the high concentration of contaminating RNA; ethylenediaminetetraacetic acid (EDTA) concentrations in both the storage buffer and the dialysis buffer were increased to 10.0 mM to minimize nuclease activity; and the T_8ES storage buffer (6 mM Tris pH 7.4, 0.1 mM EDTA, 10 mM NaCl), was replaced with TE buffer (10 mM Tris, pH 7.4, 10 mM EDTA) to decrease the salt content. The complete procedure is shown in Figure 1. DNA of a specific size range was isolated on a sucrose gradient usually 10 to 40% (w/v) (Hohn and Collins, 1980).

Cell Harvest

a. Add NaCl to 1 M (final concentration)
b. To collect cells, centrifuge 10K, 10'
c. Wash once with TE buffer
d. Weigh pellet (initially pellet in pre-weighed tube)

↓

Cell Lysis

a. Resuspend cells in TE (1 ml TE/gm cells)
b. Freeze overnight - 70°C
c. Thaw cells
d. Add 5 ml TE/gm cells
e. Transfer to centrifuge tube and measure volume
f. Add 1/10 volume lysozyme (3 mg/ml in TE)
g. Incubate on ice 15'
h. Add 1/16 volume Proteinase K (Predigested 37°C, 30') to a final concentration of 1 mg/ml.
i. Add 1/9 volume 10% Sarkosyl; Mix well
j. Incubate 37°C; 3 hours

↓

Phenol Extractions

a. Add equal volume phenol saturated with TE
b. Mix vigorously by vortexing
c. Spin 10K, 10'
d. Remove supernatant and save

↓

Figure 1. Battelle Protocol for Isolation of DCB-1 Chromosomal DNA.

Back Extraction

a. Add TE to bottom layer (equal volume)
b. Mix (vortex)
c. Spin 10K, 10'
d. Remove supernatant and add to first supernatant

↓

Continue phenol extractions until there is a clear interface between top and bottom layer

↓

Ether Extraction

a. Extract final aqueous phase with ether until both layers are clear (4 times)
b. Mix and spin 10K, 10'
c. Blow off excess ether with air

↓

Dialysis

a. Put DNA in dialysis tubing
b. Dialyze against 3 changes of 2L of:

TE = 10 mM Tris, pH 7.4, 10 mM EDTA

↓

Measure A_{260} for concentration

↓

Store at 4°C

↓

Add RNase (stock 10 mg/ml) to final concentration in solution of 20 μg/ml

↓

Let stand at room temperature 1 hour

Figure 1. (Continued).

ENZYMES

Restriction enzymes and terminal deoxynucleotidyl transferase (TdT) were purchased from BRL. Klenow fragments were purchased from Boehringer Mannheim Biochemicals (BMB). Ligations were done overnight at either 12°C or 4°C using standard procedures [International Biotechnologies, Inc. (IBI) catalog]: T_4 DNA ligase was purchased from New England Biolabs, Inc. (NEB). Cells were made competent and transformed using standard procedures such as that in the IBI catalog. Competent DH5α cells were purchased from BRL. In vitro packaging kits were purchased from Stratagene. Reagents were used according to the manufacturers' instructions.

GAS CHROMATOGRAPHY

Carbon dioxide, hydrogen, methane and nitrogen content of the gas phase of the enrichments were analyzed using a Varian Model 3700 Gas Chromatograph (Varian Instrument Group; Palo Alto, Calif.) in conjunction with a Hewlett Packard Model 3390A Integrator (Avondale, Penn.). A Carbosieve S-11 (100/120, 10 ft. x 1/8 in.; Supelco, Inc.; Bellefonte, Penn.) column was used with a helium carrier gas. Prior to use, the column was conditioned at 225°C with helium at a flow rate of 15 ml/min for 12 hours. Subsequent analyses were performed using helium at a flow rate of 30 ml/min. Gas flow rate was determined using a gas flowmeter. Gases were detected by thermal conductivity. Detector and injector port temperatures were 180°C and 200°C, respectively. Sensitivity was 160 mA at an attenuation of 4X. An autolinear program was used with an initial temperature of 35°C for 7 minutes followed by an increase in temperature at a rate of 25°C/min. to a final temperature of 175°C. Unknown gases were identified by comparison of retention time to the retention times of standard gases (99% purity). Quantification of gases was achieved by comparing the area under the curve produced by 0.5 ml of the sample to that produced by 0.5 ml of a 99% pure sample gas. Percent composition of each sample was determined by this method. Sample size was 0.5 ml for all injections.

Samples were collected from enrichments aseptically and anerobically. Sterile 1 ml gas-tight glass TB syringes (Becton Dickinson & Co., Rutherford, NJ) fitted with 22 gauge (1 in.) sterile needles were used to

remove samples of the gas phase. The 1 ml glass syringes were flushed with sterile anaerobic gas (N_2) before removing the gas sample from enrichments. The sterile anaerobic gas was delivered to a sterile 18 x 150 mm glass test tube via a 17 gauge bent needle attached to a sterile cotton-plugged 2 ml syringe. The gas was made anaerobic by passing over a heated (350°C) reduc : copper column and passed through the gas proportionator before reaching the 2 ml glass syringe (Shelton and Tiedje, 1984a). The stoppers in the cultures were flamed before sampling and an excess of gas sample (ie., greater than the 0.5 ml desired for injection) was removed from the culture. As the syringe was pulled from the culture vessel and stabbed into a black rubber stopper, the excess gas was slowly forced out in order to insure that atmospheric gas did not contaminate the sample during this transfer. Gas samples were analyzed immediately. All samples were manually injected. Due to the large gauge needle required to remove samples from the enrichments, a high temperature white septum (3/8", Alltech Associates, Inc., Deerfield, IL) was used. This septum was chosen because it displayed low bleed after repeated injections with large gauge needles.

HIGH PRESSURE LIQUID CHROMATOGRAPHY

Benzoate, 3CB and 4CB were separated, identified and quantified by high pressure liquid chromatography (HPLC). A reverse phase C18 Lichrosorb column (10 μ, 4.6 mm [ID] x 25 cm, Alltech Associates, Inc., Deerfield, IL) was used initially with a liquid phase of water:methanol:acetic acid (6:4:0.5) at a flow rate of 1.5 ml/min. The method used for the separation of benzoate and the chlorinated benzoate compounds was modified early in the study. The ratio of the solvent components was changed from 60:40:5 H_2O/methanol/acetic acid to 60:40:5 methanol/H_2O/acetic acid and the flow rate was decreased from 1.5 ml/min to 0.7 ml/min. This modification permitted us to decrease the flow rate without an increase in the retention times of compounds, and it eliminated the pressure control problems associated with the former solvent mixture. Under these modified conditions, the retention times for benzoate and 3CB were approximately 15.0 and 8.5 min. respectively. In addition, the sample volume was decreased from 250 μl to 100 μl, resulting in an increased lifespan of the guard column. The ratios of the solutions in the solvent system and the flow rate

were changed when necessary to increase the resolution of the data. Sample detection was achieved by U.V. adsorption at a wavelength of 284 nm using a LDC/Milton Roy Spectromonitor DUV detector (Laboratory Data Control Riviera Beach, FL). Sample peaks were integrated with the aid of a Hewlett Packard Model 3380A Integrator (Avondale, PA). Solvent flow was controlled with a Altex Model 420 Microprocessor (Beckman/Altex, Berkeley, CA) and a Beckman Model 11CA pump. Water was purified for HPLC use by the Milli-Q Water Purification System (Millipore Corporation, Bedford, MA). HPLC-grade methanol (Burdick & Jackson Laboratories, Muskegon, MI) and acetic acid (99+%, Aldrich Chemical Co., Milwaukee, WI) were used for the liquid phase. All glassware used in the preparation of the solvent was rinsed with HPLC water prior to use. Solvent components were measured and mixed in the proportions described and were then filtered and degassed under vacuum using a Millipore GS type filter (0.22 μm). The filtrate receptacle was rinsed with three 100 ml aliquots of filtrate before retaining the remaining filtrate for use with HPLC. Filtered solvent was stored in brown glass screw cap bottles and refiltered if not used within one week. Storage bottles were also rinsed three times with filtered solvent before being used.

Fluid culture samples for HPLC analysis were collected aseptically and anaerobically as described above using a 5 ml sterile plastic disposable syringe (Becton Dickinson & Company, Rutherford, NJ) with a 20 gauge (1 in.) sterile disposable needle. Samples were placed in plastic screw cap tubes and stored frozen until analyzed. After thawing, samples were prepared for analysis by filtering 1 ml of the sample through a 0.2 μm nylon-66 disposable filter cartridge (Alltech Associates, Inc., Deerfield, IL) into new disposable glass test tubes. Compounds of interest in the samples were identified by comparison of retention times to the retention times of authentic (high purity) compounds. These compounds were then quantified by comparing the integrated area under the curve produced by the compound in the culture sample to that produced by a 500 μM sample of the authentic compound.

NITRATE REDUCTION MEASUREMENTS

In cultures containing nitrate as the terminal electron acceptor the amount of nitrate reduction to nitrite was determined using Method 1, in the ASM Manual of Methods for General Bacteriology (p. 419 & 439). Nitrate was quantified by comparing the absorbance obtained with the sample to that of a standard curve determined with potassium nitrate. The concentration of nitrate still remaining in the fluid was determined by adding zinc powder to each assay to convert the unreduced nitrite to nitrate.

DETERMINATION OF SULFATE REDUCTION

A medium similar to the basal anaerobic medium was prepared to determine the presence of sulfate reducing organisms in the sulfate enrichments. In addition to components previously listed, (Table 1) the medium also contained (per liter) 0.1 g yeast extract, 2.0 g sodium lactate, 1.0 g sodium acetate, 0.1 g $FeSO_4$, 0.5 g sodium sulfate (as an electron accepter) and 2.0 g Bacto-agar. The medium was prepared anaerobically using standard Hungate technique, distributed into 13 x 100 mm glass test tubes and allowed to solidify at an angle to form a slant. Fluid from the anaerobic enrichments containing sulfate was aseptically and anaerobically removed as with a 1 ml sterile glass TB syringe fitted with a 22 gauge (1 in.) sterile needle. The sulfate slant medium was inoculated by stabbing the needle into the medium and injecting a small amount of culture. Air was excluded from the tubes of medium during inoculation by passing a stream of sterile anaerobic gas (N_2/CO_2; 90/10) into the tubes. Cultures were incubated at 35°C. Blackening of the medium, i.e. formation of FeS, indicated the presence of sulfate reducing organisms.

PLATING TECHNIQUES

Isolation of Dechlorinating Organisms in Pure Culture

The basic plate medium for the isolation of dechlorinating organisms had the same composition as the basal medium (Table 1), except that rumen fluid was replaced by 0.1% yeast extract and 2.0% agar was added. The following additions were made to aid in the isolation of these

microorganisms; (1) 3CB (800 μM); (2) 3CB (800 μM) plus sodium pyruvate (0.3%); (3) 3CB (800 μM) plus sodium pyruvate (0.3%), plus Volatile Fatty Acid Mix (2.0%; see Table 2) and (4) 3CB (800 μM) plus hydrogen (50%) and CO_2 (10%). Control media containing the additions, but lacking 3CB, were also prepared. The medium was prepared in a manner similar to basal medium, but after bringing the medium to a boil under the anaerobic gas phase the flask was sealed and autoclaved. The sterile medium was cooled to 50°C in a water bath, opened under the anaerobic gas phase, and the sodium bicarbonate and reducing solutions were aseptically added. The flask was resealed and transferred into the anaerobic chamber. The mouth of the flask was swabbed with 100% methanol before it was opened inside the chamber. An automatic pipetor was used to aseptically dispense 20 ml of medium into sterile plastic petri dishes (15 x 100 mm). Petri dishes and all other plastic or glassware were placed in the anaerobic chamber for at least 48 hours prior to using in order to remove traces of oxygen. Extra desiccant was placed in the chamber to aid in the drying of the plates. While still inside the anaerobic chamber, plates were sealed in anaerobic jars. The anaerobic jars contained open tubes of 2.5% sodium sulfide (placed in the jars 24 hours prior to inoculation) to insure reduction.

Cultures from which dechlorinating microorganisms were to be isolated were diluted from 1×10^{-1} to 1×10^{-6} by serially adding 0.5 ml of culture to 4.5 ml of anaerobic dilution solution (Table 4). One milliliter of the 1×10^{-4} dilution and of the 1×10^{-6} dilution was spread plated on duplicate plates of each of the media described above. Uninoculated plates were included as controls. After allowing the agar to dry, the inoculated plates were placed into anaerobic jars containing an open tube of 2.5% sodium sulfide. After sealing the jars, the anaerobic jars were taken out of the chamber and 0.2 atmospheres of N_2/CO_2 (90:10) were added via the gassing manifold to maintain a positive pressure in the jars. Jars containing plates to be incubated under hydrogen were pressurized to 0.25 atmospheres with hydrogen and additional CO_2 was added to maintain a concentration of 10%. Anaerobic jars were incubated outside the chamber at 33°C.

Isolation of Dechlorinating
Organism in Co-culture or Defined Mixed Culture

There were slight differences in the method used to obtain a co-culture versus a pure culture. The medium was the same, but the additions to the medium were different. Media containing (1) 3CB (800 μM) plus sodium sulfate (20 mM), and (2) 3CB (800 μM) plus sodium pyruvate (0.3%) plus sodium fumarate (25 mM) were prepared. Control media without 3CB were also prepared. Medium containing sodium sulfate was used for the preparation of a coculture with Desulfovibrio desulfuricans (ATCC 27774) as the terminal hydrogen-utilizing organism. Medium containing sodium fumarate was for the preparation of a coculture with Wolinella succinogenes (ATCC 29543) as the terminal hydrogen-utilizing organism. D. desulfuricans was grown in basal medium containing sodium lactate (22 mM) and sodium sulfate (25 mM). W. succinogenes was grown in basal medium containing 25 mM each of sodium formate and sodium fumarate.

Anaerobic dilution solution was used to dilute cultures containing the dechlorinating organism. An inoculum of D. desulfuricans or W. succinogenes was provided for a coculture by replacing 1.5 ml of the dilution solution with 1.5 ml of an actively growing culture of the appropriate species. Dilutions of the dechlorinating culture were prepared as described for pure culture isolation. The 1×10^{-4} and 1×10^{-6} dilutions, also containing D. desulfuricans or W. succinogenes, were plated on the appropriate medium as described above for pure culture isolation. Plates were placed inside anaerobic jars as described above and were incubated at 35°C in the incubator with the anaerobic chamber.

SECTION 4

RESULTS AND DISCUSSION

In order to properly examine the genetics of anaerobic dehalogenation, it was necessary that we have defined pure cultures. Several different approaches were used to obtain a pure culture(s) of an anaerobic dehalogenator. Because Dr. Tiejde's dehalogenating consortium and the pure culture DCB-1 were not available at the initiation of this project and because it was of interest to determine whether additional anaerobic dehalogenators could be isolated from an area other than the location in Michigan from which Dr. Tiedje obtained an inoculum source, an effort was initiated to examine Columbus sewage for dehalogenation activity. The occurrence of dehalogenation activity led to a further effort to isolate in pure culture the microorganism responsible for this activity. When Dr. Tiedje's consortium was made available to us, work was begun to isolate the dehalogenating organism(s) from the consortium. Finally, the dehalogenating organism strain DCB-1, isolated by Shelton and Tiedje, was sent to us. Since DCB-1 was a pure culture, studies with DCB-1 assumed a position of highest priority. The goal of producing a superior dehalogenating anaerobic organism can be best approached by studying the genetics and biochemistry of anaerobic dehalogenation. This sort of study can only be carried out using a pure culture. The availability of DCB-1 increased the speed at which we could move toward our goal.

ENRICHMENTS FROM COLUMBUS SEWAGE

While at the initiation of this program, there had been only one report in the literature of the isolation of an anaerobic dehalogenating microorganism in pure culture (Shelton and Tiedje, 1984b), it was likely that there would be other anaerobic organisms capable of similar activities. Because it may be possible to select for different dehalogenators by varying

the conditions of the primary enrichment, five different enrichment conditions were chosen for the experiments described in this report.

4-chlorobenzoate was chosen for one enrichment series, because Boyd and Shelton (1984) reported that cultures adapted to 4-chlorophenol were more versatile in their degradative abilities than either 2- or 3-chlorophenol adapted cultures. This may also be true of halobenzoate cultures; and a consortium containing more than one dehalogenating activity would be very useful in genetic studies. Methanogenic enrichments were prepared because the consortium capable of dehalogenation developed by Dr. Tiedje came from a methanogenic environment. Anaerobic degradation of organic compounds had been shown to occur under nitrate-reducing (Bakker, 1977; Evans, 1977; Bouwer and McCarty, 1983), sulfate-reducing (Pfenning et al., 1981; Mountfort and Bryant, 1982; Widdel, 1983) and fumarate-reducing conditions (Barik and Bryant, ASM, Ann. Abstr. Meet. 1984). Therefore, enrichments were prepared in which these compounds served as terminal electron acceptors instead of carbon dioxide (the electron acceptor in methanogenesis). A fermentative enrichment was also examined. All five conditions for anaerobic degradation of organic compounds were investigated to increase the probability of finding a variety of anaerobic dehalogenating bacteria. The isolation of a diversity of anaerobic dehalogenating bacteria in pure culture would have provided a valuable pool of genetic information for future studies.

The basal medium shown in Table 1 was used for methanogenic enrichments. Yeast extract (0.1%) or other growth supplements (ie. volatile and branched-chain fatty acids) can be used as substitutes for rumen fluid. (A 2.5% solution of sodium sulfide can be used to replace the cysteine/sulfide solution as there is little chance of loss of H_2S in the system [ie. sealed serum bottles] being used. Sulfide alone resulted in a lower E_0 and could, therefore, be more stimulatory to growth of strict anaerobes).

The nitrate reduction medium was similar to that described in Table 1. Since the E_0 of NO_3/NO_2 is +433 mV, cysteine (2.5%) replaced the cysteine/sulfide reducing solution. A minimum of 12 mM $NaNO_3$ would be

required for the complete degradation of 800 μM benzoate (800 μM x 15 = 12 mM):

$$C_7H_5O_2H + 12H_2O \rightarrow 7CO_2 + 15H_2$$
$$H_2 + NO^-_3 \rightarrow NO^-_2 + H_2O$$

The amount of $NaNO_3$ added depended upon the organic compound being used as the energy source. The N_2/CO_2 gas phase was retained as some strict anaerobes known to reduce nitrate also require CO_2 (de Vries, et al., 1974).

For the sulfate reduction enrichment, the basal medium in Table 1 was used, except that 20 mM $NaSO_4$ and 20 mM NaCl were added. Although sulfate-reducing anaerobes can be cultivated under 100 percent N_2, it has been found that sulfate-reducing anaerobes that degrade organic compounds are enriched under a gas phase containing CO_2 (Pfenning et al., 1981). The addition of NaCl and the reduction of the iron content to the level found in our medium should help to enrich for these organisms. In this case, the sodium sulfide reducing solution was used in place of the cysteine/sulfide solution.

At the time we were preparing our enrichment series, M. P. Bryant (Ann. Meet. ASM 1984) reported that fumarate could serve as a terminal electron acceptor for microorganisms that degrade benzoate. The use of fumarate would eliminate the need for an additional H_2 utilizing organism as the terminal electron sink in an enrichment. Therefore, it was of interest to determine if a dehalogenating degrader of benzoate could be obtained in pure culture if given fumarate as a terminal electron acceptor. Since the reaction of fumarate to succinate is a 2 electron pair reduction, 12 mM fumarate(the same concentration as $NO_3 \rightarrow NO_2$) would be required for complete degradation of benzoate. The basal medium described for the methanogenic enrichment was used.

The ability of fermentative organisms to dehalogenate 3CB was also examined. A number of carbohydrates were added to the basal medium for this enrichment.

Enrichment Controls

A suitable number of control enrichments were also included. Inoculated media of each type were: (1) autoclaved as a sterile control;

(2) transferred to a sterile flask and incubated aerobically to determine the need for anaerobic conditions and (3) incubated with benzoate as the energy source in order to obtain a dehalogenase negative benzoate-degrading organism. Also included was a sterile enrichment containing titanium chloride and an artificial electron donor (ie., reduced iron porphyrins; Zoro et al., 1974) in order to determine if nonbiological reductive dehalogenation would account for dehalogenation in these enrichments.

Effect of H_2 on Enrichments

The effect of H_2 on the development of consortia capable of degrading chlorinated organic compounds was of special interest and was examined. Degradation of chlorinated organics in anaerobic environments depends on the activity of three organisms. (1) a dehalogenator, (2) an organic degrader (proton reducer), (3) a H_2-utilizing organism. It has been observed that the chlorine must be removed before further degradation of the ring can occur (Suflita et al., 1983); thus, this may be an obligatory first step. The degradation of the dehalogenated intermediate requires that the H_2 produced be used by a third organism, because H_2 must be kept very low (less than 1×10^{-5} atm) to have degradation become thermodynamically feasible (ie. a negative Gibbs Free Energy) (McInerney and Bryant, 1981). H_2 would stimulate the growth of the terminal H_2-utilizing organism and probably stimulate reductive dehalogenation. However, the presence of H_2 would inhibit the degradation of the organic intermediate.

Most studies have used a N_2/CO_2 atmosphere while examining anaerobic dehalogenation. Horowitz et al. (1983) used a gas phase containing 10 percent H_2, but included a 24 hour preincubation period to remove H_2. Long lag periods (2 weeks to 6 months) have been observed before dehalogenation occurred in these studies. A possible explanation for these long lag periods may have been a result of poor availability of reducing equivalents for reductive dehalogenation. Thus, including just enough H_2 to provide reducing equivalents for dehalogenation might result in a decrease in the lag phase before dehalogenation is observed without leaving excess hydrogen to inhibit further degradation of the compound. However, the dehalogenating organism must be able to compete for hydrogen with the other H_2-utilizing anaerobes in the enrichments. The K_m for hydrogen during reductive

dehalogenation has been determined to be 30-67 μM (Suflita et al., 1983), and the affinity of methanogens and sulfate-reducing anaerobes for H_2 is 2-12 μM and 0.2-1.2 μM, respectively (Hungate et al., 1970; Robinson, 1982; Robinson and Tiedje, 1982; 1984). Thus, hydrogen would have to be added in nonlimiting amounts to be available for dehalogenation. The initial presence of the halogenated compound may also be favorable to reductive dehalogenation since these compounds are known to inhibit methanogenesis (Horowitz et al., 1983). According to the following equation:

$$2ClC_7H_4O_2^- + H_2 + 2\ H^+ \longrightarrow 2C_7H_5O_2 + 2\ H^+ + 2\ Cl^-$$

the degradation of 800 μmoles of 4-chlorobenzoate would require 400 μmoles H_2 for complete dehalogenation (at 25°C, 1 atm). This equals 9.79 ml H_2, or 16.3 percent H_2 in a 160 ml serum bottle containing 100 ml of medium.

Enrichment Results

The enrichments were periodically sampled during this study in order to determine some of the biological processes that were occurring. The enrichments were set up between 2/84 and 5/84. By 9/84, methane was detected by gas-liquid-chromatography in the gas phase of all the enrichments tested. Based upon gas production, the methanogenic enrichment was superior for the enrichment of methanogens but the methanogenic activity was almost as high when sulfate reduction enrichment medium was used. Methanogenic activity in the nitrate enrichment was very low.

HPLC analyses of culture fluid from those enrichments containing benzoate indicated that the benzoate was completely transformed within one month. In these analyses, no peak having a retention time identical to or similar to that of a reference sample of benzoate was detected. This observation indicated not total utilization but rather that all of the benzoate in the system has been at least partially degraded or transformed.

The degradation of 3CB and 4CB in the enrichments was periodically monitored. Enrichments inoculated on 8/20/84 with 10 percent Columbus sewage sludge were analyzed at various times during an 11 month period by HPLC to determine the amount of degradation of the halogenated compound. No degradation of 4CB in any of the enrichments was detected (Table 5).

Although 3CB degradation had been reported in primary enrichments to take up to 11 months to occur, 4CB degradation has not been reported in primary enrichments. Because it was unlikely that dehalogenation would occur in the 4CB enrichments after 11 months of being monitored, these enrichments were not maintained after June 30, 1985.

The degradation of 3CB was also monitored over time. Enrichments were begun on 7/13/84 with 10 percent sewage inoculum. Because a change in the analysis procedure in November resulted in some inconsistencies in the data when comparing degradation before and after 11/84, a large decrease in the concentration of 3CB was the criterion for degradation in these initial samples. Large decreases in the 3CB concentration were apparent even with the change in analysis procedure.

One of the nitrate enrichments incubated without hydrogen was the first enrichment to show significant 3CB degradation (Table 5). Once the initial amount of 3CB was no longer detectable, the culture was fed with 800 μm 3CB to confirm its ability to degrade this compound. The 3CB refed into the enrichment was utilized within one week of its addition (Figure 2a). Initially, the rate of disappearance of 3CB was 54 μmoles/liter/day, but after the third day, the rate increased to 145 μmoles/liter/day. Degradation after the third day was linear (R=0.999). A Gram stain of the enrichment was prepared. Most cells were embedded in an amorphous matrix. Gram negative short rods and cocci were present as well as refractile bodies, (ie. spores). Gram negative, thin, extremely long rods, similar to Methanospirillum hungatei, were also present. The cellular morphology of M. hungatei is quite unusual and easily recognized. The presence of M. hungatei would suggest that a methanogenic enrichment had become established. It was thought that the methanogenic enrichment had become established after the nitrate had been utilized.

The fumarate and methanogenic enrichments showed the complete absence of 3CB after 28 weeks of incubation (Table 5). There was no degradation of 3CB in either the sulfate or CCM enrichments. Enrichments showing degradation were passed to fresh medium as a 50 percent inoculum. The methanogenic enrichments were passed to basal medium. The fumarate enrichment was passed to both basal and fumarate medium in order to determine if fumarate was required for degradation of 3CB. The nitrate and

fumarate enrichments were also passed to fresh media with and without hydrogen. Passage of the nitrate enrichment to media with and without nitrate has been previously discussed. Degradation of 3CB was followed in these fresh transfers. Neither the terminal electron acceptor present in the original enrichment nor hydrogen were required for degradation. After 6 weeks, the transferred fumarate enrichment showed completed degradation of 3CB in the presence or absence of fumarate. At 10 weeks, the methanogenic enrichments showed complete degradation. Microscopic examination of the enrichments revealed a mixture of Gram negative rods of varying shape and lengths (from coccobacillus to long rod similar to M. hungatei). These enrichment experiments showed that an organism or several organisms capable of dehalogenating 3CB were present in the sewage inoculum.

TABLE 5. DEVELOPMENT OF 3-Cl-BENZOATE DEGRADING CONSORTIUM UNDER VARIOUS ENRICHMENT CONDITIONS

Enrichment Type	Time (Weeks)*	
	no H_2	$+H_2$
3-Cl-benzoate		
methanogenic	10-23+	10-23
sulfate	NDO§	NDO
nitrate	6-10	10-23
fumarate	10-23	10-23
CC fermentative	NDO	NDO
4-Cl-benzoate		
methanogenic	NDO	NDO
sulfate	NDO	NDO
nitrate	NDO	NDO
fumarate	NDO	NDO

*Weeks of incubation before degradation observed
+Degradation not observed at 10 weeks, but apparent at 23 weeks
§NDO - no degradation observed

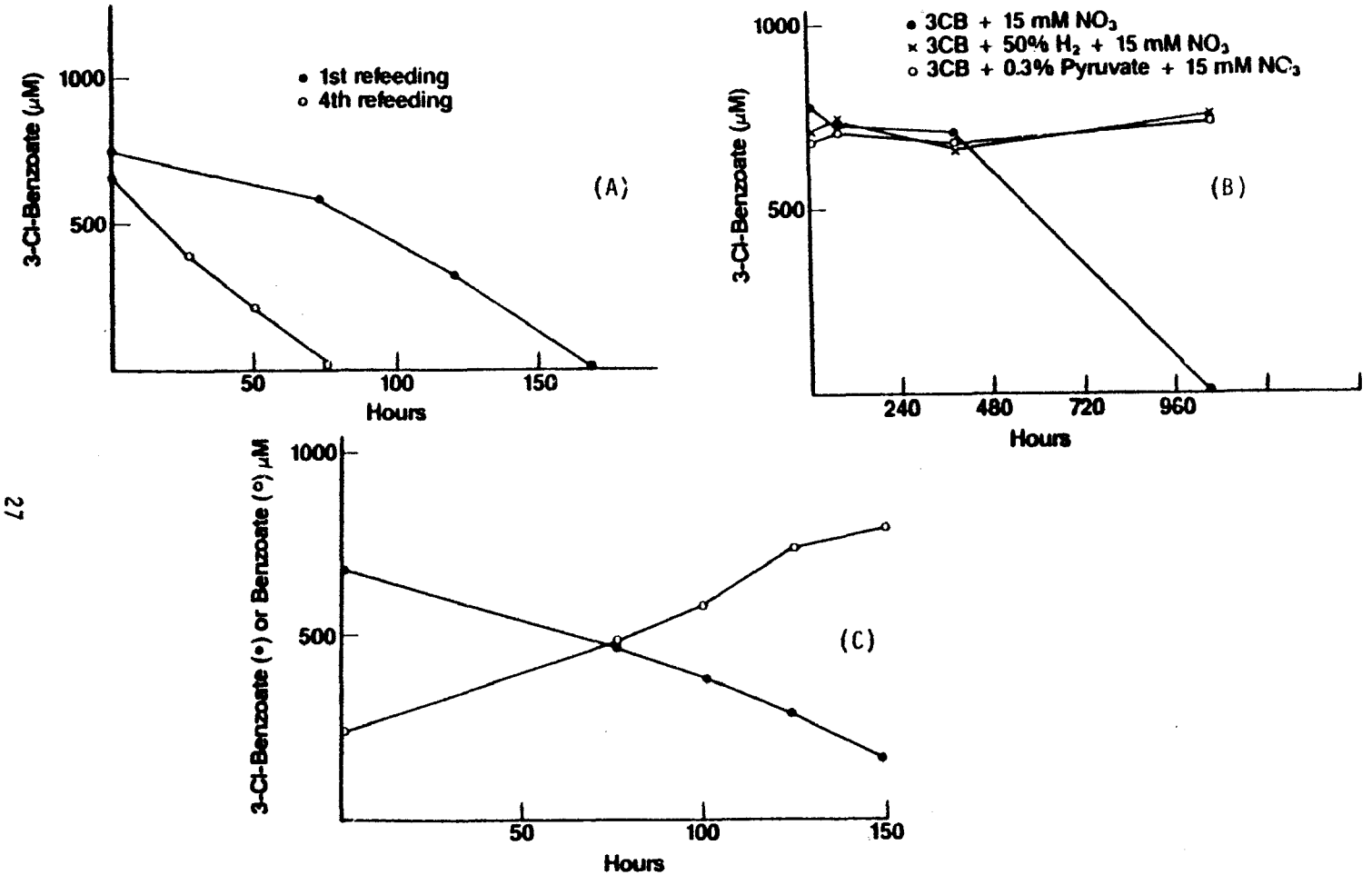


Figure 2. Dechlorination of 3CB by various cultures.

(A) 3CB/NO_3 enrichment from Columbus sewage.
(B) Response of "Tiedje" consortium to various culture conditions.
(C) 3CB/NO_3 subculture from "Tiedje" consortium.

EXAMINATION OF 3-CL-BENZOATE CONSORTIUM

The microbial consortium capable of degrading chlorobenzoates was received from Dr. James Tiedje on 10/8/84. The consortium was immediately fed 800 μM 3CB and degradation of 3CB was shown to be complete after four days. Residual benzoate was not detected. The "Tiedje" consortium was maintained by weekly feeding with 3CB and by 50 percent transfers monthly.

Published data on the "Tiedje" consortium (Shelton and Tiedje, 1984b) indicated that the dehalogenating organism isolated from this consortium grew slowly in a medium containing pyruvate and that it reduced nitrate to nitrite. This observation prompted the suggestion that it might be possible to improve the growth rate of the dehalogenating organism by providing nitrate as the terminal electron acceptor. Selectively improving the growth rate of this dehalogenating organism would aid in our attempt to isolate this organism in pure culture because of the potential enrichment of the dehalogenating microorganism. In order to enrich for the dehalogenating microorganism, the consortium was inoculated (10% v/v) into the following three types of medium:

(1) Basal medium with 800 μM 3CB and 15 mM sodium nitrate
(2) Basal medium with 800 μM 3CB, 15 mM sodium nitrate, and 0.3% sodium pyruvate
(3) Basal medium with 800 μM 3CB, 15 mM sodium nitrate, and 50% hydrogen

In these experiments, yeast extract was used instead of rumen fluid in the basal medium (see Materials and Methods). Pyruvate was added because it could provide an energy source as well as reducing potential for the reductive dechlorination of 3CB. Nitrate was added so that it could serve as a terminal electron sink to produce energy for growth.

The 3CB concentration was determined at 0, 3, 16 and 44 days (Figure 2b). At 44 days, the culture containing 3CB and 15 mM NO_3 showed no detectable 3CB, but a large peak was observed with a lower retention time (8.36 minutes) corresponding to 694 μM benzoate. This observation indicated that under these conditions 3CB was being dechlorinated, but not degraded. The entire 30 ml culture was transferred to 60 ml of fresh medium and the 3CB concentration was again determined over time (Figure 2c). It was

observed that as the 3CB concentration decreased the benzoate concentration increased. 3CB was dechlorinated at a rate of 38 μM per hour. A Gram stain of the culture showed that about 95% of the cells present were small Gram-negative coccobacilli found mostly in pairs. There were also some large Gram-negative rods. Cells with the morphology of M. hungatei were not seen. This suggests that under these conditions the benzoate degrader and the methanogens were selected against. Therefore, the dechlorinating organism was either one or both of the remaining cell types but was neither a benzoate degrader nor a methanogen. The large Gram-negative rods that were observed corresponded to the dechlorinating organism described by Tiedje, but the small coccobacilli were not described in Tiedje's report.

Since the small coccobacillus was present in much greater numbers than the large bacillus, we prepared a Most Probable Numbers (MPN) Dilution Assay (Rodina, 1972) of the culture. It was hoped that this method would allow us to determine which organism was responsible for the dechlorination activity. Dilutions of the culture were prepared from 1×10^{0} through 1×10^{-9}. Triplicate tubes of basal medium containing 800 μM 3CB and 15 mM NO_3 were inoculated with each dilution. The optical density was followed with time to determine growth. MPN data indicated that there were 1×10^{8} total cells/ml in this enrichment culture.

Dehalogenation only occurred in the lower dilutions (1×10^{-3} or less) indicating that the dehalogenator was present at a level less than 1×10^{4} cells/ml. In this enrichment the dehalogenator represented only 0.01% of the total population. Direct isolation of this organism, therefore, could be very difficult. Because no purified anaerobic dehalogenase enzyme existed and no analogous enzyme was known, probes could not be constructed which would facilitate the isolation of the anaerobic dehalogenator. Microscopic examination of the MPN cultures revealed a Gram-negative coccobacillus, commonly found in pairs and chains, as the dominant cell type in all dilutions; however, some large Gram-negative rods were present at the lower dilutions and a few smaller Gram-negative rods were present at all dilutions. The microorganisms in the lower dilutions were observed only after extended incubation to allow further growth of the culture. This incubation was followed by concentration by centrifugation. The presence of

the large gram negative rod correlated with dehalogenase activity in these dilutions.

A series of media were prepared in which 3CB, NO_3 and yeast extract (YE) were added in different combinations. This series was inoculated with one drop of the dechlorinating culture. In this manner we hoped to obtain pure cultures of the organisms or further enrich for the dehalogenating organism. The series of media included the basal medium lacking yeast extract with the following additions: 5 mM KNO_3 only; 5 mM KNO_3 + 50% H_2; 5 mM KNO_3 + 0.1% yeast extract; 5 mM KNO_3 + 50% H_2 + 0.1% yeast extract; 5 mM KNO_3 + 0.3% lactate; 5 mM KNO_3 + 0.3% lactate + 0.1% yeast extract; 0.3% lactate + 0.1% yeast extract; 3CB (800 μM) + 0.1% yeast extract; 3CB + 0.1% yeast extract + 50% H_2; 50% H_2 + 0.1% yeast extract; 0.1% yeast extract only; 3CB + 0.1% yeast extract + 5 mM KNO_3; 3CB + 5 mM KNO_3; and 3CB only.

Hydrogen was used as a possible energy source for reductive dechlorination. Lactate was used as an energy source for the nitrate reducer. Triplicate tubes of each medium were inoculated and the optical density was followed. The results of this experiment are shown in Table 6. Only tubes containing YE showed growth after one week. The optical density in tubes containing both H_2 and YE was greater (A_{600} = 0.42) than that in tubes with YE alone (A_{600} = 0.22), indicating that one or both of the organisms can utilize hydrogen. The presence of lactate inhibited growth even in the presence of yeast extract. Growth was best in media containing hydrogen in addition to yeast extract but nitrate tended to inhibit growth. After 6 weeks of incubation, dechlorination and a concomitant rise in the benzoate concentration were detected in all the media containing 3CB, except 3CB + KNO_3 and 3CB only. Apparently, yeast extract is necessary for dechlorination when KNO_3 is present. Dechlorination occurred in the defined media containing H_2 and 3CB despite a lack of visual apparent growth. To determine the population make-up of the cultures, it was necessary to streak the liquid culture onto plates. This work is described in the following section.

Isolation of Dehalogenating Organisms in Pure Culture

Since dehalogenation did not occur in the MPN cultures containing only the coccobacillus, but did occur at the lower dilutions containing the large

Gram-negative rod, it was likely that the rod and not the coccobacillus was the dehalogenating organism. Direct isolation of the dehalogenating organism was attempted from the defined H_2/3CB medium because this culture had the highest dechlorination activity per relative cell density.

TABLE 6. GROWTH AND DECHLORINATION OF 3-Cl-BENZOATE BY DECHLORINATING CONSORTIUM UNDER VARIOUS CONDITIONS

Additions*	Growth+	3-Cl-Benzoate (μM)	Benzoate (μM)
KNO_3	0.05	-	-
H_2/KNO_3	0.05	-	-
YE/KNO_3	0.22	-	-
H_2/KNO_3/YE	0.32	-	-
Lactate/KNO_3	0.08	-	-
Lactate/KNO_3/YE	0.05	-	-
Lactate/YE	0.07	-	-
3CB/YE	0.24	147.3	630.5
H_2/3CB/YE	0.47	0	752.0
H_2/YE	0.42	-	-
YE	0.22	-	-
3CB/YE/KNO_3	0.32	384.4	422.6
3CB/KNO_3	0.05	815.9	65.8
H_2/3CB	0.06	0	748.0
3CB	0.05	757.5	58.2

*Basal medium without yeast extract plus the additions indicated (see text)
+Maximum optical density (600 nm) after two weeks.

The culture was streaked onto 3CB/YE medium and incubated for 2 weeks with and without H_2. After two weeks, over one hundred colonies were picked into 3CB plus yeast extract broth. These cultures were incubated for one week and cellular morphology and dehalogenation were determined. The majority of cultures contained the coccobacillus previously described (Strain BF19, (Figure 3a), but five other morphological types were isolated. These included two coccobacilli which differed slightly from the dominant cell type. Strain BG95 (Figure 3b) was more spherical and strain BG29 (Figure 3c) did not form pairs, while chains were only periodically observed. Three Gram-negative rods were isolated, including: strain BG2, a medium rod with pointed ends (Figure 3d); strain BG49, a medium rod with round ends (Figure 3e); and a long thin rod. The presence of H_2 had little effect on the type of cell isolated. The 3CB concentration in cultures of each morphological type was determined weekly for one month, but dechlorination was not observed. A second attempt at direct isolation of a dechlorinating organism resulted in the isolation of two more types of Gram-negative rods, including a medium rod (strain BG131) which was usually found in pairs (Figure 3f) and a long oval rod (strain BG170) which tended to divide unevenly (Figure 3g). After one week, 3CB had not been dechlorinated by either of these isolates. At this point, a pure culture of the dehalogenating bacterium strain DCB-1 (Shelton & Tiedje, 1984b) became available and we could not justify screening the remaining pure cultures for dehalogenase activity.

GENETICS OF DCB-1 DEHALOGENATION

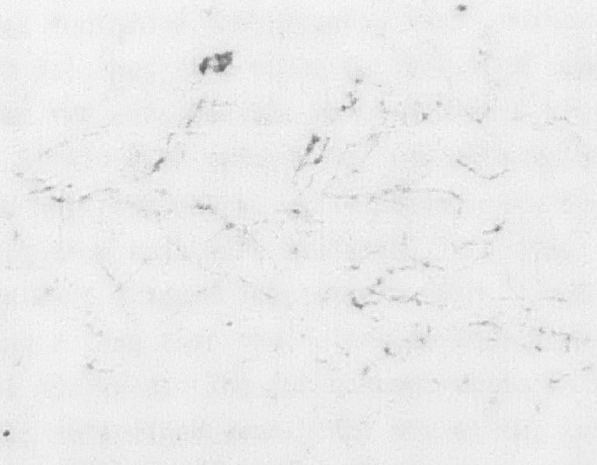
The dehalogenating organism, strain DCB-1, was received from Dr. J. M. Tiedje (Shelton & Tiedje, 1984b). DCB-1 is thought to be related to the genus Desulfovibrio. This strain was originally isolated from anaerobic digester sludge from a sewage treatment plant in Holt, Michigan. It is a Gram-negative, non-sporeforming obligate anaerobe capable of dehalogenating haloaromatic compounds by removing halogens (chloro, bromo, and iodo but not fluoro) from meta-substituted benzoate compounds. This organism grows fermentatively on pyruvate and has the ability to reduce sulfite ($SO_2^=$) or thiosulfate ($S_2O_3^=$). However, it will not dehalogenate in the presence of these inorganic sulfur electron acceptors.

3a. Strain BG19.

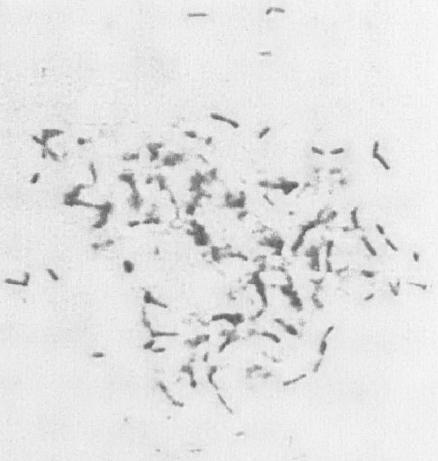
3b. Strain BG95.

3c. Strain BG29.

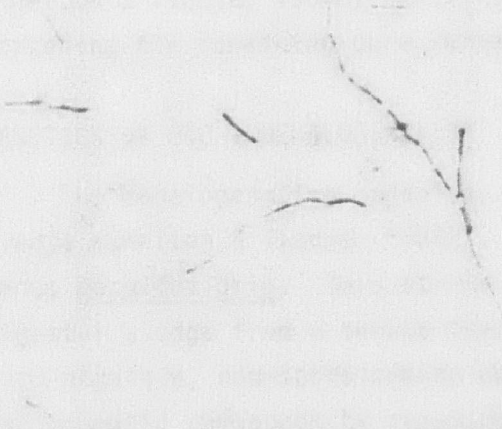
Figure 3. Photographs of the variety of cell types isolated in pure culture.



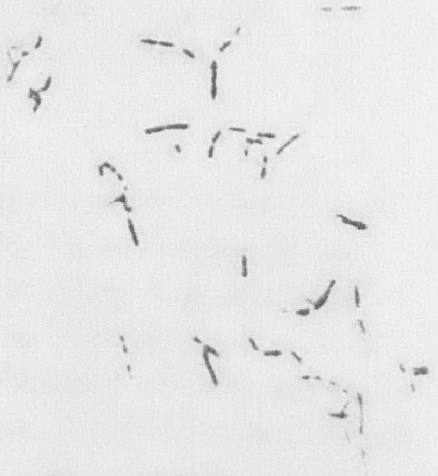
3d. Strain BG2.



3e. Strain BG49.



3f. Strain BG131.



3g. Strain BG170.

Figure 3. (Continued).

Because the purpose of this study was to investigate the genetics and biochemistry of anaerobic dehalogenation, the availability of this pure culture allowed us to begin the genetic work on anaerobic dehalogenation, without having to reisolate this dehalogenating organism from the Tiedje or the Columbus consortia. The receipt of this strain saved us a great deal of effort. Work with DCB-1 became the top priority.

DCB-1 represented the first anaerobic bacterium to be isolated in pure culture that possessed dehalogenase activity. The dehalogenase activity of DCB-1 is of intense interest because the mechanism and conditions of dehalogenation are different from the mechanisms observed in many aerobic dehalogenating microorganisms. Because of the very slow growth rate of strain DCB-1 and because strain DCB-1 is a fastidious strict anaerobe, it was decided that the best opportunity for studying anaerobic dehalogenation would be achieved through the cloning and expression of the dehalogenase encoding gene(s) from DCB-1 in an alternative host microorganism. Cloning of the DCB-1 gene(s) encoding anaerobic dehalogenation would also permit us to manipulate the environment of the enzyme in vivo and determine effects of various environmental conditions on the dehalogenation activity.

To facilitate our entry into the genetics and gene manipulation of the anaerobic dehalogenators, Dr. John Reeve (The Ohio State University), because of his successes with cloning methanogen DNA, was used as a consultant. During our conversations with Dr. Reeve, it became apparant that if any genetic engineering was to be successfully conducted, the development of an effective selection/or screen would be imperative.

Rapid Screening Method for Dehalogenase Activity

When a large number of recombinant bacteria are made in an effort to find a gene present only once in the genome, it is easiest if a screen is developed so that single colonies on a agar plate can be checked for the presence of the gene of interest. This type of testing allows a large number of recombinants to be examined rapidly; other methods are much less efficient. Because of the limited amount of research that has been done on the anaerobic dehalogenase enzyme(s), there was no way to make a DNA probe or an antibody probe for use in screening. The only way to screen for the enzyme is by expression of the dehalogenase activity in the recombinant.

After examining the literature for methods that could be used to examine large numbers of recombinant clones on agar surfaces, we were unable to find a suitable method. We selected wet chemistry as the best alternative to screening on an agar surface. We chose to test iodo-compounds in our development of a screening method. Because most biological media contain significant concentrations of chloride, the use of chlorine release, as chloride, from a chloroaromatic compound is often difficult to assess. Iodide or bromide measurements, however, usually do not present a problem in biological solutions.

A rapid qualitative assay was developed that could detect the presence of free iodide ions in liquid culture. This assay permits the rapid screening of recombinant clones for dehalogenase activity. The procedure described is a modification of the starch-iodide spot test for nitrites (Skerman, 1967). The original test depends on the formation of nitrous acid and its subsequent reaction with potassium iodide with the liberation of iodine, which turns the starch blue. In the modified test, a source of nitrite is provided as a 0.2 percent aqueous solution of KNO_2. The presence of iodide ions in the medium, due to the dehalogenation of the substrate 3-iodobenzoate, can then be detected. Quantitative analysis of the loss of 3-iodobenzoate and concomitant appearance of benzoate as determined by HPLC was used as evidence of dehalogenation. The results of the HPLC analysis were compared with the spot test reactions in order to determine the sensitivity of the spot test (Table 7).

After 23 days of incubation, all DCB-1 samples were positive for dehalogenation using the rapid starch spot test. A very strong positive reaction was evident in sample (a), the only sample shown to completely dehalogenate the 3-iodobenzoate based upon HPLC data. The remaining samples were all positive using the spot test, with 20.4 - 51.3 percent of 3 iodobenzoate remaining based on HPLC data. The development of a rapid technique to assess anaerobic dehalogenation, using 3-iodobenzoate, was a significant achievement. With this technique we will be able to screen large numbers of clones under a variety of environmental conditions. Without the development of this screen it would be extremely difficult to assess anaerobic dehalogenation.

Redox Studies

The presence of DCB-1 DNA encoding dehalogenase activity was to be detected in the host organism after cloning by the detection of dehalogenase activity. The effect of oxygen and redox potential on the anaerobic dehalogenase activity was not known.

TABLE 7. COMPARISON OF DEHALOGENASE ACTIVITY BY STANDARD HPLC METHODS AND THE RAPID SPOT SCREENING ASSAY.

Sample	Dehalogenation as Measured by HPLC						Dehalogenation as Measured by the Spot Test		
	3-iodobenzoate (% remaining)			Benzoate % formed			Starch Iodine Reaction		
	(days)			(days)			(days)		
	0	9	23	0	9	23	0	9	23
DCB-1 a	100	74.3	0	0	NT[+]	100	-	-	+++
b	100	95.6	51.3	0	NT	43.3	-	-	+
c	100	89.4	20.4	0	NT	100	-	-	+
d	100	86.2	29.5	0	NT	53.8	-	-	+
e	100	88.9	28.1	0	NT	50.0	-	-	+

Each value represents the mean of duplicate samples.

[*]A negative reaction is indicated by a minus sign and a positive reaction is indicated by one or more plus signs. A three plus reaction is one that occurred rapidly and resulted in a very dark blue color.

[+]NT = not tested.

It was, therefore, important to know that the chosen host organism, E. coli, was capable of growth at the redox potential that DCB-1 was grown at when dehalogenase activity was observed. Normally, DCB-1 is grown in a medium with an E_h of approximately -570 mV. The host E. coli strains, JM83 and AC80 were grown under varying conditions of E_h to establish the limits of

growth. Both grew at E_h values as low as -570 mV. In addition, tests with JM83 showed that growth occurred in the presence of sodium thioglycolate (<-100 mV), cysteine-HCl (-210 mV), dithiothreitol (-330 mV), and anaerobically with no extraneous reductant. Thus, the host strains ability to grow at low E_h is not a limiting factor in the potential expression of a dehalogenase gene(s).

Isolation of Plasmid DNA

Initially, it was hoped that DCB-1 might carry the genes encoding dehalogenase activity on a plasmid; the cloning of a gene or genes carried by a plasmid is generally much simpler than cloning a chromosome encoded gene(s). However, all attempts to isolate plasmid DNA (Hansen and Olsen, 1978; Birnboim and Doly, 1979) from DCB-1 were negative. There was no evidence to suggest the existence of any plasmids in DCB-1. The only way to clone the gene or genes responsible for the dehalogenase activity, therefore, was to generate a complete genomic library of DCB-1 DNA and search for the gene or genes of interest.

Isolation and Purification of DCB-1 Genomic DNA

In order to generate a complete genomic library, it was necessary to isolate large quantities of DNA. Isolation and purification of DCB-1 genomic DNA required significant modification o. existing procedures (Marmur, 1961). Initial DCB-1 genomic DNA preparations yielded low quantities of DNA which were resistant to restriction endonuclease attack and contained high levels of RNA. Two steps were taken in an effort to increase the yield of DNA. First, cells were incubated with higher concentrations of proteinase K to optimize breakage, and second, the EDTA concentration of both the storage and dialysis buffer was increased in order to inactivate nucleases. An increase in yield of genomic DNA from DCB-1 was obtained following these modifications. We observed that these DNA preparations could only be restricted by restriction endonucleases that required high salt buffers. The level of salt in the final DNA preparations was reduced by removing the sodium chloride from the storage and dialysis buffers. The DNA preparations were then sensitive to the restriction endonucleases required for cloning.

Preparation of DCB-1 Genomic Library

The goal of the cloning effort was to generate a complete DCB-1 genomic library and to screen the library for the gene (or gene complex) which encodes the dehalogenase activity. The initial set of experiments were performed in order to show that a DCB-1 genomic DNA library could be constructed using an E. coli host and vector. For these experiments, purified genomic DNA was digested with either PstI or EcoRI and ligated to PstI restricted pBR322 and EcoRI restricted pUC8, respectively. Recombinant E. coli with DCB-1 inserts were isolated (Figure 4). These clones were grown anaerobically and tested for dehalogenase activity using the 3-iodobenzoate screen. However, dehalogenase activity was not detected in any of the clones.

The successful cloning of DCB-1 DNA (small fragments) indicated that the DNA was suitable for a more extensive cloning effort. Because the isolation of DCB-1 was a slow process and the yield is relatively poor, it seemed more desirable to clone larger DNA pieces [DNA of a specific size range was isolated on a sucrose gradient (Figure 5)] into a vector such that DCB-1 DNA could then be produced in the recombinant host, E. coli, and subclones could be made then from these large inserts. A cosmid system was chosen as the primary method of generating a genomic library. Cosmids contain the cos (cohesive ends site) of the bacteriophage lambda. The presence of the cos region allows the packaging of DNA inside of lambda phage heads, which can then be further processed to become infectious particles. To be effective, the DNA located between the two cos sites must approximate the size of wild type lambda for packaging (37-52 kb). Inserting DNA into a cosmid and packaging the DNA in vitro selects for large inserts (30-45 kb). By using a cosmid cloning system, as opposed to a system requiring smaller inserts, a genomic library can be generated which contains a significantly smaller number of recombinants. The use of cosmids is also beneficial when cloning a gene complex. At this point, we do not know if the dehalogenase activity is encoded by a single gene or a set of genes. Enough recombinants must be isolated so that deletion of chromosomal material from the library is minimized. (One difficulty seen with the cosmid system is instability can occur, which can result in the deletion of cloned material). A genomic library was partially generated in the host

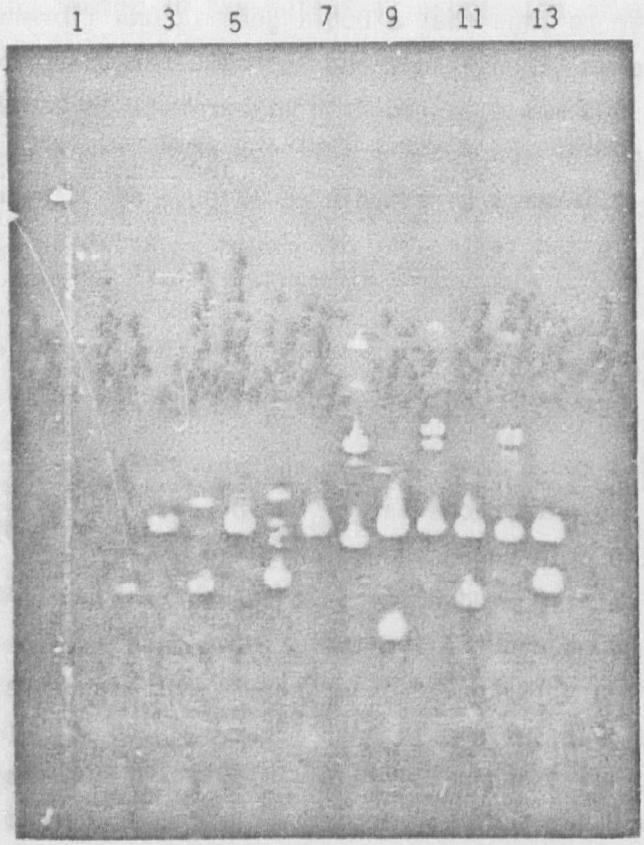


Figure 4. Agarose gel electrophoretic analysis of pUC8: DCB-1 clones in host E. coli JM83. Hind III (track 1) [23, 130 base pairs (bp), 9416 bp, 6557 bp, 4361 bp, 2322 bp, 2027bp]; plasmid vector pUC8 (track 2); pUC8 restricted with EcoRI (track 3); pUC8: DCB-1 clones unrestricted (tracks 4, 6, 8, 10 and 12); corresponding pUC8: DCB-1 clones restricted with EcoRI (tracks 5, 7, 9, 11 and 13).

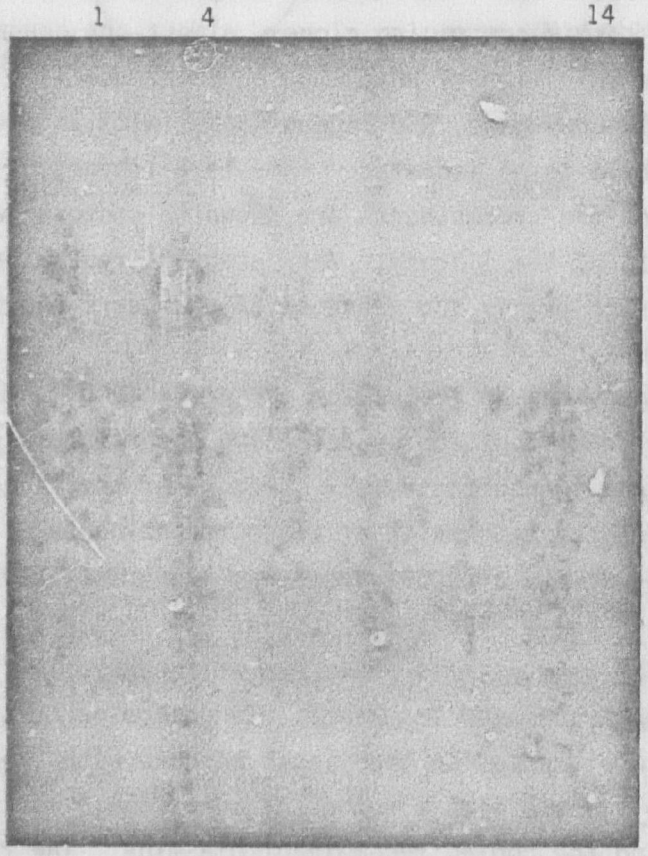


Figure 5. DCB-1 DNA run on a 10-40% (wt/vol) sucrose gradient. Lane 1- DNA restricted with Hind III; Lane 2- DNA (48,502 bp); Lane 3- DCB 1 DNA; Lane 4-14 - first 11 fractions of DNA from the sucrose gradient.

strain MM294 using the cosmid pHC79 (Hohn and Collins, 1980). The method is outlined in Figure 6. In the first round of cloning, ten recombinants were generated: these clones were stored by the standard procedure of freezing at -80°C. These clones were not recoverable after freezing. This unusual behavior may be the result of alterations in the hosts' physiology due to the presence of DCB-1 DNA. The second round of cloning has generated fifty-seven potential cosmid-containing clones, almost one genomic unit. In order to generate a genomic library which has a 95% probability of containing any particular single-copy gene, 380 recombinants (with an average insert size of 35 kb) will have to be isolated. This is a library of about five genomic units. If all of the recombinants are shown to contain large inserts, then we have about 15% of the library. The potential recombinants must be analyzed further to verify the presence of an insert and to determine the size of the insert.

Banked cosmids can be tested for the presence of the gene(s) responsible for dehalogenation by screening the recombinant bacteria for expression of dehalogenase activity. Because of the large size of the cloned DNA fragments, expression of these recombinants will depend almost entirely on the ability of DCB-1 promoters and translation initiation sites to function in E. coli. The large size of the fragments makes it unlikely that read-through from plasmid promoters will result in the synthesis of mRNA from the entire cloned fragments. To determine that these sites are functional, a complementation study must be done with the cosmid genomic library. E. coli hosts with a number of auxotrophic markers will be transformed using the pooled recombinant plasmids. The recombinant E. coli can then be screened to determine if the DCB-1 DNA can complement any of the mutations. Successful complementation would suggest that DCB-1 promoters and translation start sites can function in E. coli. Further experiments would be needed to prove that such a correlation was real. Detection of dehalogenase activity would not be limited by the need for an E. coli promoter if the DCB-1 promoters and translation start sites were active in E. coli.

Since it may be necessary to supply an E. coli promoter in order to detect expression of DCB-1 DNA in E. coli, our second strategy for cloning addresses this problem. Smaller fragments (3-9 kb) can be isolated from the

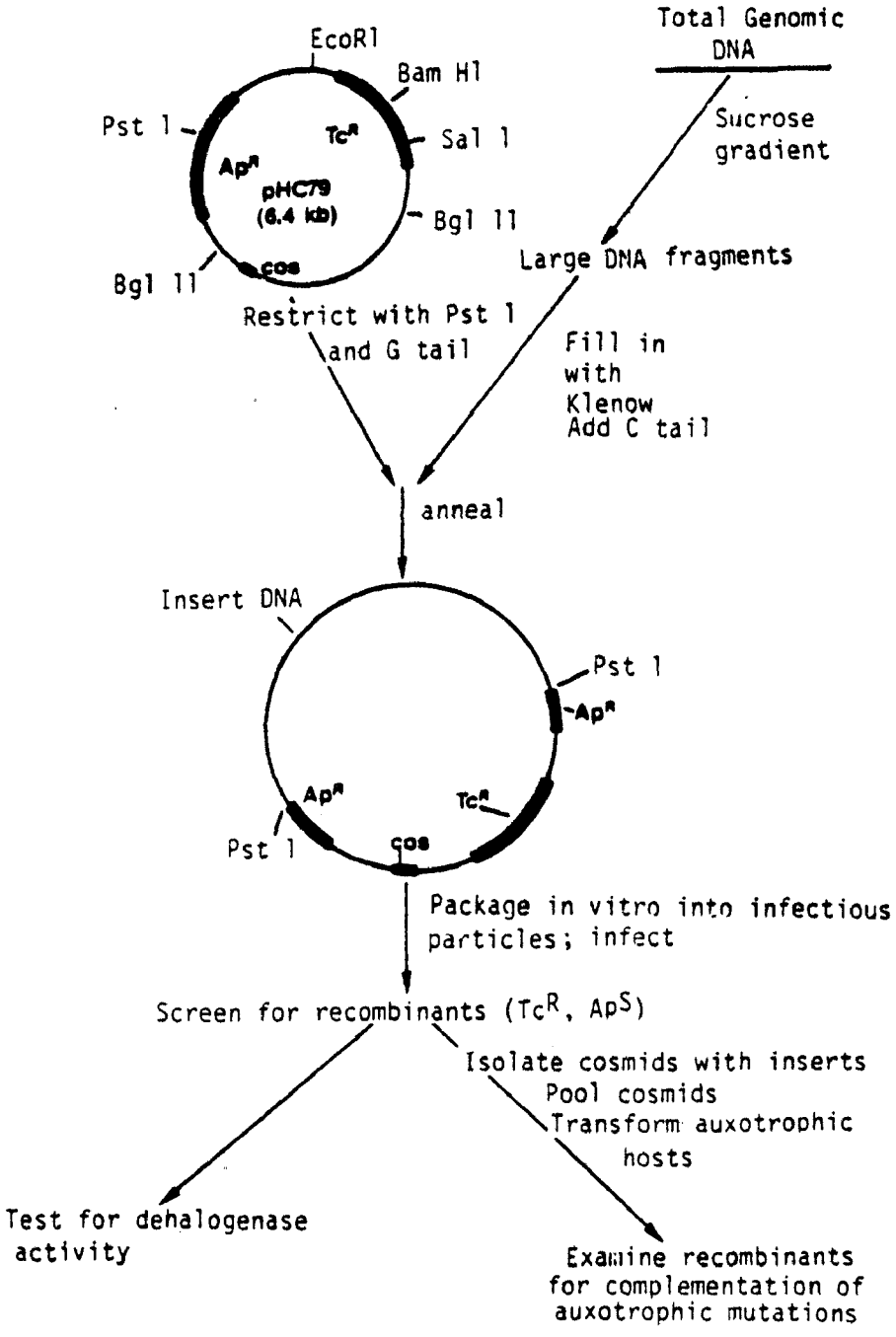


Figure 6. Construction of genomic library in cosmid vector pHC79 and screening of the library for gene expression in E. coli.

cosmids and subcloned into a restriction site, such as EcoRI, of pUC8 by the method outlined in Figure 7. Since the EcoR1 cloning site is located within the N-terminal portion of the lacZ gene, expression of fragments cloned into this site may occur from the lac promoter site. Recombinant clones will be pooled and screened for dehalogenase activity. The plasmids can then be isolated and also used in complementation studies. Because the pUC8 plasmid can be mobilized, complementation studies are not limited to an E. coli host. It should be possible to do complementation studies in Pseudomonas in order to determine if enzymes from DCB-1, an obligate anaerobe, will complement mutations in aerobic degradative pathways. These complementation studies could provide a great deal of information some of which would otherwise be provided only by mutation studies in DCB-1. Subcloning may also be useful for generating recombinants which can be stored by freezing. If it was the presence of large pieces of DCB-1 DNA which made the first set of cosmid recombinants sensitive to freezing, then it may be only possible to store either recombinants with small inserts or store the cloned DNA as purified plasmids. The first of these two methods is preferable to the second.

The size fractionation of DCB-1 DNA for cosmid cloning yielded several fractions of DNA which contained predominantly large pieces of DNA (>10kb) and yet these were pieces too small for cosmid cloning (<30kb). These pieces were reserved for cloning into pUC8 in order to further increase the size of the DCB-1 genomic library. These DNA fragments were partially restricted with the enzyme Sau3A1 to a size range of 2-5 kb and ligated into the BamHI site of pUC8. These ligation reactions were transformed into DH5α cells, and the recombinants are currently being analyzed.

Work with the recombinants will continue to verify the presence of DCB-1 DNA and to determine the best method of storage of the cloned DNA.

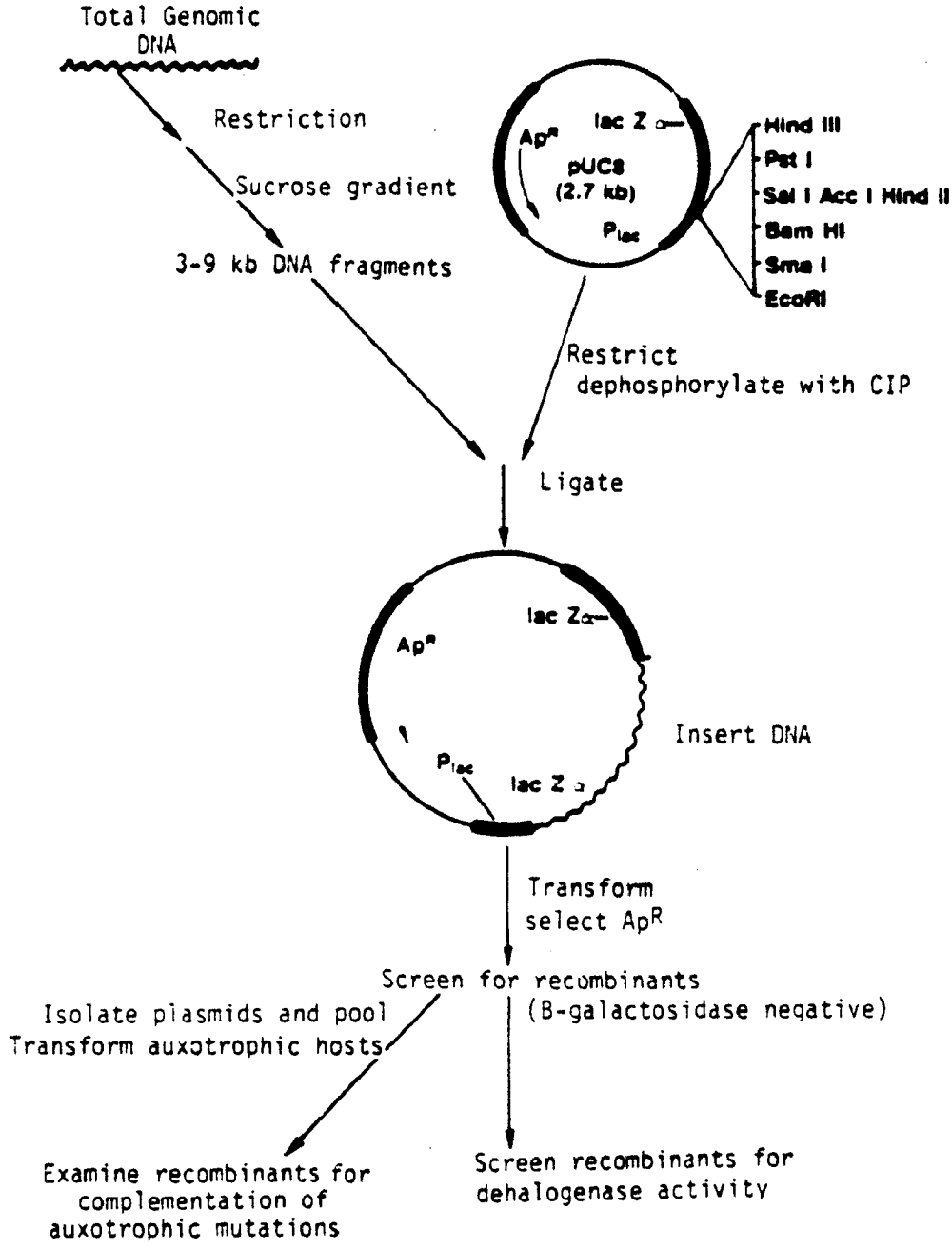


Figure 7. Construction of genomic library in pUC8 and screening of the library for gene expression in E. coli. Recombinant bacteria can be distinguished by their Ap^R, B-galactosidase negative phenotype.

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